DISSERTATION

INFORMING THE ECOLOGY AND CONSERVATION OF AMPHIBIANS IMPERILED BY CHYTRIDIOMYCOSIS

Submitted by

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ABSTRACT

INFORMING THE ECOLOGY AND CONSERVATION OF AMPHIBIANS IMPERILED BY CHYTRIDIOMYCOSIS

More than 40% of the world's amphibian species face imminent extinction, and population declines have been documented on every continent where amphibians are found. The species experiencing declines and extinctions are not random; rather, the most vulnerable species have similar life history characteristics, geographic ranges, and taxonomic associations. The observed declines are occurring more quickly than background rates of extinctions, and the fact that low-density and endemic species seem to be disproportionately affected has important implications for the conservation of global biodiversity.

Scientists have identified several non-mutually exclusive drivers of global amphibian declines, ranging from well-studied factors including habitat loss and spread of invasive species to more recently identified phenomena such as climate change and emerging infectious diseases. A growing body of literature suggests that the emerging infectious disease chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (hereafter *Bd*), is responsible for many observed frog and toad declines. Despite more than 20 years of active research on *Bd* and its amphibian hosts, large knowledge gaps remain that limit our understanding of both the ecology of this pathogen and appropriate management actions critically needed to mitigate the effects of disease.

I sought to improve our understanding of amphibian-*Bd* dynamics and to provide information for those managing landscapes and species for resilience to chytridiomycosis. First, I

outlined a modeling framework that can be used to understand host-pathogen dynamics in systems where pathogens have free-living or vectored life stages. I discuss a sampling limitation that is common in amphibian-*Bd* systems and that creates an unobservable occupancy state. Using generated data, I assessed the impacts of this unobservable state on inference using several scenarios and suggest alternative strategies and sampling methods that would ameliorate difficulties with inference.

Next, I applied the aforementioned modeling framework to a historic amphibian-*Bd* dataset on boreal toads (*Anaxyrus boreas boreas*) in the Southern Rocky Mountains (SRM). Boreal toads are highly susceptible to *Bd*, but populations demonstrate some variability in local extinction probabilities across the SRM. I evaluated several potential drivers of amphibian-*Bd* disease dynamics and identified elevation as the factor with the strongest influence on boreal toad-*Bd* dynamics. The importance of elevation suggests that an interaction between host density and pathogen physiological tolerances may result in the variation in disease risk observed in this system. Conservation biologists and managers can use these findings to prioritize sites for management intervention and to select among conservation actions including reintroductions, translocations, and habitat manipulations. Though I gained valuable information from this historic dataset, some parameters of biological interest were not estimated well due to the sampling dependencies that were present in this (and most) historic amphibian-*Bd* datasets.

Amphibian skin swabs are the primary means of detecting *Bd* in amphibian-*Bd* systems. As a result of this dependence, I encountered difficulties estimating *Bd's* distribution and persistence in the absence of amphibians. I evaluated the utility of water filtration for detecting *Bd's* aquatic flagellated zoospore in the environment to alleviate these difficulties. I used a controlled laboratory experiment to assess how water type, *Bd* concentration, and the presence of

PCR inhibitors influence detection probability of *Bd* using water filtration. I found that detection probability was low unless PCR inhibitors were removed, but collecting multiple samples in time or space could compensate for low detection and generate unbiased estimates of occurrence. In addition, I found that the abundance of *Bd* estimated from qPCR did not accurately reflect true *Bd* abundance. My work illustrates the feasibility of environmental sampling for *Bd* and other aquatic organisms, and I presented design- and model-based recommendations for those studying amphibian disease dynamics, other host-pathogen systems, and those using environmental DNA (eDNA) to understand species distributions.

