AN ABSTRACT OF THE DISSERTATION OF

Tara Chestnut for the degree of Doctor of Philosophy in Environmental Science presented on December 2, 2014.

Title: Emerging Infectious Disease in Lentic Environments: The Ecology and Biogeography of the Amphibian Chytrid Fungus *Batrachochytrium dendrobatidis*, with Perspectives on Water Quality, Limnology, and Chemical Contaminants

Abstract approved:

_____

Andrew R. Blaustein Deanna H. Olson

Biodiversity losses in terrestrial, freshwater, and marine systems are accelerating at a global scale and the most threatened vertebrate taxa are those associated with freshwater habitats. The causes of biodiversity losses are often complex and include synergistic effects of natural and human-induced stressors, such as habitat loss and fragmentation, urbanization, invasive species, contaminants, global climate change, and emerging infectious diseases. In the last 35 years, the amphibian extinction rate has been estimated to exceed 105 times the baseline expected rate for all species and in the USA, the number of occupied amphibian sites has been reported to be declining by 3.7% per year. Among the many threats to amphibians, the role of disease in population declines has been recognized increasingly over the last two decades. Numerous amphibian diseases have been identified and attributed to mass mortality events. Chytridiomycosis, the emerging infectious disease caused by the amphibian chytrid fungus, *Batrachochytrium*

dendrobatidis (Bd), is implicated as a causal agent in many recent global amphibian population declines and extinctions.

To understand the pathology and conservation implications of Bd, a greater understanding of its ecology, life history, and distribution in the wild is of paramount importance. Although it has an impact on the persistence of selected amphibian populations around the world, the full scope of the effects of chytridiomycosis on global amphibian population declines are not well understood. Most Bd research efforts have focused on Bd in amphibian hosts per se, with little attention to understand the environmental associations and dynamics of free-living Bd outside of the amphibian host. In particular, information on Bd responses to climatic variation outside of hosts is a research gap. Furthermore, as a microorganism within an aquatic environment, studies are lacking of potential water quality associations, how Bd may interact with other members of their biological communities, and how Bd responses to chemical contaminants found in aquatic environments. My research begins to fill these gaps by studying the basic ecology of free-living Bd in field settings, and investigating factors that may influence its distribution at a landscape scale, occurrence at a regional scale, and detection at a site scale.

Herein, I describe spatial and temporal patterns in the detection and density of free-living Bd in aquatic habitats in two different geographic regions of the United States, Alaska (Chapter 2) and Oregon (Chapter 3). The Alaska work examines Bd ecology at the northernmost extent of amphibian occurrence in North America, where climate

associations may be particularly relevant and where Bd occurrence may be representative of one of the most novel pathogen-host systems in the world. I also describe (Chapter 2) experimental results of Bd and amphibian response to extreme cold temperatures they may experience in continental settings, at high elevations, and at high latitudes. My Oregon studies (Chapter 3) focus on multivariate associations of free-living Bd occurrences with a suite of aquatic environmental factors, both abiotic and biotic in nature. In Chapter 4, I describe how amphibians and Bd respond to agricultural chemicals (fungicides) that they may be exposed to in field settings. These results are specific to Bd, but might also warrant consideration as fungicidal treatments for a newly described chytrid affecting salamanders; both of these amphibian chytrids have been detected in captive animals and solutions to treat trade animals for the pathogen are gaining relevancy. Finally, in Chapter 5, I reflect upon the journey of conservation biologists and herpetologists for 25 years of amphibian decline research, with global losses becoming widely recognized in 1989. In this context, my research significantly advances understanding of the geographic distribution and ecology of one potential threat factor to amphibian populations on Earth, *Batrachochytrium dendrobatidis*. The factors that I report to both promote or limit free-living Bd distribution and abundance will further inform pathogen dynamics research.

Emerging Infectious Disease in Lentic Environments: The Ecology and Biogeography of the Amphibian Chytrid Fungus *Batrachochytrium dendrobatidis*, with Perspectives on Water Quality, Limnology, and Chemical Contaminants.

by
Tara Chestnut

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented December 2, 2014
Commencement June 2015

Doctor of Philosophy dissertation of Tara Chestnut presented on December 2, 2014.

APPROVED:

_____

Co-Major Professor, representing Environmental Science

_____

Co-Major Professor, representing Environmental Science

_____

Director of the Environmental Sciences Graduate Program

_____

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

_____

Tara Chestnut, Author

ACKNOWLEDGEMENTS

WE DID IT! The sum of the parts of this body of work is greater than the whole because of my advisors, collaborators, mentors, peers, volunteers, friends, and family. My co-advisors Dede Olson and Andy Blaustein were an incredible team that provided the perfect balance of support, experience, structure, humor, humbleness and discipline that I needed to be successful. Chauncey Anderson was the string to my balloon. He kept me grounded, yet supported my outlandishly ambitious field adventures with a level head, even when it seemed that I disappeared in the Alaskan wilderness for a couple days. Radu Popa showed me that a wildlife biologist could also be microbiologist, and so much more. Together, Dede, Andy, Chauncey, Radu, and my other committee members Lisa Ganio and Joey Spatafora challenged me to be a more confident and competent scientist. I am grateful for their investment in me. Thanks to Renee Freeman, Torri Givigliano, Tara Bevandich, Traci Durrell-Khalife, and Jane Van Order for providing invaluable administrative support. The folks at COSINe IT computing services and Aaron Moffett at ROOTs IT support are my personal heroes.

My doctorate has been successful, in part, because of my collaborators and mentors. Larissa Bailey's patience, Kelly Smalling's candidness, Deb Iwanowicz's positivity, Kay Briggs' enthusiasm, Mari Reeves' ingenuousness, Meg Perdue's sincerity, Mike Adams' steadiness, and Jason Irwin's openness made for really fun, creative, and rigorous research that spanned six states and over six thousand miles. Katie Hinde, Steve Wagner, and Tiffany Garcia were my rocks during the most challenging transition in my PhD

career. I would not have finished without their support, advice and guidance. Ezel Hoed, Wo-neng Shuo, Zang Hua-ma, and Katherine Debil forced me to find strength and know the truth. I am eternally grateful to those who provided financial support for my research: U.S. Geological Survey Amphibian Research and Monitoring Program, U.S. Forest Service Pacific Northwest Research Station, OSU Provost Diversity Advancement Pipeline, Oregon Lottery Graduate Scholarship, OSU Environmental Sciences Graduate Program, and OSU Biology Teaching Assistantship.

The Blaustien lab members were a constant source of knowledge, support, constructive criticism, and provided a safe and candid place to discuss new ideas. Thank you Jennymaria Urbina, Paul Bradley, Steph Gervasi, Julia Buck, Lindsey Biga, Trang Dang, Giselle Xie, Carmen Harjoe, Nathalie Hambleck, Paul Snyder, and Delia Negru! Thanks to Sara Henderson, Eileen Fitzpatrick, Elmer Chestnut, Abe Boudouris, Jenny Urbina, Catherine Lu, Bradi Voight, and Breanne Myer for volunteering many hours of their time to help with lab experiments. Thank you also to Hannah Anderson, Mari Reeves, Meg Perdue, Sara Henderson, John Box, Julia Buck, Trang Dang, Jenny Urbina, Emily Nebergall, Abe Boudouris, Elmer Chestnut, Gillian Chestnut, Justin Connor, Bradi Voight, and Breanne Myer for assistance in the field. I am also grateful to MB, JB, AEC, JDO, and HS for much needed logistical support.

Finally, thank you to my family. There is no greater team of cheerleaders than the Chestnuts, and they will always have your back.

CONTRIBUTION OF AUTHORS

Dr. Andrew R. Blaustein and Dr. Deanna H. Olson served as my graduate co-advisors in the Environmental Sciences Graduate Program and contributed to all aspects of this research. Chauncey Anderson contributed to the design, execution and writing of Chapters 1-4. Dr. Radu Popa provided technical expertise and training in maintaining cultures and contributed to the design, execution and writing of Chapters 1, 2, and 4. Dr. Mary Voytek and Julie Kirshtein provided technical expertise, and training to perform DNA extractions and qPCR assays. Dr. Larissa Bailey provided the necessary quantitative and programming skills that contributed to the occupancy analysis in Chapter 2. Dr. Jason Irwin provided technical expertise and laboratory to conduct experiments in Chapter 2. Kelly Smalling provided technical expertise and chemical compounds for, and contributed to the design of experiments in Chapter 4. Paul Bradley contributed to Chapter 4 by collecting and rearing animals used in the experiment. Dr. Deborah Iwanowicz performed qPCR assays of some samples in Chapters 3 and 4.

TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

TABLE OF CONTENTS (CONTINUED)

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

LIST OF TABLES

DEDICATION

To those who face implicit and explicit obstacles and barriers to achieving goals, and the allies who scaled up the walls and have not pull the ladder up behind them.

CHAPTER 1 – INTRODUCTION: THE ECOLOGY OF THE PATHOGENIC FUNGUS *BATRACHOCHYTRIUM DENDROBATIDIS* IN WATERS OF NORTH AMERICA

Chauncey Anderson, Radu Popa, Andrew R. Blaustein, Mary Voytek, Deanna H. Olson, Julie Kirshtein

Adapted from: Chestnut et al. 2014. Heterogeneous occupancy and density estimates of the pathogenic fungus *Batrachochytrium dendrobatidis* in waters of North America. PLoS ONE 9:e106790.

Loss of biodiversity in terrestrial, freshwater, and marine systems is accelerating at a global scale (Vaughn 2010, Pereira et al. 2010, Jones et al. 2011, Mora and Sale 2011, Hooper et al. 2012). The causes of biodiversity losses are often complex and include synergistic effects of natural and human-induced stressors, such as habitat loss and fragmentation (Krauss et al. 2010, Hoffmann et al. 2010), urbanization (Seto et al. 2012), invasive species (Pimentel et al. 2005, Hoffmann et al. 2010, Seto et al. 2012), contaminants (Relyea 2005, 2009, Clements and Rohr 2009), global climate change (Lawler et al. 2009, Dawson et al. 2011, Mantyka-pringle et al. 2012), and emerging infectious diseases (Daszak et al. 2000, Jones et al. 2008). Among the most threatened vertebrate taxa are those with freshwater habitat associations (Vaughn 2010, World Wildlife Federation 2014), which includes the >7,000 amphibian species known today (http://amphibiaweb.org/). In the last 35 years, the amphibian extinction rate has been estimated to exceed 105 times the baseline expected rate for all species (McCallum 2007), with 32.5 to 41% of amphibian species threatened with extinction at this time, worldwide (Stuart 2004, Hoffmann et al. 2010). In the USA, the number of occupied amphibian sites has been reported to be declining by 3.7% per year (Stuart 2004, Adams et al. 2013).

Among the many threats to amphibians, increasingly, the role of disease in population declines has been recognized in the last two decades. Numerous amphibian diseases have been identified, with mass mortality events attributed to water molds (*Saprolegnia* spp.) (Blaustein et al. 1994, Romansic et al. 2011, Ault et al. 2012),

Aeromonas bacterial infections (Bradford 1991), iridoviruses (Mao et al. 1999, Chinchar 2002, Jancovich et al. 2005), alveolate infections (Davis et al. 2007, Jones et al. 2012), and malformations caused by trematodes (Johnson et al. 2002, Blaustein and Johnson 2003). Chytridiomycosis, the emerging infectious disease caused by the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (Bd), is implicated as a causal agent in many recent global amphibian population declines and extinctions (Rohr et al. 2008, Vredenburg et al. 2010, Olson et al. 2013).

Of the over 3,000 fungal species described from aquatic habitats (Shearer et al. 2007), chytrid fungi are the earliest of extant fungi to diverge phylogenetically, and now have global distribution (Hibbett et al. 2007, Adl et al. 2012). Bd is one of over 1,200 chytrid species described from freshwater, marine, and terrestrial systems occurring across temperate, tropical, and tundra environments (James et al. 2006, Ibelings et al. 2007, Gleason et al. 2011). Although chytrids function primarily as plant saprobes and parasites (Shearer et al. 2007), some also parasitize animals (Longcore et al. 1999, Kagami et al. 2004). Bd is one of only two chytrids known to infect vertebrate hosts, with each of these *Batrachochytrium* species being pathogenic to a wide but different complement of amphibian taxa (Longcore et al. 1999, Martel et al. 2013). Bd is known to infect 508 of 1,055 (48%) of amphibian species that have been sampled to detect its occurrence (Olson and Ronnenberg 2014).

To understand the pathology and conservation implications of Bd, a better understanding of its distribution, ecology, and the life history of Bd in the wild is needed. The Bd life cycle includes forms that are free-living in the aquatic environment (Berger et al. 2005, Rosenblum et al. 2010). Bd has been detected by filtering water samples to capture free-living zoospores and zoosporangia, then performing a genetic analysis on the filtered particulates (Kirshtein et al. 2007, Walker et al. 2007). Bd has not been reliably detected from sediments (Kirshtein et al. 2007). Laboratory experiments have demonstrated that Bd has survived on sterilized moist sand for up to three months and remained infective in lake water for up to seven weeks (Johnson and Speare 2003). Bd cultures can be maintained under laboratory conditions for several years (personal observation; A.R. Blaustein, personal communication), which suggests that Bd can survive in the environment without a host as long as conditions are favorable. In laboratory settings, Bd growth and reproduction depended on temperature (4–25°C, ideal 17–23°C) and pH (4–10, ideal 6–7; Piotrowski et al. 2004), and differences in both generation time and fecundity of Bd in response to different thermal regimes were observed in at least one Bd strain (Voyles et al. 2012).

A greater understanding of Bd ecology is of paramount importance to fully understand its population occurrence, dynamics, and potential threat to amphibians (Olson et al. 2013). Although it has an impact on the persistence of selected amphibian populations around the world (Vredenburg et al. 2010, Cheng et al. 2011, Adams et al. 2013), the full scope of the effects of chytridiomycosis on global amphibian population

declines is not well understood. Most Bd research efforts have focused on Bd in amphibian hosts per se, with little attention to the environmental associations and dynamics of free-living Bd outside of the amphibian host (Woodhams et al. 2008, Mitchell et al. 2008, Schmidt 2010). In amphibian hosts, Bd exhibits sensitivity to a number of environmental variables including temperature (Berger et al. 2004, Drew et al. 2006, Rohr and Raffel 2010, Raffel et al. 2010, Olson et al. 2013) and elevation (Drew et al. 2006, Seimon et al. 2007, Adams et al. 2010), suggesting a tendency to associate high-elevation areas or regions with cool temperatures with increased risk for Bd-related declines and extinctions (Fisher et al. 2009). From a large global data set of amphibians sampled for Bd infections, it was found that Bd occurrences were inversely correlated with the temperature range at sites (Olson et al. 2013). Amphibians infected with Bd exhibited seasonal patterns in the disease detection and density (Berger et al. 2004, Retallick et al. 2004, Pearl et al. 2007, Kriger and Hero 2007, Kinney et al. 2011), further implicating microclimatic associations and potential differences between strains (Gervasi et al. 2013). In wild amphibian populations from temperate areas, Bd infections appear to follow predictable patterns, with the highest prevalence and intensity in the cooler spring months, and decreasing prevalence in the warmer summer and autumn months, sometimes to non-detectable limits (Pearl et al. 2007, Kinney et al. 2011). Analogous studies are warranted to understand the basic ecology of free-living Bd. In particular, information on Bd responses to climatic variation outside of hosts is a research gap. Furthermore, as a microorganism within an aquatic environment, studies are lacking of potential water quality associations and how Bd may interact with other members of their

biological communities. For example, zooplankton commonly consume chytrids (Rasconi et al. 2014) and Buck et al. (2011) found that zooplankton are Bd predators. Very few other interactions of Bd with aquatic biota have been described.

Furthermore, to understand host-pathogen dynamics, it is imperative to investigate both the host and pathogen response to changes in environmental conditions. In particular, chemical contaminants can adversely affect organisms that occur within aquatic environments, and alter ecological processes within wetland ecosystems (Sparling et al. 2001, Relyea 2005, 2009, Relyea and Hoverman 2008, Bradford et al. 2011, Biga and Blaustein 2013). Relatively few studies have evaluated the effects of chemical contaminants such as fungicides to aquatic organisms and their disease dynamics. Fungicidal interactions with Bd are highly relevant in attempts to explain patterns of Bd occurrence in amphibians and their breeding habitats, and detection probability across the landscape, especially in light of fungicide detection in protected areas, great distances from the point of application.

Utilizing field sampling, laboratory experiments, and quantitative modeling, I sought to advance the understanding of the ecology of the amphibian chytrid fungus in the aquatic environment. In this work, I examined Bd distribution in parts of North America, Bd detection in amphibians and their breeding habitats as related to environmental conditions, and Bd response to stressors. Herein, I describe spatial and temporal patterns in occupancy and density of free-living Bd in aquatic habitats in two

different geographic regions of the United States, Alaska (Chapter 2) and Oregon (Chapter 3). The Alaska work is especially important because it examines Bd ecology at the northernmost extent of amphibian occurrence in North America, where climate associations may be particularly relevant and where Bd occurrence may be representative of one of the most novel pathogen-host systems in the world. I also describe (Chapter 2) experimental results of Bd and amphibian response to extreme cold temperatures they may experience in continental settings, at high elevations, and at high latitudes. My Oregon studies (Chapter 3) focus on multivariate associations of free-living Bd occurrences with a suite of aquatic environmental factors, both abiotic and biotic in nature. In Chapter 4 I describe how amphibian and Bd respond to agricultural chemicals (fungicides) that they may be exposed to in field settings. These results are specific to Bd, but might warrant consideration as fungicidal treatments for the newly described chytrid affecting salamanders (Martel et al. 2014); both of these amphibian chytrids have been detected in captive animals and solutions to treat trade animals for the pathogen are gaining relevancy. Finally, in Chapter 5, I reflect upon the journey of conservation biologists and herpetologists for 25 years of amphibian decline research, with global losses becoming widely recognized in 1989 (Blaustein and Wake 1990). In this context, my research has advanced understanding of the geographic distribution and ecology of one potential threat factor to amphibian populations on Earth, *Batrachochytrium dendrobatidis* and informs host-pathogen dynamics research.

References

Adams, M. J., N. D. Chelgren, D. Reinitz, R. A. Cole, L. J. Rachowicz, S. Galvan, B. McCreary, C. A. Pearl, L. L. Bailey, and J. Bettaso. 2010. Using occupancy models to understand the distribution of an amphibian pathogen, *Batrachochytrium dendrobatidis*. Ecological Applications 20:289–302.

Adams, M. J., D. A. W. Miller, E. Muths, P. S. Corn, E. H. C. Grant, L. L. Bailey, G. M. Fellers, R. N. Fisher, W. J. Sadinski, H. Waddle, and S. C. Walls. 2013. Trends in amphibian occupancy in the United States. PLoS ONE 8:e64347.

Adl, S. M., A. G. B. Simpson, C. E. Lane, J. Lukes, D. Bass, S. S. Bowser, M. W. Brown, F. Burki, M. Dunthorn, V. Hampl, A. Heiss, M. Hoppenrath, E. Lara, L. le Gall, D. H. Lynn, H. McManus, E. A. D. Mitchell, S. E. Mozley-Stanridge, L. W. Parfrey, J. Pawlowski, S. Rueckert, L. Shadwick, C. L. Schoch, A. Smirnov, and F. W. Spiegel. 2012. The revised classification of eukaryotes. Journal of Eukaryotic Microbiology 59:429–493.

Ault, K. K., J. E. Johnson, H. C. Pinkart, and R. S. Wagner. 2012. Genetic comparison of water molds from embryos of amphibians *Rana cascadae*, *Bufo boreas* and *Pseudacris regilla*. Diseases of Aquatic Organisms 99:127–137.

Berger, L., A. D. Hyatt, R. Speare, and J. E. Longcore. 2005. Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms 68:51–63.

Berger, L., R. Speare, H. Hines, G. Marantelli, A. Hyatt, K. McDonald, L. Skerratt, V. Olsen, J. Clarke, G. Gillespie, M. Mahony, N. Sheppard, C. Williams, and M. Tyler. 2004. Effect of season and temperature on mortality in amphibians due to chytridiomycosis. Australian Veterinary Journal 82:434–439.

Biga, L. M., and A. R. Blaustein. 2013. Variations in lethal and sublethal effects of cypermethrin among aquatic stages and species of anuran amphibians. Environmental Toxicology and Chemistry 32:2855–2860.

Blaustein, A. R., D. Grant Hokit, R. K. O'Hara, and R. A. Holt. 1994. Pathogenic fungus contributes to amphibian losses in the Pacific Northwest. Biological Conservation 67:251–254.

Blaustein, A. R., and P. T. Johnson. 2003. The complexity of deformed amphibians. Frontiers in Ecology and the Environment 1:87–94.

Blaustein, A. R., and D. B. Wake. 1990. Declining amphibian populations: a global phenomenon? Trends in Ecology & Evolution 5:203–204.

Bradford, D. F. 1991. Mass mortality and extinction in a high-elevation population of *Rana muscosa*. Journal of Herpetology 25:174–177.

Bradford, D. F., R. A. Knapp, D. W. Sparling, M. S. Nash, K. A. Stanley, N. G. Tallent-Halsell, L. L. McConnell, and S. M. Simonich. 2011. Pesticide distributions and population declines of California, USA, alpine frogs, *Rana muscosa* and *Rana sierrae*. Environmental Toxicology and Chemistry 30:682–691.

Buck, J. C., L. Truong, and A. R. Blaustein. 2011. Predation by zooplankton on *Batrachochytrium dendrobatidis*: biological control of the deadly amphibian chytrid fungus? Biodiversity and Conservation 20:3549–3553.

Cheng, T. L., S. M. Rovito, D. B. Wake, and V. T. Vredenburg. 2011. Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. Proceedings of the National Academy of Sciences 108:9502–9507.

Chinchar, V. G. 2002. Ranaviruses (family Iridoviridae): emerging cold-blooded killers. Archives of Virology 147:447–470.

Clements, W. H., and J. R. Rohr. 2009. Community responses to contaminants: using basic ecological principles to predict ecotoxicological effects. Environmental Toxicology and Chemistry 28:1789–1800.

Daszak, P., A. A. Cunningham, and A. D. Hyatt. 2000. Emerging infectious diseases of wildlife–threats to biodiversity and human health. Science 287:443–449.

Davis, A. K., M. J. Yabsley, M. K. Keel, and J. C. Maerz. 2007. Discovery of a novel alveolate pathogen affecting Southern Leopard Frogs in Georgia: description of the disease and host effects. EcoHealth 4:310–317.

Dawson, T. P., S. T. Jackson, J. I. House, I. C. Prentice, and G. M. Mace. 2011. Beyond predictions: biodiversity conservation in a changing climate. Science 332:53–58.

Drew, A., E. J. Allen, and L. J. S. Allen. 2006. Analysis of climatic and geographic factors affecting the presence of chytridiomycosis in Australia. Diseases of Aquatic Organisms 68:245.

Fisher, M. C., T. W. Garner, and S. F. Walker. 2009. Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. Annual Review of Microbiology 63:291–310.

Gervasi, S. S., J. Urbina, J. Hua, T. Chestnut, R. A. Relyea, and A. R. Blaustein. 2013. Experimental evidence for American Bullfrog (*Lithobates catesbeianus*)

susceptibility to chytrid fungus (*Batrachochytrium dendrobatidis*). EcoHealth:10:166-171.

Gleason, F. H., F. C. Küpper, J. P. Amon, K. Picard, C. M. Gachon, A. V. Marano, T. Sime-Ngando, and O. Lilje. 2011. Zoosporic true fungi in marine ecosystems: a review. Marine and Freshwater Research 62:383–393.

Hibbett, D. S., M. Binder, J. F. Bischoff, M. Blackwell, P. F. Cannon, O. E. Eriksson, S. Huhndorf, T. James, P. M. Kirk, R. Lücking, H. Thorsten Lumbsch, F. Lutzoni, P. B. Matheny, D. J. McLaughlin, M. J. Powell, S. Redhead, C. L. Schoch, J. W. Spatafora, J. A. Stalpers, R. Vilgalys, M. C. Aime, A. Aptroot, R. Bauer, D. Begerow, G. L. Benny, L. A. Castlebury, P. W. Crous, Y. C. Dai, W. Gams, D. M. Geiser, G. W. Griffith, C. Gueidan, D. L. Hawksworth, G. Hestmark, K. Hosaka, R. A. Humber, K. D. Hyde, J. E. Ironside, U. Kõljalg, C. P. Kurtzman, K.-H. Larsson, R. Lichtwardt, J. Longcore, J. Miadlikowska, A. Miller, J.-M. Moncalvo, S. Mozley-Standridge, F. Oberwinkler, E. Parmasto, V. Reeb, J. D. Rogers, C. Roux, L. Ryvarden, J. P. Sampaio, A. Schüssler, J. Sugiyama, R. G. Thorn, L. Tibell, W. A. Untereiner, C. Walker, Z. Wang, A. Weir, M. Weiss, M. M. White, K. Winka, Y.-J. Yao, and N. Zhang. 2007. A higher-level phylogenetic classification of the Fungi. Mycological Research 111:509–547.

Hoffmann, M., C. Hilton-Taylor, A. Angulo, M. Böhm, T. M. Brooks, S. H. M. Butchart, K. E. Carpenter, J. Chanson, B. Collen, N. A. Cox, W. R. T. Darwall, N. K. Dulvy, L. R. Harrison, V. Katariya, C. M. Pollock, S. Quader, N. I. Richman, A. S. L. Rodrigues, M. F. Tognelli, J.-C. Vié, J. M. Aguiar, D. J. Allen, G. R. Allen, G. Amori, N. B. Ananjeva, F. Andreone, P. Andrew, A. L. A. Ortiz, J. E. M. Baillie, R. Baldi, B. D. Bell, S. D. Biju, J. P. Bird, P. Black-Decima, J. J. Blanc, F. Bolaños, W. Bolivar-G, I. J. Burfield, J. A. Burton, D. R. Capper, F. Castro, G. Catullo, R. D. Cavanagh, A. Channing, N. L. Chao, A. M. Chenery, F. Chiozza, V. Clausnitzer, N. J. Collar, L. C. Collett, B. B. Collette, C. F. C. Fernandez, M. T. Craig, M. J. Crosby, N. Cumberlidge, A. Cuttelod, A. E. Derocher, A. C. Diesmos, J. S. Donaldson, J. W. Duckworth, G. Dutson, S. K. Dutta, R. H. Emslie, A. Farjon, S. Fowler, J. Freyhof, D. L. Garshelis, J. Gerlach, D. J. Gower, T. D. Grant, G. A. Hammerson, R. B. Harris, L. R. Heaney, S. B. Hedges, J.-M. Hero, B. Hughes, S. A. Hussain, J. I. M, R. F. Inger, N. Ishii, D. T. Iskandar, R. K. B. Jenkins, Y. Kaneko, M. Kottelat, K. M. Kovacs, S. L. Kuzmin, E. L. Marca, J. F. Lamoreux, M. W. N. Lau, E. O. Lavilla, K. Leus, R. L. Lewison, G. Lichtenstein, S. R. Livingstone, V. Lukoschek, D. P. Mallon, P. J. K. McGowan, A. McIvor, P. D. Moehlman, S. Molur, A. M. Alonso, J. A. Musick, K. Nowell, R. A. Nussbaum, W. Olech, N. L. Orlov, T. J. Papenfuss, G. Parra-Olea, W. F. Perrin, B. A. Polidoro, M. Pourkazemi, P. A. Racey, J. S. Ragle, M. Ram, G. Rathbun, R. P. Reynolds, A. G. J. Rhodin, S. J. Richards, L. O. Rodríguez, S. R. Ron, C. Rondinini, A. B. Rylands, Y. S. de Mitcheson, J. C. Sanciangco, K. L. Sanders, G. Santos-Barrera, J. Schipper, C. Self-Sullivan, Y. Shi, A. Shoemaker,

F. T. Short, C. Sillero-Zubiri, D. L. Silvano, K. G. Smith, A. T. Smith, J. Snoeks, A. J. Stattersfield, A. J. Symes, A. B. Taber, B. K. Talukdar, H. J. Temple, R. Timmins, J. A. Tobias, K. Tsytsulina, D. Tweddle, C. Ubeda, S. V. Valenti, P. P. van Dijk, L. M. Veiga, A. Veloso, D. C. Wege, M. Wilkinson, E. A. Williamson, F. Xie, B. E. Young, H. R. Akçakaya, L. Bennun, T. M. Blackburn, L. Boitani, H. T. Dublin, G. A. B. da Fonseca, C. Gascon, T. E. Lacher, G. M. Mace, S. A. Mainka, J. A. McNeely, R. A. Mittermeier, G. M. Reid, J. P. Rodriguez, A. A. Rosenberg, M. J. Samways, J. Smart, B. A. Stein, and S. N. Stuart. 2010. The impact of conservation on the status of the world's vertebrates. Science 330:1503–1509.

Hooper, D. U., E. C. Adair, B. J. Cardinale, J. E. Byrnes, B. A. Hungate, K. L. Matulich, A. Gonzalez, J. E. Duffy, L. Gamfeldt, and M. I. O'Connor. 2012. A global synthesis reveals biodiversity loss as a major driver of ecosystem change. Nature 486:105–108.

Ibelings, B., A. De Bruin, M. Kagami, and E. Van Donk. 2007. Diatom blooms, chytrid epidemics and the evolutionary. Journal of Phycology 43:2–2.

James, T. Y., F. Kauff, C. L. Schoch, P. B. Matheny, V. Hofstetter, C. J. Cox, G. Celio, C. Gueidan, E. Fraker, J. Miadlikowska, H. T. Lumbsch, A. Rauhut, V. Reeb, A. E. Arnold, A. Amtoft, J. E. Stajich, K. Hosaka, G.-H. Sung, D. Johnson, B. O/'Rourke, M. Crockett, M. Binder, J. M. Curtis, J. C. Slot, Z. Wang, A. W. Wilson, A. Schuszler, J. E. Longcore, K. O'Donnell, S. Mozley-Standridge, D. Porter, P. M. Letcher, M. J. Powell, J. W. Taylor, M. M. White, G. W. Griffith, D. R. Davies, R. A. Humber, J. B. Morton, J. Sugiyama, A. Y. Rossman, J. D. Rogers, D. H. Pfister, D. Hewitt, K. Hansen, S. Hambleton, R. A. Shoemaker, J. Kohlmeyer, B. Volkmann-Kohlmeyer, R. A. Spotts, M. Serdani, P. W. Crous, K. W. Hughes, K. Matsuura, E. Langer, G. Langer, W. A. Untereiner, R. Lucking, B. Budel, D. M. Geiser, A. Aptroot, P. Diederich, I. Schmitt, M. Schultz, R. Yahr, D. S. Hibbett, F. Lutzoni, D. J. McLaughlin, J. W. Spatafora, and R. Vilgalys. 2006. Reconstructing the early evolution of Fungi using a six-gene phylogeny. Nature 443:818–822.

Jancovich, J. K., E. W. Davidson, N. Parameswaran, J. Mao, V. G. Chinchar, J. P. Collins, B. L. Jacobs, and A. Storfer. 2005. Evidence for emergence of an amphibian iridoviral disease because of human-enhanced spread. Molecular Ecology 14:213–224.

Johnson, M. L., and R. Speare. 2003. Survival of *Batrachochytrium dendrobatidis* in water: quarantine and disease control implications. Emerging Infectious Diseases 9:922.

Johnson, P. T., K. B. Lunde, E. M. Thurman, E. G. Ritchie, S. N. Wray, D. R. Sutherland, J. M. Kapfer, T. J. Frest, J. Bowerman, and A. R. Blaustein. 2002. Parasite

(*Ribeiroia ondatrae*) infection linked to amphibian malformations in the western United States. Ecological Monographs 72:151–168.

Jones, J. P., B. E. N. Collen, G. Atkinson, P. W. Baxter, P. Bubb, J. B. Illian, T. E. Katzner, A. Keane, J. Loh, and E. McDonald-Madden. 2011. The why, what, and how of global biodiversity indicators beyond the 2010 target. Conservation Biology 25:450–457.

Jones, K. E., N. G. Patel, M. A. Levy, A. Storeygard, D. Balk, J. L. Gittleman, and P. Daszak. 2008. Global trends in emerging infectious diseases. Nature 451:990–993.

Jones, M. E. B., A. G. Armin, B. B. Rothermel, and A. P. Pessier. 2012. Granulomatous myositis associated with a novel alveolate pathogen in an adult southern leopard frog (*Lithobates sphenocephalus*). Diseases of Aquatic Organisms 102:163–167.

Kagami, M., E. Van Donk, A. de Bruin, M. Rijkeboer, and B. W. Ibelings. 2004. Daphnia can protect diatoms from fungal parasitism. Limnology and Oceanography 49:680–685.

Kinney, V. C., J. L. Heemeyer, A. P. Pessier, and M. J. Lannoo. 2011. Seasonal pattern of *Batrachochytrium dendrobatidis* infection and mortality in *Lithobates areolatus*: Affirmation of Vredenburg's "10,000 Zoospore Rule." PLoS One 6:e16708.

Kirshtein, J. D., C. W. Anderson, J. S. Wood, J. E. Longcore, and M. A. Voytek. 2007. Quantitative PCR detection of *Batrachochytrium dendrobatidis* DNA from sediments and water. Diseases of Aquatic Organisms 77:11-15.

Krauss, J., R. Bommarco, M. Guardiola, R. K. Heikkinen, A. Helm, M. Kuussaari, R. Lindborg, E. Öckinger, M. Pärtel, J. Pino, J. Pöyry, K. M. Raatikainen, A. Sang, C. Stefanescu, T. Teder, M. Zobel, and I. Steffan-Dewenter. 2010. Habitat fragmentation causes immediate and time-delayed biodiversity loss at different trophic levels. Ecology Letters 13:597–605.

Kriger, K. M., and J.-M. Hero. 2007. Large-scale seasonal variation in the prevalence and severity of chytridiomycosis. Journal of Zoology 271:352–359.

Lawler, J. J., S. L. Shafer, D. White, P. Kareiva, E. P. Maurer, A. R. Blaustein, and P. J. Bartlein. 2009. Projected climate-induced faunal change in the Western Hemisphere. Ecology 90:588–597.

Longcore, J. E., A. P. Pessier, and D. K. Nichols. 1999. *Batrachochytrium dendrobatidis gen et sp nov*, a chytrid pathogenic to amphibians. Mycologia 91:219–227.

Mantyka-pringle, C. S., T. G. Martin, and J. R. Rhodes. 2012. Interactions between climate and habitat loss effects on biodiversity: a systematic review and meta-analysis. Global Change Biology 18:1239–1252.

Mao, J., D. . Green, G. Fellers, and V. . Chinchar. 1999. Molecular characterization of iridoviruses isolated from sympatric amphibians and fish. Virus Research 63:45–52.

Martel, A., M. Blooi, C. Adriaensen, P. V. Rooij, W. Beukema, M. C. Fisher, R. A. Farrer, B. R. Schmidt, U. Tobler, K. Goka, K. R. Lips, C. Muletz, K. R. Zamudio, J. Bosch, S. Lötters, E. Wombwell, T. W. J. Garner, A. A. Cunningham, A. S. der Sluijs, S. Salvidio, R. Ducatelle, K. Nishikawa, T. T. Nguyen, J. E. Kolby, I. V. Bocxlaer, F. Bossuyt, and F. Pasmans. 2014. Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. Science 346:630–631.

Martel, A., A. S. der Sluijs, M. Blooi, W. Bert, R. Ducatelle, M. C. Fisher, A. Woeltjes, W. Bosman, K. Chiers, F. Bossuyt, and F. Pasmans. 2013. *Batrachochytrium salamandrivorans sp. nov*. causes lethal chytridiomycosis in amphibians. Proceedings of the National Academy of Sciences 110:15325-15329.

McCallum, M. L. 2007. Amphibian decline or extinction? Current declines dwarf background extinction rate. Journal of Herpetology 41:483–491.

Mitchell, K. M., T. S. Churcher, T. W. . Garner, and M. C. Fisher. 2008. Persistence of the emerging pathogen *Batrachochytrium dendrobatidis* outside the amphibian host greatly increases the probability of host extinction. Proceedings of the Royal Society B: Biological Sciences 275:329–334.

Mora, C., and P. F. Sale. 2011. Ongoing global biodiversity loss and the need to move beyond protected areas: a review of the technical and practical shortcomings of protected areas on land and sea. Marine Ecology Progress Series 434:251–266.

Olson, D. H., D. M. Aanensen, K. L. Ronnenberg, C. I. Powell, S. F. Walker, J. Bielby, T. W. Garner, G. Weaver, and M. C. Fisher. 2013. Mapping the global emergence of *Batrachochytrium dendrobatidis*, the amphibian chytrid fungus. PloS One 8:e56802.

Olson, D. H., and K. L. Ronnenberg. 2014. Global Bd Mapping Project: 2014 Update. Froglog 111:17–21.

Pearl, C. A., E. L. Bull, D. E. Green, J. Bowerman, M. J. Adams, A. Hyatt, and W. H. Wente. 2007. Occurrence of the amphibian pathogen *Batrachochytrium dendrobatidis* in the Pacific Northwest. Journal of Herpetology 41:145–149.

Pereira, H. M., P. W. Leadley, V. Proença, R. Alkemade, J. P. W. Scharlemann, J. F. Fernandez-Manjarrés, M. B. Araújo, P. Balvanera, R. Biggs, W. W. L. Cheung, L. Chini, H. D. Cooper, E. L. Gilman, S. Guénette, G. C. Hurtt, H. P. Huntington, G. M. Mace, T. Oberdorff, C. Revenga, P. Rodrigues, R. J. Scholes, U. R. Sumaila, and M. Walpole. 2010. Scenarios for global biodiversity in the 21st Century. Science 330:1496–1501.

Pimentel, D., R. Zuniga, and D. Morrison. 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecological Economics 52:273–288.

Piotrowski, J. S., S. L. Annis, and J. E. Longcore. 2004. Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. Mycologia 96:9–15.

Raffel, T. R., P. J. Michel, E. W. Sites, and J. R. Rohr. 2010. What drives chytrid infections in newt populations? Associations with substrate, temperature, and shade. EcoHealth 7:526–536.

Rasconi, S., B. Grami, N. Niquil, M. Jobard, and T. Sime-Ngando. 2014. Parasitic chytrids sustain zooplankton growth during inedible algal bloom. Aquatic Microbiology 5:229.

Relyea, R. A. 2005. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. Ecological Applications 15:618–627.

Relyea, R. A. 2009. A cocktail of contaminants: how mixtures of pesticides at low concentrations affect aquatic communities. Oecologia 159:363–376.

Relyea, R. A., and J. T. Hoverman. 2008. Interactive effects of predators and a pesticide on aquatic communities. Oikos 117:1647–1658.

Retallick, R. W., H. McCallum, and R. Speare. 2004. Endemic infection of the amphibian chytrid fungus in a frog community post-decline. PLoS Biology 2:e351.

Rohr, J. R., and T. R. Raffel. 2010. Linking global climate and temperature variability to widespread amphibian declines putatively caused by disease. Proceedings of the National Academy of Sciences 107:8269–8274.

Rohr, J. R., T. R. Raffel, J. M. Romansic, H. McCallum, and P. J. Hudson. 2008. Evaluating the links between climate, disease spread, and amphibian declines. Proceedings of the National Academy of Sciences 105:17436–17441.