Finally, I used the environmental sampling method validated in the laboratory (i.e., filtered water samples), in conjunction with amphibian skin swabs, to present the first estimates of *Bd* occurrence that are decoupled from amphibian populations in Chapter 4. I compared estimates of detection probabilities for the two pathogen detection methods, as well as hypothesized factors influencing these parameters. In addition, I compared decision criteria to classify samples as positive or negative, based on PCR results, and to evaluate inferential differences that result from different decision criteria. I found that heterogeneity in *Bd* occurrence was linked to the time since the predominant amphibian species was last detected at a site, rather than to environmental covariates (e.g., elevation). The decision criterion employed did not influence which covariates were deemed important but did result in different estimates for some parameters. Using a conservative decision criterion, estimates of *Bd* occurrence and swab-based detection probability were lower than with a more liberal criterion. Filtration-based detection probabilities were low and increased over the course of the season, while swab-based detection probabilities were higher and varied by season and by elevation. My work provides evidence of long-term *Bd* persistence in the environment and brings attention to the importance

of sampling the environment directly for understanding and mitigating disease-related threats to amphibian biodiversity.

In summary, my dissertation provides valuable information to researchers and managers studying disease-related declines of amphibians at local, regional, and global scales. I have brought attention to a common sampling framework in these systems that limits inferences about the distribution and dynamics of *Bd*, validated a new sampling technology that will allow for improved estimation, and implemented a landscape-level field study using this technology that provides our first estimates of *Bd* distribution and persistence independent of amphibian populations. I also used a historic dataset to evaluate competing drivers of extinction risk for boreal toads and to identify potential elevational refuges for this species of concern. My findings provide some of the most complete estimates of amphibian-*Bd* dynamics in a temperate North American system, and are being used by the partners of the Boreal Toad Recovery Team as they implement management strategies, consider boreal toad reintroductions, and continue to monitor both boreal toads and *Bd* in the SRM. The recent discovery of *Batrachochytrium salamandrivorans*, an aquatic pathogen of salamanders with many similarities to *Bd*, makes my work salient to the understanding and management of other emerging infectious diseases. I have advanced the science of amphibian disease ecology and pathogen detection, and my work will be relevant to the global community of researchers and managers striving to understand and conserve amphibian populations imperiled by disease.

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SUMMARY

Modeling of species distributions has undergone a shift from relying on equilibrium assumptions to recognizing transient system dynamics explicitly. This shift has necessitated more complex modeling techniques, but the performance of these dynamic models has not yet been assessed for systems where unobservable states exist. My work is motivated by the impacts of the emerging infectious disease chytridiomycosis, a disease of amphibians that is associated with declines of many species worldwide. Using this host-pathogen system as a general example, I first illustrate how misleading inferences can result from failing to incorporate pathogen dynamics into the modeling process, especially when the pathogen is difficult or impossible to survey in the absence of a host species. I found that traditional modeling techniques can underestimate the effect of a pathogen on host species occurrence and dynamics when the pathogen can only be detected in the host, and pathogen information is treated as a covariate. I propose a dynamic multistate modeling approach that is flexible enough to account for the detection structures that may be present in complex multistate systems, especially when the sampling design is limited by a species' natural history or sampling technology. When multistate occupancy models are used and an unobservable state is present, parameter estimation can be influenced by model complexity, data sparseness, and the underlying dynamics of the system. I

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show that, even with large sample sizes, many models incorporating seasonal variation in vital rates may not generate reasonable estimates, indicating parameter redundancy. I found that certain types of missing data can greatly hinder inference, and I make study design recommendations to avoid these issues. Additionally, I advocate the use of time-varying covariates to explain temporal trends in the data, and the development of sampling techniques that match the biology of the system to eliminate unobservable states when possible.