Romansic, J. M., P. T. Johnson, C. L. Searle, J. E. Johnson, T. S. Tunstall, B. A. Han, J. R. Rohr, and A. R. Blaustein. 2011. Individual and combined effects of multiple pathogens on Pacific treefrogs. Oecologia 166:1029–1041.

Rosenblum, E. B., J. Voyles, T. J. Poorten, and J. E. Stajich. 2010. The deadly chytrid fungus: A story of an emerging pathogen. PLoS Pathog 6:e1000550.

Schmidt, B. R. 2010. Estimating the impact of disease in species threatened by amphibian chytrid fungus: Comment on Murray et al. Conservation Biology 24:897–899.

Seimon, T. A., A. Seimon, P. Daszak, S. R. p. Halloy, L. M. Schloegel, C. A. Aguilar, P. Sowell, A. D. Hyatt, B. Konecky, and J. E Simmons. 2007. Upward range extension of Andean anurans and chytridiomycosis to extreme elevations in response to tropical deglaciation. Global Change Biology 13:288–299.

Seto, K. C., B. Güneralp, and L. R. Hutyra. 2012. Global forecasts of urban expansion to 2030 and direct impacts on biodiversity and carbon pools. Proceedings of the National Academy of Sciences 109:16083–16088.

Shearer, C., E. Descals, B. Kohlmeyer, J. Kohlmeyer, L. Marvanová, D. Padgett, D. Porter, H. Raja, J. Schmit, H. Thorton, and H. Voglymayr. 2007. Fungal biodiversity in aquatic habitats. Biodiversity and Conservation 16:49–67.

Sparling, D. W., G. M. Fellers, and L. L. McConnell. 2001. Pesticides and amphibian population declines in California, USA. Environmental Toxicology and Chemistry 20:1591–1595.

Stuart, S. N. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306:1783–1786.

Vaughn, C. C. 2010. Biodiversity losses and ecosystem function in freshwaters: emerging conclusions and research directions. BioScience 60:25–35.

Voyles, J., L. R. Johnson, C. J. Briggs, S. D. Cashins, R. A. Alford, L. Berger, L. F. Skerratt, R. Speare, and E. B. Rosenblum. 2012. Temperature alters reproductive life history patterns in *Batrachochytrium dendrobatidis*, a lethal pathogen associated with the global loss of amphibians. Ecology and Evolution 2:2241–2249.

Vredenburg, V. T., R. A. Knapp, T. S. Tunstall, and C. J. Briggs. 2010. Dynamics of an emerging disease drive large-scale amphibian population extinctions. Proceedings of the National Academy of Sciences 107:9689–9694.

Walker, S. F., M. Baldi Salas, D. Jenkins, T. W. Garner, A. A. Cunningham, A. D. Hyatt, J. Bosch, and M. C. Fisher. 2007. Environmental detection of *Batrachochytrium dendrobatidis* in a temperate climate. Diseases of Aquatic Organisms 77:105-112.

Woodhams, D. C., R. A. Alford, C. J. Briggs, M. Johnson, and L. A. Rollins-Smith. 2008. Life-history trade-offs influence disease in changing climates: strategies of an amphibian pathogen. Ecology 89:1627–1639.

World Wildlife Federation. 2014. Living Planet Report 2014. http://wwf.panda.org/about_our_earth/all_publications/living_planet_report/.

CHAPTER 2 – HOST-PATHOGEN DISEASE DYNAMICS AT HIGH LATITUDES: *BATRACHOCHYTRIUM DENDROBATIDIS* DISTRIBUTION AND OCCUPANCY IN THE ALASKA, USA BOREAL ENVIRONMENT AND ITS RESILIENCE IN HARSH CLIMATES

Deanna H. Olson, Larissa L. Bailey, Radu Popa, Chauncey W. Anderson, Jason Irwin, Andrew R. Blaustein

Abstract

Global incidence of emerging infectious diseases has increased at exceptional rates in recent years. As a result of novel diseases, populations may experience temporary declines, and in rare cases, extinction. Climate change is occurring at high latitudes at rates much faster than mid-latitude and equatorial areas, which could result in more easily observable shifts in host-pathogen community structure and dynamics. As such, high-latitude locations and species present an ideal model system for investigations of disease ecology. In this study, I investigated pathogen occupancy of hosts and their breeding habitats at high-latitudes, and pathogen response to freezing temperatures in an amphibian-fungal system. I detected the fungal pathogen *Batrachochytrium dendrobatidis* (Bd) in water collected from wood frog (*Lithobates sylvaticus*) breeding habitats throughout the Alaska, USA study area, however, there were no occasions when I detected Bd in both water and amphibians from the same site. Disease prevalence ranged from 0-4% in wild caught wood frogs. Bd occupancy of wood frog breeding habitats was highest in interior Alaska and increased as the length of the growing season decreased. I detected Bd in water from 32% (13 of 41) wood frog breeding habitats I sampled and estimated 41% of sites were occupied by Bd when accounting for imperfect detection. When Bd was present at a site, there was a 95% chance of detecting it with seven 60-ml samples of water, five 180-ml samples, three 360-ml samples, or two 600-ml samples. I observed a negative response of Bd to freezing when in culture but not in frogs infected with the pathogen. When exposed to mild freeze (-0.9 degrees C) Bd density in culture was 12.5 times lower than cultures that were not frozen, which may suggest it is

susceptible to osmotic and temperature shock when it is not protected from freezing. I did not observe a difference in Bd infection loads in experimentally infected wood frogs that were either frozen or held above freezing temperatures, suggesting that at high latitudes wood frogs my function as a cryoprotectant for Bd. Climate-induced changes to abiotic (e.g., increased temperature, altered hydrology) and biotic conditions or processes (e.g., arrival of new hosts and diseases expanding ranges) will likely alter the existing host-pathogen dynamics. Because these changes are occurring more rapidly at high latitudes, opportunities may exist to study disease theory as it occurs in real-time in novel systems.

Introduction

In the last 50 years, the global incidence of emerging infectious diseases has increased at unprecedented rates, yet current knowledge of pathogen distribution, diversity and abundance in the environment is largely lacking (Daszak et al. 2000, Jones et al. 2008, Smith et al. 2009b). Emerging infectious diseases can influence wildlife populations by causing temporary declines, and in some cases extinction (De Castro and Bolker 2005, Smith et al. 2009a, Vredenburg et al. 2010, Best et al. 2012, McCallum 2012). Understanding the distribution of pathogens in the environment, and the underlying causes of disease emergence is critical to biological conservation. High-latitude locations and species present an ideal field-model system for investigations of disease ecology. In the last 10-20 thousand years glacial retreat in North America revealed new habitats uninfluenced by pre-existing communities and the relatively low biodiversity of pioneer colonizers provide a relatively simple system for study (Calkin 1988, Calkin et al. 2001). The northern half of North America was glaciated during the late Wisconsinan until the late Pleistocene Epoch, approximately 10,000-18,000 years ago, and much of present day Alaska, USA has retained mountain glaciers (Rutter et al. 2012, Hughes et al. 2013). As glaciers retreated north and west, habitats were rapidly colonized by pioneer species expanding their ranges (Lee-Yaw et al. 2008, Breen et al. 2012, Zigouris et al. 2013). As a result, the species (both hosts and pathogens) that occur presently at high latitudes in North America are likely the earliest of the pioneer species and the first colonizers following glacial retreat (Hoberg 2005, Morin and Chuine 2006, Shafer et al. 2010, Breen et al. 2012). From an evolutionary perspective, compared to

other systems, relatively little time has passed for these colonizers to evolve and co-evolve in their new environments, and as such this is an ideal system to examine host-pathogen dynamics, especially in the context of emerging infectious disease wherein novel pathogens may be infecting hosts at the margins of their ranges. Novel host-pathogen dynamics along species range boundaries at high latitudes may offer insights to patterns observed elsewhere. Edges of species ranges may be marginal habitats for species, adding physiological and ecological stress to them in a variety of ways. Furthermore, there is evidence for climate change occurring at high latitudes at rates much faster than mid-latitude and equatorial areas (Hinzman et al. 2005, Tucker et al. 2011, Ezard et al. 2011), which could result in more easily observable shifts in host-pathogen community structure and dynamics (Lobitz et al. 2000). In this study, I investigated the ecology of an amphibian-fungal pathogen system at high latitudes using both observational and experimental approaches.

Some species, such as the wood frog (*Lithobates sylvaticus*) rapidly colonized northern latitudes following glacial retreat, originating from the northern plains near present day Wisconsin, Michigan and Ohio USA, and Ontario, Canada (Lee-Yaw et al. 2008). This frog may be one of the earliest vertebrates to colonize Alaska, and is the only amphibian species that occurs in interior Alaska (Lee-Yaw et al. 2008). Wood frog survival in these extreme environments is due to adaptations which allow them to freeze in winter; in interior Alaska, they can survive temperatures as low as -16 C (Costanzo et al. 2013, 2014, Larson et al. 2014).

Amphibian populations in North America and Europe reportedly harbor the fungal pathogen, *Batrachochytrium dendrobatidis* (Bd) to approximately 60 degrees North in latitude (Reeves and Green 2006, Reeves 2008, Scalera et al. 2008, Slough 2009, Schock et al. 2010). Bd can cause the emerging infectious disease chytridiomycosis, which is implicated as a contributing factor in amphibian declines worldwide (Longcore et al. 1999, Rosenblum et al. 2010). Recent efforts have mapped detections and produced landscape-scale models of the global distribution of Bd in amphibians (Olson et al. 2013). Globally, Bd detection in amphibians decreased as the air temperature range increased (Olson et al. 2013), and models predicted a relatively low probability of occurrence at high latitudes. However, the global Bd data available for these models were limited with respect to high latitude Bd occurrences, as sampling efforts were biased towards mid-latitudes. Nevertheless, consistent with the modeled projection, the mean annual air temperature range difference in interior Alaska between summer and winter is one of the most marked on the planet, more than 40 C (Slaughter and Viereck 1986). As such, Bd occurrence at high latitudes may be explained by one of two mechanisms; resistance to environmental conditions (i.e., constant growth and maintenance) or resilience to environmental conditions (i.e., periods of decline and regrowth).

As a taxon, chytrid fungi are common soil microbes that have a variety of ecophysiological patterns with temperature, for example, they demonstrate tolerance to

extreme temperatures and conditions in some settings (Gleason et al. 2004, 2008, 2010). However, their physiology can leave them susceptible to osmotic and temperature shock associated with freezing durations and cycles typical at high latitudes (Hibbett et al. 2007). Bd exhibits life history variation with temperature; at higher temperatures (17-25 C) zoospores encyst and develop into zoosporangium (the reproductive life stage) faster, but at lower temperatures (4-10 C) zoosporangium produce more zoospores and they encyst at slower rates and remain infective for longer periods of time (Woodhams et al. 2008, Voyles et al. 2012). Bd life history at temperatures lower than 4 C are yet unknown. Alaskan winters are characterized by prolonged extreme cold temperatures, with milder temperatures in coastal regions and the coldest temperatures in the interior (Slaughter and Vierecl 1986, Walsh et al. 2008). In interior Alaska, surface soil temperatures may be below freezing for up to 8 months of the year and soils may experience 30 or more freeze/thaw cycles during a season (Slaughter and Viereck 1986). At high latitudes, lentic water temperatures can be similar to adjacent soil temperatures and may be moderated based on basin physiography (Hinzman et al. 2005, Carroll et al. 2011), which could be relevant to where Bd occurs (Gleason et al. 2010). As such, Bd distribution at high latitudes may differ between interior zones and coastal areas where the temperature ranges and freeze regimes are less extreme, with more widespread occurrence on the coast.

This study used a three-phase approach to investigate Bd ecology and disease patterns at high latitudes, with wood frogs in Alaska as my model system. The first phase

was field-based, where I sampled for Bd in wood frogs and water at known frog breeding sites, then used occupancy models to estimate the probability of Bd occupancy of wood frog breeding sites and Bd detection probability at a site given that it is occupied. Because species ranges are defined as the extent of occurrence or the area of occupancy (Gaston 1991, He and Gaston 2000), distribution models that only include detections and do not account for non-detections may result in an underestimation of range size and extent (MacKenzie et al. 2002, MacKenzie 2006). There are two potential states of non-detections, 'absent' or 'present but not detected', and occupancy models account for both of these states. Non-detections when a species is present may be the result of low abundance, insufficient sampling, sampling biases, or non-detections may result when a species was truly absent at the time of collection (MacKenzie et al. 2002, MacKenzie 2006, Bailey et al. 2014). As such, I examined associations of Bd occupancy and detection probabilities with site-level metrics (e.g., air temperature, precipitation, number of growing degree days, number of frost-free days) and sample-location level covariates (i.e., water temperature, pH, specific conductance, and turbidity at the time of sampling, and volume of water filtered). The second phase of my research was laboratory-based, where I conducted multiple controlled experiments to examine if Bd in culture (independent of the host) or in frogs infected with the pathogen can persist through sub-zero temperatures that are typical of the Alaskan winter. I investigated response of Bd in culture to both short-term freezing at mild temperatures (between -0.9 C and -2.5 C) and long-term freezing at extreme cold temperatures (-80 C). I also examined response of Bd in infected frogs to short-term freezing at a mild temperature (-0.9 C). The third phase

investigated the presence of Bd in wood frog museum specimens to determine whether there is any evidence that Bd occurred in Alaska prior to the first field observation in 2002 (Reeves and Green 2006).

In the first phase, using field collected data, I asked the following questions: what proportion of amphibian habitats are occupied by Bd; is there a relationship between Bd occupancy and site-level climate metrics; is there a relationship between Bd detection in amphibian habitats and water quality; and is there a difference in Bd detection between sampling methods (sampling amphibians or water from their breeding habitats)? I hypothesized that the distributional range of Bd was restricted due to hypothesized limited adaptation to extreme climates. I predicted Bd prevalence in wood frogs and their aquatic habitats would correspond to climate gradients, with the highest prevalence near the coast (with a more restricted mean annual temperature range, e.g., 25 C) and lowest in the interior (with an expanded mean annual temperature range, e.g., 40 C; (Slaughter and Viereck 1986, Walsh et al. 2008).

In the second phase, I used an experimental approach to investigate whether freezing temperatures inhibit Bd growth. In natural settings, I hypothesized that wood frogs (and other potential hosts or reservoirs that are not yet identified) may function as cryoprotectants that allow Bd to persist through harsh high latitude winters. I asked the questions: does Bd in culture survive a mild (-0.9 C) short-term freeze; does a cyroprotectant aid survival through an extreme (-80 C) long-term freeze; and do wood

frogs infected with Bd reduce infection loads when they experience a mild (-2.5 C) short-term freeze? I predicted Bd would survive a mild short-term freeze but growth would be inhibited, and it would not survive an extreme long-term freeze without a cryoprotectant because of limited adaptations in some chytrids to survive such temperatures (Boyle et al. 2003, Hibbett et al. 2007, Houseknecht et al. 2012). I also predicted that when wood frogs are infected with Bd then frozen, infection loads would decrease, but the infection would not be eliminated because the frog may serve as a cryoprotectant that buffers Bd cells from osmotic shock associated with freezing, owing to the unique physiology of the species, in which they freeze to survive the extreme cold (Larson et al. 2014),

In the third phase, where I assessed the occurrence of Bd in wood frog museum specimens collected in Alaska. I asked the questions: do specimens test positive for Bd, and if so, in what proportions; and what is the earliest date of detection?

Methods

Phase 1: Field Study Design and Data Collection

I conducted a latitudinal survey of Bd in frogs and water from 2009 to 2011, from the Kenai Peninsula, Alaska (60° North latitude) to the northern limit of the wood frog in Alaska (~67° North, Figure 2.1). I sampled in one-degree increments, (i.e., latitudinal strata) with a minimum of two sites per stratum for a total of 41 sites across the 7-degree gradient. Sites were selected from a list of known wood frog breeding sites provided by the Alaska Department of Fish and Game. Sites on private land were eliminated if

landowner permission was not granted. Final site selection was subject to the participation of the land manager or collaborator, therefore my scope of inference is limited to the sites I sampled and is not representative of the entire range of the wood frog within state of Alaska, however, the area sampled encompasses the range of the species. From south to north regions per stratum, sampling included: 60°N, Kenai National Wildlife Refuge (n=3); 61°N, Anchorage (n=3); 62°N, Talkeetna (n=2), Tetlin National Wildlife Refuge (n=4); 63.5°N, Denali National Park (n=8); 65°N, Fort Wainwright, Fairbanks (n=7); 66°N, Yukon Flats National Wildlife Refuge (n=4); and 67°N, Coldfoot and Wiseman (n=5), Arctic National Wildlife Refuge (n=5). In June-July 2009, I sampled from 60°N to 63°N, and at Arctic National Wildlife Refuge (ANWR, 67°N). In August 2010, I sampled from 64°N to 67°N, and at Tetlin National Wildlife Refuge (62°N). In May-June 2011, I made repeat visits to sites sampled in previous years Denali (n=4), Fort Wainwright (n=2), Wiseman (n=4), and sampled new sites from Yukon Flats National Wildlife Refuge and Fort Wainwright. Unevenness in my sampling scheme was due to logistic constrains related to the wide geographic distribution and remoteness of the sites.

In all years, water samples were collected to detect Bd in amphibian breeding habitats. When Bd water samples were collected, I measured water temperature, pH, specific conductance, and turbidity at the sample location. In 2009 and 2011, skin swabs were collected from amphibians. Samples from amphibians were collected from ANWR but water samples were not collected. To ensure detection in wood frogs when infection prevalence was low, attempts were made to swab a minimum of 60 animals when

possible (Skerratt et al. 2008). To assess Bd in wood frog breeding habitats, I collected three spatial replicate water filter samples at each site where water was sampled, and recorded the volume of water sampled. We filtered water and performed DNA extractions following standard protocols described by Kirshtein et al. (2007), with modifications to improve DNA recovery described in Chestnut et al. (2014). Amphibians were captured by hand or with a dipnet. Bd samples from amphibians were collected with a sterile synthetic swab by rubbing post-metamorphic frogs 25 times (five strokes on the pelvic patch, thighs, and toes and webbing of each hind foot) with a sterile synthetic swab (MW113, Medical Wire and Equipment, Corsham, Wiltshire, UK) or rubbing the mouthparts of larvae with five full rotations of the swab. Swabs were placed into a sterile 2.0 microcentrifuge tube and stored on ice until return to the laboratory. I extracted DNA from the swabs by adding 100 μL of Prepman Ultra to each tube, heating to 100°C for 10 min and cooling for 3 min before centrifuging at 10,000 rpm for 3 min. I then immediately pipetted the supernatant into 96-well stock plates, which were kept frozen at -20 C. Bd genomic equivalents from water filters and swabs were estimated in triplicate following standard protocols for quantitative PCR analysis using the Qiagen QuantiTect SYBR Green PCR kit (Boyle et al. 2004, Kirshtein et al. 2007).

Phase 1 Data Analysis

I examined the relationship between the probability of Bd occupancy and detection in wood frog breeding habitats, and hypothesized covariates using occupancy models implemented in program Presence 4.4 (http://www.mbr-

pwrc.usgs.gov/software/presence.html). Only water samples were included in this analysis, swabs were not. As multiple observations are required per site, I considered individual Bd water samples from each site as an independent observation for a total of 3 observations per visit. I used a single-season occupancy model to estimate the site-level probability of Bd occurrence (i.e., occupancy) in amphibian habitats (Ψ), and the probability of detecting Bd when it is present in amphibian breeding habitats (p). I investigated annual and seasonal air temperature and precipitation covariates that I hypothesized were related to the probability of occurrence (Ψ), and water quality covariates that I hypothesized were related to detection probability (p) in amphibian habitats (Table 2.1).

The site-level air temperature and precipitation covariates of Bd occupancy I investigated were generated by the Scenarios Network for Alaska and Arctic Planning (SNAP, http://www.snap.uaf.edu/). I used historical climate data produced by SNAP, downscaled to 771-m grid cells via the delta method (Walsh et al. 2008). The climate covariates I included in the Ψ model included decadal (1999-2009) averages of annual and seasonal (i.e., winter, spring, summer and autumn) air temperatures and precipitation. I also included wood frog detection at the time of sampling as a predictor of Bd occupancy. Covariates of detection probability (p) were recorded at the time of sampling (i.e., volume of water filtered, and water temperature, pH, specific conductance, and turbidity at the location the sample was collected). Model strength was evaluated based on Akaike's Information Criteria (AIC) and the resulting Akaike's weights (Burnham and Anderson 2002). ΔAIC values <2 indicated similar support between models, and the AIC

weights indicated the strength of evidence for top performing models (Burnham and Anderson 2002). I estimated the detection probability based on the number of water samples collected and the volume of the water filtered per sample using the maximum likelihood estimates from the top-performing model.

In my initial analysis, I developed a candidate model set including 20 models specifying different covariate relationships for occupancy and detection probabilities. Initial occupancy was modeled without any covariates to focus my investigation on detection before exploring predictors of occupancy. In this candidate set, detection probability was modeled as a constant or as a function of volume, temperature, pH, pH squared, specific conductance, turbidity, and all covariates of p combined. Next, to test whether a single-season model structure was appropriate, I included year as a covariate of Ψ and fit models with and without this covariate to determine if occupancy varied among years. My final candidate model set evaluated annual and seasonal covariates of temperature and precipitation, to determine the appropriate scale for my questions. I assumed that this candidate set contained at least one model that adequately fit my data. Bd data were archived in the USGS Western Ecological Research Center Multitaxa Database, headquartered in Sacramento, CA, USA. For data summaries, I used R 2.14.0 (R Core Team 2012).

Phase 2: Laboratory Experimental Study Design and Data Collection

Experiment 1: I examined Bd response to freezing by exposing recent wood frog metamorphs to Bd, and then freezing the infected frogs at temperatures they would

experience in nature. As Bd only infects keratinized structures in amphibians (Marantelli et al. 2004), which are lacking in frog embryos, I collected egg masses from the Kenai Peninsula in June 2013 to ensure no prior Bd infection. Following hatching, larval animals were raised in an environmental chamber housed in a laboratory setting set at the temperature and photoperiod from the location they were collected (60.52696, -150.06953, WGS84). Larval wood frogs were raised in 40-L glass aquaria filled with dechlorinated water treated with 2.5-ml each of NovAqua and AmQuel that was aged for a minimum of one day to ensure temperature equilibrium. Floating plastic cover objects were provided, and algae were allowed to colonize the aquaria walls and cover objects to supplement food provided every other day (1:1:1 pulverized fish flakes, kelp flakes and rabbit chow). Water was changed every 10-14 days, and 1-L of water from the previous tank was transferred to the new tanks, along with the cover objects, to provide algal inoculum. As larvae metamorphosed, they were transferred into a 40-L aquaria lined with a single layer of wet unbleached paper towels and sphagnum moss, and elevated by approximately 2-cm on one side to create a pool. Metamorphs were fed fruit flies and pin-head crickets ad libitum every one to two days.

I exposed 20 wood frogs 1-2 months post-metamorphosis to 10,000 Bd zoospores, estimated using a cell-counting chamber, by placing them in individual 50-ml beakers with a 2-ml bath (5,000 zoospores/ml) for 24 hours. I chose this amount because a previous study saw high mortality in wood frogs exposed to higher zoospore densities (Searle et al. 2011a) and my goal was to infect the animals but not to induce mortality. Individuals were then randomly assigned to one of two treatments (frozen or not

frozen/control, 10 animals each) and placed with the Bd inoculum in a 15-cm petri dish lined with moist sphagnum moss. Animals were incubated at 5 C for five days before they were swabbed to establish the pre-treatment Bd infection load. One day after swabbing, animals were placed in sterile 50-ml falcon tubes. Tubes with control animals were not frozen and held at 4 C and tubes with treatment animals were placed in an ethanol bath set to 0 C. Frozen animals had a small (approximately 1 cm^3) piece of ice at the bottom of the falcon tube to catalyze freezing and were cooled at a rate of -0.04 degrees per hour (approximately 1 C per day) until -2.5 C was reached. They were held at this temperature for 4 hours to ensure temperature equilibrium was reached. Animals were then removed from the ethanol bath and allowed to thaw until soft in the tubes, approximately 4 hours. Once soft, all animals were returned to clean 15-cm petri dishes lined with moist sphagnum moss, and recovery was monitored visually. I assessed Bd infection load following the same methodology as described above for collecting field samples. Animals were swabbed before freezing and on days 6, 12, and 19 post freezing.

Experiment 2: I tested the response of Bd in culture to short-term freezing at a mild (-0.9 C) temperature. Using two experimental treatments, frozen and not frozen, I measured Bd response by counting Bd colonies post-freezing on petri plates with 1% tryptone agar. Samples (9 per treatment) were each prepared in 15-ml Pyrex tubes filled with 7-ml of 1% tryptone and inoculated with 1 ml of 1% tryptone containing 2500 Bd zoospores. To estimate Bd zoospores, I grew Bd for six days on petri plates with 1% tryptone agar and harvested zoospores after flooding the plate with 1% tryptone broth. I

pooled the inoculum by transferring it aseptically to a sterile 50-ml plastic Falcon tube and estimated the number of zoospores in the pooled inoculum using a cell counting chamber. Pyrex tubes were randomly assigned to a treatment, placed in a stainless steel rack and allowed to incubate for one week at room temperature (approximately 18 C). I then placed the rack in an environmental chamber for one week and reduced the temperature by 2 C per day until 4 C was reached. Prior to freezing, all Pyrex tubes were placed in sterile 50-ml falcon tubes. Unfrozen tubes were held at 4 C and frozen tubes were submerged in an ethanol bath held at -0.9 C for two days. Following freezing all Pyrex tubes were removed from the Falcon tubes and returned to the stainless steel rack, held at 4 C for one week, and incubated at room temperature for one month. A 0.5-ml sample from each tube was transferred aseptically to individual petri plates prepared with 1% tryptone agar and Bd growth was monitored for 3 weeks. I estimated the number of colonies on each plate by obtaining independent counts from three different observers.

Experiment 3: I tested Bd response to long-term freezing with and without cryoprotection at extreme cold temperatures using three experimental treatments: two cryopreservation methods plus a tryptone-only treatment with no cryoprotectant. I prepared Bd cultures (strain JEL 630) using 10% Dimethyl sulfoxide (DMSO)/10% Fetal Calf Serum (FCS), 10% Glycerol in 1% culture media, and a 1% tryptone-only. I chose the DMSO/FCS protocol because it is considered the optimal cryoprotectant for Bd (Boyle et al. 2003), and the glycerol treatment to simulate cryoprotectants in wood frogs. The tryptone-only treatment represented free-living Bd in the aquatic environment

without a cryoprotectant. Samples (18 per treatment) were prepared in 2.0-ml microcentrifuge tubes, inoculated with 1 ml 1% tryptone containing 2500 Bd zoospores, estimated using a cell counting chamber. Each tube was randomly assigned to a treatment and placed in a polystyrene freezer box, then frozen in a -80° C freezer for three months. All samples were thawed according to the protocol described by (Boyle et al. 2003) and transferred aseptically to individual petri plates prepared with 1% tryptone agar. Bd growth was monitored for three weeks. Recovery was considered positive if one or more Bd colonies were observed with the naked eye growing on the petri plates after three weeks.

Phase 2 Data Analysis

Experiment 1: I fit a linear mixed-effects model that included the fixed effects of treatment and day, random effect of individual, and a repeated measures covariance structure with an alpha of 0.05. Models were fit with constant variance for each position (day) and no within group (treatment) correlations between days.

Experiment 2: I compared the mean number of Bd colonies between the frozen and non-frozen treatments with a two-sample t-test.

Experiment 3: I report the final observations, which did not warrant statistical analysis.

All analysis and data summarization were performed using the software R, Version 3.1.0 (R Core Team 2012). Models were fit with the nlme package, version 3.1-117.

Phase 3: Museum Specimen Data Collection and Analysis

To investigate historical wood frog specimens for Bd I sampled 50 contemporary ethanol-preserved wood frog specimens collected between 1994 and 2002 accessioned in the University of Alaska Fairbanks Museum of the North. I prepared histological slides by collecting skin scrapings from the toes and webbing of preserved specimens with a scalpel blade treated with ethanol and flamed between each specimen. Skin scrapings were stained with Congo Red (Briggs and Burgin 2004), and permanently mounted on glass slides with Permount adhesive. Additionally, I collected skin swabs from the toes and webbing on the opposite foot used for slide preparation and performed qPCR analysis following protocols described by (Cheng et al. 2011). I report the final observations from the histological slides and qPCR assays; statistical analyses were not needed.

Results

Phase 1: Field Surveys

I detected Bd in wood frogs and their breeding habitats throughout the study area, however, there were no occasions when I detected Bd in both water and amphibians from the same site. I detected Bd at 13 of 41 (32%) wood frog breeding sites sampled by filtering water; however with occupancy modeling, I estimated 41% (SE = 10%, CI = 25%-61%) of these sites were occupied by Bd. This suggests that I would underestimate the proportion of sites that are occupied by Bd in Alaska by 9 percentage points if I did

not take into account imperfect detection. The highest Bd densities in water samples were in the spring months, and occupancy tended to be higher in the interior (Figure 2.1). At eight sites, Bd detections were from only one of the three water filter samples collected during a sampling event. At one site, I detected Bd from two of three water filters, and at four sites I recovered Bd from all three water filters.

Wood frogs tested positive for Bd at four sites including one in Arctic National Wildlife Refuge (Tables 2.2 & 2.3). All Bd detections in wood frogs occurred in 2009 and infection intensities were low, <10 zoospore equivalents in all samples. In 2009, 11 of 297 (4%) swabs were Bd-positive (1 from Arctic National Wildlife Refuge, 3 from Denali National Park, and 7 from Kenai National Wildlife Refuge) and infection prevalence at a site ranged from 7-24%. At the sites with Bd detections in frogs, we encountered larvae at three sites, and metamorphs at one site (Tables 2.2 & 2.3). At three of the four sites where frogs tested Bd-positive, water samples were Bd-negative (Table 2.2). Water samples were not collected from the fourth site (Table 2.3). In 2011, 0 of 283 swabs were Bd-positive. Most frogs encountered and sampled in 2011 were either recent hatchlings or new metamorphs. Logistics precluded sampling at other times of the year.

My candidate model set first explored predictors of detection and had strong support that volume of water sampled should be retained in models predicting occupancy (model weight = 0.9467, ΔAIC comparing to second ranking model = 7.00) therefore, I continued my investigations of predictors of occupancy with volume included in the models. The water quality variables I hypothesized to relate to p (Table 2.1) were not strong predictors of detection probability. In my candidate models for predicting

occupancy, there was no difference among models that included year, thus I determined that a single season model structure was adequate. My candidate models for occupancy also provided support for investigating air temperature and precipitation at the annual scale, rather than at season scales. My final model set included one predictor of detection: volume of water sampled; and four covariates for occupancy: length of growing season; mean annual decadal precipitation; wood frog detection at the time of sampling; and constant occupancy across sites. The best-supported final models were those that included length of growing season and wood frog detection at the time of sampling (Table 2.4), but the model weight was stronger for the model that included only length of growing season (0.61). The probability of a site being occupied by Bd was highest at sites with the shortest growing season (Figure 2.2). Sites at the highest latitudes, near the communities of Wiseman and Coldfoot, AK had the highest probability of Bd occupancy, with individual site estimates between 59-67%. The probability that an individual site in Denali National Park and Preserve was occupied by Bd was between 44-55%. Between 23-29% of individual sites near Fairbanks and in Tetlin National Wildlife Refuge were estimated to be occupied by Bd. Sites near Talkeetna and on the Kenai Peninsula had the lowest probability of Bd occupancy, with individual site estimates between 2-5%, although the confidence intervals for these estimates were quite wide.

The probability of detecting Bd when it was present varied according to the volume of water and number of samples collected (Figure 2.3). To achieve a 95% detection probability seven samples of 60 ml of water, five samples of 180 ml, three samples of 360 ml, or two samples of 600 ml were necessary. The volume an observer

was able to pass through a filter depended on the turbidity of the water. The mean volume of water I was able to pass through a filter in this study was 330 ml.

Phase 2: Laboratory Experiments

Experiment 1: There was no evidence to support a difference between median Bd infection load between the frozen and non-frozen treatments (Table 2.5), therefore I reject my hypothesis that exposure to a mild freeze (-2.5 C) may reduce Bd infection in wood frogs (Table 2.6). I found strong evidence of an effect of day in this experiment ($F_{3,46}$ 44.38, p=<0.0001), with both treatments having reduced Bd loads on Day 6. Although there was no difference between the median Bd infection loads between the frozen and non-frozen treatments, I observed marked differences in the mean and range of Bd GE between the two groups. On day 0, the median and mean infection loads between the frozen and non-frozen treatments were similar as expected, and while there was no difference in the median infection loads between the two treatments on any subsequent day, However, while the median Bd infection loads were similar to each other on each day, the variation differed as reflected by the mean (Table 2.5) and the ratio of the difference in means between the frozen and non-frozen treatments on any day (Figure 2.4). On day 6, Bd mean infection load was three times lower in the frozen treatment, on day 12 the mean was 24 times lower, and day 19 mean Bd infection loads were six times lower in the frozen treatment. The maximum infection loads in the frozen treatment on days 6, 12, and 19 post-freezing were 2.23 GE, 8.95 GE, and 19.76 GE, respectively,

while the maximum infection loads on the same days in the non-frozen treatment were 5.80 GE, 480.32 GE, and 220.06 GE (Table 2.6).

In addition to variation in Bd densities over time, I observed variation in detection among individual wood frogs infected with Bd in this experiment. Prior to the freezing treatment, all 20 wood frogs exposed to Bd had infections above my detection limit (0.1 GE). At day 6, six of 10 wood frogs in the frozen treatment, and three of 10 frogs in the non-frozen treatment had infection levels below the detection limit. Three animals in the freezing treatment did not survive past day 6. At day 12, one of seven wood frogs in the frozen treatment and two of 10 frogs in the non-frozen treatment had infection levels below the detection limit, these were different individuals than on day 6. At day 19, all seven wood frogs from the frozen treatment had Bd infections above the detection limit, and one of 10 animals from the non-frozen treatment had an infection below the detection limit. Individuals did not have infections below the detection limit more than once (i.e., all animals that had infection loads below the detection limit on day 6 had an infection load above the detection limit on days 12 and 19).

Experiment 2: I found strong evidence that freezing significantly reduced Bd survival in culture ($t = -9.59$, $p = <0.0001$). Bd colony counts on plates were 12.5 times more dense in the non-frozen treatment (mean = 160) than in the frozen treatment (mean = 13; Figure 2.5).

Experiment 3: Nine of 18 (50%) replicates in each treatment recovered from the DMSO/FCS treatment. Bd did not survive freezing in either the glycerol or tryptone-only treatments.

Phase 3: Museum Specimens

I detected Bd from 2 of 50 (4%) of museum specimens using qPCR. Bd was not detected in Congo Red stained histological slides. Both Bd-positive samples were from adult wood frogs collected in the vicinity of Goose Bay (61.42528, -149.98583; datum:WGS84) in August 1999 (Table 2.7).

Discussion

Emerging infectious diseases at high latitudes are understudied and this system may provide useful models for understanding host-pathogen ecology in harsh environments (Kutz et al. 2009, 2014, Davidson et al. 2011). Bd occupancy of amphibian breeding habitats in this study (41%) were lower than observed at mid-latitudes in North America (61%) and densities in Alaska were at least an order of magnitude lower (Chestnut et al. 2014). These general results support earlier models of Bd occurrence in amphibians being less at high latitudes (Olson et al. 2013), however, these models did not take into account Bd occurrence in the environment independent of the host. Although the pathogen in my study area was relatively widespread, I report intricacies in patterns of host and parasite ecology related to time (e.g., seasonality in detection), space (e.g., occupancy of sites in interior versus coast), and host life stage that cannot be easily teased apart without a further studies of Bd ecology outside of amphibian hosts.

Although I detected Bd throughout the wood frog range, I detected it more often in the environment and less often in the host. The free-living portion of the Bd life cycle in this system appears protracted. This observation alone might suggest that Bd over-winters in wood frog breeding habitats, surviving either as a free-living saprobe or as a parasite on an alternate host, and that wood frogs may clear Bd infections during the winter months. Experimentally, I observed Bd susceptibility to short-term and long-term freezing in culture, suggesting its density could be reduced in the environment when exposed to mild freezing temperatures, and potentially eliminated when temperatures are extremely cold for several months. This could also explain lower Bd occupancy of amphibian habitats and densities observed throughout the study area as compared to observations in temperate North America (Chestnut et al. 2014). Bd is known to overwinter in aquatic amphibian habitats in the temperate environment (Chestnut et al. 2014), but my results demonstrate that mild freezing may effectively reduce the density in the environment, and prolonged deep freeze may eliminate it from the environment in the absence of a cryoprotectant.

Bd prevalence in wood frogs in my study ranged from 0% in 2011 to 4% in 2009. Although the prevalence I observed was low, it was consistent with Bd prevalence in other wood frog populations in North America (6.6%, Ouellet et al. 2005; 0%, Chestnut et al. 2008; 6.4%, Reeves 2008; 0.83%, Zellmer et al. 2008). Contrary to my prediction, mild freezing (-2.5 C) did not reduce the intensity of Bd infections in wood frogs, which could support the hypothesis that wood frogs may act as a cryoprotectant and reservoir for Bd overwinter. I did not observe a difference in Bd infections between infected wood

frogs that were frozen and not frozen, but I did observe individual variation in Bd infection loads, suggesting factors other than freezing influence the occurrence and intensity of the disease in this species at high latitudes.

Amphibian response to Bd is different according to species and life stage (Rachowicz and Vredenburg 2004, Blaustein et al. 2005, Searle et al. 2011a, Bancroft et al. 2011). In both years I sampled wood frogs for Bd, I encountered the larval life stage most often (Table 2.3). In 2009, most wood frogs I sampled were in late Gosner states (e.g. hind limb buds developing) and in 2011 the majority of the larval encounters were early Gosner stages (e.g. recent hatchlings; Gosner 1960). The probability of detecting Bd in larval life stages when it is present is lower than in adult amphibians. (Adams et al. 2010), possibly due to limited keratin in the tissues at this early life stages (Marantelli et al. 2004). Additionally, recent hatchlings may not have been exposed to free-living Bd for a duration that was long enough to develop an infection. Thus, the Bd prevalence I observed in my study area may be representative of larval life stages but is likely an underestimate of overall Bd prevalence within the populations I sampled.

This study affirms the utility of using both field observations and experiments to explore and explain the patterns and processes in the natural world. The field results showed differences in pathogen distribution and occupancy of wood frog breeding habitats, but in the opposite direction from my prediction. I predicted that the probability of Bd occupancy would be highest in the coastal regions and lowest in the interior due to moderate coastal climates, but I observed the highest probability of occupancy at the interior sites, which experiences extreme cold temperatures in winter months.

Experimentally, freezing did not reduce Bd infections in hosts but in culture Bd responded negatively to mild freezing; fewer colonies grew in the frozen treatments but it was not eliminated except when it was exposed to extreme cold temperatures in the absence of a cryoprotectant. This suggests that Bd may persist at high latitudes by overwintering in amphibians, living saprobically in aquatic environments protected from extreme cold temperatures (e.g., spring-fed systems and other waterbodies that do not freeze solid in winter), or possibly utilizing alternative hosts that function as cryoprotectants. The higher density of Bd I observed in the field in the spring months may be attributed to pathogen dynamics that are independent of the host (e.g., free-living Bd that survived the winter increase growth in response to increased temperatures), dependant on the host (e.g., the arrival or activity of infected hosts from uplands releasing pathogen into the environment), an interaction between the host and pathogen (e.g., the arrival of hosts stimulate the growth of the pathogen in the environment that survived the winter), or most likely, a combination of all three of these scenarios.

Winter is thought to be when amphibians in many parts of the world succumb to Bd infections (Savage et al. 2011, Phillott et al. 2013); however, these observations are from temperate, tropical and subtropical areas that do not experience such dramatic temperature fluctuations that include extreme cold and heat, as is characteristic of the boreal environment. Previous studies of wood frogs at high latitudes showed relatively low occurrence of Bd in wild populations (Chestnut et al. 2008, Reeves 2008, Schock et al. 2010). The unique physiology of wood frogs, which allows them to survive freezing to -16 C (Larson et al. 2014), may protect them from succumbing to the disease by halting

disease development overwinter. This benefit, however, may be short in duration as Bd growth continues in wood frogs after they thaw.

Climate change induced wetland drying is observed in Alaska at a landscape-level (Klein et al. 2005, Riordan et al. 2006, Lu and Zhuang 2011), which can have direct and indirect effects on the host-pathogen disease dynamics. Wetland drying is pronounced on the Kenai Peninsula (Klein et al. 2005), where I observed the lowest Bd occupancy in wetlands. Wood frogs breed primarily in shallow, fishless wetlands that are often ephemeral (Berven 1990). Wetland drying is one of the main drivers of population structure for wood frogs because the eggs and larvae are especially vulnerable to desiccation in the shallow habitats where they breed (Berven 1990, 2009). Bd is also vulnerable to desiccation (Johnson et al. 2003), although its fate in temporary wetlands and vernal pools is largely unknown. Whereas I did not monitor hydroperiod in this study, wood frogs within my study area typically occurred in closed-basin ponds and wetlands that dry seasonally. These small, shallow wetlands may be more susceptible to climate-induced desiccation and appear to be rapidly disappearing from the boreal landscape (Klein et al. 2005, Smol and Douglas 2007, Carroll et al. 2011). Bd survival in seasonal wetlands that dry versus wetlands with permanent, year-round water is unknown. It is possible that Bd survival may be reduced or eliminated in seasonal wetlands when they dry, and are inoculated by frogs returning in the spring to breed. Deep-water lakes and spring-fed systems that do not freeze solid are present within my study area, and may be possible reservoirs for Bd.

The incidence of disease and mortality of hosts are often linked to environmental changes such as temperature (Altizer et al. 2006). Poikilothermic hosts may be especially sensitive to temperature (Rohr et al. 2011, Blaustein et al. 2012). Temperature can influence disease outcomes by mediating host immune responses and changing pathogen reproductive rates (Altizer et al. 2006, Rollins-Smith et al. 2011, Voyles et al. 2012). This is true for the amphibian-Bd disease system, where temperature is an important regulator for amphibian immune responses to Bd (Raffel et al. 2006, Ramsey et al. 2010). Incidence of chytridiomycosis in wild amphibian populations can be associated with changes in environmental temperatures (Berger et al. 2004, Woodhams and Alford 2005, Bosch et al. 2007). Whereas the mechanisms that drive thermal effects on amphibian immune response and Bd growth are complex, it is clear that temperature is a key factor for disease development in tropical and temperate regions (Becker and Zamudio 2011, Savage et al. 2011, Knapp et al. 2011). It is less clear how temperature influences disease dynamics in amphibians at high latitudes. Bd generation time and fecundity are influenced by temperature, and Bd exhibits differences in long-term responses when exposed to different thermal regimes (Woodhams et al. 2008, Voyles et al. 2012). When cold-adapted Bd is exposed to warmer temperatures, zoospores develop faster than warm-adapted Bd of the same strain, however, in the cold-adapted strain overall zoospore developmental time is longer (Voyles et al. 2012). Zoospores in the cold adapted strain may take up to 4 times longer to encyst, which could increase effective exposure time to hosts, and take 6 times longer to mature and release zoospores (Voyles et al. 2012). These adaptations (longer time to encystment and maturity) to a cold environment could explain

the high occupancy rates but low density I observed in wetlands (e.g. Bd is present but takes longer to develop in the boreal environment, and low prevalence in wood frog life stages sampled).

While the boreal zone is characterized by extreme cold and harsh winter conditions, the summer months also may be inhospitable due to heat. Conditions in the boreal zone during summer months are demonstrated to limit some parasites (Hoar et al. 2012). This study revealed a pattern that was the reverse of my prediction: Bd occupancy of a site was highest at sites with the shortest growing season. However, the geographic scale of these temperature data (771 m grid cells) fails to capture the fine-scale factors that may affect Bd occurrence at a site, e.g., microhabitat, surface temperatures, and solar radiation. The day length in the spring months is approximately the same throughout my study area (13-14 hours), but as summer progresses, the northern parts of the study area may have 4-6 hours more day length than the southern portions (18-20 hours in the south versus 22-24 hours in the north). This difference in day length can result in higher surface temperatures that are not reflected by air temperature. The surface temperatures experienced by both host and pathogen in this system are considerably warmer than the air temperature would suggest (Kutz et al. 2014). As such, these warmer surface temperatures in the interior resulting from longer day length in the summer months may stimulate Bd growth and result in higher Bd occupancy rates of wetlands in the interior. Amphibians can also reduce or clear Bd infections by elevating their body temperature (Woodhams et al. 2003, Forrest and Schlaepfer 2011). While average monthly temperatures in the summer months within my study area are typically below 20 C

(Walsh et al. 2008), daily temperatures may be high enough (27-30 C) to reduce or clear Bd infections in basking wood frogs (Woodhams et al. 2003, Forrest and Schlaepfer 2011), which could provide one explanation for the low prevalence I observed in the animals I sampled in the field.

An alternate explanation for the low prevalence in wild caught wood frogs, and absence of a difference in the infection loads of wood frogs experimentally exposed to Bd then exposed to different temperature regimes, is host-parasite co-evolution. There is evidence that Bd is endemic in parts of its range (Rosenblum et al. 2013) and its evolutionary history suggests that the Globally Pandemic Lineage 1 (GPL-1) clade is primarily North American (Schloegel et al. 2012). While modern amphibians diverged from a common ancestor in the Devonian Period about 370 million years ago, Bd diverged from a common ancestor approximately 100,000 years before present (Rosenblum *et al.*, 2013). Modern Bd strains evolved 25,000 years before present (10,000- to 40,000-y range), which predates wood frog colonization of Alaska. It is likely that some North American amphibians co-evolved with GLP-1 Bd strains, and possible that wood frogs carried Bd with them as they colonized the boreal north. The extreme cold winter temperatures may limit Bd from reaching high densities in the environment and infection intensities in wood frogs, although the effect of increased temperatures resulting from recent, rapid climate change is not clear. Wood frog populations at high latitudes appear to be persisting despite widespread occurrence of Bd in their breeding habitats, although in the absence of long-term monitoring programs it is impossible to

know how wood frogs (and their diseases) will respond to changes in habitat availability and suitability, increased temperatures, and other stressors.

Climate-induced changes to abiotic (e.g. increased temperature, altered hydrology) and biotic processes (e.g. arrival of new hosts and diseases expanding ranges) will likely alter the existing host-pathogen dynamics in the boreal environment. Increased temperatures and altered hydroperiods may cause some previously ideal habitats to become uninhabitable and new habitats may become available for existing species to occupy. Species range expansions from the south and east may diversify both the pool of candidate hosts, and introduce novel pathogens, or new strains of existing pathogens with varied virulence. As these processes are occurring more rapidly at high latitudes than in other parts of the world, opportunities exist to develop tools to predict emerging infectious disease outbreaks that may be applicable at a global scale. The relatively simple system is also ideal to investigate disease theory such as the Red Queen hypothesis (evolutionary arms race between host and pathogen; Van Valen 1973, Liow et al. 2011), and the dilution effect (increased biodiversity protects against pathogens because some hosts are less efficient that others, which in turn lowers the overall prevalence in a community; Schmidt and Ostfeld 2001, Johnson and Thieltges 2010, Searle et al. 2011b), in the face of rapid climate change, as it occurs in real-time in novel systems.

References

Adams, M. J. et al. 2010. Using occupancy models to understand the distribution of an amphibian pathogen, *Batrachochytrium dendrobatidis*. - Ecol. Appl. 20: 289–302.

Altizer, S. et al. 2006. Seasonality and the dynamics of infectious diseases. - Ecol. Lett. 9: 467–484.

Bailey, L. L. et al. 2014. Advances and applications of occupancy models. - Methods Ecol. Evol. 5: 1269–1279.

Bancroft, B. A. et al. 2011. Species-level correlates of susceptibility to the pathogenic amphibian fungus *Batrachochytrium dendrobatidis* in the United States. - Biodivers. Conserv. 20: 1911–1920.

Becker, Cg. and Zamudio, K. R. 2011. Tropical amphibian populations experience higher disease risk in natural habitats. - Proc. Natl. Acad. Sci. USA 108: 9893–9898.

Berger, L. et al. 2004. Effect of season and temperature on mortality in amphibians due to chytridiomycosis. - Aust. Vet. J. 82: 434–439.

Berven, K. A. 1990. Factors affecting population fluctuations in larval and adult stages of the wood frog (*Rana sylvatica*). - Ecology 71: 1599–1608.

Berven, K. A. 2009. Density Dependence in the Terrestrial Stage of Wood Frogs: Evidence from a 21-Year Population Study.

Best, A. et al. 2012. Local transmission processes and disease-driven host extinctions. - Theor. Ecol. 5: 211–217.

Blaustein, A. R. et al. 2005. Interspecific variation in susceptibility of frog tadpoles to the pathogenic fungus *Batrachochytrium dendrobatidis*. - Conserv. Biol. 19: 1460–1468.

Blaustein, A. R. et al. 2012. Ecophysiology meets conservation: understanding the role of disease in amphibian population declines. - Philos. Trans. R. Soc. B Biol. Sci. 367: 1688–1707.

Bosch, J. et al. 2007. Climate change and outbreaks of amphibian chytridiomycosis in a montane area of Central Spain; is there a link? - Proc. R. Soc. B Biol. Sci. 274: 253–260.

Boyle, D. et al. 2003. Cryo-archiving of *Batrachochytrium dendrobatidis* and other chytridiomycetes. - Dis. Aquat. Organ. 56: 59–64.

Boyle, D. G. et al. 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. - Dis. Aquat. Organ. 60: 141–148.

Breen, A. L. et al. 2012. Genetic consequences of glacial survival: the late Quaternary history of balsam poplar (*Populus balsamifera* L.) in North America. - J. Biogeogr. 39: 918–928.

Briggs, C. and Burgin, S. 2004. Congo Red, an effective stain for revealing the chytrid fungus, *Batrachochytrium dendrobatidis*, in epidermal skin scrapings from frogs. - Mycologist 18: 98–103.

Burnham, K. P. and Anderson, D. R. 2002. Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach. - Springer Science & Business Media.

Calkin, P. E. 1988. Holocene glaciation of Alaska (and adjoining Yukon Territory, Canada). - Quat. Sci. Rev. 7: 159–184.

Calkin, P. E. et al. 2001. Holocene coastal glaciation of Alaska. - Quat. Sci. Rev. 20: 449–461.

Carroll, M. L. et al. 2011. Shrinking lakes of the Arctic: Spatial relationships and trajectory of change. - Geophys. Res. Lett. 38: L20406.

Cheng, T. L. et al. 2011. Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. - Proc. Natl. Acad. Sci. 108: 9502–9507.

Chestnut, T. et al. 2008. Results of amphibian chytrid (*Batrachochytrium dendrobatidis*) sampling in Denali National Park, Alaska, USA. - Herpetol. Rev. 39: 202–204.

Chestnut, T. et al. 2014. Heterogeneous occupancy and density estimates of the pathogenic fungus *Batrachochytrium dendrobatidis* in waters of North America. - PLoS ONE 9: e106790.

Costanzo, J. P. et al. 2013. Hibernation physiology, freezing adaptation and extreme freeze tolerance in a northern population of the wood frog. - J. Exp. Biol. 216: 3461–3473.

Costanzo, J. P. et al. 2014. Seasonality of freeze tolerance in a subarctic population of the wood frog, *Rana sylvatica*. - Int. J. Zool. 2014:e750153

Daszak, P. et al. 2000. Emerging infectious diseases of wildlife–threats to biodiversity and human health. - Science 287: 443–449.

Davidson, R. et al. 2011. Arctic parasitology: why should we care? - Trends Parasitol. 27: 239–245.

De Castro, F. and Bolker, B. 2005. Mechanisms of disease-induced extinction. - Ecol. Lett. 8: 117–126.

Ezard, T. H. G. et al. 2011. Interplay between changing climate and species' ecology drives macroevolutionary dynamics. - Science 332: 349–351.

Forrest, M. J. and Schlaepfer, M. A. 2011. Nothing a hot bath won't cure: infection rates of amphibian chytrid fungus correlate negatively with water temperature under natural field settings. - PLoS ONE 6: e28444.

Gaston, K. J. 1991. How large is a species' geographic range? - Oikos 61: 434–438.

Gleason, F. H. et al. 2004. Some Chytridiomycota in soil recover from drying and high temperatures. - Mycol. Res. 108: 583–589.

Gleason, F. H. et al. 2008. Freeze tolerance of soil chytrids from temperate climates in Australia. - Mycol. Res. 112: 976–982.

Gleason, F. H. et al. 2010. Can zoosporic true fungi grow or survive in extreme or stressful environments? - Extremophiles 14: 417–425.

Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. - Herpetologica 16: 183–190.

He, F. and Gaston, K. J. 2000. Occupancy-abundance relationships and sampling scales. - Ecography 23: 503–511.

Hibbett, D. S. et al. 2007. A higher-level phylogenetic classification of the Fungi. - Mycol. Res. 111: 509–547.

Hinzman, L. D. et al. 2005. Evidence and implications of recent climate change in Northern Alaska and other Arctic regions. - Clim. Change 72: 251–298.

Hoar, B. M. et al. 2012. Development and availability of the free-living stages of *Ostertagia gruehneri*, an abomasal parasite of barrenground caribou (*Rangifer tarandus groenlandicus*), on the Canadian tundra. - Parasitology 139: 1093–1100.

Hoberg, E. P. 2005. Coevolution and biogeography among Nematodirinae (Nematoda: Trichostrongylina), Lagomorpha, and Artiodactyla (Mammalia): Exploring determinats of history and structure for the Northern fauna across the Holarctic. - J. Parasitol. 91: 358–369.

Houseknecht, J. L. et al. 2012. Viability of fastidious Phytophthora following different cryopreservation treatments. - Fungal Biol. 116: 1081–1089.

Hughes, P. D. et al. 2013. Timing of glaciation during the last glacial cycle: evaluating the concept of a global “Last Glacial Maximum” (LGM). - Earth-Sci. Rev. 125: 171–198.

Johnson, P. T. J. and Thieltges, D. W. 2010. Diversity, decoys and the dilution effect: how ecological communities affect disease risk. - J. Exp. Biol. 213: 961–970.

Johnson, M. L. et al. 2003. Fungicidal effects of chemical disinfectants, UV light, desiccation and heat on the amphibian chytrid, Batrachochytrium dendrobatidis. - Dis. Aquat. Organ. 57: 255–260.

Jones, K. E. et al. 2008. Global trends in emerging infectious diseases. - Nature 451: 990–993.

Kirshtein, J. D. et al. 2007. Quantitative PCR detection of *Batrachochytrium dendrobatidis* DNA from sediments and water. - Dis. Aquat. Organ. 77: 11.

Klein, E. et al. 2005. Wetland drying and succession across the Kenai Peninsula Lowlands, south-central Alaska. - Can. J. For. Res. 35: 1931–1941.

Knapp, R. A. et al. 2011. Nowhere to hide: impact of a temperature-sensitive amphibian pathogen along an elevation gradient in the temperate zone. - Ecosphere 2: art93.

Kutz, S. J. et al. 2009. The Arctic as a model for anticipating, preventing, and mitigating climate change impacts on host–parasite interactions. - Vet. Parasitol. 163: 217–228.

Kutz, S. J. et al. 2014. A walk on the tundra: Host–parasite interactions in an extreme environment. - Int. J. Parasitol. Parasites Wildl. in press.

Larson, D. J. et al. 2014. Wood frog adaptations to overwintering in Alaska: New limits to freezing tolerance. - J. Exp. Biol.: jeb.101931.

Lee-Yaw, J. A. et al. 2008. Postglacial range expansion from northern refugia by the wood frog, *Rana sylvatica*. - Mol. Ecol. 17: 867–884.

Liow, L. H. et al. 2011. Red Queen: from populations to taxa and communities. - Trends Ecol. Evol. 26: 349–358.

Lobitz, B. et al. 2000. Climate and infectious disease: Use of remote sensing for detection of *Vibrio cholerae* by indirect measurement. - Proc. Natl. Acad. Sci. 97: 1438 – 1443.

Longcore, J. E. et al. 1999. *Batrachochytrium dendrobatidis gen et sp nov*, a chytrid pathogenic to amphibians. - Mycologia 91: 219–227.

Lu, X. and Zhuang, Q. 2011. Areal changes of land ecosystems in the Alaskan Yukon River Basin from 1984 to 2008. - Environ. Res. Lett. 6: 034012.

MacKenzie, D. I. 2006. Occupancy estimation and modeling: inferring patterns and dynamics of species occurrence. - Academic Press.

MacKenzie, D. I. et al. 2002. Estimating site occupancy rates when detection probabilities are less than one. - Ecology 83: 2248–2255.

Marantelli, G. et al. 2004. Distribution of the amphibian chytrid Batrachochytrium dendrobatidis and keratin during tadpole development. - Pac. Conserv. Biol. 10: 173.

McCallum, H. 2012. Disease and the dynamics of extinction. - Philos. Trans. R. Soc. B Biol. Sci. 367: 2828–2839.

Morin, X. and Chuine, I. 2006. Niche breadth, competitive strength and range size of tree species: a trade-off based framework to understand species distribution. - Ecol. Lett. 9: 185–195.

Olson, D. H. et al. 2013. Mapping the global emergence of *Batrachochytrium dendrobatidis*, the amphibian chytrid fungus. - PloS One 8: e56802.

Ouellet, M. et al. 2005. Historical evidence of widespread chytrid infection in North American amphibian populations. - Conserv. Biol. 19: 1431–1440.

Phillott, A. D. et al. 2013. Chytridiomycosis and seasonal mortality of tropical stream-associated frogs 15 Years after introduction of *Batrachochytrium dendrobatidis*. - Conserv. Biol. 27:1058-1068.

Rachowicz, L. J. and Vredenburg, V. T. 2004. Transmission of *Batrachochytrium dendrobatidis* within and between amphibian life stages. - Dis. Aquat. Organ. 61: 75–83.

Raffel, T. R. et al. 2006. Negative effects of changing temperature on amphibian immunity under field conditions. - Funct. Ecol. 20: 819–828.

Ramsey, J. P. et al. 2010. Immune defenses against *Batrachochytrium dendrobatidis*, a fungus linked to global amphibian declines, in the South African Clawed Frog, *Xenopus laevis*. - Infect. Immun. 78: 3981–3992.

R Core Team 2012. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Reeves, M. K. 2008. *Batrachochytrium dendrobatidis* in wood frogs (Rana sylvatica) from three national wildlife refuges in Alaska, USA. - Herpetol. Rev. 39: 68.

Reeves, M. K. and Green, D. E. 2006. *Rana sylvatica* chytridiomycosis. - Herpetol. Rev. 37: 450.

Riordan, B. et al. 2006. Shrinking ponds in subarctic Alaska based on 1950–2002 remotely sensed images. - J. Geophys. Res. Biogeosciences 111: G04002.

Rohr, J. R. et al. 2011. Frontiers in climate change–disease research. - Trends Ecol. Evol. 26: 270–277.

Rollins-Smith, L. A. et al. 2011. Amphibian immune defenses against chytridiomycosis: impacts of changing environments. - Integr. Comp. Biol. 51: 552–562.

Rosenblum, E. B. et al. 2010. The deadly chytrid fungus: a story of an emerging pathogen. - PLoS Pathog 6: e1000550.

Rosenblum, E. B. et al. 2013. Complex history of the amphibian-killing chytrid fungus revealed with genome resequencing data. - Proc. Natl. Acad. Sci. 110: 9385–9390.

Rutter, N. et al. 2012. The glacial and loess record of North America. - In: Glaciations in North and South America from the Miocene to the Last Glacial Maximum. Springer, pp. 35–49.

Savage, A. E. et al. 2011. Disease dynamics vary spatially and temporally in a North American amphibian. - Biol. Conserv. 144: 1910–1915.

Scalera, R. et al. 2008. Occurrence of *Batrachochytrium dendrobatidis* in amphibian populations in Denmark. - Herp Rev 39: 199–200.

Schloegel, L. M. et al. 2012. Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. - Mol. Ecol. 21: 5162–5177.

Schmidt, K. A. and Ostfeld, R. S. 2001. Biodiversity and the dilution effect in disease ecology. - Ecology 82: 609–619.

Schock, D. M. et al. 2010. Amphibian chytrid fungus and ranaviruses in the Northwest Territories, Canada. - Dis. Aquat. Organ. 92: 231–240.

Searle, C. L. et al. 2011a. Differential host susceptibility to *Batrachochytrium dendrobatidis*, an emerging amphibian pathogen. - Conserv. Biol. 25: 965–974.

Searle, C. L. et al. 2011b. A dilution effect in the emerging amphibian pathogen *Batrachochytrium dendrobatidis*. - Proc. Natl. Acad. Sci. 108: 16322–16326.

Shafer, A. B. A. et al. 2010. Of glaciers and refugia: a decade of study sheds new light on the phylogeography of northwestern North America. - Mol. Ecol. 19: 4589–4621.

Slaughter, C. W. and Viereck, L. A. 1986. Climatic characteristics of the taiga in interior Alaska. - In: Forest Ecosystems in the Alaskan Taiga. Springer, pp. 9–21.

Slough, B. G. 2009. Amphibian chytrid fungus in Western Toads (*Anaxyrus boreas*) in British Columbia and Yukon, Canada. - Herpetol. Rev. 40: 319.

Smith, K. G. et al. 2009a. Selecting for extinction: nonrandom disease-associated extinction homogenizes amphibian biotas. - Ecol. Lett. 12: 1069–1078.

Smith, K. F. et al. 2009b. The role of infectious diseases in biological conservation. - Anim. Conserv. 12: 1–12.

Smol, J. P. and Douglas, M. S. V. 2007. Crossing the final ecological threshold in high Arctic ponds. - Proc. Natl. Acad. Sci. 104: 12395–12397.

Tucker, C. J. et al. 2011. Climate change, extreme weather events, and fungal disease emergence and spread. - Fungal Dis. Emerg. Threat Hum. Anim. Plant Health Workshop Summ.: 324.

Van Valen, L. 1973. A new evolutionary law. - Evol. Theory 1: 1–30.

Voyles, J. et al. 2012. Temperature alters reproductive life history patterns in *Batrachochytrium dendrobatidis*, a lethal pathogen associated with the global loss of amphibians. - Ecol. Evol. 2: 2241–2249.

Vredenburg, V. T. et al. 2010. Dynamics of an emerging disease drive large-scale amphibian population extinctions. - Proc. Natl. Acad. Sci. 107: 9689–9694.

Walsh, J. E. et al. 2008. Global Climate Model Performance over Alaska and Greenland. - J. Clim. 21: 6156–6174.

Woodhams, D. C. and Alford, R. A. 2005. Ecology of chytridiomycosis in rainforest stream frog assemblages of tropical Queensland. - Conserv. Biol. 19: 1449–1459.

Woodhams, D. et al. 2003. Emerging disease of amphibians cured by elevated body temperature. - Dis. Aquat. Organ. 55: 65–67.

Woodhams, D. C. et al. 2008. Life-history trade-offs influence disease in changing climates: strategies of an amphibian pathogen. - Ecology 89: 1627–1639.

Zellmer, A. J. et al. 2008. Low prevalence of *Batrachochytrium dendrobatidis* across *Rana sylvatica* populations in Southeastern Michigan. - Herpetol. Rev. 39: 196–199.

Zigouris, J. et al. 2013. Phylogeography and post-glacial recolonization in wolverines (*Gulo gulo*) from across their circumpolar distribution. - PLoS ONE 8: e83837.

Tables

Table 2.1. Habitat and sample attributes hypothesized to relate to the probability of detection (p) or occurrence (Ψ) of *Batrachochytrium dendrobatidis* (Bd) in amphibian breeding habitats in Alaska, USA. Bold **X** indicates attribute was included in the best fitting models, based on AIC value and weight; - indicates variable was not included in the model

Covariate abbreviation and description	Probability of occurrence (Ψ)	Detection probability (p)	Data source
vol = volume of water filtered from an amphibian habitat to the nearest L	-	**X**	Field measurement
temp = temperature in degrees Celsius to the nearest tenth at the location a sample was collected	-	X	Field measurement
ph = pH at the location a sample was collected	-	X	Field measurement
sp_cond = Specific conductance in microsiemens (uS), standardized to 25°C, at the location a sample was collected	-	X	Field Measurement
turb = turbidity in Formazin Nephelometric Unit (FNU)	-	X	Field Measurement
year = year field sampling was conducted	X	-	Calendar
frog = wood frogs in any life stage detected at the time of sampling	X	-	Field Measurement
growing = decadal average of mean length of growing season in days	**X**	-	Scenarios Network for Alaska and Arctic Planning (SNAP), Walsh et al. 2008
tempmean = decadal average of mean annual temperature in degrees C	**X**	-	SNAP, Walsh et al. 2008
tempwin = decadal average of mean winter (Dec-Feb) temperature in degrees C	X	-	SNAP, Walsh et al. 2008
tempspr = decadal average of mean spring (Mar-May) temperature in degrees C	X	-	SNAP, Walsh et al. 2008
Tempsum = decadal average of mean summer (Jun-Aug) temperature in degrees C	X	-	SNAP, Walsh et al. 2008
tempfal = decadal average of mean fall (Sep-Nov) temperature in degrees C	X	-	SNAP, Walsh et al. 2008
precipannual = decadal average of mean annual precipitation in mm	X	-	SNAP, Walsh et al. 2008

Precipwin = decadal average of mean winter (Dec-Feb) precipitation in mm	X	-	SNAP, Walsh et al. 2008
Precipspr = decadal average of mean spring (Mar-May) precipitation in mm	X	-	SNAP, Walsh et al. 2008
Precipsum = decadal average of mean summer (Jun-Aug) precipitation in mm	X	-	SNAP, Walsh et al. 2008
precipfal = decadal average of mean fall (Sep-Nov) precipitation in mm	X	-	SNAP, Walsh et al. 2008

Table 2.2. Locations where environmental samples were taken for *Batrachochytrium dendrobatidis* (Bd) from water collected from *Lithobates sylvaticus* breeding habitats in Alaska. Frogs were swabbed for Bd when animals were encountered and life stage was recorded. Bd detections in bold face. Life stage: Tad = tadpole; Ad = adult.

				Water samples		**Frogs swabbed**		
Site	**Latitude**	**Longitude**	**Date**	***Bd* det.**	**Total**	***Bd* det.**	**Total**	**Life stage**
KEN03	60.71422	-150.81543	**30 Jun 2009**	**0**	**3**	**8**	**33**	**Tad**
KEN02	60.73232	-150.61922	30 Jun 2009	0	3	0	21	Tad
KEN01	60.75063	-150.50337	**30 Jun 2009**	**0**	**3**	**1**	**5**	**Tad**
ANC02	61.18892	-149.84764	3 Jul 2009	0	1			
TAL01	62.23035	-150.05415	2 Aug 2010	0	3			
TAL02	62.23035	-150.05415	2 Aug 2010	0	3			
SC15	62.638138	-141.045552	**19 Aug 2010**	**1**	**3**			
SC02	62.638996	-141.03895	19 Aug 2010	0	3			
SC01	62.639369	-141.035344	19 Aug 2010	0	3			
HW20	62.78673	-141.31056	19 Aug 2010	0	3			
DNP17	63.44144	-150.56743	**26 Jun 2009**	0	3	**2**	**15**	**Tad**
DNP15	63.44213	-150.56554	**26 Jun 2009**	**1**	**3**	0	15	Tad
			2 Jun 2011	**3**	**3**	0	30	Tad
DNP04	63.44341	-150.71692	21 Jun 2009	0	3	0	12	Tad
			3 Jun 2011	**0**	**3**			
DNP03	63.44342	-150.71402	21 Jun 2009	0	3	0	16	Tad
DNP05	63.47335	-150.83560	22 Jun 2009	0	3	0	15	Tad
			2 Jun 2011	**1**	**3**	0	30	Tad
DNP06	63.47584	-150.83893	22 Jun 2009	0	3	0	16	Tad
DNP10	63.48057	-150.85991	24 Jun 2009	0	3	0	15	Tad
DNP11	63.48080	-150.85681	**24 Jun 2009**	0	3	**1**	**15**	**Tad**
DNP08	63.48161	-150.86206	**23 Jun 2009**	**1**	**1**	0	15	Tad
			1 Jun 2011	0	3	0	31	Tad
FBK04	64.84765	-147.57971	3 Aug 2010	0	3			
			7 Jun 2011	**1**	**3**	0	2	Ad
FBK01	64.85451	-147.52885	7 Jun 2011	0	2			
FBK02	64.8549	-147.52994	3 Aug 2010	0	3			
FBK03	64.85536	-147.53217	3 Aug 2010	0	3			

				Water samples		Frogs swabbed		
Site	**Latitude**	**Longitude**	**Date**	***Bd* det.**	**Total**	***Bd* det.**	**Total**	**Life stage**
FBK07	64.85691	-147.61613	7 Jun 2011	0	2			
FBK06	64.85818	-147.61641	**7 Jun 2011**	**1**	**3**	0	12	Tad
0_18_5	66.330214	-148.952891	30 Jun 2011	0	1			
0_18_6	66.333333	-148.9686111	28 Jun 2011	0	1			
0_18_3	66.334167	-148.96888	28 Jun 2011	0	1			
0_18_2	66.385	-149.1361111	24 Jun 2011	0	1			
RN06	67.24969	-150.17737	5 Aug 2010	0	3			
			26 May 2011	2	3			
RN01	67.42212	-150.11705	5 Aug 2010	0	3			
			26 May 2011	**2**	**3**			
RN02	67.43517	-150.13261	5 Aug 2010	0	3			
			25 May 2011	**1**	**3**	0	1	Ad
RN03	67.43668	-150.15256	5 Aug 10	0	3			
			25 May 2011	**1**	**3**	0	1	Ad
DH09	67.60558	-149.78485	28 May 2011	**2**	**3**	0	1	Ad

Table 2.3. Locations where *Lithobates sylvaticus* were swabbed for *Batrachochytrium dendrobatidis* (Bd). Bd detections in bold face. Water samples were not taken at these sites. Life stages: Tad = tadpole; Met = metamorph; Juv = juvenile; Ad = adult.

			Frogs sampled			
Site	Latitude (degrees)	Longitude (degrees)	Date	Bd detected	Total Swabbed	Life stage
Arctic Circle						
ANC01	61.14617	-149.96458	17 June 2009	0	15	Tad
ARC02	**67.14222**	**-142.1999**	**22 July 2009**	**1**	**10**	**Met**
ARC07	67.21586	-142.18792	19 July 2009	0	7	Met, 1 Ad
ARC20	67.21586	-142.18792	18 July 2009	0	10	Met
ARC24	67.19384	-142.18822	21 July 2009	0	12	Met
ARC25	67.19441	-142.18454	21 July 2009	0	9	Met
ARC27	67.19646	-142.16574	21 July 2009	0	10	Met
Denali National Park						
DNP06	63.47584	-150.83893	2 June 2011	0	30	Tad
DNP07	63.51852	-150.84836	22 June 2009	0	14	Tad
DNP09	63.47559	-150.85095	24 June 2009	0	2	Ad
DNP10	63.48057	-150.85991	1 June 2011	0	35	Tad
DNP11	63.48080	-150.85681	1 June 2011	0	31	Tad
DNP12	63.45802	-150.89537	25 June 09	0	12	Tad
DNP13	63.45682	-150.88776	25 June 09	0	4	Tad
Yukon River basin						
YRB01	66.455179	-147.90461	25 July 2011	0	1	Juv
YRB02	66.46191	-147.90272	24 July 2011	0	1	Juv
YRB03	66.459984	-147.90221	25 July 2011	0	1	Juv
YRB04	66.461759	-147.90243	30 July 2011	0	1	Juv
YRB05	65.977639	-144.55792	5 Aug 2011	0	1	Juv
YRB06	65.978619	-144.55975	6 Aug 2011	0	1	Juv
YRB07	66.219222	-146.45566	2 Aug 2011	0	1	Juv
YRB08	65.4116207	-148.22051	29 July 2011	0	1	Juv
YRB09	66.232407	-145.26532	16 June 2011	0	1	Juv
YRB10	66.321185	-148.03411	20 June 2011	0	1	Juv
YRB11	66.319847	-148.04093	19 June 2011	0	1	Juv
YRB12	66.31975	-148.04075	22 June 2011	0	1	Juv
YRB13	66.321131	-148.03404	23 June 2011	0	1	Juv
YRB14	66.325559	-148.01429	25 June 2011	0	1	Juv

YRB15	65.976736	-144.72386	2 July 2011	0	1	Juv
YRB16	65.976736	-144.72386	2 July 2011	0	1	Juv
YRB17	66.295046	-148.71601	28 July 2011	0	1	Juv
YRB18	65.976824	-144.72308	20 June 2011	0	1	Juv
YRB19	66.152554	-148.85396	9 June 2011	0	1	Juv
YRB20	66.152554	-148.85396	9 June 2011	0	1	Juv
YRB21	66.147663	-148.85542	7 June 2011	0	1	Ad
YRB22	66.147577	-148.85586	7 June 2011	0	1	Juv
YRB23	66.147942	-148.85613	10 June 2011	0	1	Ad
YRB24	66.125322	-144.9161	16 June 2011	0	1	Juv
YRB26	66.331287	-148.97593	28 June 2011	0	1	Ad
YRB27	66.220424	-146.68996	9 Aug 2011	0	1	Juv
YRB28	66.197368	-146.77322	4 Aug 2011	0	1	Juv
YRB29	66.31326	-149.04305	6 June 2011	0	1	Juv
YRB30	66.313193	-149.04338	10 June 2011	0	1	Juv
YRB31	66.257369	-148.72126	15 June 2011	0	1	Juv
YRB32	66.257369	-148.72126	15 June 2011	0	1	Juv
YRB33	66.257369	-148.72126	15 June 2011	0	1	Juv
YRB34	66.256643	-148.75805	15 June 2011	0	1	Juv
YRB35	66.336425	-148.26548	27 July 2011	0	1	Juv
YRB36	66.338805	-148.2662	27 July 2011	0	1	Juv
YRB37	66.331814	-148.28507	27 July 2011	0	1	Juv
YRB38	66.331814	-148.28507	27 July 2011	0	1	Juv
YRB39	66.335673	-148.26966	27 July 2011	0	1	Juv
YRB40	66.159586	-147.73766	6 Aug 2011	0	1	Ad
YRB41	66.306276	-148.68701	29 July 2011	0	1	Juv
YRB42	66.157446	-147.73862	31 July 2011	0	1	Ad
YRB43	66.161487	-148.22811	2 Aug 2011	0	1	Juv
YRB44	66.161487	-148.22811	2 Aug 2011	0	1	Ad
YRB45	66.157084	-148.20379	3 Aug 2011	0	1	Ad
YRB46	66.23191	-146.45381	10 Aug 2011	0	1	Juv
YRB47	66.151283	-147.71738	31 July 2011	0	1	Ad
YRB48	66.232458	-146.44371	8 Aug 2011	0	1	Juv
YRB49	66.305141	-148.69245	29 July 2011	0	1	Juv

Table 2.4. Model selection statistics for a prioiri models relating to occupancy (psi) of *Batrachochytrium dendrobatidis* from 41 wood frog (*Lithobates sylvaticus*) breeding habitats in Alaska, USA with decadal average means of 4 environmental covariates derived from the Scenarios Network for Alaska and Arctic Planning (Walsh et al. 2008), where detection probability (p) is a function of the volume of water sampled (vol); growing (length of growing season in days); precip (mean annual precipitation in mm), frog (wood frogs in any life stage detected at the time of sampling); (.) = occupancy is constant across sites.

Model	AIC	ΔAIC	AIC weight	Model Likelihood	Number of parameters	-2*LogLike
Psi(growing),p(vol)	89.65	0	0.6081	1	3	83.65
Psi(growing + frogs),p(vol)	91.65	2	0.2237	0.3679	4	83.65
Psi(frogs),p(vol)	94.57	4.92	0.0520	0.0854	3	88.57
Psi(precip),p(vol)	94.71	5.06	0.0484	0.0797	3	88.71
Psi(.),p(vol)	94.72	5.07	0.0482	0.0793	3	88.72
Psi(precip + frogs),p(vol)	96.52	6.87	0.0196	0.0322	4	88.52

Table 2.5. Results of linear mixed-effects model comparing the difference in *Batrachochytrium dendrobatidis* (Bd) log genomic equivalents (GE) of wood frogs (*Lithobates sylvaticus*) exposed to two temperature treatments, a mild freeze (-2.5 C), and not frozen (4 C) among days. GE were measured by swabbing frogs at three time points for 19 days post freezing.

Comparison of the difference between treatments by day	Estimate	Standard Error	t-value	DF	p-value
Day 0	0.17	0.53	0.31	18	0.76
Day 6	0.81	0.79	1.03	18	0.32
Day 12	1.59	1.37	1.15	18	0.26
Day 19	0.49	1.11	0.44	18	0.66

Table 2.6. Summary of *Batrachochytrium dendrobatidis* (Bd) genomic equivalents detected in wood frogs experimentally infected with Bd zoospores and exposed to two temperature treatments, a mild freeze (-2.5 C), and not frozen (4 C) over time. The detection limit was 0.01 genomic equivalents.

	Bd Genomic Equivalents									
Day of Sampling	Frozen (-2.5 C)					Not Frozen (4 C)				
	mean	median	st dev	min	max	mean	median	st dev	min	max
Day 0 – Before freezing	12.0	4.6	21.5	2.07	72.41	13.8	7.6	16.9	0.95	53.62
Day 6 – Post freezing	0.4	0.0	0.7	<0.01	2.23	1.2	0.3	1.9	<0.01	5.80
Day 12 – Post freezing	2.9	1.1	4.1	<0.01	8.95	71.1	3.3	149.2	<0.01	480.32
Day 19 – Post freezing	6.8	6.9	7.2	<0.01	19.76	39.0	6.0	76.3	<0.01	220.06

Table 2.7. Locations where museum specimens of *Lithobates sylvaticus* that were sampled for *Batrachochytrium dendrobatidis* were originally collected. All specimens are from the University of Alaska – Fairbanks Museum of the North herpetology collection. Bd detections in bold face.