INTRODUCTION

Drawing inferences about spatial and temporal dynamics of species' distributions is central to our understanding of ecology, as well as to making effective conservation and management decisions. Unfortunately, our understanding of the biotic and abiotic factors structuring species' distributions are often based on static patterns that assume that species are at equilibrium with the environment, despite the fact that both species distributions and the environment are dynamic and distributional responses to change are not instantaneous (Yackulic et al. 2015, Clement et al. 2016). Many ecosystems under pressure from climate change, invasive species, habitat fragmentation, or emerging infectious disease are likely experiencing transient dynamics, where species distributions are not at equilibrium (Ovaskainen and Hanski 2002). In these cases, static distribution models can miss important dynamics and yield misleading biological inferences (Yackulic et al. 2015). Direct observations of the transient dynamics should yield stronger inferences, but such studies can be hindered by an inability to identify the system's state or to appropriately account for important environmental variables. To address these challenges, scientists have developed complex multistate occupancy models that incorporate the dynamic nature of habitats, multiple interacting species, and other factors of interest, while accounting for species' nondetection or state misclassification (Nichols et al. 2007, Richmond et

al. 2010, Miller et al. 2012a). However, increasing the number of mutually exclusive states in a system can result in cases where the true species distribution is unknown or even unobservable.

State uncertainty arises when the state of a study unit cannot be ascertained perfectly by an observer (Pradel 2005). An unobservable state is an extreme case of state uncertainty where it is impossible to properly assign the unit's true state (state-specific detection probability = p_{state} = 0). State uncertainty and its consequences have been well-studied for individual-based mark-recapture models (Kendall and Nichols 2002, Conn and Cooch 2009, Bailey et al. 2010), but less so for multistate occupancy models where state uncertainty may also occur (but see (Royle and Link 2006, Miller et al. 2011). Regardless of the model class, unobservable states may lead to misleading biological inferences due to parameter bias, lack of precision, or parameter redundancy (Catchpole and Morgan 1997, Cole et al. 2010, Hubbard et al. 2014).

Multistate occupancy models assume that an unknown underlying species distribution describes a population of sample sites (Nichols et al. 2007, MacKenzie et al. 2009). Multiple occupied states are defined by an investigator depending on the biological questions of interest and may include reproductive status (Martin et al. 2009), disease state (Elmore et al. 2014), relative abundance (Falke et al. 2010), competitor occurrence (Steen et al. 2014), and/or habitat features (Miller et al. 2012a). Imperfect state assignment occurs during the observation process, as sites are repeatedly surveyed over a period of time when the state of the site is assumed to be static. Though the state does not change, the observations by the observers, which are imperfect, may change.

The direct estimation of detection and classification probabilities to account for state uncertainty assumes that detection is possible for all occupied states (MacKenzie et al. 2009). This assumption may be violated in some scenarios, resulting in an unobservable state, but the

repercussions of this violation have not yet been explored in occupancy models. Unobservable states may stem from limitations in the sampling design or the nature of the system. For example, it may be difficult or impossible to identify certain life stages of species due to lack of technological ability or lack of knowledge about the system.

Host-pathogen systems are one case where unobservable occupancy states may exist. For example, in the simplest case with a single target host and single free-living or vectored pathogen, the pathogen-only state cannot be observed if detection of the pathogen is conditional on host occurrence and detection unless alternative host species are sampled. Ignoring the unobservable state may lead to misleading conclusions regarding host-pathogen dynamics. I use expected values data generated under a simple host-pathogen scenario to explore the bias in parameters estimated when the unobservable state is "ignored" and pathogen detection is instead treated as a dynamic covariate influencing host occurrence. Next, I adopt a 2-species occupancy modeling approach (a special case of a multistate occupancy model; Richmond et al. 2010) where the probability of detecting the pathogen is fixed at 0 both when hosts are present but not detected, and when the pathogen occurs in isolation outside of the target host.

I used analytic and numeric approaches to explore how parameter values, sample size, and amount of missing data influence precision, bias, and parameter redundancy in this simple host-pathogen example with wide applicability to other systems. Obtaining unbiased estimates of system dynamics is necessary to correctly identify factors influencing changes in species distributions over time. These inferences and estimates are central to our ability to understand transient system dynamics and evaluate the effects of proposed management and conservation actions using predictive models. This work is the first to explore the impacts of unobservable

occupancy states on inference in multistate occupancy systems, and will be useful to those making conservation decisions in host-pathogen and other complex systems.