Location collected					
Latitude (degrees)	Longitude (degrees)	Date collected	Record number	Bd detected	Life stage
61.22500	-149.65000	8 Aug 1994	162	No	Adult
61.26667	-149.68333	29 July 1994	164	No	Adult
61.29083	-149.62611	11 Aug 1994	273	No	Adult
61.30778	-149.77889	10 Aug 1994	278	No	Adult
61.36556	-143.44250	6 Aug 2002	125	No	Adult
			126	No	Adult
61.42528	**-149.98583**	3 Aug 1999	**45**	**Yes**	**Adult**
			47	No	Adult
			48	**Yes**	**Adult**
61.45578	-143.78953	4 Aug 2002	127	No	Adult
		5 Aug 2002	138	No	Adult
61.45694	-143.79611	4 Aug 2002	136	No	Adult
			137	No	Adult
		5 Aug 2002	129	No	Adult
			130	No	Adult
61.45806	-143.80944	5 Aug 2002	128	No	Adult
61.76083	-157.29694	15 Aug 1999	264	No	Adult
61.79389	-157.39278	4 Aug 1999	262	No	Adult
61.96972	-151.19667	6 Aug 1999	33	No	Adult
61.99389	-152.07278	20 July 1999	28	No	Adult
			29	No	Adult
			30	No	Adult
			31	No	Adult
62.24333	-150.24972	30 July 2002	153	No	Adult
62.26833	-150.24417	30 July 2002	156	No	Adult

Location collected					
Latitude (degrees)	Longitude (degrees)	Date collected	Record number	Bd detected	Life stage
62.31372	-141.18088	22 July 2001	296	No	Adult
62.38472	-150.72389	2 Aug 2002	146	No	Adult
			147	No	Adult
			148	No	Adult
62.38472	-150.72389	2 Aug 2002	149	No	Adult
			150	No	Adult
		1 Aug 2002	151	No	Adult
63.92778	-151.49194	15 July 2002	144	No	Adult
		17 July 2002	140	No	Adult
			141	No	Adult
63.92917	-151.50278	16 July 2002	132	No	Adult
63.92944	-151.49583	15 July 2002	142	No	Adult
		18 July 2002	131	No	Adult
		16 July 2002	134	No	Adult
			135	No	Adult
63.93028	-151.50000	15 July 2002	143	No	Adult
65.32885	-143.11543	16 Aug 2001	112	No	Adult
65.35475	-142.95708	10 Aug 2001	116	No	Adult
65.35547	-143.18225	17 Aug 2001	107	No	Adult
65.37639	-142.53167	15 Aug 2001	288	No	Adult
			289	No	Adult
65.89052	-149.73983	25 Aug 2001	279	No	Adult
68.13333	-151.75000	Prior to 1999 No date on specimen	216	No	Adult

Figures

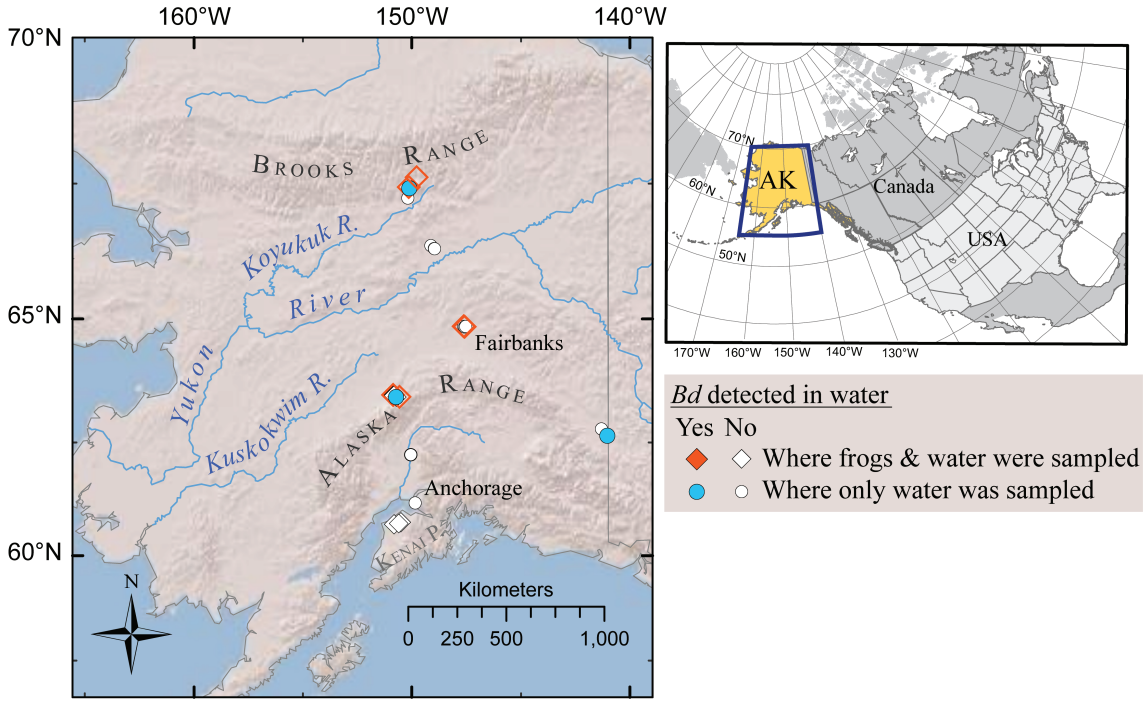

Figure 2.1. Sampling locations (n=34, with 9 sites visited twice; 43 sampling occasions) for environmental samples for *Batrachochytrium dendrobatidis* (Bd) collected from water in wood frog (*Lithobates sylvaticus*) breeding habitats in Alaska, USA, with results of Bd detections.

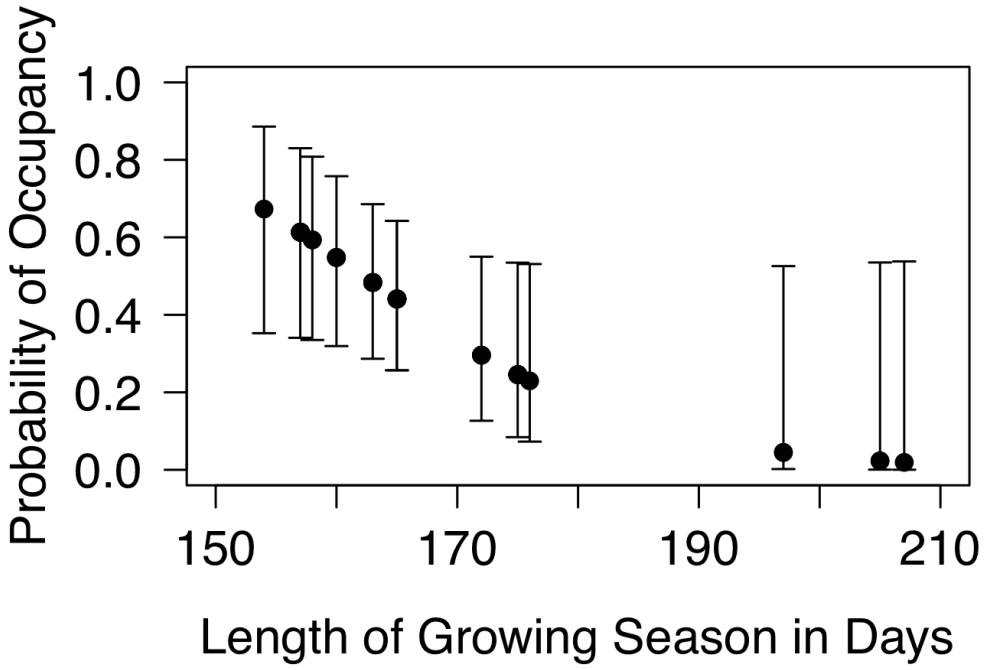

Figure 2.2. Variation in the probability of *Batrachochytrium dendrobatidis* occupancy of wood frog (*Lithobates sylvaticus*) breeding habitats as a function of the length of the growing season in days.

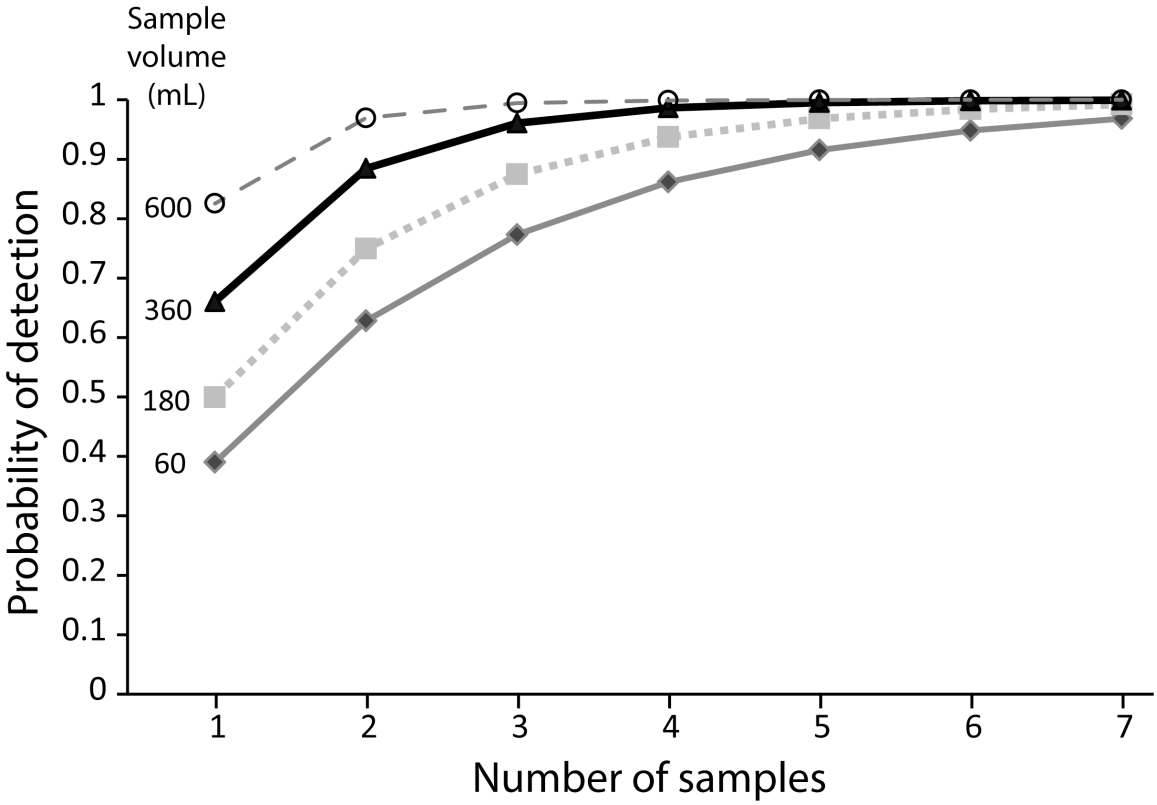

Figure 2.3. Variation in detection probability of Batrachochytrium dendrobatidis (Bd) collected from wood frog (Lithobates sylvaticus) breeding habitats as a function of (i) the number of samples and (ii) the volume of water collected. Volumes are representative of field collected samples.

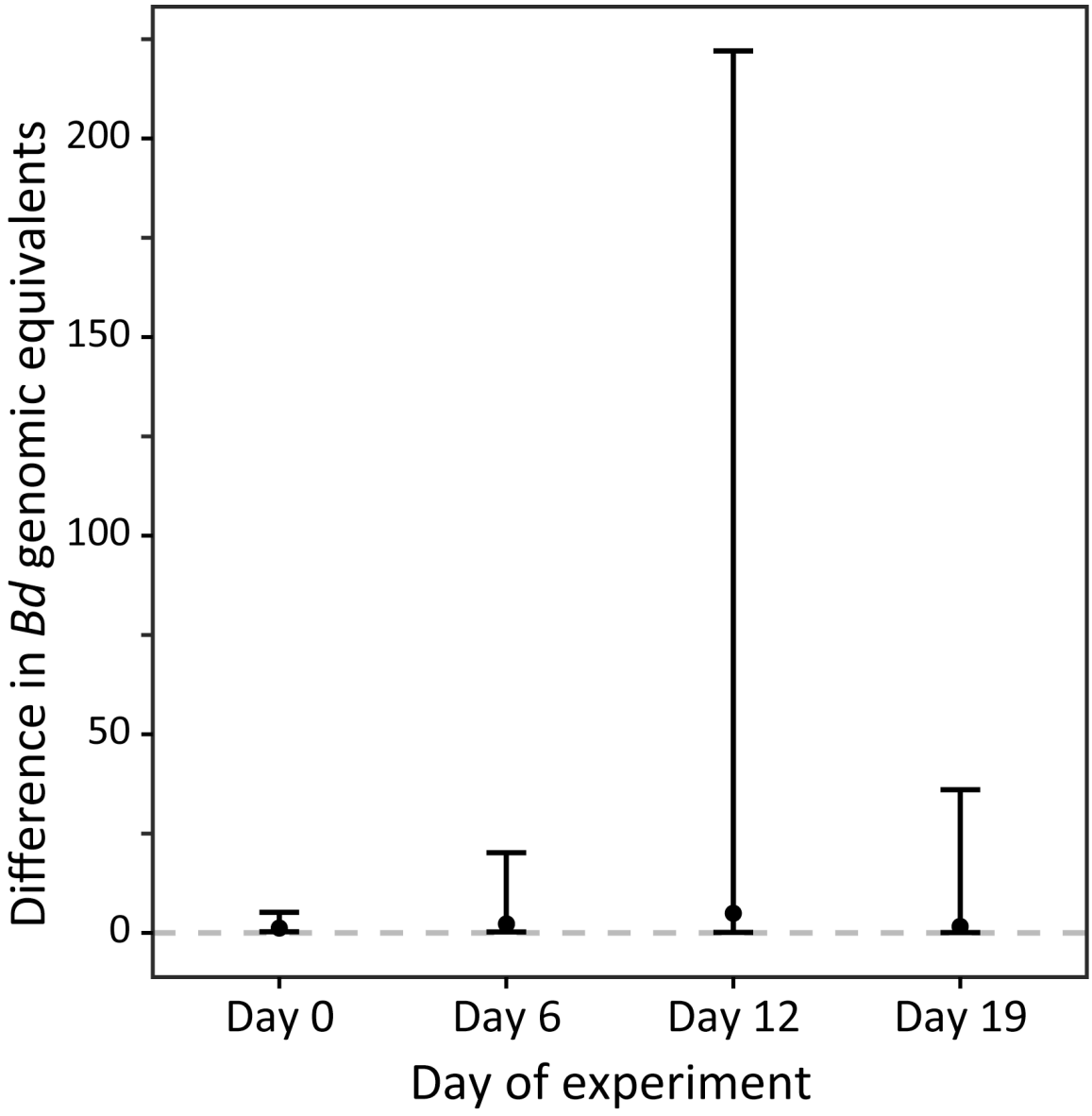

Figure 2.4. Comparison of the ratio of the mean difference in *Batrachochytrium dendrobatidis* (Bd) genomic equivalents (GE) in wood frogs (*Lithobates sylvaticus*) experimentally infected with Bd then frozen (-2.5 degrees C) or not frozen (4 degrees C), with 95% confidence intervals.

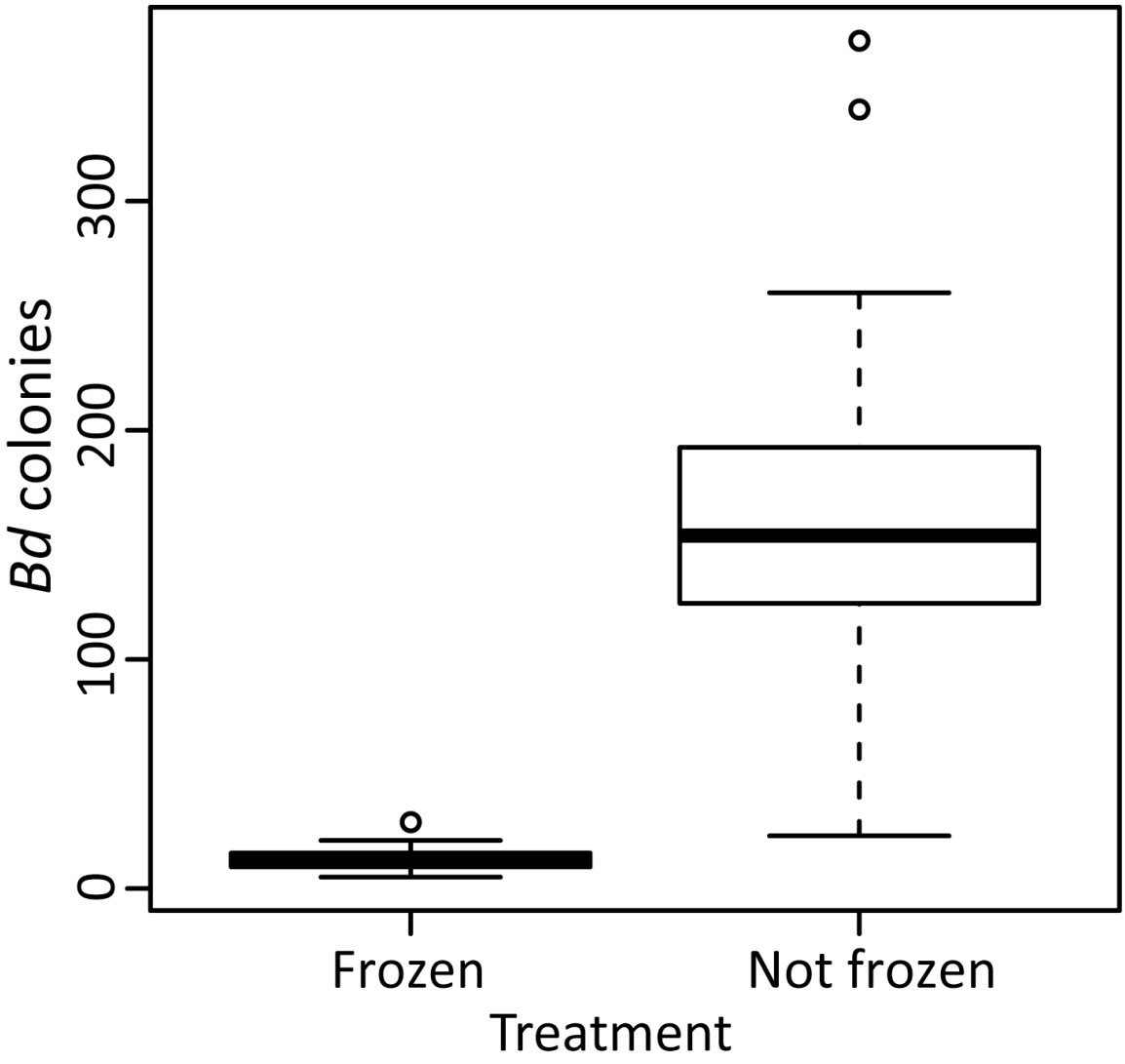

Figure 2.5. Number of *Batrachochytrium dendrobatidis* colonies (median and quartiles) growing on 1% tryptone agar plates one month after cultures were exposed to one of two temperature treatments, frozen (-0.9 C) or not frozen (4.0 C), for two days.

CHAPTER 3 – HOST-PATHOGEN DISEASE DYNAMICS AT MID-LATITUDES: ECOLOGY OF THE AMPHIBIAN CHYTRID FUNGUS *BATRACHOCHYTRIUM DENDROBATIDIS* IN LENTIC SYSTEMS: PERSPECTIVES ON WATER QUALITY, LIMNOLOGY, AND POTENTIAL ALTERNATIVE NON-AMPHIBIAN HOSTS

Deanna H Olson, Chauncey W Anderson, Deborah Iwanowicz, and AR Blaustein

Abstract

Globally, emerging infectious disease (EID) events have increased significantly over the last several decades, most caused by non-vector borne pathogens. Emerging fungal pathogens are included in disease outbreaks and infect a wide variety of hosts. I investigated the ecology of the emerging infectious chytrid fungal pathogen *Batrachochytrium dendrobatidis* (Bd) in amphibians and their breeding habitats. I conducted an exploratory analysis using machine-learning methods (boosted regression trees) to investigate biotic and abiotic variables that may relate to the occurrence of Bd in amphibian habitats. At 16 randomly selected sites in the Willamette Valley, Oregon, USA, I found that amphibian density, species richness, and Bd infection in amphibians sampled were not associated with Bd detection in the aquatic environment, suggesting that Bd is not tightly cued to amphibian hosts in these wetlands. Among five other environmental variables addressed, Bd detection in amphibian breeding habitats was associated with low organic carbon (<7.3 mg/L), and high euglenoid density (>116). If Bd is associated with low organic carbon conditions in the aquatic environment, it may have multiple strategies for accessing vital nutrients, and may assimilate both organic and inorganic matter to obtain nutrients (i.e., nitrogen). This would allow Bd to persist in a diversity of habitats when amphibians are absent, and could offer an explanation for its widespread distribution and year-round persistence in aquatic habitats. The relationship between Bd and euglenoids is unclear. I provide preliminary but inconclusive experimental evidence that Bd zoospores may utilize euglenoids, either as a parasite or saprobe. This study supports the hypothesis that both abiotic and biotic factors

independent of amphibians may influence Bd occurrence in aquatic settings. Understanding the ecology of this pathogen outside of amphibian hosts, as well as factors related to disease persistence and environmental transmission is vital to unraveling this disease system, and informing conservation decisions.

Introduction

In the last 60 years, emerging infectious disease (EID) events have increased significantly and the majority of contemporary EID events were caused by non-vector borne pathogens (Dobson and Foufopoulos 2001, Jones et al. 2008). The patterns of global emergence are often related to environmental and ecological factors, especially anthropogenic change and wildlife host species richness (Jones et al. 2008). Although fungal pathogens only represented ~6% of these EID events, the scope of impacts caused by fungal pathogens is widespread which suggests a high capacity for many fungal pathogens to establish in diverse environments and host species (Desprez-Loustau et al. 2007, Tucker et al. 2011, Taylor and Gurr 2014). For example, the chestnut blight fungus, *Cryphonectria parasitica*, introduced to North America from Asia in the early 1900s, caused the functional extinction of the dominant forest tree species (*Castenea dentata*) in eastern North America in less than 50 years (Dutech et al. 2012). The cascading effects of this pathogen, which removed mature *C. dentata* from 3.6 million hectares, changed the structure and function of eastern forests and is still being realized, (Anagnostakis 1987). The fungal pathogen *Aspergillus sydowii* is attributed to massive population declines in Caribbean corals (e.g., *Gorgonia* spp.), yet the origin of the pathogen, which is typically a terrestrial fungus, is unclear (Alker et al. 2001, Rypien et al. 2008). First described in 2006, an EID in North American bats, *Geomyces destructans*, is spreading rapidly across eastern North America, causing regional bat population collapses. It is predicted to lead to regional extinction of one of the most common bat species in North America, *Myotis lucifugus*, the little brown bat (Frick et al. 2010).

Finally, the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (Bd), is linked to recent global amphibian population declines and possible extinctions in Australia, Central America, and other regions of the world (Bosch et al. 2001, Muths et al. 2003, La Marca et al. 2005, Lips et al. 2005, Collins et al. 2009, Murray et al. 2009). Commonalities among these four pathogens include multiple genetic lineages and introduction events (Alker et al. 2001, Rypien et al. 2008, Frick et al. 2010, Schloegel et al. 2012, Dutech et al. 2012). This highlights the need to understand: 1) the biological and epidemiological factors involved with these widespread fungal pathogens; 2) the challenges that complicate the identification of disease emergence; and 3) the relative risk fungal diseases pose to wildlife and their habitats (Woolhouse 2002).

Many pathogens infect multiple host species (Woolhouse et al. 2001). Whereas disease can reduce populations to low numbers, either temporarily or permanently, it is rare for a pathogen to cause extinction (Gerber et al. 2005, De Castro and Bolker 2005, McCallum 2012). In many disease systems, the potential impact of a pathogen is related to both the density of the host and pathogen (Anderson et al. 1979, May and Anderson 1979). As such, the impact of a pathogen on a declining population should be ameliorated when a host population reaches a density threshold, where the host population is too small for a pathogen to effectively find susceptible hosts (McCallum and Dobson 1995). However, extinction attributed to disease is possible when alternate hosts or environmental reservoirs are present (Gerber et al. 2005, McCallum 2012) or when the disease effect is severe and fast-acting relative to the hosts' potential compensatory

response. In cases like this, where disease transmission is frequency-dependent, pathogen density can remain high when specific host populations become rare. Importantly, when biotic and abiotic reservoirs exist, a pathogen can persist at high incidence regardless of host density, increasing the probability of exposure when host density is low (Thrall and Burdon 1997, Rudolf and Antonovics 2005).

There are very few cases where disease has been implicated as the direct cause in the extinction of a species, yet there are several contemporary examples of the amphibian chytrid fungus causing local amphibian extinctions, and possibly species extirpation (Bradford 1991, Pounds and Crump 1994, La Marca et al. 2005, Lips et al. 2005, Pounds et al. 2006, McCallum 2007, Collins et al. 2009, Collins 2010, Best et al. 2012). Bd has been associated with mass amphibian mortality episodes and local extinctions in both disturbed and protected landscapes in temperate and tropical regions (Lips et al. 2006, Rachowicz et al. 2006, James et al. 2009, Cheng et al. 2011). Species, individuals, and life stages demonstrate variation in susceptibility to Bd; although rapid mortality may occur after exposure, some animals may carry very high infection loads without signs of disease (Daszak et al. 2004, Blaustein et al. 2005, Garcia et al. 2006, Pearl et al. 2007, Bancroft et al. 2011, Searle et al. 2011, Gervasi et al. 2013b, 2013a). Furthermore, Bd may not cause direct mortality, although in some cases, the infection may induce behaviors that reduce fitness or increase exposure to predation (Muths et al. 2003, Parris and Cornelius 2004, Pilliod et al. 2010, Han et al. 2011), resulting in declines indirectly related to disease. In infected amphibians, Bd occurrence has been found to be associated

with a number of environmental variables including temperature, elevation, rainfall, and season (Young et al. 2001, Collins and Storfer 2003, Berger et al. 2004, Drew et al. 2006, Kriger and Hero 2007, Olson et al. 2013). In temperate areas, seasonal variation in Bd infection prevalence in amphibian populations has been found, with the highest prevalence in the early spring and lowest in the autumn (Retallick et al. 2004, Pearl et al. 2007).

Bd can occur year-round in the aquatic environment (Chestnut et al. 2014), but little is known about how Bd survives in the aquatic environment outside of amphibian hosts. In aquatic systems, occurrence of free-living Bd has been associated with elevation, but the effects of temperature, precipitation and season in that study were not evident (Chestnut et al. 2014). The observed physiological tolerance of Bd for temperature and pH in laboratory settings (Piotrowski et al. 2004), generally mirrors the thermal and pH tolerance of many amphibian species (Karns 1992, Sadinski and Dunson 1992, Wyman and Jancola 1992), although variation in Bd growth and fecundity is observed according to temperature (Voyles et al. 2012). In one study, free-living Bd occupied 61% of amphibian breeding habitats sampled (Chestnut et al. 2014). This study also found wide variation in Bd density among seasons, and among different samples taken at a single time, demonstrating its potential for both temporal and spatial heterogeneity that may be unrelated to host density. In water collected from amphibian habitats, Bd prevalence was also observed to be highest in the spring (Chestnut et al. 2014), although peaks in free-living Bd densities were observed among all seasons.

The effect of season on Bd occurrence may be associated with by abiotic and biotic factors. Bd growth and fecundity is influenced by temperature (Voyles et al. 2012) and in many regions, the spring season temperatures are often within the range for optimal Bd growth (Piotrowski et al. 2004). Additionally, Bd-infected animals likely shed zoospores into the water when they return to breeding sites, presumably increasing Bd densities in the water. Infected animals also likely transmit the infection to each other as they assemble for breeding. This increase in host abundance may elicit a chemotaxis response from free-living Bd, which could result in an increase in the zoospore density on the skin. The variation in Bd density observed in the aquatic environment among seasons also may be attributed to alternate hosts or reservoir species that may be present; for example, crayfish and nematodes may be potential alternate hosts for Bd (Shapard et al. 2011, McMahon et al. 2013).

This research extends the use of environmental sampling methodology to assess patterns of Bd occurrence with environmental conditions in amphibian breeding habitats. The goals of my research were to conduct an exploratory analysis of biotic (e.g., amphibian assemblage and aquatic flora) and abiotic (e.g., nutrients, carbon) factors that may relate to the occurrence of Bd in amphibian habitats. Specifically, I was interested in whether Bd presence in amphibian breeding habitats was related to nutrients (nitrogen and phosphorus species), total organic carbon, amphibian species richness, amphibian density, the presence of Bd in amphibians, or phytoplankton assemblages. I predicted that

if Bd occurrence in the aquatic environment is primarily host-dependent, then amphibian richness, amphibian density, and the presence of Bd-infected amphibians would be identified as more important variables to Bd detection in amphibian habitats. If, however, Bd occurrence in aquatic systems is a function of environmental factors independent of amphibian hosts, then I predicted that nutrients, organic carbon, and phytoplankton assemblage would be more important variables related to Bd detection in amphibian breeding habitats. If Bd prevalence is a function of both host-dependant and non-host related environmental variables, then I predicted a combination of amphibian and non-amphibian biotic or abiotic variables would be associated with the presence of Bd in amphibian habitats. This study was exploratory in nature, designed as a means of developing new hypotheses regarding the ecological relationships with Bd occurrence in amphibian breeding habitats.

Fungi serve a central role in energy flow and they make nutrients such as nitrogen accessible by transforming it into forms that other organisms can use (e.g. by reducing organic nitrogen during decomposition). Some fungi may use both organic and inorganic sources of nitrogen, but those that use inorganic sources require specialized enzymes to obtain nutrients (Jennings 1995, Boddy et al. 2011). Carbon-to-nitrogen ratios also may control fungal contributions to organic decomposition in aquatic ecosystems (Wurzbacher et al. 2014). When organic carbon levels are high, generally organic nitrogen levels are also high, but when organic carbon is depleted the primary sources of nitrogen are typically inorganic, which is limiting for species that cannot assimilate

inorganic nitrogen (Jennings 1995, Boddy et al. 2011). Data on how zoosporic fungi utilize nitrogen are extremely limited, as is information about the role of different nitrogen species in fungal survival and growth in the environment. Some chytrids are efficient in converting inorganic compounds into organic compounds (Gleason et al. 2008) but it is not known whether Bd can assimilate inorganic compounds. In this study, I examined associations of free-living Bd with nutrients (ammonium, nitrate, nitrite, total dissolved nitrogen, total nitrogen, total phosphorous, organophosphorus, and total organic carbon) in the aquatic environment to assess whether they may be related to Bd occurrence. Associations with organic carbon or particular nitrogen species may provide insights to the role of Bd in the degradation of organic matter derived from algae and other sources, and if free-living Bd may be functioning as an aquatic saprophyte, parasite, or both.

Homolaphlyctis polyrhiza (Hp) is the closest known relative to Bd, and it occurs in lentic systems (isolated from a lake in Maine, USA; Longcore et al. 2011). Hp is a non-pathogenic saprobe and does not survive on amphibian skin (Joneson et al. 2011). It is not known if Bd has traits to allow a saprobic existence in the absence of amphibian hosts, however phytoplankton parasitism by other chytrid fungi is well-documented (Lefevre et al. 2012, Rasconi et al. 2012). In wetlands, phytoplankton populations exhibit seasonal patterns in species assemblages and density (Euliss et al. 2004) that could influence seasonal patterns in chytrid species assemblages and density. Chytrid abundance and biomass was also found to be higher in eutrophic systems (Rasconi et al.

2012), e.g., in wetlands where many lentic-associated amphibians breed. Because Bd can occur year-round in the aquatic environment and variation in Bd density was observed among seasons (Chestnut et al. 2014), that appeared to be unrelated to amphibian host density, I was interested in investigating whether Bd occurrence could be related to phytoplankton assemblages and density. In this study, in addition to examining amphibian and Bd associations, I assessed associations of free-living Bd with aquatic nutrients (organic carbon, nitrogen, and phosphorous) and phytoplankton density (bluegreen algae, chrysophyte, cryptophyte, diatom, dinoflagellate, eugelnoid, green algae, total taxa richness, and total species richness). Following the results of the field study, I conducted a laboratory experiment to investigate the potential for direct interaction between Bd and phytoplankton.

Methods

This study occurred in the Willamette Valley of western Oregon, USA, a broad structural depression between the Coast Range and Cascade Range, approximately 200 km in length and between 30 and 50 km in width, with a north-south orientation (Franklin and Dyrness 1988, Urich and Wentz 1999). The area has a maritime climate characterized by wet, mild winters with prolonged cloudy periods, relatively dry summers, and a long frost-free season. Average annual temperature is 11.3 C (January 3.8 C, July 19 C), and average annual precipitation is ~1,000 mm, most of which falls as rain October through March (dry summers average 51 mm of rain). Average annual snowfall is 19 mm. The landscape has been shaped by human activities (e.g., fire control, clearing, logging, row crops, and grazing) and is dominated by urban areas, agriculture at

low elevations, and forestry at higher elevations. The dominant habitat types can be classified as oak woodland (*Quercus garryana, Arbutus menziesii*), and prairie (*Agrostis* spp., *Bromus* spp., *Festuca* spp., and a number of forbs). Conifer stands (*Pseudotsuga mellziesii, Abies grandis*) occur in patches throughout the valley and forests are common in the foothills. *Acer macrophylillum* is also widespread. *Populus trichocarpa, Fraxinus latifolia*, and *Salix* spp. are characteristic of riparian and seasonally flooded areas.

Site Selection

I selected sites by a stratified random design using geographic information systems (GIS) software, with sites classified as permanent lakes or wetlands in the Willamette Valley and foothills from the USGS National Water Quality database, grouped by land use (i.e. urban, agriculture, forested). Candidate sites were limited to an elevational band between 0 and 180 m above sea-level, which a previous study identified as influencing Bd site occupancy of amphibian habitats (Chestnut et al. 2014). I conducted preliminary screening to verify land use, determine accessibility and assess suitability of amphibian habitat. If sites were not accessible or unlikely breeding habitats for amphibians (e.g. stocked fishing ponds, or stormwater catchments) they were eliminated as candidate sites. To avoid geographic clustering, especially for sites in the urban land use category which were concentrated in the north of the Willamette Valley (near the Portland metropolitan area), I stratified the sites into three latitudinal sections representing the north, mid, and southern portions of the valley prior to final selection. Attempts were made to include at least one site from each land use category in each of

these three north-south strata, although no agricultural sites occurred in the northern strata. From the final list of potential sites, five to six were randomly selected from each land use type for a total of 16 sites (Figure 3.1). I sampled each site quarterly over a year in random order by generating a list of random numbers in the software R (R Core Team 2012). The first five sites were sampled in the first month of the quarter, the next five were sampled in the second month of the quarter, and the remaining six sites were sampled in the third month of the quarter. If a site was dry during a seasonal sampling event, it was not sampled or replaced with another site, and sampling continued when it held water again in a later season.

Environmental Bd Sampling Methods

Water at field sites was sampled for Bd quarterly, four times over a single year in 2011-2012. During each sampling event, three replicate Bd water samples were collected. In small wetlands (<~2 ha), the three samples were taken equidistant to one another. In larger wetlands and lakes (>~2 ha), the three samples were taken from the same shoreline alcove at least 50 meters apart. Water samples were taken from the same location during each quarterly visit. Each sample was collected from shallow water, less than 1 meter in depth, typically between 5 and 20 cm below the water surface using an established protocol (Kirshtein et al. 2007), with modifications to improve DNA recovery and sampling efficiency (Chestnut et al. 2014). Samples were kept cool until returning from the field and then were immediately refrigerated until DNA extraction occurred, within three months of sample collection. Genomic equivalents (GE) were estimated using quantitative PCR in triplicate.

Aquatic Nutrients and Phytoplankton Sampling Methods

Nutrient, carbon and phytoplankton samples were collected using standard USGS protocols (Moulton et al. 2002, U.S. Geological Survey 2010). Nutrient and carbon samples were processed by the USGS National Water Laboratory (Denver, Colorado, USA). Phytoplankton were identified by Aquatic Analysts (Friday Harbor, Washington, USA). Nine abiotic and 12 biotic metrics (Table 3.1) were evaluated for associations with free-living Bd.

Amphibian Sampling

Amphibians were sampled concurrently with water sampling using aquatic funnel traps (Olson and Leonard 1997). Fifteen traps were set per site in clusters of three groups of five traps located in <1 m of water and left overnight. Traps were set at least one meter apart in the vicinity where the Bd water samples were taken. When possible, traps were set in less than 30 cm of water to ensure an air pocket was available to animals respiring with lungs. To assess amphibian density and species richness, I quantified and visually identified (species and life stage) all amphibians captured in the aquatic funnel traps. Skin samples for Bd were collected from amphibians by rubbing the ventral surfaces (five times each on abdomen, hind legs, and toes/webbing) or the oral disk of larval anurans (five full rotations) with a sterile synthetic swab (Advantage Bundling/Medical Wire Co. fine tip, catalog number MW113), which was then placed dry in a sterile 2.0 ml microcentrifuge tube. To address Bd detection when prevalence is low (≥5%), I attempted to collect samples from 60 individuals of each species encountered per sampling event (Skerratt et al. 2008). If fewer than 60 animals were encountered, samples

were collected from all animals captured. DNA was extracted and GE quantified using qPCR assay (Boyle et al. 2004).

Analytical Methods

I used a machine-learning approach, which uses an algorithm to learn the relationship between the response (Bd presence) and predictor (biotic and abiotic) variables (Breiman 2001). Machine-learning techniques attempt to learn these relationships and find dominant patterns, where the assumptions are that the relationships are complex and unknown. These methods do not assume independence or an appropriate data model typical of conventional (e.g., linear regression) statistical approaches (Elith et al. 2008).

I used boosted regression trees (BRT) to detect and describe patterns that may not be evident with conventional modeling techniques aimed at fitting a single model, which may be too simplistic for many real-life situations (Elith et al. 2008). Boosted regression trees were developed by combining the strengths of two algorithms; classification and regression tree (decision tree) group of models and boosting. Decision tree models are built according to optimal decision rules based on how binary decisions best accommodate a given dataset (De'ath 2007, Elith et al. 2008). Boosting builds and combines a collection of models, and selects the tree that minimizes the loss function (Elith et al. 2008). Although BRT models are complex, partial dependency plots can visualize the contribution or relative importance of individual explanatory variables, which can provide powerful ecological insights.