METHODS

Study System

Though my work is widely applicable to a suite of biological systems, it is motivated by impacts of chytridiomycosis, an emerging infectious disease of amphibians worldwide (Skerratt et al. 2007). The causative agent of chytridiomycosis is the pathogen *Batrachochytrium dendrobatidis* (*Bd*), a fungus with a free-living aquatic zoospore that can infect amphibian skin (Berger et al. 2005). Understanding changes in the transient dynamics of both amphibian hosts and *Bd* is necessary to inform management during and after the onset of a chytridiomycosis-linked perturbation to the host population or metapopulation. Of particular interest to us is the boreal toad (*Anaxyrus boreas boreas*) and *Bd* system in the Southern Rocky Mountains (SRM). Historically, sampling in the SRM consisted of detection-nondetection records for the host species (the boreal toad), with opportunistic swabbing of toads as the only means of detecting the pathogen (*Bd*), resulting in an unobservable, pathogen-only state. This scenario is typical of many long-term amphibian-*Bd* datasets. During a given season, each sampled potential breeding site must be in one of four mutually exclusive states: occupied by the target amphibian (host only, state A), occupied by *Bd* (pathogen only, state B), not occupied by either amphibian host or *Bd* (unoccupied, state U), or occupied by both the target amphibian and *Bd* (host and pathogen, state AB).

Modeling Framework

To explore the benefits and limitations of dynamic multispecies modeling, I assumed that the dynamics of my host-pathogen system were consistent with a 2-species occupancy model

(MacKenzie et al. 2004, Richmond et al. 2010), where pathogen detection depends on host occurrence and detection. Accordingly, I used the conditional binomial parameterization outlined by (Richmond et al. 2010) with a robust design extension to investigate dynamics across years. The model estimates the unconditional probability of initial host species (species A) presence (ψ^A) and the probability of pathogen (species B) presence, conditional on host presence (ψ^{BA}) or absence (ψ^{Ba}). Estimates of other state probabilities of interest can be calculated from these estimates, for instance the initial state distribution vector (for states A, B, U, and AB), φ_0:

$$\varphi_0 = [\psi^A(1-\psi^{BA}) \quad (1-\psi^A)\psi^{Ba} \quad (1-\psi^A)(1-\psi^{Ba}) \quad \psi^A\psi^{BA}]$$

Transitions among the four states (Figure 1.1) can be expressed as a transition probability matrix φ_t, with rows denoting the state of the site at time t and columns denoting the state of the site at time $t+1$.Transitions are parameterized as a function of colonization (γ) and extinction (ε) probabilities similar to those presented in Miller et al. (2012a), and are state dependent (Table 1.1). For instance, the probability of a site transitioning from state AB (host and pathogen) in year t to state B (pathogen only) in year $t+1$ is the product of the probabilities of host extinct and pathogen persistence, given the presence of both species (i.e., $\varepsilon^{AB}(1-\varepsilon^{BA})$).

Multiple state-specific detection parameters allow investigators to explore scenarios where the detection of the pathogen species (B) may vary as a function of the presence and/or detection of the host species (A). The detection matrix p_t gives the probability of observing each possible state (columns) given a corresponding true state (rows; Table 1.2). Detection probabilities denoted p^x indicate detection of species x when it occurs alone, while probabilities denoted r^y indicate detection when species co-occur. Species B's detection probability may differ depending on whether species A is present and detected ($y = BA$) or present and undetected ($y = Ba$). This model assumes that false positive detections do not occur. I

constrained the probability of detecting the pathogen when it occurs alone (p^B) or with an undetected host (r^{Ba}) to 0 based on my SRM boreal toad-*Bd* dataset, where boreal toad swabs are the only means of detecting *Bd*. I generated expected values data, and analyzed those data using various model types and structures, described below.

Single-species Model

To quantify the bias induced when the unobservable state is "ignored" and pathogen detection is simply treated as a dynamic covariate, I generated expected values data using the 2-species model described above in Program GenPRES (Hines 2006) using parameter values that I deemed realistic for the SRM boreal toad-*Bd* system (Table 1.3). I generated detection-nondetection records for 80 sites over 4 seasons, assuming 2 surveys were conducted per season. The generated data were designed to mimic the SRM boreal toad-*Bd* system and assume a high initial occupancy of toads (ψ^A =0.90) because the historic dataset conditions on boreal toad breeding sites rather than a random sample of potential breeding sites. The selected "true" parameters simulate a decline in host from 0.90 to 0.71 over 4 years (Table 1.3), which reflects of the status of SRM toad populations (unpublished dataset, Boreal Toad Recovery Team).

The generated data were converted to a dynamic single-species dataset for host occurrence, with annual pathogen detection (1) or nondetection (0) information used as a time-varying covariate. First, I used the initial occupancy parameterization of the dynamic single-species occupancy model (MacKenzie et al. 2003), ran a single model consistent with the true model ($\psi_1(.)\gamma(Bd)\varepsilon(Bd)p(Bd)$), and compared resulting estimates to the true parameter values used to generate the expected values data. Next, using an alternative parameterization ($\psi(Bd)\varepsilon(Bd)p(Bd)$; MacKenzie et al. 2003), I modeled host occupancy and extinction

probabilities as a function of annual pathogen detection and investigated the magnitude and direction of the estimated effect of *Bd* on boreal toad occurrence.

2-species Model

The identifiability of the 2-species occupancy model has not been assessed when an unobservable state is present. Parameter redundancy, or non-identifiability, may occur when a dataset lacks information to estimate unique values for some model parameters. Redundancy can be either intrinsic, stemming from inherent model properties, or extrinsic, stemming from data sparseness (Gimenez et al. 2004). Several methods have been used to assess parameter redundancy in mark-recapture models (Gimenez et al. 2004) and I employ three to explore redundancy in my host-pathogen system: symbolic differentiation (Catchpole and Morgan 1997, Cole et al. 2010), an analytic-numeric method (Burnham 1987), and data cloning (Lele et al. 2007).

Symbolic differentiation is an analytic method that assesses the rank of a derivative matrix (the Jacobian) for a model using symbolic algebra (Catchpole and Morgan 1997, Cole et al. 2010), and is the preferred method for assessing parameter redundancy (Gimenez et al. 2004, Bailey et al. 2010). Full-rank matrices indicate that all parameters in a model are uniquely identifiable, while rank-deficient matrices indicate parameter redundancy. In addition to calculating the number of identifiable parameters, the identifiable parameter combinations may also be obtained based solely on model structure (Catchpole et al. 1998). Using symbolic differentiation, I evaluated models where all vital rates were constant or time-varying, and detection probabilities varied by survey, season, both, or were constant, creating 8 different models. I used a hybrid symbolic-numeric method with the symbolic algebra software MAPLE

to overcome memory issues in MAPLE and obtain final parameter redundancy results (Choquet and Cole 2012).