I developed BRT models using a multi-stage process. First, we developed three candidate BRT models for 53 sampling events at 16 sites using Bd detection (presence) as the response variable. All candidate models included season, amphibian species richness, amphibian density, and Bd-positive amphibians as predictor variables. The first BRT candidate model also included eight nutrient variables (ammonium, nitrate, nitrite, total dissolved nitrogen, total nitrogen, total phosphorous, organophosphorus, and total organic carbon). The second BRT candidate model included seven phytoplankton variables measured in absolute density as cells/mL (bluegreen algae, chrysophyte, cryptophyte, diatom, dinoflagellate, eugelnoid, green algae), plus total phytoplankton taxa richness and total phytoplankton species richness. The third BRT candidate model included all variables. I used a bag fraction of 0.75, interaction depth of 1, and a learning rate of 0.001, with a tree complexity of 5. A bag fraction is the percentage of data randomly selected for development of each tree. The interaction depth is the number of splits on a tree starting with a single node, which increases the total number of nodes by three, and the number of terminal nodes by two with each split, i.e., the left node, right node and NA node. The trees had a total of 4 nodes, with 3 terminal nodes in the trees. The learning rate influences the total number of trees evaluated for the model, while the tree complexity controls whether interactions are fitted. A value of 5 allows assessment of up to 5-way interactions. Variable relative importance (VRI) was calculated using the formula developed by (Friedman 2001), and implemented in the R (version) gbm library to estimate the relative influence of predictor variables. Calculations of VRI are based on the number of times a variable is selected for splitting, weighted by the squared

improvement to the models as a result of each split, averaged over all trees. The relative importance of each variable is scaled so that the sum adds to 100, with higher numbers indicating stronger influence on the modeled response. The final BRT models were pruned by using a combination of VRI scores and partial dependency responses following the approach outlined by (Elith et al. 2008) to avoid over fitting. In the final model, I included only those response variables with a 10% or greater VRI score identified by any of the candidate models.

Laboratory Experiment

Based on the results of the BRT analysis, which revealed a potential relationship between Bd presence and eugelnoid density, I conducted a common garden experiment to examine whether Bd parasitizes euglenoids in culture. I selected the two euglenoid species that were commercially available in pure culture, *Chlamydomonas richarsonii* (+ and -) and *Euglena gracilis* (Carolina Biological Supply). Using sterile technique, I harvested bacteria-free culture slants of *C. richarsonii* (+ and -) and *E. gracilis* and introduced them to a sterile 50-ml falcon tube with 15-ml of 0.2% tryptone. I inoculated the tubes with 100,000 Bd zoospores (JEL 427), quantified using a cell counter, then vortexed each tube for 10 seconds to homogenize the samples and break up colonies. Tubes were incubated at 18-20 C under a 12-hour light/dark cycle. Samples from each tube were harvested (300 uL) daily for four days, placed in clean ceramic well plates, stained with 15-uL 1% Congo Red, and scanned under a compound microscope at 400x and 1000x.

Results

In the final BRT models examining ecological associations with Bd detection (presence) in amphibian breeding habitats, I retained five variables: amphibian density, season, total organic carbon, euglenoid density, and cryptophyte density. Total organic carbon had the highest VRI score (40%), followed by eugelnoid density (30.5%), amphibian density (12%), season (11%), and cryptophyte density (6.5%). The partial dependency plots revealed the response per variable, where the y-axis is the fitted function of Bd presence in amphibian breeding habitats, based on the individual effect of each explanatory variable (Figure 3.2). Bd detection was related to low organic carbon (<7.3 mg/L), and high euglenoid density (>116). In all candidate models, the VRI score for Bd-positive amphibians was <1%. There was no correlation among variables (amphibian density, Bd-positive animals, season, total organic carbon, euglenoid density, and cryptophyte density) in the final model (r^2 = <0.10).

I observed differences in Bd detection between amphibians and their breeding habitats, where detection in amphibians was lower. Bd was detected in amphibians at 8 of 16 (50%) sites during 15 of 53 (28%) sampling events (GE range = 0.06-608, mean = 19, median = 0.95). In amphibians, 136 of 1200 (11%) individuals tested positive for Bd including American bullfrog (*Lithobates catesbeianus*, n=121), rough-skinned newt (*Taricha granulosa*, n=9), long-toed salamander (*Ambystoma macrodactylum*, n=5), and Pacific chorus frog (*Pseudacris regilla*, n=1). Bd was not detected in Northwestern salamander (*Ambystoma gracile*) or Red-legged frog (*Rana aurora*). Bd was detected in

amphibians in all seasons, with the highest number of Bd detections in autumn, September-November (Figure 3.3). Amphibians were detected at least once at all but one site, and amphibian species richness (range 0-5) varied among seasons and sites. There were no sites where all six possible species were detected. Generally fewer than 100 animals were captured per visit (mean 43, median 6), but there were two sampling events in the autumn where >400 animals, primarily bullfrog larvae, were captured. In water collected from amphibian habitats, Bd was detected at 14 of 16 (88%) sites sampled during 24 of 53 (55%) sampling events (GE range 0.03-1072 GE/L of water sampled, mean 160 GE/L, median 15 GE/L). Bd density in water achieved the highest densities in the summer months (June-August), but was highly variable among seasons and sites (Figure 3.4).

I observed relatively little variation in the total dissolved nitrogen, total nitrogen, ammonium, total phosphorous, organophosphorus, and total organic carbon among seasons (Figure 3.5). Bd density was generally highest when total organic carbon was less than 10 mg/L, regardless of season (Figure 3.6). Nitrate and nitrite were highest in the winter months. In phytoplankton assemblages, density varied by season and taxonomic group (Figure 3.7). High densities were observed for: bluegreen algae in the summer months; chrysophytes in spring and autumn; cryptophytes in summer and autumn; dinoflagellates in autumn; and eugelnoids in summer and autumn. Diatom and green algae densities were high in all seasons. Bd density was generally highest when eugelnoid density was also high, regardless of season (Figure 3.8).

In the laboratory experiment, I observed Bd zoospores on the surface of *Chlamydomonas richarsonii* two and three days following inoculation. The zoospores appeared to be in the beginning stages of encystment (Figures 3.9 and 3.10) but parasitism was inconclusive.

Discussion

Examination of Bd ecology separate from amphibian hosts is a relatively new frontier of inquiry. In general, there are many aspects of chytrid ecology that are not well understood, particularly the effects of the physical and biological environment on population size, and interactions between parasitic chytrids and their hosts (Gleason et al. 2008). Some chytrids are efficient in converting inorganic compounds into organic compounds (Gleason et al. 2008) and many species are observed growing in field conditions with very little organic matter (Digby et al. 2010). In this study, the top two variables identified in the models examining associations of environmental metrics with free-living Bd detection were total organic carbon concentration and euglenoid density, which had a combined VRI score of over 70%. Inorganic nutrient concentrations were otherwise unimportant. Total organic carbon levels remained relatively consistent year round (mean = 10.93 mg/L, median = 8.50 mg/L), but Bd detection was highest when total organic carbon was less than the mean or median concentrations (<7.3 mg/L; Figure 3.6). Depleted organic carbon suggests reduced levels of organic nitrogen, which can limit fungi occurrence and growth (Digby et al. 2010), however, I did not find an

association with the nitrogen species I examined. If Bd growth is optimal in conditions with low organic carbon in the aquatic environment, Bd may be flexible in how it accesses nutrients in the saprobic life stage, and may be able to assimilate both organic and inorganic matter to obtain nutrients. This strategy would allow Bd to persist in diverse habitats when amphibian hosts are absent, and could offer an explanation for its catholic global distribution.

My analysis also found Bd detection was related to high euglenoid density. Euglenoids are flagellates common in freshwater systems, especially those high in organic matter. Many are autotrophic, producing energy through photosynthesis, although some species are heterotrophs, obtaining energy through phagocytosis or diffusion (Kiss et al. 1987). Among chytrid taxa more broadly, chytrid-phytoplankton parasitism is a well-documented phenomenon, and larger phytoplankton are often more heavily infected (Lefevre et al. 2012, Rasconi et al. 2012). In this study, euglenoid density was highest in summer and autumn, although its relative contribution to the overall phytoplankton density in these months was small, 4.3% and 19% respectively; Bd density was also highest in the summer (Figure 3.8). The common garden experiment provided suggestive evidence that Bd may interact directly with euglenoids (Figures 3.9 and 3.10). In the aquatic environment, phytoplankton could serve as an alternative host for Bd in the absence of amphibian hosts, where Bd could function as either a parasite or a saprobe. Hp, closest known relative of Bd, is an aquatic saprobe (Longcore et al. 2011), yet little is known about the strategies it utilizes to survive and grow saprobically in the aquatic

environment, or if it shares these traits with Bd. It is possible that Bd may be exploiting euglenoids as a food source in wetlands, especially as the availability of amphibian hosts decreases in the summer and fall as amphibian young-of-the-year metamorphose and move to terrestrial habitats. If Bd was using phytoplankton opportunistically in these study sites, I would expect it to use larger and more abundant phytoplankton species, although the cell structure, protein or carbohydrate synthesis processes, and other aspects of eugleniod biology may be relevant. It is possible that Bd has a specialized relationship with euglenoids in the study area, though the nature of the relationship is unknown. The three common eugleniod species detected in the study area are relatively small in size (*Euglena* spp. 50-70 um, *Phacus* spp. 32-50 um, *Trachelomonas* spp. 12-25 um; Olenina 2006)), especially when compared to the size of Bd (zoospore 3-5 um, zoosporangium 40 um, (Longcore et al. 1999). Additionally, the overall eugelnoid density I observed during the study was low compared to other phytoplankton taxa present (mean = 7.5%, median = 1.4%), suggesting a more specific relationship with Bd. This observation and the specific interaction between Bd and euglenoids warrants additional research. Genomic evidence suggests Bd has an obligate rather than an opportunistic association with amphibian hosts (Farrer et al. 2013), but this does not preclude Bd from utilizing non-amphibian hosts (McMahon et al. 2013). In amphibians, Bd parasitizes keratinized structures (Marantelli et al. 2004), which are lacking in euglenoids. It is not clear from these results whether the nature of the relationship between Bd and euglenoids could be saprobic or parasitic, or possibly spurious. Several chytrid species are considered promiscuous parasites (Paterson 1956, 1958, Gromov et al. 1999), and this may be true of Bd. Additionally, there is

emerging evidence that divergent eukaryotic pathogens (e.g., the malaria parasite *Plasmodium falciparum* and the plant pathogen *Phytophthora infestans*) share common mechanisms of pathogenicity and use equivalent host-targeting signals (Haldar et al. 2006); these shared features may provide opportunities for pathogens to shift host species and taxa.

Variation in host susceptibility, host response, and in the occurrence and density of free-living Bd in the environment make this a complex disease system to unravel. Bd infects multiple amphibian host species: 695 of 1377 (50%) global amphibian species tested Bd-positive (Olson and Ronnenberg 2014). Some species are more efficient hosts and can maintain high Bd infection loads without mortality (Searle et al. 2011, Gervasi et al. 2013a), which may influence Bd occurrence and density in the environment. For example, the American bullfrog, a frog species native to the eastern U.S., was introduced to many locations throughout the world for commercial uses and the pet trade beginning in the early 1900s (Moyle 1973, Hayes and Jennings 1986, Ficetola et al. 2007, Bai et al. 2010). Bullfrogs are considered a reservoir host for Bd (Daszak et al. 2004, Garner et al. 2006, Schloegel et al. 2010), and Bd occupancy of lentic-breeding sites increases when they are present (Adams et al. 2010). In this study, bullfrogs accounted for 89% of Bd detections in amphibians. Although it is important to note that American bullfrogs are not universal carriers and can exhibit symptoms of chytridiomycosis, including mortality, when infected with some Bd strains (Gervasi et al. 2013b). Pacific chorus frogs can maintain high Bd infection loads without succumbing to the disease, and some have

suggested they may be a reservoir host in parts of their native range (Reeder et al. 2012, Gervasi et al. 2013a). Less than 1% of the amphibians that tested Bd-positive in this study were Pacific chorus frogs, which suggests that bullfrogs may be a more important reservoir host for Bd in this system.

Within the broader geographic region that includes the study area, Bd is widespread in amphibians and their breeding habitats (Pearl et al. 2007, Chestnut et al. 2014). Die-off events attributed to Bd in the region have not been reported in the literature, and amphibian populations appear to persist with relatively high Bd prevalence (Pearl et al. 2007, 2009, Deguise and Richardson 2009, Adams et al. 2010, Padgett-Flohr and Hayes 2011). This may be related to amphibian co-evolution with Bd strains present in the region. It is likely that Bd is endemic in some parts of its range (Rosenblum et al. 2013). Two Bd strains isolated from Oregon (JEL 626 and JEL 630) are in the GPL-1 clade, a lineage found primarily in North America (Schloegel et al. 2012), which may support the hypothesis that Bd is endemic to the study area.

Bd exhibits seasonality in amphibians and their aquatic habitats. In amphibians, Bd prevalence is reported to be highest in the spring and lowest in the summer and fall (Kriger and Hero 2006, Adams et al. 2010, Schmidt et al. 2013, Chestnut et al. 2014). In amphibian habitats, Bd density was also reported highest in the spring, however, increases in density were observed in summer, fall, and winter months (Chestnut et al. 2014). In this study, I detected Bd in amphibians in all seasons, and the highest number

of detections occurred in the fall (Figure 3.3), which is a new finding compared to previous studies. Bd density in water was highest in the summer months (Figure 3.4). Bd detection in amphibians from swabs in this study (11%) mirrored detection from a previous study conducted within part of the study area (11.4%, (Adams et al. 2010). However, host life stage is an important consideration when interpreting these results. The amphibian sampling methodology (aquatic funnel traps) was biased toward capturing larval and aquatic (e.g., neotenic adult) amphibian life stages. The probability of detecting Bd in larval amphibians is lower than in other life stages (Adams et al. 2010), therefore, it is likely that Bd prevalence is underestimated in the multi-life stages of amphibian populations in the study area.

This study demonstrates that both abiotic and biotic factors independent of amphibians relate to Bd occurrence in aquatic settings. Understanding the ecology of this pathogen outside of amphibian hosts, as well as factors related to disease persistence and transmission, is vital to unraveling this system and informing conservation decisions. Extinction events attributed solely to disease rarely occur in systems where transmission is density-dependent, and are often explained by frequency-dependent disease transmission, or a combination of the two (McCallum 2012). The amphibian-Bd model is an example where Bd exposure to amphibians may occur by either density-dependent transmission (e.g., contact with an infected individual) or frequency-dependent transmission (e.g., contact with free-living Bd in the environment). In systems where disease transmission occurs through both density-dependent and frequency-dependent

exposure pathways, even small amounts of frequency-dependent exposure can drive populations to extinction (Ryder et al. 2007). Additionally, environmental factors including habitat alteration and chemical contaminants can influence disease dynamics by decreasing host immune response which can result in increased susceptibility to a pathogen (Nürnberger et al. 2004, Loker 2012, Hua and Relyea 2014).

The patterns and processes influencing Bd occurrence in hosts and the environment may be unique to the region where it occurs, and may vary over space and time. Amphibian response to the pathogen may depend on the number and diversity of strains within a site, whether Bd strains are introduced or endemic, if non-native hosts or reservoirs are introduced, and the time since new strains or hosts were introduced. Multiple Bd strains can be present in a single site; for example, JEL626 and JEL630 were recovered from different frogs collected at the same time from a small wetland in the Willamette Valley, Oregon (Schloegel et al. 2012). Bd exhibits great diversity in its phenotypic responses to environmental stress (Woodhams et al. 2008), and apparent rapid rates of adaptive evolution, which may explain its success in infecting a diversity of hosts across biomes (Farrer et al. 2013). Widespread genomic variation within and amongst Bd isolates is apparent, and rapid mutations occur during recombination that can generate genome diversity within the timescale of a single host infection (Farrer et al. 2013). This highlights the importance of avoiding generalizations regarding Bd ecology, and considering its potential ecologic and epidemiological plasticity. Such variation needs consideration in host management and conservation planning, as Bd patterns across

space and time may change faster than policy implementation timelines. This is especially important in our current age of globalization, where the movement of people, water, and wildlife may occur at a rapid pace. A key component in early detection and rapid response to EID events is initiating surveillance systems with adequate diagnostic tools and coordination at local, regional, national, and global scales.

Literature Cited

Adams, M. J., N. D. Chelgren, D. Reinitz, R. A. Cole, L. J. Rachowicz, S. Galvan, B. McCreary, C. A. Pearl, L. L. Bailey, and J. Bettaso. 2010. Using occupancy models to understand the distribution of an amphibian pathogen, *Batrachochytrium dendrobatidis*. Ecological Applications 20:289–302.

Alker, A. P., G. W. Smith, and K. Kim. 2001. Characterization of *Aspergillus sydowii* (Thom et Church), a fungal pathogen of Caribbean sea fan corals. Hydrobiologia 460:105–111.

Anagnostakis, S. L. 1987. Chestnut Blight: The classical problem of an introduced pathogen. Mycologia 79:23–37.

Anderson, R. M., R. M. May, and others. 1979. Population biology of infectious diseases: Part I. Nature 280:361–367.

Bai, C., T. W. Garner, and Y. Li. 2010. First evidence of *Batrachochytrium dendrobatidis* in China: discovery of chytridiomycosis in introduced American bullfrogs and native amphibians in the Yunnan Province, China. EcoHealth 7:127–134.

Bancroft, B. A., B. A. Han, C. L. Searle, L. M. Biga, D. H. Olson, L. B. Kats, J. J. Lawler, and A. R. Blaustein. 2011. Species-level correlates of susceptibility to the pathogenic amphibian fungus *Batrachochytrium dendrobatidis* in the United States. Biodiversity and Conservation 20:1911–1920.

Berger, L., R. Speare, H. B. Hines, G. Marantelli, A. D. Hyatt, K. R. McDonald, L. F. Skerratt, V. Olsen, J. M. Clarke, and G. Gillespie. 2004. Effect of season and temperature on mortality in amphibians due to chytridiomycosis. Australian Veterinary Journal 82:434–439.

Best, A., S. Webb, J. Antonovics, and M. Boots. 2012. Local transmission processes and disease-driven host extinctions. Theoretical Ecology 5:211–217.

Blaustein, A. R., J. M. Romansic, E. A. Scheessele, B. A. Han, A. P. Pessier, and J. E. Longcore. 2005. Interspecific variation in susceptibility of frog tadpoles to the pathogenic fungus *Batracochytrium dendrobatidis*. Conservation Biology 19:1460–1468.

Boddy, L., R. Marchant, and D. J. Read. 2011. Nitrogen, Phosphorus and Sulphur Utilisation by Fungi. British Mycological Society, University of Birmingham. Cambridge University Press. 330p. ISBN: 9780521106245

Bosch, J., I. Martínez-Solano, and M. García-París. 2001. Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. Biological Conservation 97:331–337.

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

Bradford, D. F. 1991. Mass mortality and extinction in a high-elevation population of *Rana muscosa*. Journal of Herpetology 25:174–177.

Breiman, L. 2001. Random Forests. Machine Learning 45:5–32.

De Castro, F., and B. Bolker. 2005. Mechanisms of disease-induced extinction. Ecology Letters 8:117–126.

Cheng, T. L., S. M. Rovito, D. B. Wake, and V. T. Vredenburg. 2011. Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. Proceedings of the National Academy of Sciences 108:9502–9507.

Chestnut, T., C. Anderson, R. Popa, A. R. Blaustein, M. Voytek, D. H. Olson, and J. Kirshtein. 2014. Heterogeneous occupancy and density estimates of the pathogenic fungus *Batrachochytrium dendrobatidis* in waters of North America. PLoS ONE 9:e106790.

Collins, J. P. 2010. Amphibian decline and extinction: What we know and what we need to learn. Diseases of Aquatic Organisms 92:93–99.

Collins, J. P., M. L. Crump, and T. E. Lovejoy III. 2009. Extinction in Our Times: Global Amphibian Decline: Global Amphibian Decline. Oxford University Press, USA.

Collins, J. P., and A. Storfer. 2003. Global amphibian declines: sorting the hypotheses. Diversity and Distributions 9:89–98.

Daszak, P., A. Strieby, A. A. Cunningham, J. E. Longcore, C. C. Brown, and D. Porter. 2004. Experimental evidence that the bullfrog (*Rana catesbeiana*) is a potential carrier of chytridiomycosis, an emerging fungal disease of amphibians. Herpetological Journal 14:201–207.

De'ath, G. 2007. Boosted trees for ecological modeling and prediction. Ecology 88:243–251.

Deguise, I., and J. S. Richardson. 2009. Prevalence of the chytrid fungus (*Batrachochytrium dendrobatidis*) in western toads in southwestern British Columbia, Canada. Northwestern Naturalist 90:35–38.

Desprez-Loustau, M.-L., C. Robin, M. Buée, R. Courtecuisse, J. Garbaye, F. Suffert, I. Sache, and D. M. Rizzo. 2007. The fungal dimension of biological invasions. Trends in Ecology & Evolution 22:472–480.

Digby, A. L., F. H. Gleason, and P. A. McGee. 2010. Some fungi in the Chytridiomycota can assimilate both inorganic and organic sources of nitrogen. Fungal Ecology 3:261–266.

Dobson, A., and J. Foufopoulos. 2001. Emerging infectious pathogens of wildlife. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences 356:1001–1012.

Drew, A., E. J. Allen, and L. J. S. Allen. 2006. Analysis of climatic and geographic factors affecting the presence of chytridiomycosis in Australia. Diseases of Aquatic Organisms 68:245.

Dutech, C., B. Barrès, J. Bridier, C. Robin, M. G. Milgroom, and V. Ravigné. 2012. The chestnut blight fungus world tour: successive introduction events from diverse origins in an invasive plant fungal pathogen. Molecular Ecology 21:3931–3946.

Elith, J., J. R. Leathwick, and T. Hastie. 2008. A working guide to boosted regression trees. Journal of Animal Ecology 77:802–813.

Euliss, N. H., J. W. LaBaugh, L. H. Fredrickson, D. M. Mushet, M. K. Laubhan, G. A. Swanson, T. C. Winter, D. O. Rosenberry, and R. D. Nelson. 2004. The wetland continuum: A conceptual framework for interpreting biological studies. Wetlands 24:448–458.

Farrer, R. A., D. A. Henk, T. W. J. Garner, F. Balloux, D. C. Woodhams, and M. C. Fisher. 2013. Chromosomal copy number variation, selection and uneven rates of recombination reveal cryptic genome diversity linked to pathogenicity. PLoS Genet 9:e1003703.

Ficetola, G. F., C. Coïc, M. Detaint, M. Berroneau, O. Lorvelec, and C. Miaud. 2007. Pattern of distribution of the American bullfrog *Rana catesbeiana* in Europe. Biological Invasions 9:767–772.

Franklin, J. F., and C. T. Dyrness. 1988. Natural vegetation of Oregon and Washington. Portland, OR., Pacific Northwest Forest and Range Experiment Station, Forest Service, U.S. Dept. of Agriculture.

Frick, W. F., J. F. Pollock, A. C. Hicks, K. E. Langwig, D. S. Reynolds, G. G. Turner, C. M. Butchkoski, and T. H. Kunz. 2010. An emerging disease causes regional population collapse of a common North American bat species. Science 329:679–682.

Friedman, J. H. 2001. Greedy function approximation: a gradient boosting machine. Annals of Statistics:1189–1232.

Garcia, T. S., J. M. Romansic, and A. R. Blaustein. 2006. Survival of three species of anuran metamorphs exposed to UV-B radiation and the pathogenic fungus *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms 72:163.

Garner, T. W., M. W. Perkins, P. Govindarajulu, D. Seglie, S. Walker, A. A. Cunningham, and M. C. Fisher. 2006. The emerging amphibian pathogen *Batrachochytrium dendrobatidis* globally infects introduced populations of the North American bullfrog, *Rana catesbeiana*. Biology Letters 2:455–459.

Gerber, L. R., H. McCallum, K. D. Lafferty, J. L. Sabo, and A. Dobson. 2005. Exposing extinction risk analysis to pathogens: Is disease just another form of density dependence? Ecological Applications 15:1402–1414.

Gervasi, S., C. Gondhalekar, D. H. Olson, and A. R. Blaustein. 2013a. Host identity matters in the amphibian-*Batrachochytrium dendrobatidis* system: Fine-scale patterns of variation in responses to a multi-host Pathogen. Plos One 8:e54490.

Gervasi, S. S., J. Urbina, J. Hua, T. Chestnut, R. A. Relyea, and A. R. Blaustein. 2013b. Experimental evidence for American bullfrog (*Lithobates catesbeianus*) susceptibility to chytrid fungus (*Batrachochytrium dendrobatidis*). EcoHealth:1–6.

Gleason, F. H., M. Kagami, E. Lefevre, and T. Sime-Ngando. 2008. The ecology of chytrids in aquatic ecosystems: roles in food web dynamics. Fungal Biology Reviews 22:17–25.

Gromov, B. V., A. V. Plujusch, and K. A. Mamkaeva. 1999. Morphology and possible host range of *Rhizophydium algavorum* sp. nov.(Chytridiales)—an obligate parasite of algae. Protistology 1:62–65.

Haldar, K., S. Kamoun, N. L. Hiller, S. Bhattacharje, and C. van Ooij. 2006. Common infection strategies of pathogenic eukaryotes. Nature Reviews Microbiology 4:922–931.

Han, B. A., C. L. Searle, and A. R. Blaustein. 2011. Effects of an infectious fungus, *Batrachochytrium dendrobatidis*, on amphibian predator-prey interactions. PLoS One 6:e16675.

Hayes, M. P., and M. R. Jennings. 1986. Decline of ranid frog species in western North America: Are bullfrogs (*Rana catesbeiana*) responsible? Journal of Herpetology 20:490.

Hua, J., and R. Relyea. 2014. Chemical cocktails in aquatic systems: Pesticide effects on the response and recovery of >20 animal taxa. Environmental Pollution 189:18–26.

Olenina, S. H. 2006. Biovolumes and size-classes of phytoplankton in the Baltic Sea. Baltic Sea Environment Proceedings, 144 p. ISSN 0357-2994

James, T. Y., A. P. Litvintseva, R. Vilgalys, J. A. T. Morgan, J. W. Taylor, M. C. Fisher, L. Berger, C. Weldon, L. du Preez, and J. E. Longcore. 2009. Rapid global expansion of the fungal disease chytridiomycosis into declining and healthy amphibian populations. PLoS Pathogens 5:e1000458.

Jennings, D. H. 1995. The Physiology of Fungal Nutrition. Cambridge University Press.

Jones, K. E., N. G. Patel, M. A. Levy, A. Storeygard, D. Balk, J. L. Gittleman, and P. Daszak. 2008. Global trends in emerging infectious diseases. Nature 451:990–993.

Joneson, S., J. E. Stajich, S.-H. Shiu, and E. B. Rosenblum. 2011. Genomic transition to pathogenicity in chytrid fungi. PLoS Pathogens 7:e1002338.

Karns, D. R. 1992. Effects of acidic bog habitats on amphibian reproduction in a northern Minnesota peatland. Journal of herpetology:401–412.

Kirshtein, J. D., C. W. Anderson, J. S. Wood, J. E. Longcore, and M. A. Voytek. 2007. Quantitative PCR detection of *Batrachochytrium dendrobatidis* DNA from sediments and water. Diseases of Aquatic Organisms 77:11-15.

Kiss, J. Z., A. C. Vasconcelos, and R. E. Triemer. 1987. Structure of the euglenoid storage carbohydrate, paramylon. American Journal of Botany 74:877.

Kriger, K. M., and J.-M. Hero. 2007a. The chytrid fungus *Batrachochytrium dendrobatidis* is non-randomly distributed across amphibian breeding habitats. Diversity and Distributions 13:781–788.

Kriger, K. M., and J.-M. Hero. 2007b. Large-scale seasonal variation in the prevalence and severity of chytridiomycosis. Journal of Zoology 271:352–359.

Lefevre, E., P. M. Letcher, and M. J. Powell. 2012. Temporal variation of the small eukaryotic community in two freshwater lakes: emphasis on zoosporic fungi. Aquatic Microbial Ecology 67:91–105.

Lips, K. R., F. Brem, R. Brenes, J. D. Reeve, R. A. Alford, J. Voyles, C. Carey, L. Livo, A. P. Pessier, and J. P. Collins. 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proceedings of the National Academy of Sciences of the United States of America 103:3165–3170.

Lips, K. R., P. A. Burrowes, J. R. Mendelson, and G. Parra-Olea. 2005. Amphibian declines in Latin America: Widespread population declines, extinctions, and impacts. Biotropica 37:163–165.

Loker, E. S. 2012. Macroevolutionary immunology: a role for immunity in the diversification of animal life. Frontiers in Immunology 3:25.

Longcore, J. E., P. M. Letcher, and T. Y. James. 2011. *Homolaphlyctis polyrhiza* gen. et sp nov., a species in the Rhizophydiales (Chytridiomycetes) with multiple rhizoidal axes. Mycotaxon 118:433–440.

Longcore, J. E., A. P. Pessier, and D. K. Nichols. 1999. *Batrachochytrium dendrobatidis* gen et sp nov, a chytrid pathogenic to amphibians. Mycologia 91:219–227.

Marantelli, G., L. Berger, R. Speare, and L. Keegan. 2004. Distribution of the amphibian chytrid *Batrachochytrium dendrobatidis* and keratin during tadpole development. Pacific Conservation Biology 10:173.

La Marca, E., K. R. Lips, S. Lötters, R. Puschendorf, R. Ibáñez, J. V. Rueda-Almonacid, R. Schulte, C. Marty, F. Castro, and J. Manzanilla-Puppo. 2005. Catastrophic population declines and extinctions in Neotropical harlequin frogs (Bufonidae: Atelopus). Biotropica 37:190–201.

May, R. M., and R. M. Anderson. 1979. Population biology of infectious diseases: Part II. Nature 280:455–461.

McCallum, H. 2012. Disease and the dynamics of extinction. Philosophical Transactions of the Royal Society B: Biological Sciences 367:2828–2839.

McCallum, H., and A. Dobson. 1995. Detecting disease and parasite threats to endangered species and ecosystems. Trends in Ecology & Evolution 10:190–194.

McCallum, M. L. 2007. Amphibian decline or extinction? Current declines dwarf background extinction rate. Journal of Herpetology 41:483–491.

McMahon, T. A., L. A. Brannelly, M. W. H. Chatfield, P. T. J. Johnson, M. B. Joseph, V. J. McKenzie, C. L. Richards-Zawacki, M. D. Venesky, and J. R. Rohr. 2013. Chytrid fungus *Batrachochytrium dendrobatidis* has nonamphibian hosts and releases chemicals

that cause pathology in the absence of infection. Proceedings of the National Academy of Sciences 110:210–215.

Moulton, S. R., J. G. Kennen, R. M. Goldstein, and J. A. Hambrook. 2002. Revised Protocols for Sampling Algal, Invertebrate, and Fish Communities as Part of the National Water-Quality Assessment Program. United States Geological Survey. Available online: http://pubs.usgs.gov/of/2002/ofr-02-150/

Moyle, P. B. 1973. Effects of Introduced Bullfrogs, *Rana catesbeiana*, on the Native Frogs of the San Joaquin Valley, California. Copeia 1973:18.

Murray, K. A., L. F. Skerratt, R. Speare, and H. McCallum. 2009. Impact and dynamics of disease in species threatened by the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*. Conservation Biology 23:1242–1252.

Muths, E., P. Stephen Corn, A. P. Pessier, and D. Earl Green. 2003. Evidence for disease-related amphibian decline in Colorado. Biological Conservation 110:357–365.

Nürnberger, T., F. Brunner, B. Kemmerling, and L. Piater. 2004. Innate immunity in plants and animals: striking similarities and obvious differences. Immunological Reviews 198:249–266.

Olson, D. H., D. M. Aanensen, K. L. Ronnenberg, C. I. Powell, S. F. Walker, J. Bielby, T. W. Garner, G. Weaver, and M. C. Fisher. 2013. Mapping the global emergence of *Batrachochytrium dendrobatidis*, the amphibian chytrid fungus. PLoS One 8:e56802.

Olson, D. H., W. P. Leonard, and R.B. Bury. 1997. Sampling Amphibians in Lentic habitats. Northwest Fauna 4:1–21.

Olson, D. H., and K. L. Ronnenberg. 2014. Global Bd Mapping Project: 2014 Update. Froglog 111:17–21.

Padgett-Flohr, G. E., and M. P. Hayes. 2011. Assessment of the vulnerability of the Oregon spotted frog (*Rana pretiosa*) to the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*). Herpetological Conservation and Biology 6:99–106.

Parris, M. J., and T. O. Cornelius. 2004. Fungal pathogen causes competitive and developmental stress in larval amphibian communities. Ecology 85:3385–3395.

Paterson, R. A. 1956. Additions to the phycomycete flora of the Douglas Lake region. II. New chytridiaceous fungi. Mycologia:270–277.

Paterson, R. A. 1958. On the planktonic chytrids *Zygorhizidium melosirae* Canter and *Z. planktonicum* Canter. Transactions of the British Mycological Society 41:457–460.

Pearl, C. A., J. Bowerman, M. J. Adams, and N. D. Chelgren. 2009. Widespread occurrence of the chytrid fungus *Batrachochytrium dendrobatidis* on Oregon spotted frogs (*Rana pretiosa*). EcoHealth 6:209–218.

Pearl, C. A., E. L. Bull, D. E. Green, J. Bowerman, M. J. Adams, A. Hyatt, and W. H. Wente. 2007. Occurrence of the amphibian pathogen *Batrachochytrium dendrobatidis* in the Pacific Northwest. Journal of Herpetology 41:145–149.

Pilliod, D. S., E. Muths, R. D. Scherer, P. E. Bartelt, P. S. Corn, B. R. Hossack, B. A. Lambert, R. McCaffery, and C. Gaughan. 2010. Effects of amphibian chytrid fungus on individual survival probability in wild boreal toads. Conservation Biology 24:1259–1267.

Piotrowski, J. S., S. L. Annis, and J. E. Longcore. 2004. Physiology of *Batrachochytrium dendrobatidi*s, a chytrid pathogen of amphibians. Mycologia 96:9–15.

Pounds, A.J., M. R. Bustamante, L. A. Coloma, J. A. Consuegra, M. P. L. Fogden, P. N. Foster, E. La Marca, K. L. Masters, A. Merino-Viteri, R. Puschendorf, S. R. Ron, G. A. Sánchez-Azofeifa, C. J. Still, and B. E. Young. 2006. Widespread amphibian extinctions from epidemic disease driven by global warming. Nature 439:161–167.

Pounds, J. A., and M. L. Crump. 1994. Amphibian declines and climate disturbance: the case of the golden toad and the harlequin frog. Conservation Biology 8:72–85.

Rachowicz, L. J., R. A. Knapp, J. A. Morgan, M. J. Stice, V. T. Vredenburg, J. M. Parker, and C. J. Briggs. 2006. Emerging infectious disease as a proximate cause of amphibian mass mortality. Ecology 87:1671–1683.

Rasconi, S., N. Niquil, and T. Sime-Ngando. 2012. Phytoplankton chytridiomycosis: community structure and infectivity of fungal parasites in aquatic ecosystems. Environmental Microbiology 14:2151–2170.

Reeder, N.M.M, A.P. Pessier, and V.T. Vredenburg. 2012. A reservoir species for the emerging amphibian pathogen *Batrachochytrium dendrobatidis* thrives in a landscape decimated by disease. PLoS One 7:e33567.

Retallick, R. W., H. McCallum, and R. Speare. 2004. Endemic infection of the amphibian chytrid fungus in a frog community post-decline. PLoS Biology 2:e351.

Rosenblum, E. B., T. Y. James, K. R. Zamudio, T. J. Poorten, D. Ilut, D. Rodriguez, J. M. Eastman, K. Richards-Hrdlicka, S. Joneson, T. S. Jenkinson, J. E. Longcore, G. Parra Olea, L. F. Toledo, M. L. Arellano, E. M. Medina, S. Restrepo, S. V. Flechas, L. Berger, C. J. Briggs, and J. E. Stajich. 2013. Complex history of the amphibian-killing chytrid

fungus revealed with genome resequencing data. Proceedings of the National Academy of Sciences 110:9385-9390.

Rudolf, V. H., and J. Antonovics. 2005. Species coexistence and pathogens with frequency-dependent transmission. The American Naturalist 166:112–118.

Ryder, J. J., M. R. Miller, A. White, R. J. Knell, and M. Boots. 2007. Host-parasite population dynamics under combined frequency- and density-dependent transmission. Oikos 116:2017–2026.

Rypien, K. L., J. P. Andras, and C. D. Harvell. 2008. Globally panmictic population structure in the opportunistic fungal pathogen *Aspergillus sydowii*. Molecular Ecology 17:4068–4078.

Sadinski, W. J., and W. A. Dunson. 1992. A multilevel study of effects of low pH on amphibians of temporary ponds. Journal of Herpetology:413–422.

Schloegel, L. M., C. M. Ferreira, T. Y. James, M. Hipolito, J. E. Longcore, A. D. Hyatt, M. Yabsley, A. M. C. R. P. F. Martins, R. Mazzoni, A. J. Davies, and P. Daszak. 2010. The North American bullfrog as a reservoir for the spread of *Batrachochytrium dendrobatidi*s in Brazil. Animal Conservation 13:53–61.

Schloegel, L. M., L. F. Toledo, J. E. Longcore, S. E. Greenspan, C. A. Vieira, M. Lee, S. Zhao, C. Wangen, C. M. Ferreira, M. Hipolito, A. J. Davies, C. A. Cuomo, P. Daszak, and T. Y. James. 2012. Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. Molecular Ecology 21:5162–5177.

Schmidt, B. R., M. Kéry, S. Ursenbacher, O. J. Hyman, and J. P. Collins. 2013. Site occupancy models in the analysis of environmental DNA presence/absence surveys: a case study of an emerging amphibian pathogen. Methods in Ecology and Evolution 4:646–653.

Searle, C. L., S. S. Gervasi, J. Hua, J. I. Hammond, R. A. Relyea, D. H. Olson, and A. R. Blaustein. 2011. Differential host susceptibility to *Batrachochytrium dendrobatidis*, an emerging amphibian pathogen. Conservation Biology 25:965–974.

Shapard, E. J., A. S. Moss, and M. J. San Francisco. 2012. *Batrachochytrium dendrobatidis* can infect and cause mortality in the nematode *Caenorhabditis elegans*. Mycopathologia 173:121–126.

Skerratt, L. F., L. Berger, H. B. Hines, K. R. McDonald, D. Mendez, and R. Speare. 2008. Survey protocol for detecting chytridiomycosis in all Australian frog populations. Diseases of Aquatic Organisms 80:85–94.

Taylor, C., and S. Gurr. 2014. Fungal pathogenesis: Past, present and future. Fungal Biology Reviews 28:24–28.

Thrall, P. H., and J. J. Burdon. 1997. Host-pathogen dynamics in a metapopulation context: The ecological and evolutionary consequences of being spatial. The Journal of Ecology 85:743.

Tucker, C. J., K. Yager, A. Anyamba, and K. J. Linthicum. 2011. Climate Change, Extreme Weather Events, and Fungal Disease Emergence and Spread. Fungal Diseases: An Emerging Threat to Human, Animal, and Plant Health: Workshop Summary. http://ntrs.nasa.gov/archive/nasa/casi.ntrs.nasa.gov/20110012851.pdf

U.S. Geological Survey. 2010. National field manual for the collection of water-quality data: U.S. Geological Survey Techniques of Water-Resources Investigations, book 9, chaps. A1-A9. US Department of the Interior, US Geological Survey, Online only, http://pubs.water.usgs.gov/twri9A.

Voyles, J., L. R. Johnson, C. J. Briggs, S. D. Cashins, R. A. Alford, L. Berger, L. F. Skerratt, R. Speare, and E. B. Rosenblum. 2012. Temperature alters reproductive life history patterns in *Batrachochytrium dendrobatidis*, a lethal pathogen associated with the global loss of amphibians. Ecology and Evolution 2:2241–2249.