Symbolic differentiation is often difficult to employ for complex models (Forcina 2008, Hunter and Caswell 2009) and in these cases alternatives exist (Choquet and Cole 2012, Cole 2012) that can assess parameter redundancy. In addition to using the hybrid symbolic-numeric method, I also assessed bias, precision, and parameter redundancy using a numeric-analytic approach (Burnham 1987) and data cloning (Lele et al. 2007). To accomplish this, I generated expected values data under a "true" model and then analyzed the resulting dataset using different model structures (Burnham 1987, Schaub et al. 2004, Bailey et al. 2010). Bias and precision were assessed by comparing model estimates to the true parameter values and by using these metrics to investigate issues related to intrinsic or extrinsic parameter redundancy. If the identifiability of a parameter was questionable based on the numeric-analytic results (e.g., the standard error for a parameter was very large, but potentially reasonable), data cloning was used (Lele et al. 2007). This method artificially "clones" the observed data to increase sample size and provides a way to determine the identifiability of parameters in a model by approximating the asymptotic standard error of maximum likelihood estimates (Lele et al. 2010). If the standard error of the parameter of interest decreases predictably toward zero as the number of clones increases, the parameter is estimable. If not, the parameter is considered unidentifiable. Data cloning for select models was implemented in Program MARK using 1000 clones. Numeric methods are not prone to the computer memory problems that symbolic differentiation encounters, but can be subject to inaccuracies (Gimenez et al. 2004, Hunter and Caswell 2009).

I generated expected values data using parameter values from the SRM boreal toad-*Bd* system above (n = 80) and then: (1) decreased the quantity of data by introducing 'missing

values' to explore impacts of data sparseness, (2) increased the sample size two-fold (n = 160) to investigate how a reasonable increase in sample size might alter precision, and (3) increased the sample size one-hundred-fold (n = 8000) to separate intrinsic from extrinsic identifiability issues. In the missing values dataset, I considered the original 80 sites, where 20 sites were sampled for both species in every season (year), 20 sites were sampled in even seasons only, 20 sites were sampled in odd seasons only, and 20 sites were sampled each season for boreal toads but never for *Bd*. This sampling scheme reflects the tradeoffs that managers and biologists commonly make with limited time or financial resources. Finally, I explored a new set of parameter values for a different hypothetical host-pathogen system to determine how differences in host-pathogen dynamics may influence identifiability, bias, and precision for two sample sizes (n = 80, and n = 8000). This new system reflects a rare host species that is highly susceptible to disease (hereafter "rare and susceptible host system", $\varepsilon^{AB} = 0.9$) and assumes a decline in host occurrence from 0.20 to 0.07 over 4 years (Table 1.3).

I investigated bias, precision, and parameter redundancy in these 6 unique scenarios by fitting a suite of forty-eight 2-species models to each generated dataset using RMark (Laake 2013), a formula-based interface for Program MARK (White and Burnham 1999). The models included annual variation or constant structures for vital rates, and detection structures that were constant or variable by season or survey (Table 1.4). To reduce the number of models in the candidate set, estimated detection parameters (p^A, r^A, and r^{BA}) were constrained to have the same structure within a given model. Vital rate pairs (γ^A and γ^{AB}, γ^B and $\gamma^{BA}, \varepsilon^A$and ε^{AB}, ε^B and ε^{BA}) were also constrained to have identical structures within the same model (Table 1.4). I ran the simplest model first, using simulated annealing, and fit new models using initial values from the simplest model to reduce computing time and to improve convergence.

RESULTS

Single-species Model

I found that using a covariate for an imperfectly detected pathogen resulted in bias in several vital rate parameters, using the initial occupancy parameterization (Table 1.5). Colonization of the host species in the absence of the pathogen (γ^A) was underestimated and colonization of the host in the presence of the pathogen (γ^{AB}) was overestimated, leading to the inappropriate conclusion that host colonization is independent of pathogen presence (true difference in colonization probability = 0.65, estimated difference = 0.02). In addition, the extinction probability of hosts in the absence of the pathogen (ε^A) and the detection probability of hosts in the presence of the pathogen (r^A) were both overestimated (Table 1.5). When I used the alternative parameterization and modeled host occupancy as a function of pathogen detection, the estimated relationship between *Bd* and host occurrence was positive ($\hat{\beta}$ =19.90) and was poorly estimated. This result is clearly incorrect, as the data were generated under the assumption that *Bd* negatively influences host occurrence. However, because *Bd* is only detected when the host is detected, all sites with *Bd* detections were also known to be occupied by the host, yielding the erroneous positive relationship with host occupancy.