Woodhams, D. C., R. A. Alford, C. J. Briggs, M. Johnson, and L. A. Rollins-Smith. 2008. Life-history trade-offs influence disease in changing climates: strategies of an amphibian pathogen. Ecology 89:1627–1639.

Woolhouse, M. E. J. 2002. Population biology of emerging and re-emerging pathogens. Trends in Microbiology 10:s3–s7.

Woolhouse, M. E. J., L. H. Taylor, and D. T. Haydon. 2001. Population biology of multihost pathogens. Science 292:1109–1112.

Wyman, R. L., and J. Jancola. 1992. Degree and scale of terrestrial acidification and amphibian community structure. Journal of Herpetology:392–401.

Young, B. E., K. R. Lips, J. K. Reaser, R. Ibáñez, A. W. Salas, J. R. Cedeño, L. A. Coloma, S. Ron, E. La Marca, and J. R. Meyer. 2001. Population declines and priorities for amphibian conservation in Latin America. Conservation Biology 15:1213–1223.

Table 3.1 Biotic and abiotic metrics evaluated for associations with free-living *Batrachochytrium dendrobatidis* in amphibian breeding habitats in the Willamette Valley, Oregon, USA.

Environmental Metrics	
Abiotic	Ammonium (NH4+)
	Nitrate (NO3-)
	Nitrite (NO2-)
	Total Dissolved Nitrogen (TDN)
	Total Nitrogen (TN)
	Total Phosphorous (TP)
	Organophosphorous (OP)
	Total Organic Carbon (TOC)
	Season
Biotic	Amphibian density
	Amphibian species richness
	Bd-positive amphibians present
	Blue-green algae density (cell count/mL)
	Chrysophyte (cell count/mL)
	Cryptophyte (cell count/mL)
	Diatom (cell count/mL)
	Dinoflagellate (cell count/mL)
	Eugelnoid (cell count/mL)
	Green algae (cell count/mL)
	Total phytoplankton taxa richness
	Total phytoplankton species richness

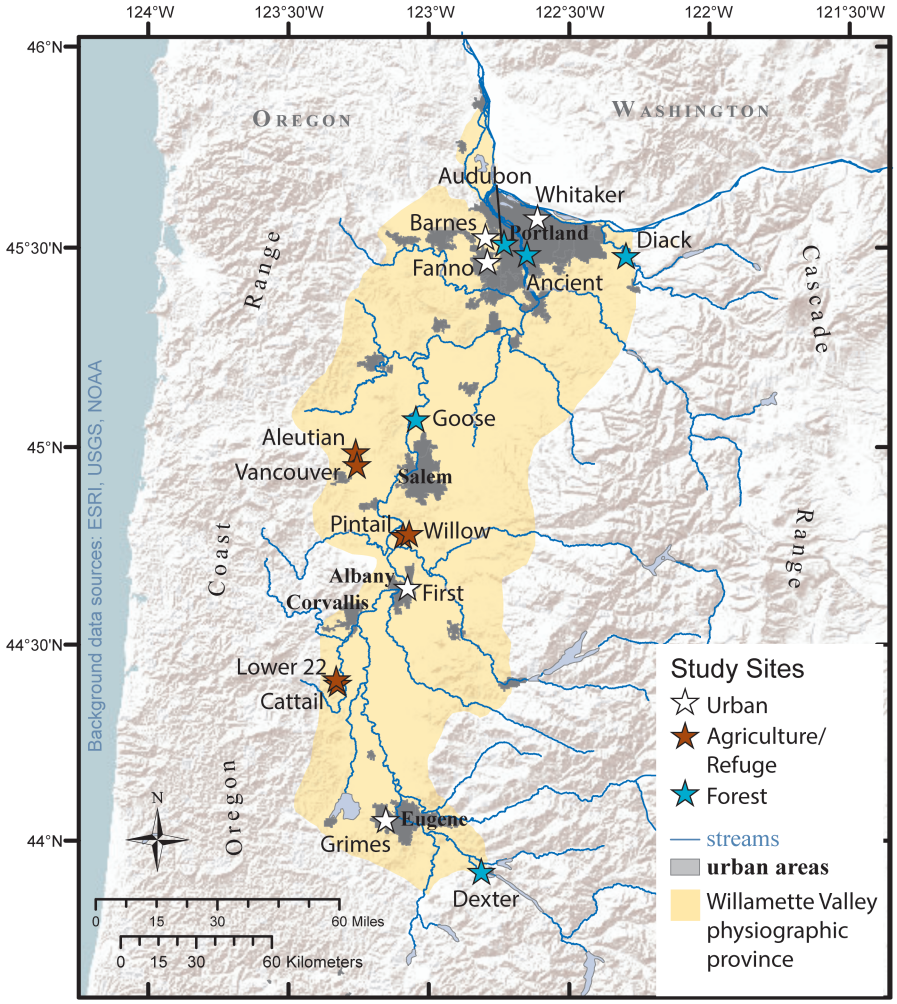

Figure 3.1. Sampling locations for *Batrachochytrium dendrobatidis* (Bd) in amphibians and their breeding habitats in the Willamette Valley, Oregon, USA. Sites are stratified by land use (urban, agricultural, forested) and were randomly sampled quarterly for 12 months (2011-2012).

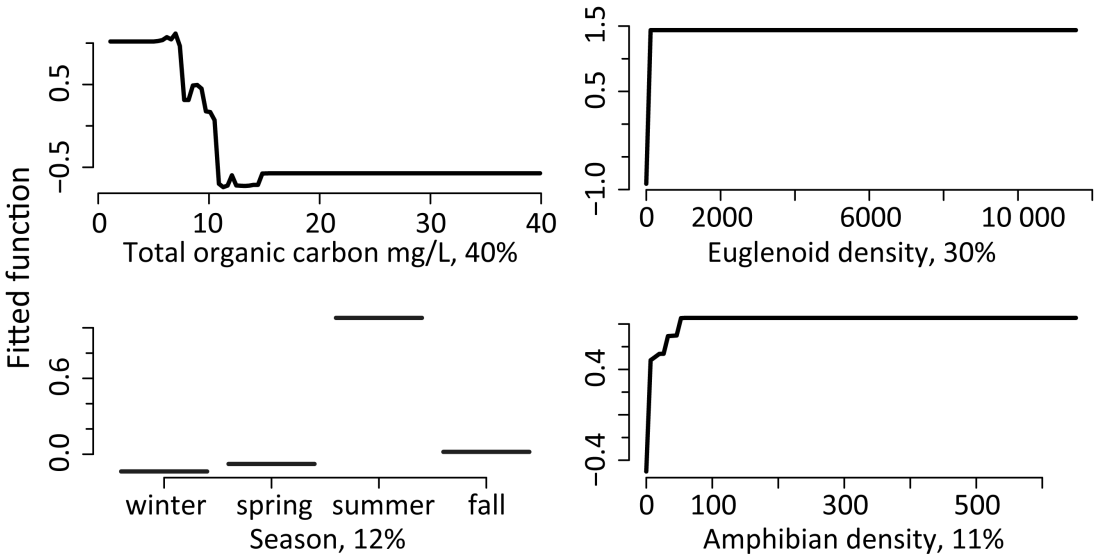

Figure 3.2. Partial dependency plots for variables in boosted regression tree (BRT) model for *Batrachochytrium dendrobatidis* (Bd) detection in water collected from amphibian breeding habitats in the Willamette Valley, Oregon, USA. BRT partial dependency plots show the response of Bd detection (y-axis = fitted function of Bd detection) based on the effect of individual explanatory variables with the response of all other variables removed. Shown in order of model importance with the relative contribution of each explanatory variable shown in parentheses, top row: total organic carbon, euglenoid density; bottom row: season, amphibian density

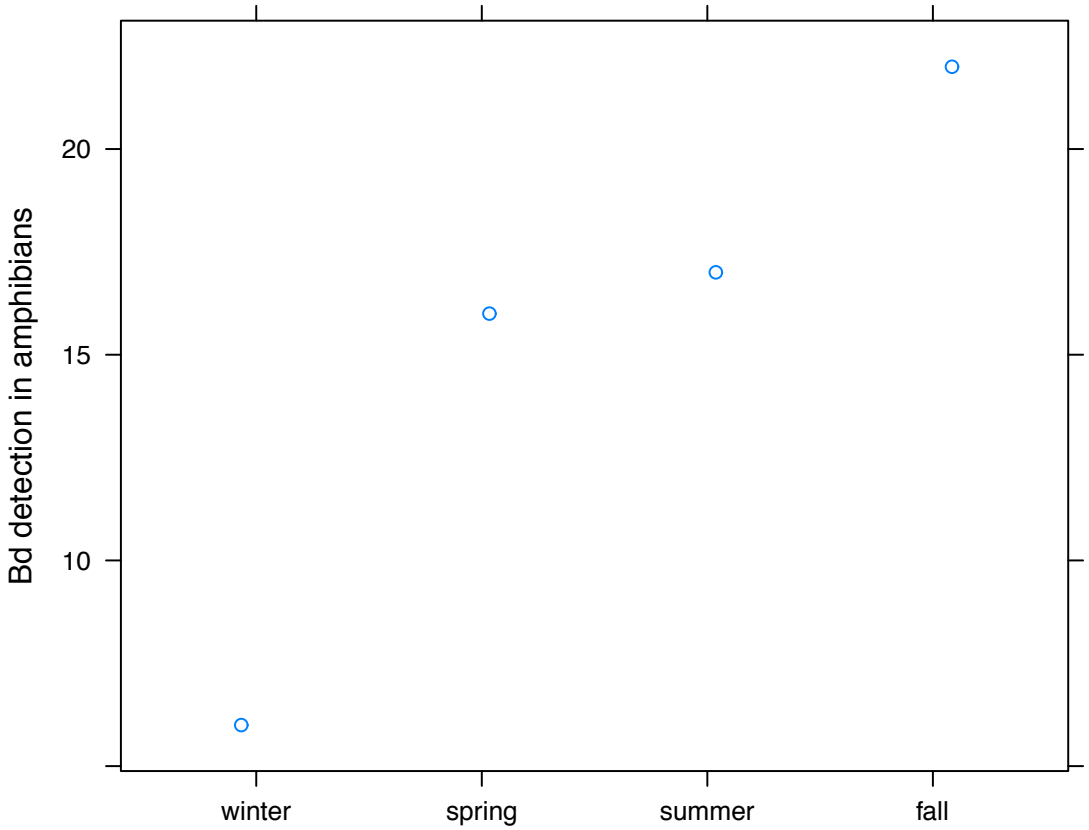

Figure 3.3. Total number of *Batrachochytrium dendrobatidis* (Bd) detections (presence) in amphibians from 16 sites in the Willamette Valley, Oregon, USA, sampled quarterly for 12 months. Seasons: winter = December-February, spring = March-May, summer = June-August, fall = September-November.

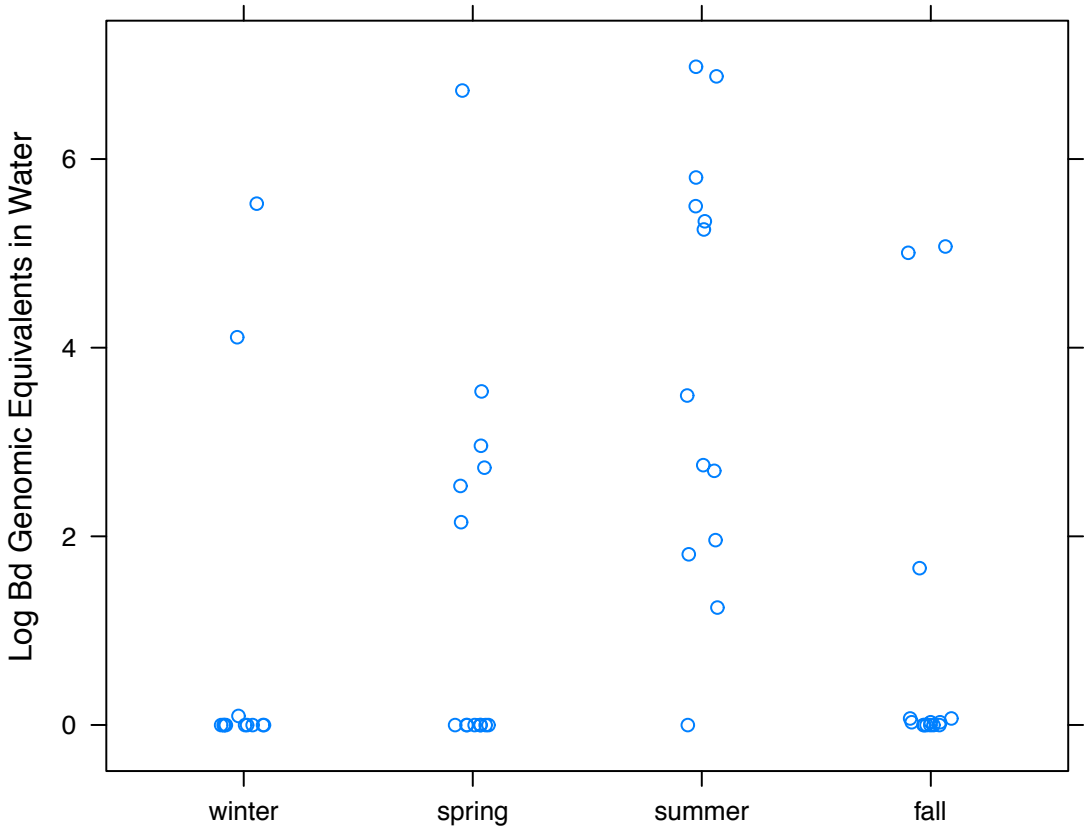

Figure 3.4. Log genomic equivalents of *Batrachochytrium dendrobatidis* (Bd) detected in water filtered from 16 amphibian breeding sites in the Willamette Valley, Oregon, USA, sampled quarterly for 12 months. Seasons: winter = December-February, spring = March-May, summer = June-August, fall = September-November.

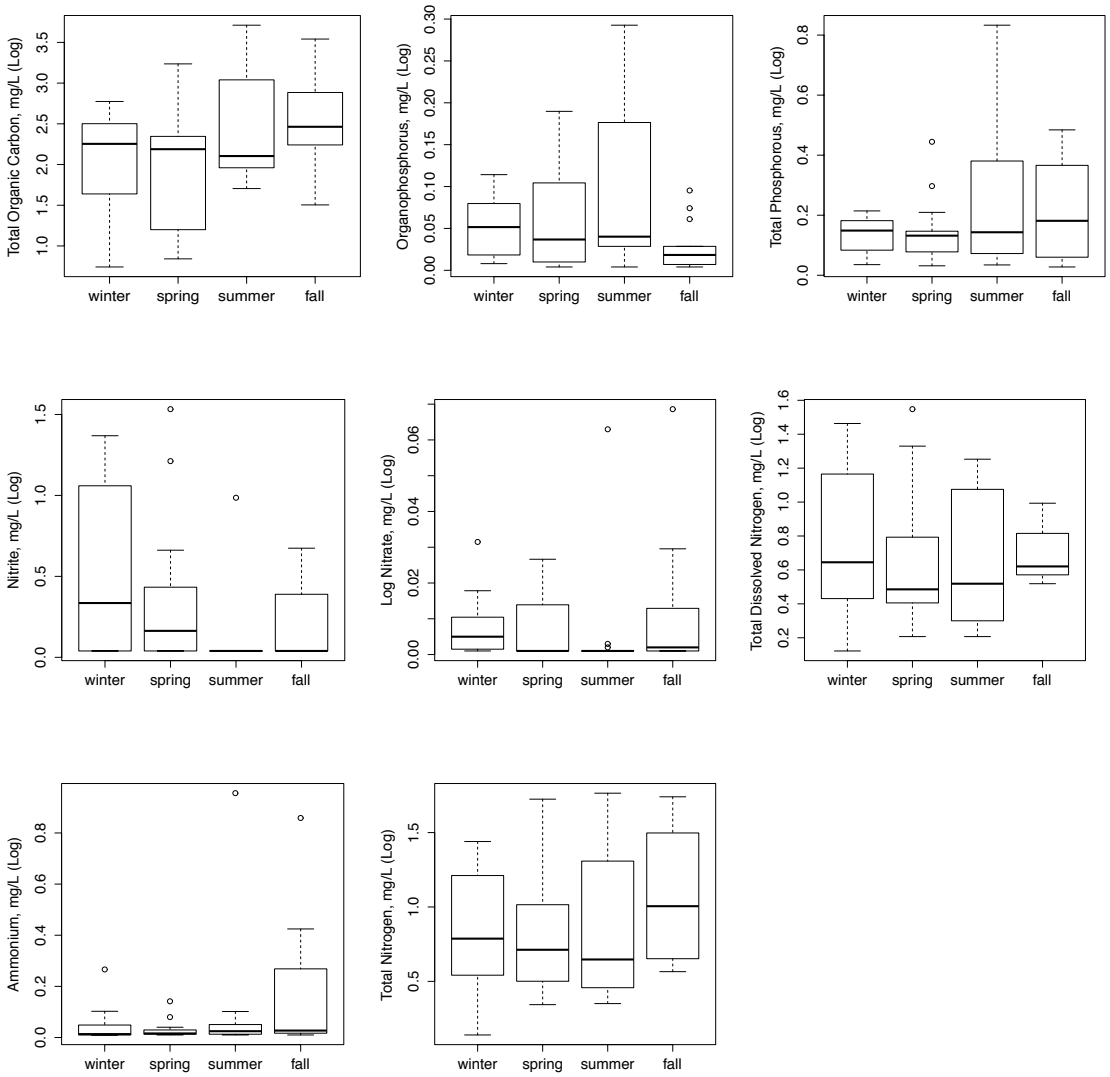

Figure 3.5. Seasonal variation in water chemistry (total dissolved nitrogen, total nitrogen, ammonium, nitrate, nitrite, total phosphorous, organophosphorus, and total organic carbon) measured in mg/L from 16 amphibian breeding sites in the Willamette Valley, Oregon, USA, sampled quarterly for 12 months. Seasons: winter = December-February, spring = March-May, summer = June-August, fall = September-November.

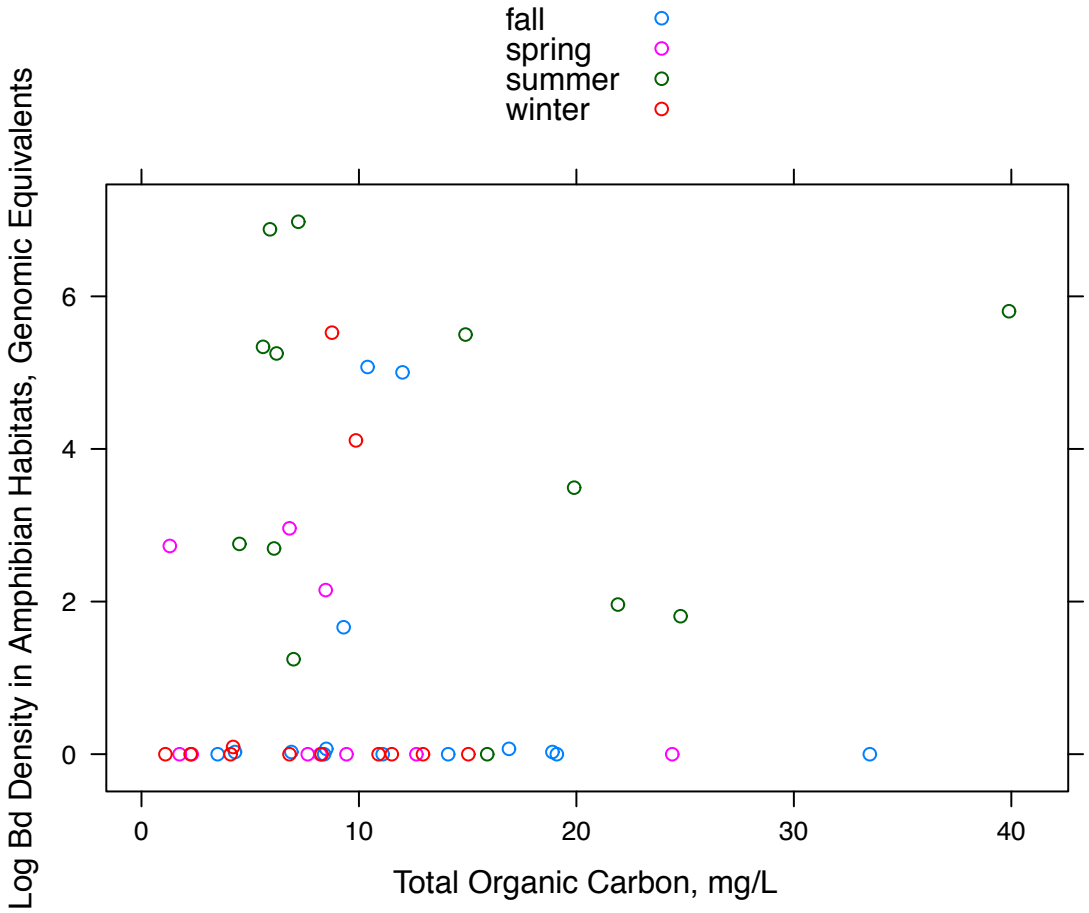

Figure 3.6. Log *Batrachochytrium dendrobatidis* (Bd) zoospore density related to total organic carbon by season from 16 amphibian breeding sites in the Willamette Valley, Oregon, USA, sampled quarterly for 12 months. Bd detection in amphibian breeding habitats could be related to low organic carbon (<7.3 mg/L). Seasons: winter = December-February, spring = March-May, summer = June-August, fall = September-November.

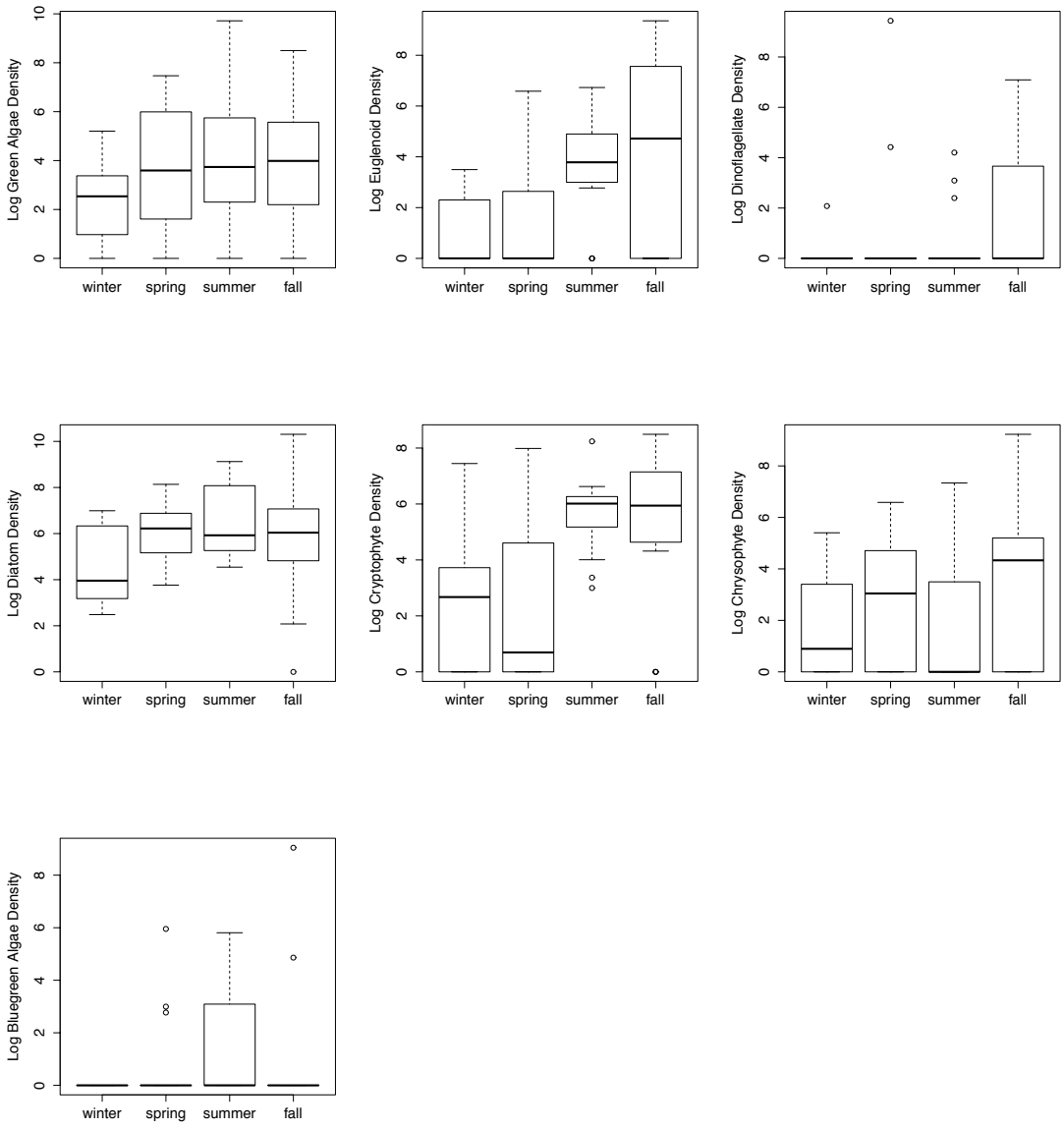

Figure 3.7. Seasonal variation in phytoplankton counts (bluegreen algae, chrysophyte, cryptophyte, diatom, dinoflagellate, eugelnoid, green algae) from 16 amphibian breeding sites in the Willamette Valley, Oregon, USA, sampled quarterly for 12 months. Seasons: winter = December-February, spring = March-May, summer = June-August, fall = September-November.

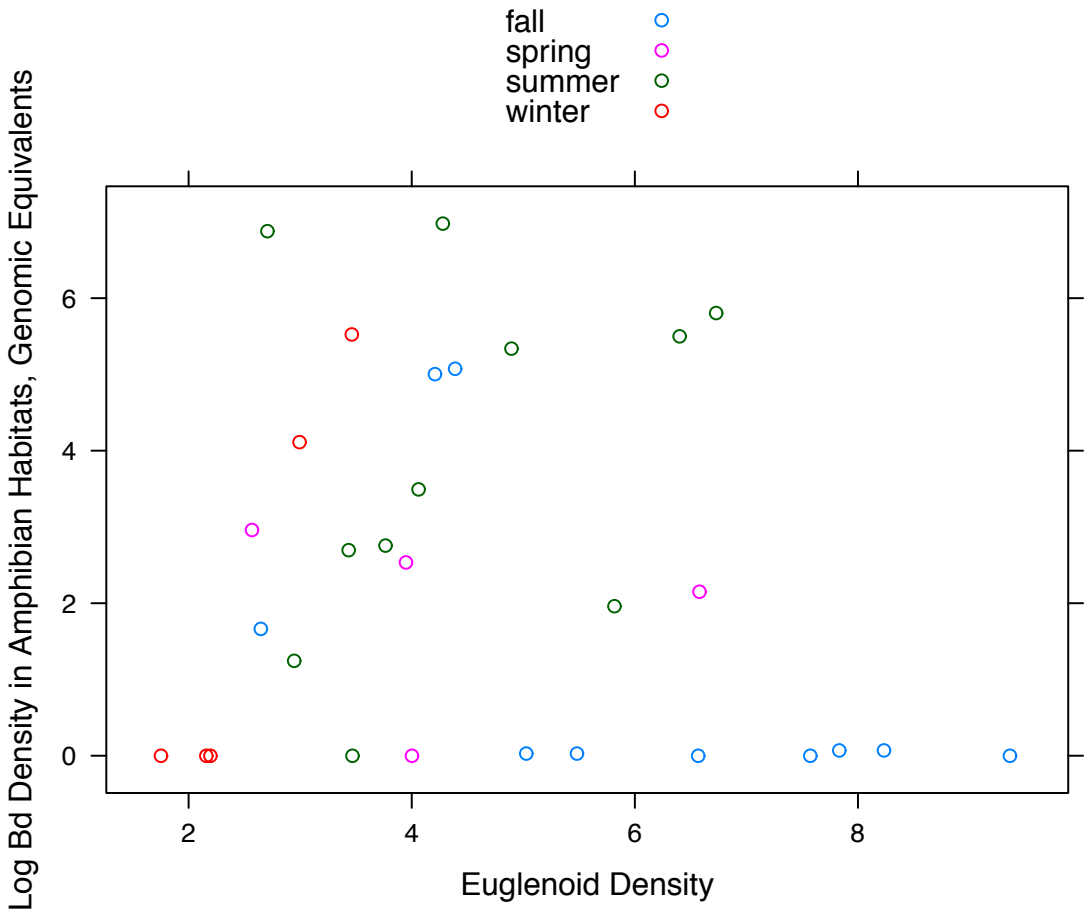

Figure 3.8. Log *Batrachochytrium dendrobatidis* (Bd) zoospore density related to log euglenoid density (cell count/mL) by season from 16 amphibian breeding sites in the Willamette Valley, Oregon, USA, sampled quarterly for 12 months. Bd detection in amphibian breeding habitats could be related to high euglenoid density (>116). Seasons: winter = December-February, spring = March-May, summer = June-August, fall = September-November.

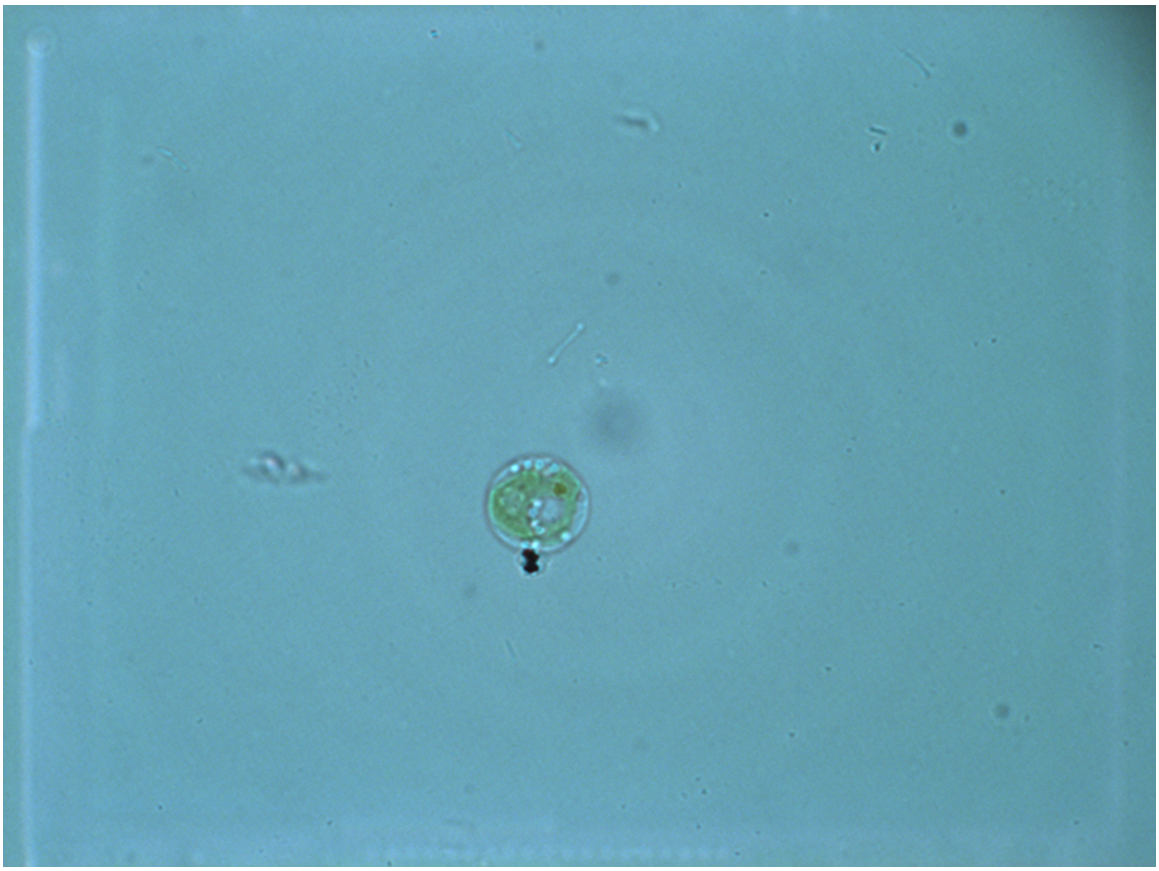

Figure 3.9. *Batrachochytrium dendrobatidis* (Bd) zoospores on the surface of *Chlamydomonas richarsonii*, possibly in the early stages of encysting, three days following inoculation in sterile 0.2% tryptone broth. Bd was stained with 0.5% Congo red to aide visualization (compound microscope, 1000x).

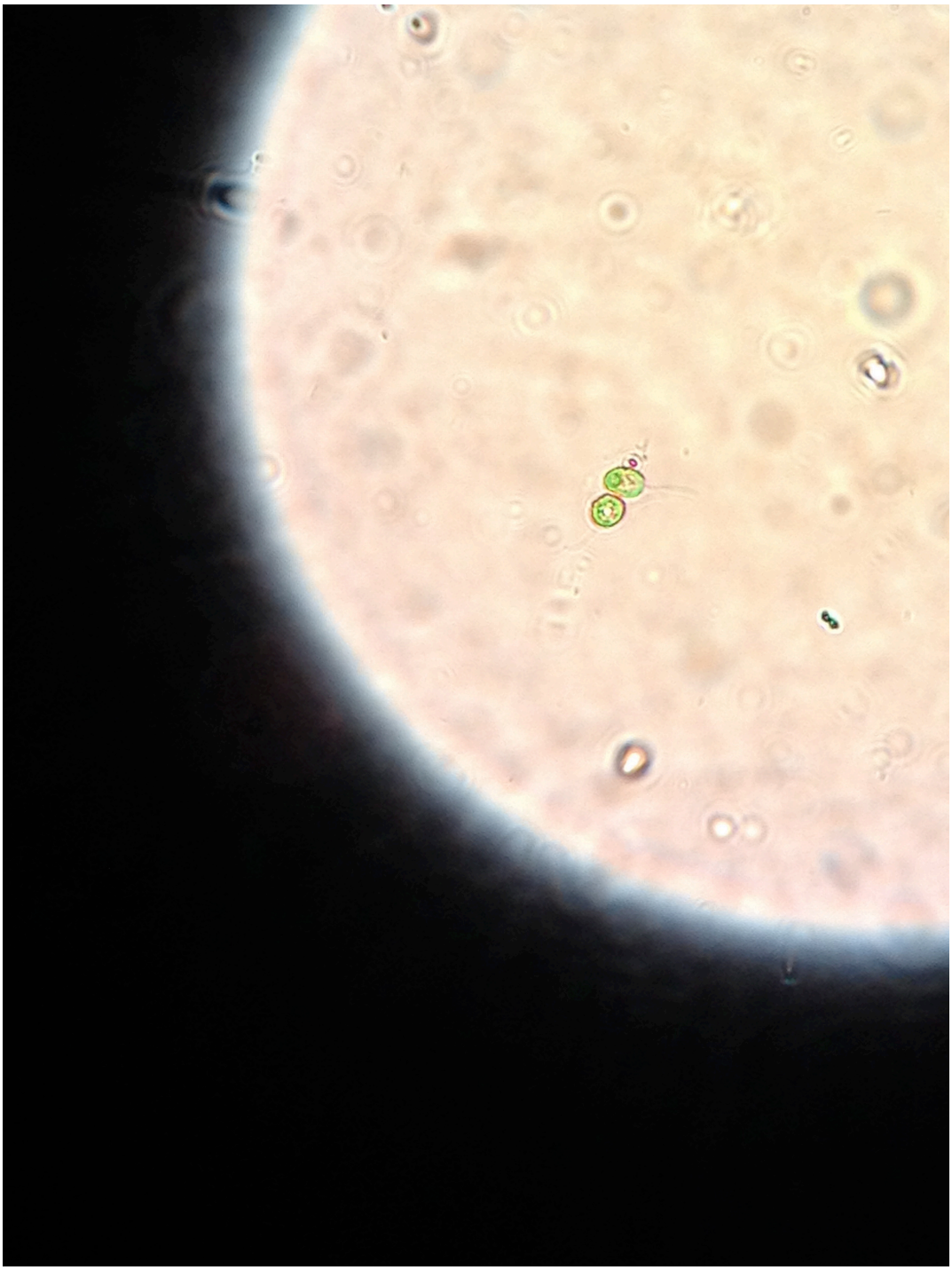

Figure 3.10. *Batrachochytrium dendrobatidis* (Bd) zoospores on the surface of *Chlamydomonas richarsonii*, possibly in the early stages of encysting, 24-hours following inoculation in sterile 0.2% tryptone broth. Bd was stained with 0.5% Congo red to aide visualization (compound microscope, 400x).

CHAPTER 4. RESPONSE OF THE FUNGAL PATHOGEN *BATRACHOCHYTRIUM DENDROBATIDIS* TO ENVIRONMENTAL STRESSORS: IS BD INHIBITED BY CURRENT-USE FUNGICIDES IN CULTURE OR INFECTED AMPHIBIANS?

Deanna H. Olson, Kelly L. Smalling, Chauncey W. Anderson, Radu Popa, Paul W. Bradley, Deborah D. Iwanowicz, Andrew R. Blaustein

Abstract

Aquatic systems are reservoirs for chemical contaminants which are non-point source pollution from industry and agriculture. Fungicides are detected in 75% of surface waters sampled in the United States. These contaminants can adversely affect organisms that occur within these systems, and alter ecological interactions. Relatively few studies have evaluated the effects of fungicides on aquatic organisms and their disease dynamics. I evaluated the response of the aquatic fungus *Batrachochytrium dendrobatidis* (Bd), a pathogen infecting amphibians throughout the world, to five current-use fungicides (azoxystrobin, boscalid, dimethomorph, fludioxinil, and tebuconazole) at five different concentrations using a completely randomized design. I also examined the effect of one fungicide, tebuconazole, on Bd infection of the Pacific chorus frog, *Pseudacris regilla*. I observed trends that suggest Bd responds negatively to only some of the compounds tested. Bd response to different concentrations was monotonic in some compounds, and non-monotonic in other compounds. Tebuconazole cleared Bd infections in *P. regilla* and did not result in frog mortality. Our study sheds light on the effects of fungicides to aquatic organisms and how exposure to fungicides may influence disease dynamics in aquatic systems.

Introduction

Pesticides are applied widely in agricultural areas and are non-point source pollution in the water and sediments of wetland habitats in these settings (Amweg et al. 2005, Bradford et al. 2010). They are also detected in wilderness areas great distances

from the point of application, transported atmospherically as vapor or on particulates (Sparling et al. 2001, Fellers et al. 2004, Davidson 2004, Bradford et al. 2010, 2011, Coscollà et al. 2010, Smalling et al. 2013). As many as 41 pesticide compounds have been detected in air samples, with little difference in detection between urban and rural areas (Coscollà et al. 2010). Some compounds (e.g., tebuconazole) are detected in air samples in the gas phase, which suggests shorter atmospheric half-lives and less susceptibility to long-distance transport whereas compounds detected in the particle phase (e.g., atrazine) suggest longer atmospheric half-lives and more susceptibility to transport over long distances (Atkinson et al. 1999). For example, simazine, a triazine herbicide used for broad spectum weed control, was observed in precipitation near areas it was applied, in more distant high alpine areas during snow melt, and in high alpine frog tissues months after application (Vogel et al. 2008, Bradford et al. 2011, Smalling et al. 2013). Little is known about the fate and transport of many current-use pesticides in the environment, especially fungicides, in part because several compounds are newly developed. In recent studies from the U.S. and Europe, at least 12 current-use fungicide compounds have been detected from air, water, sediment or tissue samples (Coscollà et al. 2010, Smalling et al. 2013).

In the last 20 years, trends in herbicide application rates increased from the early 1990s to present, while insecticide and fungicide applications decreased (Stone et al. 2014). A minimum of 20 million pounds of fungicides were applied annually in the U.S. over this time period. A peak in fungicide use occurred in the mid-1990s, with nearly 80

million pounds applied. Applications declined through the mid-2000s, but recently, trends in fungicide use in the U.S. have increased (Stone et al. 2014). Fungicides are applied to treat diseases and increase crop yields. In one study in the U.S., at least one fungicide compound was detected in 75% of the surface waters sampled. Azoxystrobin and boscalid were detected in more than 50% of the samples collected (Reilly et al. 2012). Boscalid was the most frequently detected fungicide, in 72% of surface waters (maximum 110 ng L^{-1}/0.11 ppb, median 22.5 ng L^{-1} /0.2 ppb). Azoxystrobin was detected in 51% of surface water samples (maximum 59.8 ng L^{-1}/0.06 ppb, median 30.6 ng L^{-1}/0.03 ppb). Chlorothalonil, pyraclostrobin, and pyrimethanil were detected less frequently, between 28 and 40% of sites. Fludioxinil was detected infrequently, at 3% of sites (maximum and median 3.3 ng L^{-1}/ 0.003 ppb). Given the ubiquity of pesticides in surface waters, it is important to assess the exposure risk to aquatic organisms.

Negative effects of pesticides to non-target organisms such as amphibians can include reduced fitness (e.g., reduced size at metamorphosis), disruption to endocrine function (e.g., hemaphroditism, feminization of male frogs), and mortality (Hayes et al. 2002, 2006, Fellers et al. 2004, Davidson 2004, Davidson and Knapp 2007, Biga and Blaustein 2013). Amphibians are an especially vulnerable group, because they have permeable skin, several pathways of exposure (e.g., in wetlands during breeding, and in uplands post breeding), and over two-thirds of species worldwide display population declines (Collins and Storfer 2003, Stuart 2004, Collins 2010, Rohr and Raffel 2010, Butchart et al. 2010, Hoffmann et al. 2010, Brühl et al. 2013). The effect of herbicides

and insecticides on amphibian survival and physiology has been an area of focused research, as has the interactions of these pesticides with species and ecological processes within wetland ecosystems (Sparling et al. 2001, Relyea 2005a, 2009, Relyea and Hoverman 2008, Bradford et al. 2011, Biga and Blaustein 2013). For example, exposure to the herbicide glyphosate or its commercial formulations can be lethal to amphibians and influence their behavior (Relyea 2005b, 2005c, Jones et al. 2010). Atrazine, a widely used herbicide detected in the environment, is an endocrine disruptor that demasculinizes and feminizes the gonads of male amphibians (Hayes et al. 2010, 2011, Vandenberg et al. 2012). However, different amphibian species and life stages respond differently to contaminants and pesticides. For example, the insecticide carbaryl delayed metamorphosis in Ranid and Hylid frogs, and increased the growth rate of a Hylid (Buck et al. 2012). When exposed to cypermethrin, a synthetic pyrethroid insecticide, early larval stage *Rana cascadae* showed an increase in mortality but not later larval stages, whereas all larval stages of *Pseudacris regilla* experienced increased mortality (Biga and Blaustein 2013).

The exposure risk and effects of fungicides to amphibians are less well studied than herbicides and insecticides. In the California Sierra Nevada Range, *P. regilla* was found to bioaccumulate nine different pesticides and three pesticide degradates from remote sites (Smalling et al. 2014). Tebuconazole and pyraclostrobin were the most frequently detected fungicides in their tissues. *Bufo cognatus* larvae exposed to the fungicide strobilurin responded differently to different formulations, where some

formulations caused mortality and others caused an increase in developmental rate (Hooser et al. 2012, Hartman et al. 2014). Exposure to the fungicide thiophanate-methyl also facilitated growth and development in *Lithobates sphenocephalus* (Hanlon et al. 2012). Neither low (<1.8 μg L^{-1}) nor high (>32 μg L^{-1}) concentrations of chlorothalonil affected *Osteopilus septentrionalis* survival (McMahon et al. 2013).

Adding complexity to the problem of evaluating the effects of environmental contaminants to aquatic organisms such as amphibians is how they respond when they are simultaneously infected with a disease, and further, how the pathogen responds when it is exposed to a contaminant. Exposure to contaminants can have positive and negative effects on pathogen transmission, infectivity, and host susceptibility (Rohr et al. 2008, Clements and Rohr 2009, Blaustein et al. 2011). In some cases, exposure to a pesticide does not alter amphibian survival when they are then exposed to a disease (Groner et al. 2013), and some pesticides (e.g., glyphosate) may ameliorate the effects of a disease (Hanlon and Parris 2012, Gahl et al. 2012).

The effects of current-use fungicides to amphibians and their diseases are understudied, despite the fact that many amphibian population declines, massive mortality events and extinctions are attributed to fungal or fungal-like pathogens (e.g., *Saprolegnia* spp.; *Batrachochytrium dendrobatidis,* Bd*; B. salamandrivorans*, Bs). The water mold, *Saprolegnia,* typically invades amphibian eggs and dead or dying amphibians, though it can also negatively affect healthy animals of all life stages,

especially when they are exposed to stressors (Romansic et al. 2009, Ault et al. 2012). Bd causes the emerging infectious disease, chytridiomycosis, implicated as a causal agent in global amphibian population declines (Mendelson et al. 2006, Fisher et al. 2012, Olson et al. 2013). Bd in amphibians and in culture showed a nonlinear response to exposure to the fungicide chlorothalonil, where the low and high concentrations inhibited growth more so than intermediate concentrations (McMahon et al. 2013). Thiophanate-methyl at a concentration of 0.6 mg L^{-1} cleared Bd from *Lithobates sphenocephalus*.

I was interested in investigating the effects of current-use fungicides on amphibians and on Bd. Because Bd occurs in the aquatic environment both within a host and as a free-living stage (e.g., zoospores and zoosporangium) independent of a host, I assessed its response to five current-use fungicides in culture, and in infected animals. My goals were to investigate the relationship between Bd growth and fungicide concentration to determine if the current-use fungicides I tested: 1) inhibit the growth of free-living *Bd* in culture at different concentrations; and 2) reduce or eliminate Bd infections in amphibians. My results may shed light on patterns of Bd outbreaks observed in the field, which may be influenced by direct fungicide applications, as well as pesticide drift and atmospheric deposition. My results also may informed controlled biosecurity approaches, such as fungicides to trial for treatment of captive amphibians with fungal infections.

Materials and Methods

In a completely randomized design, I measured the response of free-living Bd in culture to five current-use fungicides (azoxystrobin, boscalid, dimethomorph, fludioxinil, and tebuconazole; Table 4.1) in separate experiments, and the effect of one fungicide (tebuconazole) on Bd-infected larval amphibians. The response variables I measured were density in Bd cultures, and Bd genomic equivalents in amphibians. I was interested in these compounds because Bd may be exposed to them in the field either directly in water or sediments, or indirectly in Bd-infected amphibians. Based on the modes of action (Table 4.1), I predicted Bd would respond negatively to each of these compounds. Compounds were suspended in triethylene glycol in 1% tryptone broth using a Hewlett Packard 8452A spectrophotometer at 480 nm wavelength. Each compound had five replicates and five treatment concentrations, using a serial dilution from 1 mg/ml to 0.0001 mg/ml added to 15-ml pyrex tubes filled with 6 ml sterile 1% tryptone broth. The tubes were inoculated with 1-ml of 1% tryptone containing 12,000 *Bd* zoospores per ml harvested from 6 day old plates, followed by the addition of 100 μl of the fungicide suspended in triethylene glycol, and incubated in a controlled setting (room temperature 22 degrees C, 12-hour light/dark cycle). The final fungicide concentrations in each treatment were 14000 ppb, 1400 ppb, 140 ppb, 14 ppb, and 1 ppb. Light absorbance was measured on day 0 (before and after inoculation), 7, 14, and 21. I assessed bacterial contamination by visually inspecting each tube for cloudiness before measurements were taken. I plated all experimental units at the end of the experiment or if cloudiness was

observed during visual inspection. I observed the plates for one week for bacterial growth.

To compare the mean average growth of *Bd* per treatment to the control over time, I used linear mixed-effects models that included the fixed effects of fungicide concentration and day and a repeated-measures covariance structure. I used Akaike's Information Criteria (AIC) to determine the appropriate covariance structure and considered that covariance models within 2 ΔAIC had similar degrees of support. I fit regression lines with the lm model in base R version 2.15.2 in R Studio 0.97.306.

To examine the response of amphibians and *Bd* to current-use fungicides, I used *P. regilla* as a model species and chose one fungicide, tebuconazole, as the treatment. I chose tebuconazole because it has been detected in tissues from wild-caught *P. regilla* (Smalling et al. 2013), and is in the same class as itraconazole, a compound used to treat Bd infections in amphibians (Jones et al. 2012, Woodhams et al. 2012, Brannelly et al. 2012). As such, I predicted tebuconazole would reduce Bd infections in *P. regilla*. I collected egg masses (Oregon coast site: 44.348626, -124.094889, WGS84) to ensure no prior Bd infection; Bd only infects keratinized tissue in amphibians, which embryos lack (Marantelli et al. 2004). After hatching, animals were raised in low densities in aquaria (~20 tadpoles per 40-L glass aquaria) filled with dechlorinated water treated with 2.5 ml each of NovAqua and AmQuel and aged for a minimum of one day to ensure temperature equilibrium at 14 degrees C. Food was provided every other day (1:1:1 pulverized fish

flakes, kelp flakes and rabbit chow) and algae was allowed to colonize the aquarium walls. Tanks were cleaned every 10-14 days, and 1-L of water from the previous tank was transferred to the new tanks to provide algal inoculum. Individuals were randomly assigned to one of seven treatments (18 larvae per treatment) which included two tebuconazole concentrations (high: 1 mg/ml, and low: 0.01 mg/ml) with and without Bd exposure and three controls; treated water, triethylene glycol (the fungicide carrier), and a Bd only exposure. Bd-exposed *P. regilla* larvae were placed in individual 600 ml beakers filled with 350 ml of treated water and exposed to 15,000 *Bd* zoospores for 24 hours, estimated using a cell counting chamber. Unexposed larvae were also placed in beakers and exposed to a sham treatment. After 24 hours, I added tebuconazole (or an additional sham treatment) to each experimental unit to achieve the desired fungicide concentration. The high concentration was 1 mg/ml and the low concentration was 0.1 mg/ml, for a final concentration in the beaker of 0.00014 mg/ml (14.29 parts per billion), and 0.000014 mg/ml (1.43 parts per billion). I monitored the larvae for mortality at 4 hours, 8 hours, 12 hours, 18 hours, 24 hours, 36 hours, 48 hours, then daily for a total of 21 days. While in the beakers, individuals were fed *ad libitum* every other day. On day 10 I refreshed the water by adding 150 ml of oxygen saturated treated water to the beakers.

At the end of the experiment, I humanely euthanized the animals with buffered MS-222 then swabbed the mouthparts of each animal with a sterile synthetic swab (Advantage Bundling/Medical Wire Co. fine tip, catalog number MW113). Infection loads were determined by running a quantitative PCR assay to estimate the Bd genomic

equivalents in/on each animal. To test for a difference between the mean Bd genomic equivalents among treatments, I fit a linear mixed-effects model fit by restricted maximum likelihood, which included the fixed effects of Bd concentration and day, and the random effects of the individual animal. I again fit regression lines with the lm model in base R version 2.15.2 in R Studio 0.97.306.

I conducted this study in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the approved Institutional Animal Care and Use Committee of Oregon State University (ACUP # 4184). Amphibian eggs were collected with permission from to Oregon Department of Fish and Wildlife (2012 Oregon Scientific Taking Permit #006-12 issued to A.R. Blaustein).

Results

I observed a general negative trend in Bd growth in response to the increasing concentration of some but not all fungicide compounds tested. I observed negative trends in some concentrations, and in most cases, the declining trend in Bd growth was apparent after day 7 but Bd recovered such that there were no differences between the treatments and control groups by day 21.

Azoxystrobin, Bd in culture

There was a declining trend in the change in Bd growth over time in the two highest azoxystrobin concentrations (14,000 ppb and 1,400 ppb, Figure 4.1). However, I saw no evidence of a significant difference in mean Bd density between any of the azoxystrobin concentrations and the triethylene glycol control on any day (Table 4.2). There was strong evidence for an effect of day in all of the azoxystrobin treatments (14,000 ppb: $F_{3,23}$ 122.42, p=<0.0001; 1,400 ppb: $F_{3,24}$ 5.64, p=0.005; 140 ppb: $F_{3,24}$ 12.40, p=<0.0001; 14 ppb: $F_{3,24}$ 33.35, p=<0.0001; 1.4 ppb: $F_{3,24}$ 8.28, p=0.0006).

Boscalid, Bd in culture

In the two highest boscalid concentrations (14,000 ppb and 1,400 ppb), I observed a declining trend in Bd density that was apparent on day 14 (Figure 4.1). I saw evidence for a difference in mean Bd density between these two treatments and the control on day 14 (Table 4.2). I also observed strong evidence for an interaction between treatment and day in all of the boscalid treatments (14,000 ppb: $F_{3,24}$ 8.33, p=0.0006; 1,400 ppb: $F_{3,24}$ 105.92, p=<0.0001; 140 ppb: $F_{3,24}$ 5.01, p=0.008; 14 ppb: $F_{3,24}$ 34.93, p=<0.0001; 1.4 ppb: $F_{3,24}$ 9.33, p=0.0003).

Dimethomorph, Bd in culture

In all of the dimethomorph concentrations, there was a declining trend in Bd density over time (Figure 4.1). Evidence of a difference in mean Bd density was evident between all of the dimethomorph concentrations (14,000 ppb, 1,400 ppb, 140 ppb, 14 ppb, and 1.4 ppb) and the triethylene glycol control on Day 14 but not on any other day

(Table 4.2). There was strong evidence for an interaction between treatment and day in all of the dimethomorph concentrations (1 mg/ml: $F_{3,24}$ 21.65, p=<0.0001; 0.1 mg/ml: $F_{3,24}$ 7.83, p=0.0008; 0.01 mg/ml: $F_{3,24}$ 75.59, p=<0.0001; 0.001 mg/ml: $F_{3,24}$ 32.20, p=<0.0001; 0.0001 mg/ml: $F_{3,24}$ 22.16, p=<0.0001).

Fludioxinil, Bd in culture

I observed a difference in mean Bd density between most of the fludioxinil treatments and the triethylene glycol control that varied based on concentration. The highest concentration of fludioxinil (14,000 ppb) resulted in an increase in Bd density on all days (Table 4.2). In all of the other concentrations that I tested, I observed a declining trend in Bd density (Figure 4.1). Evidence for a difference between the treatments and control was variable according to the concentration of the compound and day. Bd exposed to 1,400 ppb fludioxinil was reduced on day 7 and there was strong evidence for a difference in mean density between the treatment and control on day 14, but there was no difference on day 21 (Table 4.2). In the 14 ppb fludioxinil concentration, Bd density was reduced on day 7, but not on days 14 or 21 (Table 4.2). I observed no evidence for a difference in Bd density between the 140 ppb or 1.4 ppb fludioxinil concentrations and the triethylene control (Table 4.2). Evidence for an interaction between treatment and day was apparent in all but one of the fludioxinil treatments (14,000 ppb: $F_{3,24}$ 4.88, p=0.009; 1,400 ppb: $F_{3,24}$ 44.94, p=<0.0001; 140 ppb: $F_{3,24}$ 0.87, p=0.47; 14 ppb: $F_{3,24}$ 16.41, p=<0.0001; 1.4 ppb: $F_{3,24}$ 19.28, p=<0.0001). In the 140 ppb fludioxinil concentration, I observed an effect of day without an interaction with treatment ($F_{3,24}$ 3.86, p=0.02).

Tebuconazole, Bd in culture

I observed a declining trend in Bd density in response to all of the tebuconazole concentrations I tested (Figure 4.1). I did not see evidence for a difference between the treatments and control in the two highest tebuconazole concentrations (14,000 ppb and 1,400 ppb, Table 4.2). However, I found strong evidence for a difference between the treatments and control in the three lowest tebuconazole concentrations I tested (140 ppb, 14 ppb, and 1.4 ppb) on day 14 but not on days 7 or 21 (Table 4.2). There was strong support for an interaction between treatment and day in all but one of the tebuconazole concentrations that I tested (14,000 ppb: $F_{3,21}$ 1.20, p=<0.34; 1,400 ppb: $F_{3,24}$ 15.38, <p=0.0001; 140 ppb: $F_{3,24}$ 6.96, p=<0.002; 14 ppb: $F_{3,24}$ 19.90, p=<0.0001; 1.4 ppb: $F_{3,24}$ 14.59, p=<0.0001). In the 0.01 mg/ml tebuconazole treatment, I found evidence for an effect of day without an interaction with treatment ($F_{3,21}$ 17.24, p=<0.0001).

Tebuconazole, Bd in Pseudacris regilla

Tebuconazole had no effect on *P. regilla* survival. All but one control animal survived to the end of the experiment. I found strong evidence that exposure to tebuconazole cleared *Bd* infections in *P. regilla* larvae ($F_{6,119}$ 4.51, p=0.0004). Only one of 18 larvae in each of the low and high tebuconazole treatments was infected with Bd at the end of the experiment (Figure 4.2). Of the animals that tested Bd-positive (1 high tebuconazole-Bd exposed, 1 low tebuconazole-Bd exposed, and 9 Bd exposed controls), Bd genomic equivalents were typically between 30 and 40 units (Figure 4.2).

Discussion

Several compounds inhibit Bd growth in culture and in amphibians infected with Bd (Johnson et al. 2003, Buck et al. 2012, Hanlon and Parris 2012, Jones et al. 2012). However, most studies have not investigated Bd response to fungicidal agents over time. In my study, I assessed the effects of five concentrations of five different fungicides on Bd in culture for three weeks. I also assessed the effects of two concentrations of one fungicide on the survival and disease status of *P. regilla*. My results suggest that whereas some fungicides may inhibit Bd growth, the fungus can recover in a relatively short period of time (days to weeks), at least to the fungicides I used in my study. Furthermore, in one instance Bd had the opposite response to what was expected; it increased growth in response to exposure to a high concentration to a compound that inhibits transduction pathways (fludioxinil), which may reduce or prevent reproduction (PPDB 2014). In *P. regilla* exposed to Bd, the compound I tested (tebuconazole) did not affect survival, and it cleared Bd infections at both low and high concentrations.

Importantly, I observed dynamic trends in Bd responses to fungicides that changed over time. If I had only measured Bd response at one time point (e.g., 21 days, at the end of the experiment), I would have missed the finer-scale trends in how these fungicides influence Bd growth. It is important to note, however, that the methods I used to evaluate Bd density measured light absorbance, or essentially the cloudiness of a

sample, and did not measure whether Bd was alive or dead. If an increase in light absorbance was observed, and bacterial contamination was not confirmed, I assumed the increase in light absorbance was due to Bd growth.

The differential responses I observed in Bd response to fungicides may be attributed to the mode of action or aqueous photolysis time (degradation time in water) of the compounds tested. Two of the compounds I tested, azoxystrobin and boscalid, act by inhibiting cellular respiration. The aqueous photolysis time for azoxystrobin is 9 days and for boscalid it is 30 days (PPDB 2014). I observed a declining trend in Bd response to azoxystrobin and the two highest concentrations of boscalid. Bd growth also was inhibited by another fungicide (chlorothalonil) that effects cellular respiration (McMahon et al. 2013). However, the responses reported by McMahon et al (2013) (daily response over eight days) were at a much finer time scale than our study (weekly response over three weeks). Dimethomorph acts by inhibiting cell wall synthesis, and the aqueous photolysis time is 70 days (PPDB 2014). The longer stability of this compound in water may explain why I saw evidence of Bd inhibition in all of the dimethomorph concentrations that I tested. Fludioxinil acts by inhibiting signal transduction pathways (Hagiwara et al. 2009), which could inhibit spore germination (Rosslenbroich and Stuebler 2000). Like azoxystrobin, it has a relatively short time to aqueous photolysis: 10 days (PPDB 2014). If Bd reproduction is reduced by fludioxinil but only for 10 days, this may explain why I saw a decline in Bd density on days 7 or 14 but not day 21. Tebuconazole inhibits sterol synthesis, and its time to aqueous photolysis is 28 days,

which is more similar to boscalid. I observed a negative trend in Bd growth in response to tebuconazole, although it is unclear why the response was apparent in the intermediate and lower but not the higher concentrations. Some fungi (e.g., yeasts and filamentous ascomycetes) have been observed as contributors to biodegradation of fungicides over time (Coppola et al. 2011). It is possible that Bd contributed to the biodegradation of the compounds I tested, which could also explain the general pattern I observed where Bd densities in many of the treatments declined until day 14 but then recovered by day 21.

Our hypothesis that tebuconazole would clear Bd infections in *P. regilla* larvae was supported by our experimental results. All but two *P. regilla* larvae exposed to tebuconazole, one from the high-dose and one from the low-dose treatment, had no sign of Bd infection. Tebuconazole and itraconazole, which is effective in treating Bd infections in amphibians, (Jones et al. 2012, Brannelly et al. 2012) inhibit sterol synthesis, but the inhibitory effect of azoles may differ according to the compound (Marichal et al. 1985). Both fungi and animals synthesize sterols, although the sterols they synthesize are different; the major sterol that fungi synthesize is ergosterol, whereas animals synthesize cholesterol (Gaulin et al. 2010). This difference in sterols may explain why Bd in *P. regilla* was inhibited by tebuconazole, but the frogs did not experience mortality.

I expected to see a monotonic, or linear response, to the different fungicide concentrations I tested, where the highest concentrations would be more inhibitory to Bd

than the lowest concentrations. In three of the compounds I tested, azoxystrobin, boscalid, and dimethomorph, this was generally the pattern I observed. The two remaining compounds I tested, fludioxinil and tebuconazole, revealed non-monotonic patterns, where there was a nonlinear relationship between the fungicide dose and the effect to *Bd*. The highest concentration of fludioxinil I tested (14,000 ppb) resulted in an increase in *Bd* growth that was markedly higher than our controls which resembled a masting event in response to environmental stress (e.g., plants respond with high seed production in response to water stress; Piovesan and Adams 2005). The lower four concentrations I tested (1400, 140, 14 and 1.4 ppb) showed a monotonic response, as I expected. In the tebuconazole concentrations, *Bd* showed no trend in response to the highest two concentrations (14,000 and 1400 ppb) but had a negative trend in growth in response to the intermediate and lowest concentrations (140, 14 and 1.4 ppb). The negative trends I observed in response to the lower concentrations of tebuconazole were apparent in both experiments, where I evaluated *Bd* response in culture and in frog larvae infected with *Bd*. These lower concentrations are closer to the values recovered from aquatic habitats (Battaglin et al. 2011, Reilly et al. 2012b, 2012a, Smalling et al. 2012, 2013), and may be more relevant to predicting *Bd* response in aquatic settings. Dose-response curves for effects on biota are available for many pesticides, but assumptions are made based on concentrations (e.g., assumptions that a response to high doses predicts the response to low doses of a contaminant). These assumptions do not reflect differential effects to concentrations that are non-linear in nature. Non-monotonic patterns of response are becoming more apparent in toxicology studies, where the effects

of low doses cannot be predicted by the effects observed at high doses (Vandenberg et al. 2012). For example, the effects of the fungicide chlorothalonil to *Bd* growth, a non-monotonic response was observed, where the intermediate doses had the highest inhibitory effect (McMahon et al. 2013).

In practice, multiple pesticides and fertilizers are often applied in an agricultural landscape rather than a single compound, and these mixtures may affect aquatic communities differently than just one compound alone (Boone et al. 2007, Relyea 2009, Mann et al. 2009). For example, when aquatic communities are exposed to multiple herbicides, a direct effect to phytoplankton and periphyton may be observed, as well as indirect effects to zooplankton and larval amphibians as the availability of food for grazers is reduced (Relyea 2009). In some cases, the effect depends on the mixture of compounds; e.g., mixtures of up to six herbicides did not have negative long-term effects to amphibian survival or metamorphosis, but mixtures of insecticides, and mixtures combining herbicides and insecticides resulted in high mortality to a Ranid species but not a Hylid species (Relyea 2009). The effect of pesticide mixtures that include fungicides to aquatic communities is understudied, but I would expect similar direct and indirect effects in systems where fungicides are detected.

Fungi are important elements in the environment with key ecological functions (Shearer et al. 2007, Heilmann-Clausen et al. 2014). In aquatic systems, chytrids specifically are an important food source for grazers such as zooplankton. Chytrids also

convert inorganic matter into organic matter that becomes accessible as food to other organisms, decompose organic matter, and serve a role as parasites to plants and animals (Gleason et al. 2008). The reduction or absence of chytrid fungi caused by non-point source pollution of agricultural fungicides or by intentional fungicidal treatment in an attempt to eradicate pathogenic fungi such as *Bd* from a system could result in unanticipated cascading effects. For example, there is evidence that chronic exposure to tebuconazole affects aquatic microbial communities such that their capacity to break down leaf litter in aquatic systems is reduced (Artigas et al. 2012). Furthermore, pathogenic fungi exposed to fungicides as non-point source pollution can develop resistance to compounds (e.g., azoles such as tebuconazole), which can have implications to aquatic systems and human health (e.g., azole resistance in the human pathogen *Aspergillus fumigatus* may be a side-effect of environmental fungicide use; Verweij et al. 2009).

Although Bd has devastated amphibian populations in many parts of the world, its ecology in the aquatic environment is vastly understudied. Bd appears to be present in ponds and wetlands year round and the density varies according to season (Chestnut et al. 2014). Bd occupancy of amphibian breeding sites is higher when an invasive host species (i.e., American bullfrog, *Lithobates catesbeianus*) is present (Adams et al. 2010). Bd is also a food source for zooplankton (Buck et al. 2011), and there is experimental evidence that zooplankton can consume Bd zoospores to such a degree that transmission to amphibian is reduced (Searle et al. 2013).

There is great interest in finding ways to combat the effects of aquatic diseases to plants and animals. Due to the complex ecological roles of aquatic fungi, the use of anti-fungal agents to control aquatic fungal diseases in the wild have not been widely considered (Gleason and Marano 2011, Hanlon and Parris 2012). In general, research on the effects of anti-fungal agents, including fungicides, to zoosporic fungi are limited to a few species that are typically plant or animal pathogens. Given the non-monotonic responses of Bd to fungicides that are evident in this and other studies (McMahon et al. 2013), it cannot be assumed that there will be a generalized, linear response from such a diverse group of organisms.

Fungal diversity and functions, and the ecosystems services fungi provide are important considerations when evaluating strategies to address problems in conservation biology (Heilmann-Clausen et al. 2014). While 100,000 fungal species have been described, it is estimated that at least 1.5 million and possibly as many as 3-5 million species occur worldwide (Blackwell 2011, Scheffers et al. 2012). The mycoloop, described as nutrient transfer from phytoplankton to zooplankton, which includes the consumption of fungal parasites of phytoplankton, plays an understated yet vital role in aquatic food web dynamics (Kagami et al. 2014). Chytrid zoospores can be quite dense in aquatic systems, and they provide exceptional nutritional resources to zooplankton, which can shape aquatic ecosystems (Gleason et al. 2008, Kagami et al. 2014). The effect of widespread fungicide occurrence in surface waters is unknown relative to fungal diversity

in aquatic systems, the mycoloop, or Bd density at site. However, there is evidence that fungicides can induce declines in freshwater biodiversity via both top-down and bottom-up effects such that ecosystems functions and services are altered (McMahon et al. 2012). Using fungicides to treat one problem (e.g., treating wild amphibian populations to combat chytridiomycosis) may have unintended and unpredicted consequences to aquatic ecosystems. However, the threat Bs poses to North America salamanders is much more dire than Bd and this study may inform development of potential treatments that might be suitable for use on animals in captive situations (e.g. to preserve private and public collections or continue captive breeding programs aimed at recovering and releasing rare species). Treating terraria and enclosures with fungicidal agents may be effective in reducing or eliminating these pathogens. However, variation in how Bd responded to different concentrations of different fungicides presents a challenge in developing a prescription for treating Bd and Bs-infected animals and their habitats. Experimental work investigating the development of fungicide resistance in these pathogens would aid in evaluating the feasibility of fungicide treatments.

References

Adams, M. J., N. D. Chelgren, D. Reinitz, R. A. Cole, L. J. Rachowicz, S. Galvan, B. McCreary, C. A. Pearl, L. L. Bailey, and J. Bettaso. 2010. Using occupancy models to understand the distribution of an amphibian pathogen, *Batrachochytrium dendrobatidis*. Ecological Applications 20:289–302.

Amweg, E. L., D. P. Weston, and N. M. Ureda. 2005. Use and toxicity of pyrethroid pesticides in the Central Valley, California, USA. Environmental Toxicology and Chemistry 24:966–972.

Artigas, J., J. Majerholc, A. Foulquier, C. Margoum, B. Volat, M. Neyra, and S. Pesce. 2012. Effects of the fungicide tebuconazole on microbial capacities for litter breakdown in streams. Aquatic Toxicology 122–123:197–205.

Atkinson, R., R. Guicherit, R. A. Hites, W.-U. Palm, J. N. Seiber, and P. D. Voogt. 1999. Transformations of Pesticides in the Atmosphere: A State of the Art. Pages 219–243 *in* H. F. G. V. Dijk, W. A. J. V. Pul, and P. D. Voogt, editors. Fate of Pesticides in the Atmosphere: Implications for Environmental Risk Assessment. Springer Netherlands.

Ault, K. K., J. E. Johnson, H. C. Pinkart, and R. S. Wagner. 2012. Genetic comparison of water molds from embryos of amphibians *Rana cascadae*, *Bufo boreas* and *Pseudacris regilla*. Diseases of Aquatic Organisms 99:127–137.

Battaglin, W. A., M. W. Sandstrom, K. M. Kuivila, D. W. Kolpin, and M. T. Meyer. 2011. Occurrence of Azoxystrobin, Propiconazole, and Selected Other Fungicides in US Streams, 2005–2006. Water, Air, & Soil Pollution 218:307–322.

Biga, L. M., and A. R. Blaustein. 2013. Variations in lethal and sublethal effects of cypermethrin among aquatic stages and species of anuran amphibians. Environmental Toxicology and Chemistry 32:2855–2860.

Blackwell, M. 2011. The Fungi: 1, 2, 3 … 5.1 million species? American Journal of Botany 98:426–438.

Blaustein, A. R., B. A. Han, R. A. Relyea, P. T. J. Johnson, J. C. Buck, S. S. Gervasi, and L. B. Kats. 2011. The complexity of amphibian population declines: understanding the role of cofactors in driving amphibian losses. Annals of the New York Academy of Sciences 1223:108–119.

Boone, M. D., R. D. Semlitsch, E. E. Little, and M. C. Doyle. 2007. Multiple stressors in amphibian communities: effects of chemical contamination, bullfrogs, and fish. Ecological Applications 17:291–301.

Bradford, D. F., E. M. Heithmar, N. G. Tallent-Halsell, G.-M. Momplaisir, C. G. Rosal, K. E. Varner, M. S. Nash, and L. A. Riddick. 2010. Temporal Patterns and Sources of Atmospherically Deposited Pesticides in Alpine Lakes of the Sierra Nevada, California, U.S.A. Environmental Science & Technology 44:4609–4614.

Bradford, D. F., R. A. Knapp, D. W. Sparling, M. S. Nash, K. A. Stanley, N. G. Tallent-Halsell, L. L. McConnell, and S. M. Simonich. 2011. Pesticide distributions and population declines of California, USA, alpine frogs, Rana muscosa and Rana sierrae. Environmental Toxicology and Chemistry 30:682–691.

Brannelly, L., C. Richards-Zawacki, and A. Pessier. 2012. Clinical trials with itraconazole as a treatment for chytrid fungal infections in amphibians. Diseases of Aquatic Organisms 101:95–104.

Brühl, C. A., T. Schmidt, S. Pieper, and A. Alscher. 2013. Terrestrial pesticide exposure of amphibians: An underestimated cause of global decline? Scientific reports 3.

Buck, J. C., E. A. Scheessele, R. A. Relyea, and A. R. Blaustein. 2012. The effects of multiple stressors on wetland communities: pesticides, pathogens and competing amphibians. Freshwater Biology 57:61–73.

Buck, J. C., L. Truong, and A. R. Blaustein. 2011. Predation by zooplankton on *Batrachochytrium dendrobatidis*: biological control of the deadly amphibian chytrid fungus? Biodiversity and Conservation 20:3549–3553.

Butchart, S. H. M., M. Walpole, B. Collen, A. van Strien, J. P. W. Scharlemann, R. E. A. Almond, J. E. M. Baillie, B. Bomhard, C. Brown, J. Bruno, K. E. Carpenter, G. M. Carr, J. Chanson, A. M. Chenery, J. Csirke, N. C. Davidson, F. Dentener, M. Foster, A. Galli, J. N. Galloway, P. Genovesi, R. D. Gregory, M. Hockings, V. Kapos, J.-F. Lamarque, F. Leverington, J. Loh, M. A. McGeoch, L. McRae, A. Minasyan, M. H. Morcillo, T. E. E. Oldfield, D. Pauly, S. Quader, C. Revenga, J. R. Sauer, B. Skolnik, D. Spear, D. Stanwell-Smith, S. N. Stuart, A. Symes, M. Tierney, T. D. Tyrrell, J.-C. Vié, and R. Watson. 2010. Global Biodiversity: Indicators of Recent Declines. Science 328:1164–1168.

Chestnut, T., C. Anderson, R. Popa, A. R. Blaustein, M. Voytek, D. H. Olson, and J. Kirshtein. 2014. Heterogeneous occupancy and density estimates of the pathogenic fungus *Batrachochytrium dendrobatidis* in waters of North America. PLoS ONE 9:e106790.

Clements, W. H., and J. R. Rohr. 2009. Community responses to contaminants: Using basic ecological principles to predict ecotoxicological effects. Environmental Toxicology and Chemistry 28:1789–1800.

Collins, J. P. 2010. Amphibian decline and extinction: What we know and what we need to learn. Diseases of Aquatic Organisms 92:93–99.

Collins, J. P., and A. Storfer. 2003. Global amphibian declines: sorting the hypotheses. Diversity and distributions 9:89–98.

Coppola, L., F. Comitini, C. Casucci, V. Milanovic, E. Monaci, M. Marinozzi, M. Taccari, M. Ciani, and C. Vischetti. 2011. Fungicides degradation in an organic biomixture: impact on microbial diversity. New Biotechnology 29:99–106.

Coscollà, C., P. Colin, A. Yahyaoui, O. Petrique, V. Yusà, A. Mellouki, and A. Pastor. 2010. Occurrence of currently used pesticides in ambient air of Centre Region (France). Atmospheric Environment 44:3915–3925.

Davidson, C. 2004. Declining downwind: amphibian population declines in California and historical pesticide use. Ecological Applications 14:1892–1902.

Davidson, C., and R. A. Knapp. 2007. Multiple stressors and amphibian declines: dual impacts of pesticides and fish on Yellow-legged frogs. Ecological Applications 17:587–597.

Fellers, G. M., L. L. McConnell, D. Pratt, and S. Datta. 2004. Pesticides in mountain yellow-legged frogs (*Rana muscosa*) from the Sierra Nevada Mountains of California, USA. Environmental Toxicology and Chemistry 23:2170–2177.

Fisher, M. C., J. Stajich, and R. A. Farrer. 2012. Emergence of the chytrid fungus *Batrachochytrium dendrobatidis* and global amphibian declines. Evolution of Virulence in Eukaryotic Microbes:461.

Gahl, M. K., J. E. Longcore, and J. E. Houlahan. 2012. Varying Responses of Northeastern North American Amphibians to the Chytrid Pathogen *Batrachochytrium dendrobatidis*. Conservation Biology 26:135–141.

Gaulin, E., A. Bottin, and B. Dumas. 2010. Sterol biosynthesis in oomycete pathogens. Plant Signaling & Behavior 5:258–260.

Gleason, F. H., M. Kagami, E. Lefevre, and T. Sime-Ngando. 2008. The ecology of chytrids in aquatic ecosystems: roles in food web dynamics. Fungal Biology Reviews 22:17–25.

Gleason, F. H., and A. V. Marano. 2011. The effects of antifungal substances on some zoosporic fungi (Kingdom Fungi). Hydrobiologia 659:81–92.

Groner, M. L., J. C. Buck, S. Gervasi, A. R. Blaustein, L. K. Reinert, L. A. Rollins-Smith, M. E. Bier, J. Hempel, and R. A. Relyea. 2013. Larval exposure to

predator cues alters immune function and response to a fungal pathogen in post-metamorphic wood frogs. Ecological Applications 23:1443–1454.

Hagiwara, D., Y. Asano, J. Marui, A. Yoshimi, T. Mizuno, and K. Abe. 2009. Transcriptional profiling for Aspergillus nidulans HogA MAPK signaling pathway in response to fludioxonil and osmotic stress. Fungal Genetics and Biology 46:868–878.

Hanlon, S. M., J. L. Kerby, and M. J. Parris. 2012. Unlikely Remedy: Fungicide Clears Infection from Pathogenic Fungus in Larval Southern Leopard Frogs (*Lithobates sphenocephalus*). PloS One 7:e43573.

Hanlon, S. M., and M. J. Parris. 2012. The impact of pesticides on the pathogen *Batrachochytrium dendrobatidis* independent of potential hosts. Archives of Environmental Contamination and Toxicology 63:137–143.

Hartman, E. A. H., J. B. Belden, L. M. Smith, and S. T. McMurry. 2014. Chronic effects of strobilurin fungicides on development, growth, and mortality of larval Great Plains toads (*Bufo cognatus*). Ecotoxicology 23:396–403.

Hayes, T. B., L. L. Anderson, V. R. Beasley, S. R. de Solla, T. Iguchi, H. Ingraham, P. Kestemont, J. Kniewald, Z. Kniewald, V. S. Langlois, and others. 2011. Demasculinization and feminization of male gonads by atrazine: consistent effects across vertebrate classes. The Journal of Steroid Biochemistry and Molecular Biology 127:64–73.

Hayes, T. B., P. Case, S. Chui, D. Chung, C. Haeffele, K. Haston, M. Lee, V. P. Mai, Y. Marjuoa, J. Parker, and others. 2006. Pesticide mixtures, endocrine disruption, and amphibian declines: are we underestimating the impact? Environmental Health Perspectives 114:40.