2-species Models

Using symbolic differentiation, I found that the 2-species model had no intrinsic parameter redundancy when all four states were observable, even when vital rate and detection probabilities varied over time and/or surveys. However, when one of the states was unobservable, as in the SRM boreal toad-*Bd* system, the matrix was full-rank only when all vital rate parameters were time-constant and there were at least 3 seasons of data, with 2 or more surveys per season. Models with vital rate parameters that varied with time showed some

parameter redundancy, however, I was unable to obtain the estimable parameter combinations and determine which parameters were inestimable due to computational complexity and memory problems in MAPLE (Appendix 1.1). I was unable to identify a natural reparameterization for this model that would prove intrinsic parameter redundancy (e.g., as illustrated for different models in Cole 2012).

Our numeric-analytic results corroborated the symbolic differentiation results, showing that the 3 models with time-constant vital rates provided unbiased estimates for all parameters regardless of the sample size, amount of missing data, parameter values, or detection structures used. In the SRM boreal toad-*Bd* system, all parameters in these models were estimated with a reasonable level of precision (SE < 0.50) at the smallest sample size evaluated (n = 80), and ψ^{Ba}, γ^{A}, and ε^{B} had the largest standard errors among the parameters estimated (Chapter 1 Appendix 1.2). In the rare and susceptible host-pathogen system, ψ^{Ba}, γ^{B}, and ε^{B}had the largest standard errors, which were > 0.5 at the smallest sample size evaluated (Appendix 1.3). In general, standard errors were larger in the rare and susceptible system than in the boreal toad system, and ψ^{Ba} was the least precise parameter in both systems. In both the SRM-boreal toad and rare and susceptible systems, precision improved with sample size, and precision was quite high when 8000 sites were used (Appendices 1.2 and 1.3) indicating that some parameters may only be extrinsically identifiable at sample sizes that are not plausible in the real world. The introduction of missing data increased standard errors but did not create bias in time-constant models. For example, the average standard error for ψ^{Ba} in time-constant models in the SRM-boreal toad system was 0.34 with a sample size of 80 sites, 0.50 when missing data were considered in the 80 site dataset, 0.24 using 160 sites, and 0.03 using 8000 sites (recall the true and estimated ψ^{Ba} value was 0.05).

Of the 45 models with time variation in vital rates, 27 models appeared to be completely identifiable based on numeric-analytic and data cloning results (Appendices 1.2 and 1.3). This finding was independent of the dataset used. Despite many models being intrinsically identifiable, only 9 models with time variation yielded reasonable standard errors (SE < 0.50 on the probability scale) for all parameters in the SRM system at the smallest sample size, and no models were completely without precision issues for the rare and susceptible host system for the same sample size (n = 80). Other models, despite being identifiable, had extreme precision issues at small sample sizes. The 9 models providing valid inference for all parameters in the SRM system included models with time variation in host extinction (ε^{A} and ε^{AB}) and/or pathogen colonization (γ^{B} and γ^{BA}), but without time variation in other parameters. Though all parameters were technically identifiable in these models, the standard errors for $\psi^{Ba}$and $\varepsilon^{B}_{t=1}$ were large (>0.30), especially at small sample sizes. For the remaining models with time variation (18 models), a comparison of results from the smallest and largest sample sizes, in conjunction with data cloning, imply that a subset of initial occupancy (ψ^{Ba}) and vital rate parameters at the beginning of the time series ($\gamma^{A}_{t=1}$, $\gamma^{AB}_{t=1}$, $\gamma^{B}_{t=1}$and $\varepsilon^{B}_{t=1}$) are often unidentifiable in models with complex time structures. These parameters are frequently biased with inestimable standard errors (Appendices 1.2 and 1.3). Patterns were consistent between the SRM and rare and susceptible host systems, but precision for identifiable parameters worsened with the inclusion of missing data and decreasing sample size.