Hayes, T. B., V. Khoury, A. Narayan, M. Nazir, A. Park, T. Brown, L. Adame, E. Chan, D. Buchholz, T. Stueve, and others. 2010. Atrazine induces complete feminization and chemical castration in male African clawed frogs (*Xenopus laevis*). Proceedings of the National Academy of Sciences 107:4612–4617.

Hayes, T., K. Haston, M. Tsui, A. Hoang, C. Haeffele, and A. Vonk. 2002. Herbicides: feminization of male frogs in the wild. Nature 419:895–896.

Heilmann-Clausen, J., E. S. Barron, L. Boddy, A. Dahlberg, G. W. Griffith, J. Nordén, O. Ovaskainen, C. Perini, B. Senn-Irlet, and P. Halme. 2014. A Fungal Perspective on Conservation Biology. Conservation Biology doi: 10.1111/cobi.12388.

Hoffmann, M., C. Hilton-Taylor, A. Angulo, M. Böhm, T. M. Brooks, S. H. M. Butchart, K. E. Carpenter, J. Chanson, B. Collen, N. A. Cox, W. R. T. Darwall, N. K.

Dulvy, L. R. Harrison, V. Katariya, C. M. Pollock, S. Quader, N. I. Richman, A. S. L. Rodrigues, M. F. Tognelli, J.-C. Vié, J. M. Aguiar, D. J. Allen, G. R. Allen, G. Amori, N. B. Ananjeva, F. Andreone, P. Andrew, A. L. A. Ortiz, J. E. M. Baillie, R. Baldi, B. D. Bell, S. D. Biju, J. P. Bird, P. Black-Decima, J. J. Blanc, F. Bolaños, W. Bolivar-G, I. J. Burfield, J. A. Burton, D. R. Capper, F. Castro, G. Catullo, R. D. Cavanagh, A. Channing, N. L. Chao, A. M. Chenery, F. Chiozza, V. Clausnitzer, N. J. Collar, L. C. Collett, B. B. Collette, C. F. C. Fernandez, M. T. Craig, M. J. Crosby, N. Cumberlidge, A. Cuttelod, A. E. Derocher, A. C. Diesmos, J. S. Donaldson, J. W. Duckworth, G. Dutson, S. K. Dutta, R. H. Emslie, A. Farjon, S. Fowler, J. Freyhof, D. L. Garshelis, J. Gerlach, D. J. Gower, T. D. Grant, G. A. Hammerson, R. B. Harris, L. R. Heaney, S. B. Hedges, J.-M. Hero, B. Hughes, S. A. Hussain, J. I. M, R. F. Inger, N. Ishii, D. T. Iskandar, R. K. B. Jenkins, Y. Kaneko, M. Kottelat, K. M. Kovacs, S. L. Kuzmin, E. L. Marca, J. F. Lamoreux, M. W. N. Lau, E. O. Lavilla, K. Leus, R. L. Lewison, G. Lichtenstein, S. R. Livingstone, V. Lukoschek, D. P. Mallon, P. J. K. McGowan, A. McIvor, P. D. Moehlman, S. Molur, A. M. Alonso, J. A. Musick, K. Nowell, R. A. Nussbaum, W. Olech, N. L. Orlov, T. J. Papenfuss, G. Parra-Olea, W. F. Perrin, B. A. Polidoro, M. Pourkazemi, P. A. Racey, J. S. Ragle, M. Ram, G. Rathbun, R. P. Reynolds, A. G. J. Rhodin, S. J. Richards, L. O. Rodríguez, S. R. Ron, C. Rondinini, A. B. Rylands, Y. S. de Mitcheson, J. C. Sanciangco, K. L. Sanders, G. Santos-Barrera, J. Schipper, C. Self-Sullivan, Y. Shi, A. Shoemaker, F. T. Short, C. Sillero-Zubiri, D. L. Silvano, K. G. Smith, A. T. Smith, J. Snoeks, A. J. Stattersfield, A. J. Symes, A. B. Taber, B. K. Talukdar, H. J. Temple, R. Timmins, J. A. Tobias, K. Tsytsulina, D. Tweddle, C. Ubeda, S. V. Valenti, P. P. van Dijk, L. M. Veiga, A. Veloso, D. C. Wege, M. Wilkinson, E. A. Williamson, F. Xie, B. E. Young, H. R. Akçakaya, L. Bennun, T. M. Blackburn, L. Boitani, H. T. Dublin, G. A. B. da Fonseca, C. Gascon, T. E. Lacher, G. M. Mace, S. A. Mainka, J. A. McNeely, R. A. Mittermeier, G. M. Reid, J. P. Rodriguez, A. A. Rosenberg, M. J. Samways, J. Smart, B. A. Stein, and S. N. Stuart. 2010. The impact of conservation on the status of the world's vertebrates. Science 330:1503–1509.

Hooser, E. A., J. B. Belden, L. M. Smith, and S. T. McMurry. 2012. Acute toxicity of three strobilurin fungicide formulations and their active ingredients to tadpoles. Ecotoxicology 21:1458–1464.

Johnson, M. L., L. Berger, L. Phillips, and R. Speare. 2003. Fungicidal effects of chemical disinfectants, UV light, desiccation and heat on the amphibian chytrid, *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms 57:255–260.

Jones, D. K., J. I. Hammond, and R. A. Relyea. 2010. Roundup® and amphibians: the importance of concentration, application time, and stratification. Environmental Toxicology and Chemistry 29:2016–2025.

Jones, M. E., D. Paddock, L. Bender, J. L. Allen, M. S. Schrenzel, and A. P. Pessier. 2012. Treatment of chytridiomycosis with reduced-dose itraconazole. Diseases of Aquatic Organisms 99:243–249.

Kagami, M., T. Miki, and G. Takimoto. 2014. Mycoloop: chytrids in aquatic food webs. Frontiers in Microbiology 5.

Mann, R. M., R. V. Hyne, C. B. Choung, and S. P. Wilson. 2009. Amphibians and agricultural chemicals: Review of the risks in a complex environment. Environmental Pollution 157:2903–2927.

Marantelli, G., L. Berger, R. Speare, and L. Keegan. 2004. Distribution of the amphibian chytrid *Batrachochytrium dendrobatidis* and keratin during tadpole development. Pacific Conservation Biology 10:173.

Marichal, P., J. Gorrens, and H. V. Bossche. 1985. The action of itraconazole and ketoconazole on growth and sterol synthesis in *Aspergillus fumigatus* and *Aspergillus niger*. Sabouraudia 23:13–21.

McMahon, T. A., N. T. Halstead, S. Johnson, T. R. Raffel, J. M. Romansic, P. W. Crumrine, and J. R. Rohr. 2012. Fungicide-induced declines of freshwater biodiversity modify ecosystem functions and services. Ecology Letters 15:714–722.

McMahon, T. A., J. M. Romansic, and J. R. Rohr. 2013. Nonmonotonic and monotonic effects of pesticides on the pathogenic fungus *Batrachochytrium dendrobatidis* in culture and on tadpoles. Environmental Science & Technology 47:7958–7964.

Mendelson, J. R., K. R. Lips, R. W. Gagliardo, G. B. Rabb, J. P. Collins, J. E. Diffendorfer, P. Daszak, R. Ibáñez, K. C. Zippel, and D. P. Lawson. 2006. Biodiversity-Confronting amphibian declines and extinctions. Science 313:48–48.

Olson, D. H., D. M. Aanensen, K. L. Ronnenberg, C. I. Powell, S. F. Walker, J. Bielby, T. W. Garner, G. Weaver, and M. C. Fisher. 2013. Mapping the global emergence of *Batrachochytrium dendrobatidis*, the amphibian chytrid fungus. PloS One 8:e56802.

Piovesan, G., and J. M. Adams. 2005. The evolutionary ecology of masting: does the environmental prediction hypothesis also have a role in mesic temperate forests? Ecological Research 20:739–743.

PPDB. 2014. Pesticide Properties Database http://sitem.herts.ac.uk/aeru/footprint/index2.htm. Agriculture & Environment Research Unit (AERU), University of Hertfordshire.

Reilly, T. J., K. L. Smalling, J. L. Orlando, and K. M. Kuivila. 2012a. Occurrence of boscalid and other selected fungicides in surface water and groundwater in three targeted use areas in the United States. Chemosphere 89:228–234.

Reilly, T. J., K. L. Smalling, E. R. Wilson, and W. A. Battaglin. 2012b. Dissolved Pesticides, Dissolved Organic Carbon, and Water-quality Characteristics in Selected Idaho Streams, April-December 2010. US Department of the Interior, US Geological Survey.

Relyea, R. A. 2005a. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. Ecological Applications 15:618–627.

Relyea, R. A. 2005b. The lethal impact of Roundup on aquatic and terrestrial amphibians. Ecological Applications 15:1118–1124.

Relyea, R. A. 2005c. The lethal impacts of Roundup and predatory stress on six species of North American tadpoles. Archives of Environmental Contamination and Toxicology 48:351–357.

Relyea, R. A. 2009. A cocktail of contaminants: how mixtures of pesticides at low concentrations affect aquatic communities. Oecologia 159:363–376.

Relyea, R. A., and J. T. Hoverman. 2008. Interactive effects of predators and a pesticide on aquatic communities. Oikos 117:1647–1658.

Rohr, J. R., and T. R. Raffel. 2010. Linking global climate and temperature variability to widespread amphibian declines putatively caused by disease. Proceedings of the National Academy of Sciences 107:8269–8274.

Rohr, J. R., T. R. Raffel, S. K. Sessions, and P. J. Hudson. 2008. Understanding the net effects of pesticides on amphibian trematode infections. Ecological Applications 18:1743–1753.

Romansic, J. M., A. A. Waggener, B. A. Bancroft, and A. R. Blaustein. 2009. Influence of ultraviolet-B radiation on growth, prevalence of deformities, and susceptibility to predation in Cascades frog (*Rana cascadae*) larvae. Hydrobiologia 624:219–233.

Rosslenbroich, H.-J., and D. Stuebler. 2000. *Botrytis cinerea* — history of chemical control and novel fungicides for its management. Crop Protection 19:557–561.

Scheffers, B. R., L. N. Joppa, S. L. Pimm, and W. F. Laurance. 2012. What we know and don't know about Earth's missing biodiversity. Trends in Ecology & Evolution 27:501–510.

Searle, C. L., J. R. Mendelson, L. E. Green, and M. A. Duffy. 2013. Daphnia predation on the amphibian chytrid fungus and its impacts on disease risk in tadpoles. Ecology and Evolution 3:4129–4138.

Shearer, C., E. Descals, B. Kohlmeyer, J. Kohlmeyer, L. Marvanová, D. Padgett, D. Porter, H. Raja, J. Schmit, H. Thorton, and H. Voglymayr. 2007. Fungal biodiversity in aquatic habitats. Biodiversity and Conservation 16:49–67.

Smalling, K. L., G. M. Fellers, P. M. Kleeman, and K. M. Kuivila. 2013. Accumulation of pesticides in pacific chorus frogs (*Pseudacris regilla*) from California's Sierra Nevada Mountains, USA. Environmental Toxicology and Chemistry 32:2026–2034.

Smalling, K. L., J. L. Orlando, D. Calhoun, W. A. Battaglin, and K. M. Kuivila. 2012. Occurrence of pesticides in water and sediment collected from amphibian habitats located throughout the United States, 2009–10. US Geological Survey Data Series 707:36.

Sparling, D. W., G. M. Fellers, and L. L. McConnell. 2001. Pesticides and amphibian population declines in California, USA. Environmental Toxicology and Chemistry 20:1591–1595.

Stuart, S. N. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306:1783–1786.

Vandenberg, L. N., T. Colborn, T. B. Hayes, J. J. Heindel, D. R. Jacobs Jr, D.-H. Lee, T. Shioda, A. M. Soto, F. S. vom Saal, W. V. Welshons, and others. 2012. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocrine Reviews 33:378–455.

Verweij, P. E., E. Snelders, G. H. Kema, E. Mellado, and W. J. Melchers. 2009. Azole resistance in *Aspergillus fumigatus*: a side-effect of environmental fungicide use? The Lancet Infectious Diseases 9:789–795.

Vogel, J. R., M. S. Majewski, and P. D. Capel. 2008. Pesticides in rain in four agricultural watersheds in the United States. Journal of Environment Quality 37:1101.

Woodhams, D. C., C. C. Geiger, L. K. Reinert, L. A. Rollins-Smith, B. Lam, R. N. Harris, C. J. Briggs, V. T. Vredenburg, and J. Voyles. 2012. Treatment of amphibians infected with chytrid fungus: learning from failed trials with itraconazole, antimicrobial peptides, bacteria, and heat therapy. Diseases of Aquatic Organisms 98:11–25.

Tables

Table 4.1. Compound, trade and chemical names of the fungicides used in this study, including mode of action and Fungicide Resistance Action Committee (FRAC) group number.

Compound Name	Trade Names	Chemical Name	Mode of Action	FRAC Group
Azoxystrobin	Heritage, Amistar	methoxyacrylates	inhibits respiration	11
Boscalid	Emerald, Endura, Pristine	Pyridine-carboxamides	inhibits respiration	7
Dimethomorph	Acrobat	cinnamic acid amides	inhibits cell wall synthesis	40
Fludioxinil	Maxim, Medallion	Phenylpyrroles	inhibits signaling	12
Tebuconazole	Elite	Triazoles	inhibits sterol synthesis	3
Triethylene Glycol	TEG	Dihydroxy alcohol	solvent for fungicides	NA

Table 4.2. *Batracochytrium dendrobatidis (Bd)* response to 5 concentrations of 5 current-use agricultural fungicides over time (three weeks) in separate experiments. Degrees of freedom in all treatments = 8. **bold** indicates a significant difference and * indicates a near significant difference in mean *Bd* density between the fungicide treatment and the triethylene glycol control.

		Day 0		Day 7		Day 14		Day 21	
Compound	Concentration	t-value	p-value	t-value	p-value	t-value	p-value	t-value	p-value
Azoxystrobin	14000 ppb	0.15	0.88	-0.98	0.36	-1.75	0.12	-1.61	0.15
	1400 ppb	-0.32	0.76	-1.47	0.18	-1.52	0.17	-1.22	0.26
	140 ppb	-0.42	0.68	-1.32	0.22	-0.66	0.53	0.34	0.73
	14 ppb	-0.06	0.95	-1.00	0.35	-0.74	0.48	-0.60	0.56
	1.4 ppb	-0.07	0.95	-0.52	0.62	0.01	0.99	0.41	0.69
Boscalid	14000 ppb	0.35	0.74	-1.52	0.17	-2.41	**0.04**	-0.57	0.59
	1400 ppb	0.50	0.63	-1.13	0.29	-2.48	**0.04**	-1.06	0.32
	140 ppb	-0.64	0.54	-0.98	0.36	-1.47	0.18	0.01	0.99
	14 ppb	-0.51	0.62	-1.19	0.27	0.70	0.50	-0.96	0.37
	1.4 ppb	-0.19	0.85	-0.39	0.70	0.11	0.91	1.16	0.28
Dimethomorph	14000 ppb	-0.09	0.93	-1.24	0.25	-3.28	**0.01**	-0.52	0.62
	1400 ppb	-0.99	0.35	-1.33	0.22	-2.76	**0.02**	0.30	0.78
	140 ppb	-0.48	0.64	-1.33	0.22	-2.15	0.06*	0.016	0.99
	14 ppb	-1.12	0.29	-1.71	0.13	-2.40	**0.04**	-0.13	0.90
	1.4 ppb	-0.57	0.58	-0.86	0.43	-2.48	**0.04**	0.13	0.90
Fludioxinil	14000 ppb	-0.14	0.89	2.74	**0.03**	2.41	**0.04**	3.17	**0.03**
	1400 ppb	-0.17	0.87	-2.05	0.07*	-2.88	**0.02**	-1.36	0.21
	140 ppb	-0.16	0.88	-1.45	0.19	-1.28	0.23	-0.47	0.66
	14 ppb	0.27	0.80	-0.52	**0.04**	-1.08	0.31	0.06	0.95
	1.4 ppb	-0.28	0.79	-1.87	0.10	-0.36	0.73	0.33	0.75
Tebuconazole	14000 ppb	-0.18	0.87	-1.38	0.21	-1.43	0.20	-1.25	0.25
	1400 ppb	-0.24	0.81	-1.91	0.09	-2.02	0.08	-0.09	0.93

140 ppb	0.05	0.96	-1.57	0.16	-2.74	**0.03**	-1.02	0.34
14 ppb	-0.38	0.71	-1.18	0.27	-3.21	**0.01**	-1.02	0.34
1.4 ppb	-0.00	1.00	-0.26	0.07*	-2.29	**0.05**	-1.04	0.33

Figures

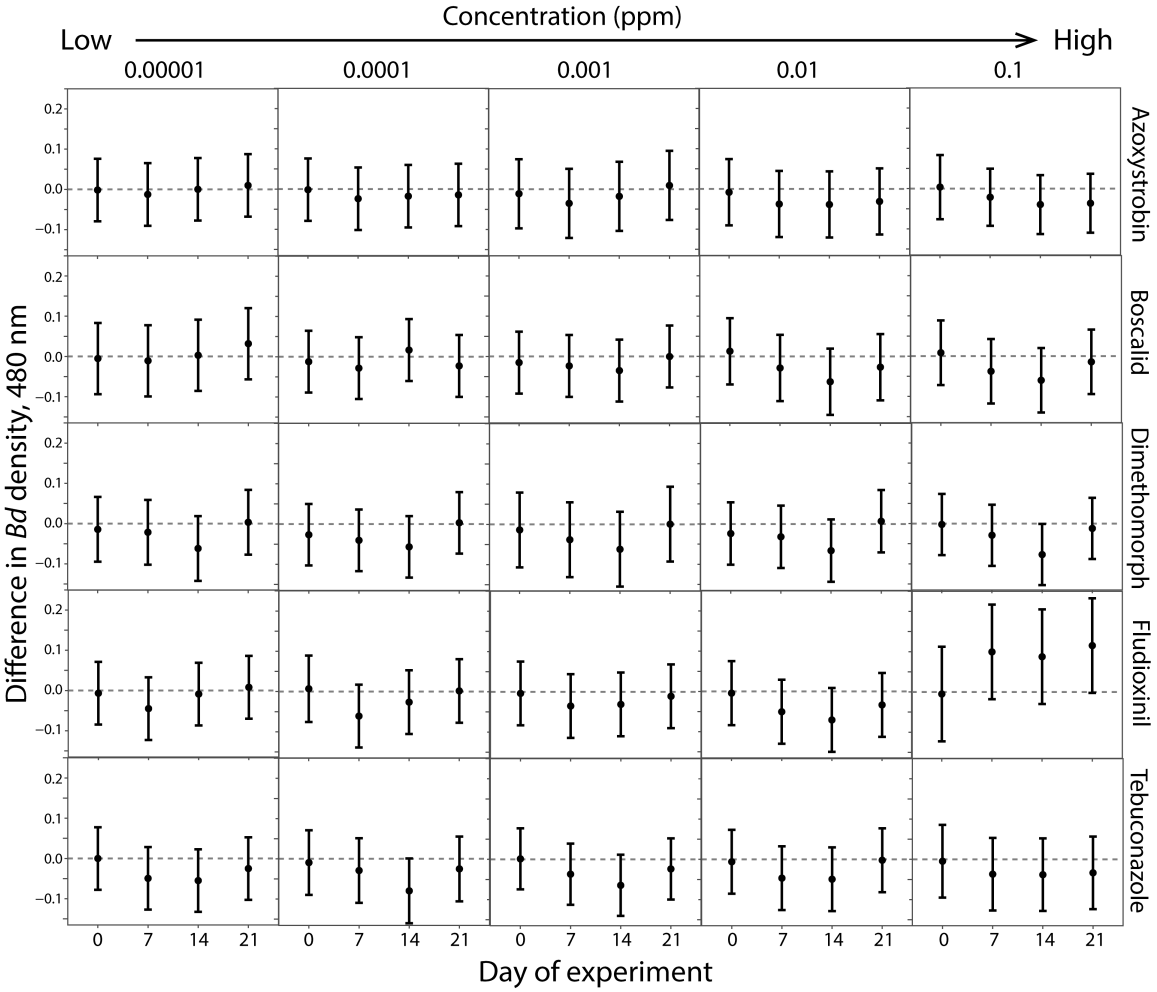

Figure 4.1. Observed difference in mean density with 95% confidence intervals between treatment and control of *Batrachochytrium dendrobatidis* (*Bd)* in culture in response to five concentrations of five current-use fungicides at seven-day intervals for three weeks.

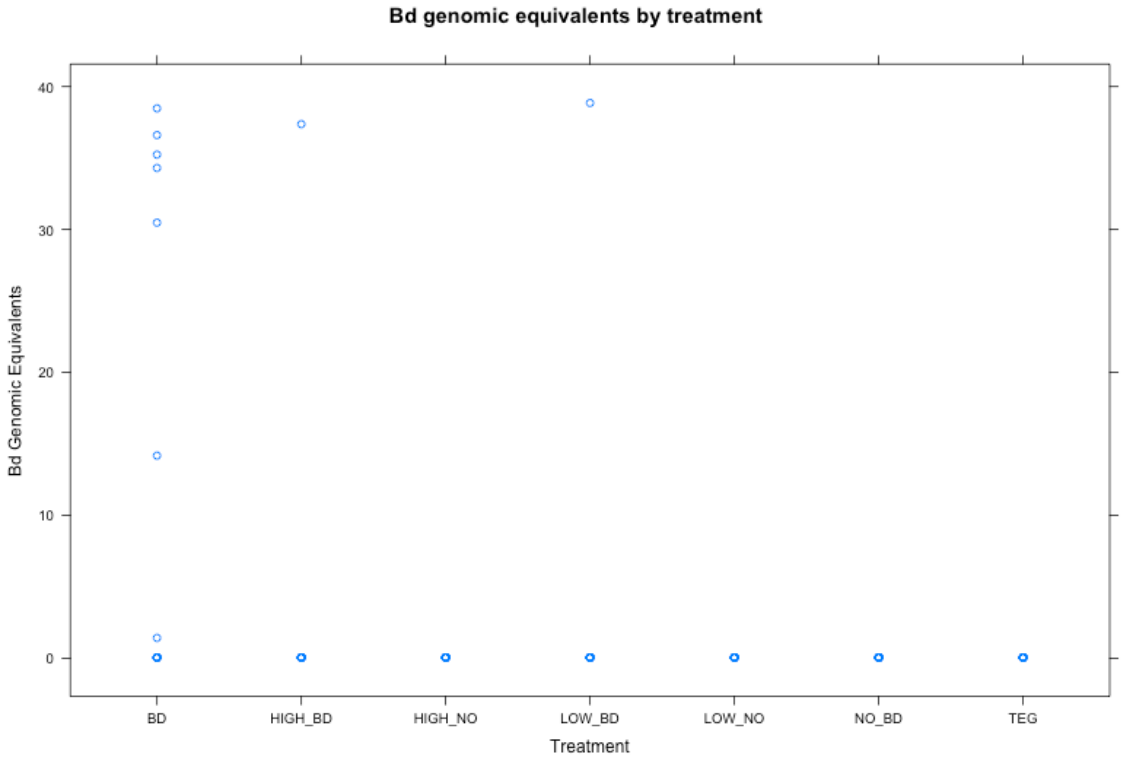

Figure 4.2. *Batrachochytrium dendrobatidis* (*Bd)* genomic equivalents (GE) estimated using quantitative PCR in *Pseudacris regilla* exposed to two tebuconazole concentrations: high = 14.29 parts per billion; low = 1.43 parts per billion.

CHAPTER 5. CONCLUSIONS: TROUBLE IN THE AQUATIC WORLD – HOW WILDLIFE PROFESSIONALS ARE BATTLING AMPHIBIAN DECLINES

Deanna H. Olson

The Wildlife Professional, p. 28-31

Winter 2014

A parasitic fungus similar to *Batrachochytrium dendrobatidis,* which caused the extinction of numerous tropical frog and toad species, is killing salamanders in Europe. Scientists first identified the fungus, *Batrachochytrium salamandrivorans,* in 2013 as the culprit behind the death of fire salamanders (*Salamandra salamandra*) in the Netherlands (Martel et al. 2013) and are now exploring its potential impact to other species. Although the fungus, which kills the amphibians by infecting their skin, has not yet spread to the U.S., researchers believe it's only a matter of time before it does and, when that happens, the impact on salamander populations could be devastating (Martel et al. 2014).

Reports of worldwide declines of amphibians began a quarter of a century ago (Blaustein and Wake 1990). Globally, some amphibian population declines occurred in the late 1950s and early 1960s, and declining trends continued in North America (Houlahan et al. 2000). In the earlier years, population declines were attributed primarily to overharvest due to unregulated supply of species such as the northern leopard frog (*Lithobates pipiens*) for educational use (Dodd 2013). In later years, however, causes of decline were less evident. In 1989, herpetologists at the First World Congress of Herpetology traded alarming stories of losses across continents and in seemingly protected landscapes, making it clear that amphibian population declines were a "global phenomenon." In response to these reports, in 1991, the International Union for Conservation of Nature or IUCN established the Declining Amphibian Populations Task Force to better understand the scale and scope of global amphibian declines. Unfortunately, the absence of long-term monitoring data and targeted studies made it difficult for the task force to compile information.

Today, according to AmphibiaWeb.org, there are 7,342 amphibian species in the world, double the number since the first alerts of declines, making the situation seem deceptively less dire. In fact, our understanding of genetic diversity significantly raises the stakes, and we are at risk of losing far more species than we believed only a few years ago. According to the IUCN, amphibians now lead the list of vertebrate taxa affected by the larger "biodiversity crisis" and sixth major mass-extinction event on Earth (Wake and Vredenburg 2008, Keith et al. 2014).

Decline and Mitigation

Across the world, numerous factors are responsible for the ongoing decline in amphibian populations such as habitat loss, invasive species, chemical contaminants, diseases, climate change, and synergisms among several of these factors occurring together.

The Oregon spotted frog (*Rana pretiosa*), recently listed as threatened under the Endangered Species Act (Federal Register 2014), is an example of a species facing combined threats. Once common in large, relatively warm wetlands with permanent water across the Pacific Northwest, Oregon spotted frog populations are believed lost from at least 78 percent of their former range. Factors (several of which are driven by human-caused changes to the landscape) such as loss of wetlands, hydrological changes, disease, and depredation by non-native predators including introduced trout and bullfrogs have contributed to declining populations.

Yet, for each of these threat factors alone or in concert, science, management, and the public are playing key roles in the form of research, management, and monitoring. The following is a sampling of ongoing efforts to address and mitigate threats facing amphibians in the U.S.

Research

One stressor that has been gaining attention, with significant research contributions over recent years, is the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*, which causes the disease chytridiomycosis. This pathogen, described 15 years ago (Longcore et al. 1999), can be lethal under some circumstances, which has been the case for the federally endangered mountain yellow-legged frog (*Rana muscosa*). The species appears to be more susceptible to chytridiomycosis than other frogs in the region, and exposure to pesticides may weaken its immune response.

In response, researchers are working to understand geographic and biologic occurrence of the fungus along with pathogenicity patterns. The U.S. Forest Service, with the help of world scientists, professional ecologists, resource managers, and volunteer citizen scientists, has developed occurrence maps reflecting the 1,377 species that have been inventoried for the fungus. It's widespread, found about half the time overall, yet at most sites with the fungus, amphibians do not show symptoms of chytridiomycosis. Now, emerging science suggests that some strains of the fungus may be located exclusively in North America (Schloegel et al. 2012). Consequently, it's likely that some North American amphibians co-evolved with some of these strains, which would explain why

we see amphibian populations that test positive for chytrid infection, but without disease-related die-offs. Given the ubiquity and antiquity of the global pandemic lineages of the amphibian chytrid, the question of whether some subtle environmental or strain change has occurred to trigger symptoms becomes extremely relevant.

New research aimed at studying the ecology of the fungus has also helped us to understand patterns of occurrence. This aquatic fungus appears to be sensitive to temperature conditions, having reduced occurrences in areas that get extremely hot and cold. In at least one strain of the fungus, researchers found that differences in generation time and fecundity were observed in response to different thermal regimes (Voyles et al. 2012). We pursued the fungus at high latitudes to see if its range might be limited there due to extreme cold temperatures. In Alaska, we found the fungus throughout the range of the wood frog (*Lithobates sylvaticus*), the only amphibian species that inhabits the Alaska interior and a unique frog species in that they "freeze" in winter. Our research showed that frogs can largely clear the pathogen during this process, although upon warming, lingering fungal zoospores appeared to be able to quickly re-establish infection.

In another recent advance in research, the occurrence of the amphibian chytrid fungus can now be detected as a free-living form by filtering water, in addition to swabbing animals to detect it infecting their skin. As part of our research, we described water sampling across the U.S. where we reported this fungus persisting year-round, with variable densities in the environment (Chestnut et al. 2014). Water is moved between watersheds for a number of management and conservation needs such as fire fighting, fish hatchery production, and reintroduction programs. Well-meaning nature enthusiasts

and teachers have relocated animals and released pets and classroom animals that may be infected with or carry the amphibian chytrid fungus. In addition, amphibians are part of an enormous world trade for food and pets, with hundreds of millions of animals, and water they live in, moving across borders every year. Hence, infected waters may be a concern for amphibian health as well as infected individual amphibians. In fact, the stakes for salamander conservation have been raised this year, given the deadly consequences of the newly described chytrid fungus, *Batrachochytrium salamandrivorans*, for many of the world's salamanders. The movement of animals or the water in which they're kept could prove lethal to native U.S. species such as forest-dwelling newts. The methods we used to sample wetlands for *Batrachochytrium dentrobatidis* can easily be modified to provide early detection for *Batrachochytrium salamandrivorans* and other aquatic diseases.

Monitoring and Modeling Tools

Using occupancy modeling, statistical methods that account for imperfect detection, in 2013 researchers with the U.S. Geological Survey Amphibian Research and Monitoring Initiative provided the first estimate of the rate of amphibian declines in the U.S. (Adams et al. 2013). They studied amphibian occupancy of sites on federal lands and reported that those populations from across the nation were declining at a rate of 3.7 percent per year, noting that salamanders were declining at a faster rate than frogs. Further, their research showed that amphibian occupancy of sites declined in all parts of the U.S., with the south experiencing greatest declines.

Today, researchers can better quantify species decline metrics with monitoring conducted by a diversity of professional ecologists, citizen scientists, and land managers. Standardized survey protocols have been established based on habitats and life history attributes of species in various regions and compiled into a manual developed by Partners in Amphibian and Reptile Conservation (Graeter et al. 2013). In 2000, Congress established the U.S. Geological Survey Amphibian Research and Monitoring Initiative to investigate status and trends of amphibians, identify causes of amphibian declines, and provide critical information to natural resource managers to support effective management actions to address declines. However, with our current knowledge of a variety of threat factors and their potential interactions, wildlife professionals might consider increasing the scope and scale of routine amphibian monitoring.

On-the-Ground Management

With ongoing management measures, wildlife professionals are already seeing signs of success. For example, research shows that habitat restoration can result in increases in spotted frog populations, and scientists noted that between 1991 and 2011, Columbia spotted frog populations (*Rana luteiventris*) in the northwestern U.S. grew rapidly in response to wetland restoration in areas with historical population declines (Hossack et al. 2013). Still, the long-term benefits of management efforts for many species are currently unknown, however, experts agree that no single action is enough to recover most species. Further, some threats are challenging to control such as the effects

of non-native fish and bullfrogs. For example, the Oregon spotted frog requires permanent year-round water and, as a result, management actions such as altering hydrologic regimes that would reduce or eradicate harmful predators could also hurt the frogs and other aquatic organisms.

In terms of threats from disease, federal and state wildlife agencies, in collaboration with the Woodland Park Zoo in Washington State have implemented reintroduction programs that screen Oregon spotted frogs for the amphibian chytrid fungus before release to new locations. In the wild, Oregon spotted frogs have tested positive for several diseases of concern including the amphibian chytrid fungus, the fungus-like pathogen *Saprolegnia* spp., and trematode parasite *Ribeiroia ondatrae*, which causes limb malformations. However, it isn't clear if these diseases are a threat to populations because the strains that occur throughout the range along with the effect of co-infections are not known. Strain differences warrant identification and study for differential pathogenicity. Screening animals prior to reintroduction is a sound precaution that will prevent the introduction of virulent strains that may present in the area where animals were collected but may be novel to areas they are released.

Collaborative Efforts

Ultimately, partnerships and joint efforts are critical in managing amphibian populations. For a host of considerations such as ethical, aesthetic, biomedical, ecological, or One-Health (a worldwide strategy for expanding interdisciplinary collaborations and communications for the health of humans and the environment) we are

no longer free to consider species as we once have. Our role has shifted from exploiting species for various uses toward becoming their stewards. The bridging of science, management, and the public to address amphibian declines is creating a new platform for conservation biology, where partnerships and open communication pathways expedite the pace of science and its application to field settings. It's a bottom-up approach where local human communities are making great strides to affect their local wildlife communities, and a top-down approach where programs that span regions and continents can have strong ripple effects. This is especially evident in the U.S., where a variety of state, federal, tribal, and private lands are being managed for ecosystem services inclusive of amphibian diversity.

Public and private coalitions are building as neighboring landowners are puzzling out where to establish protected areas, and how each group may contribute to addressing amphibian declines and identifying solutions, which may span a larger spatial context. As landowners and managers coordinate efforts, each entity contributes resources that allow species inventories across a much broader area than could be achieved by one landowner alone. This cooperative process facilitates greater understanding of amphibian species ecology, recognition of new species, understanding of known or suspected threats, and implementation of multi-agency protections. In some cases, these cooperative efforts and conservation strategies have made formal protections such as listing under the Endangered Species Act less necessary.

A prime example of this process has been the development of multi-agency conservation strategies among federal agencies, which have helped preclude formal

decisions to list species under the ESA. The tri-agency Conservation Strategy for the forest-dependent Siskiyou Mountains salamander (*Plethodon stormi*) is one example of this approach, where targeted surveys for animals and habitats resulted in discovery of a new species—the Scott Bar salamander (*Plethodon asupak*)—and the designation of high priority sites for long-term management of the Siskiyou Mountains salamander across a swath of Forest Service and Bureau of Land Management Lands, in cooperation with the U.S. Fish and Wildlife Service.

What Lies Ahead

We are entering a new age of information transfer about wildlife threats and population status, which enables an improved response of both research and management to a variety of stressors. E-communications and real-time web portals for information are being developed for a variety of purposes, and this will be changing how we aggregate and assess data, conduct risk assessments, and respond to critical issues. The fates of amphibians and other imperiled species are not random. There is a human link to most known amphibian threats and, as a result, we have a role in both their imperilment and stewardship. We expect to continue to test new tools for amphibian conservation that may have application for broader wildlife consideration. Amphibians also serve as ideal tools to teach future generations how wildlife are integrated with their environment, and how our actions affect their futures and ours. In this way, amphibians are helping to bind science, management, and the public into a new alliance for conservation.

References

Adams, M. J., D. A. W. Miller, E. Muths, P. S. Corn, E. H. C. Grant, L. L. Bailey, G. M. Fellers, R. N. Fisher, W. J. Sadinski, H. Waddle, and S. C. Walls. 2013. Trends in amphibian occupancy in the United States. PLoS One 8:e64347.

Blaustein, A. R., and D. B. Wake. 1990. Declining amphibian populations: a global phenomenon? Bulletin of the Ecological Society of America 5:203–204.

Chestnut, T., C. Anderson, R. Popa, A. R. Blaustein, M. Voytek, D. H. Olson, and J. Kirshtein. 2014. Heterogeneous occupancy and density estimates of the pathogenic fungus *Batrachochytrium dendrobatidis* in waters of North America. PLoS One 9:e106790.

Dodd, C. K. D. 2013. *Lithobates pipiens* (Schreber, 1782). pp. 578-608 *In:* Frogs of the United States and Canada, 2-vol. set. Johns Hopkins University Press, Baltimore. 1032p.

Federal Register. 2014. Endangered and Threatened Wildlife and Plants; Threatened Status for Oregon Spotted Frog; Final Rule. 79 FR 168 (August 29, 2014) pp.51658-51710. http://www.fws.gov/wafwo/species/osf/OSF_ListingFinalRule_29Aug2014.pdf

Graeter G. J., K. A. Buhlmann, L. R. Wilkinson, and J. W. Gibbons. 2013. Inventory and Monitoring: Recommended Techniques for Reptiles and Amphibians, with application to the United States and Canada. http://parcplace.org/parcplace/publications/inventory-and-monitoring-guide.html

Hossack, B. R., W. H. Lowe, J. L. Ware, and P. S. Corn. 2013. Disease in a dynamic landscape: Host behavior and wildfire reduce amphibian chytrid infection. Biological Conservation 157:293–299.

Houlahan, J. E., C. S. Findlay, B. R. Schmidt, A. H. Meyer, and S. L. Kuzmin. 2000. Quantitative evidence for global amphibian population declines. Nature 404:752–755.

Keith, D. A., M. Mahony, H. Hines, J. Elith, T. J. Regan, J. B. Baumgartner, D. Hunter, G. W. Heard, N. J. Mitchell, K. M. Parris, T. Penman, B. Scheele, C. C. Simpson, R. Tingley, C. R. Tracy, M. West, and H. R. Akçakaya. 2014. Detecting extinction risk from climate change by IUCN Red List criteria. Conservation Biology 28:810–819.

Longcore, J. E., A. P. Pessier, and D. K. Nichols. 1999. Batrachochytrium dendrobatidis gen et sp nov, a chytrid pathogenic to amphibians. Mycologia 91:219–227.

Martel, A., M. Blooi, C. Adriaensen, P. V. Rooij, W. Beukema, M. C. Fisher, R. A. Farrer, B. R. Schmidt, U. Tobler, K. Goka, K. R. Lips, C. Muletz, K. R. Zamudio, J. Bosch, S. Lötters, E. Wombwell, T. W. J. Garner, A. A. Cunningham, A. S. der Sluijs, S. Salvidio, R. Ducatelle, K. Nishikawa, T. T. Nguyen, J. E. Kolby, I. V. Bocxlaer, F. Bossuyt, and F. Pasmans. 2014. Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. Science 346:630–631.

Martel, A., A. S. der Sluijs, M. Blooi, W. Bert, R. Ducatelle, M. C. Fisher, A. Woeltjes, W. Bosman, K. Chiers, F. Bossuyt, and F. Pasmans. 2013. *Batrachochytrium salamandrivorans sp. nov*. causes lethal chytridiomycosis in amphibians. Proceedings of the National Academy of Sciences:110:15325-15329.

Schloegel, L. M., L. F. Toledo, J. E. Longcore, S. E. Greenspan, C. A. Vieira, M. Lee, S. Zhao, C. Wangen, C. M. Ferreira, M. Hipolito, A. J. Davies, C. A. Cuomo, P. Daszak, and T. Y. James. 2012. Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. Molecular Ecology 21:5162–5177.

Voyles, J., L. R. Johnson, C. J. Briggs, S. D. Cashins, R. A. Alford, L. Berger, L. F. Skerratt, R. Speare, and E. B. Rosenblum. 2012. Temperature alters reproductive life history patterns in *Batrachochytrium dendrobatidis*, a lethal pathogen associated with the global loss of amphibians. Ecology and Evolution 2:2241–2249.

Wake, D. B., and V. T. Vredenburg. 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proceedings of the National Academy of Sciences 105:11466–11473.