DISCUSSION

When the detection of one species is related to the presence and/or detection of another, using an imperfectly detected covariate to describe a relationship in occurrence will often result in biased estimates of the effect being studied and thus misleading inferences. This problem

stems from the fact that what is actually being modeled is the relationship between the occurrence of one species and the detection of the other, rather than the independent occurrences of both species. While this problem has not been addressed explicitly until now, the issue has been intuited by ecologists for some time. For instance, initial work on barred and spotted owls in the Pacific Northwest of the US focused on using barred owl detection/nondetection data to model the occurrence of northern spotted owls (Kelly et al. 2003, Olson et al. 2005). Later, static 2-species models demonstrated that barred owl detection was imperfect and potentially influenced by northern spotted owl detection (Bailey et al. 2009). Current research in this system uses a multispecies occupancy approach, similar to the methods I employed, to model variation in detection probabilities based on species co-occurrence and to estimate the distributional dynamics of both species (Yackulic et al. 2014, Dugger et al. 2016).

Here, I examined an extreme case where detection of one species is completely dependent upon the occurrence and detection of another, resulting in an unobservable occupancy state. Though I use an amphibian-*Bd* system to describe my approach, this situation may exist in cryptic predator-prey systems, systems with territorial species, and many other host-pathogen systems. Using an imperfectly detected pathogen covariate resulted in underestimation of the effect of the pathogen on host colonization probability in one parameterization. My SRM example was based on data from historic breeding sites at the start of an epidemic, where most sites were occupied by the host at the beginning of the study, leaving few unoccupied sites to be colonized. For other systems with different initial state distributions, the parameters that are biased may vary, suggesting that the nature of the biological system likely influences which parameters are most difficult to estimate. Using the alternative parameterization, where occupancy is modeled directly as a function of the *Bd* detection as a covariate, I estimated an

erroneous positive effect of *Bd* on amphibian occurrence. My work may explain the puzzling findings of other studies where the effect *Bd* on host amphibians appears to be minimal or even slightly positive (Grant et al. 2016a) in regions with local extinctions attributed to *Bd*. Underestimating or misidentifying the impact of one species on another could result in host declines going undetected for long periods of time, and the failure to detect a negative pathogen effect when one exists. Management and conservation actions may be triggered late, or not at all, resulting in a higher likelihood of species extinction. I recommend that biologists explore potential biases in their system following the framework that I have outlined in this manuscript to avoid making erroneous inference.

Though challenges arising from computational complexity and computer memory made symbolic differentiation difficult, I was able to use a combination of methods to determine that several parameters were intrinsically unidentifiable for models with complex time-varying parameter structures. More importantly, I found that some models with simple forms of time variation were both intrinsically and extrinsically identifiable, even for realistic sample sizes. My results inform researchers working in similar host-pathogen systems about which hypotheses can be reliably investigated and also about which parameters may be biased or extremely imprecise. In particular, I found that estimates of vital rate parameters at the beginning of the time series, the extinction probability of the pathogen (ε^{B}), and the initial probability of the pathogen when it occurs alone (ψ^{Ba}) often suffered from bias, imprecision, and unidentifiability depending on model structure.

The mark-recapture literature has shown that transitions to and from unobservable states (temporary emigration) are not identifiable at the end of a time series when these parameters are time-specific (Kendall et al. 1997). Additionally, survival of unobservable individuals is only

estimable if it is constrained to be equivalent to observable individuals (Kendall and Nichols 2002). My work demonstrates a similar parallel for multistate occupancy models, as I found that state and vital rate parameters that condition on the unobservable state (the pathogen-only state in my example), are unidentifiable in many models that incorporate time variation in vital rates. Setting the first transition parameters equal to transition parameters for a later season, similar to a suggestion in the mark-recapture literature (Kendall et al. 1997), made all parameters identifiable (including ψ^{Ba}), but this remedy should be applied cautiously. The decision of which vital rates in the time series to set equal is an important one, based on the biological knowledge of the system. If substantial time variation in vital rates exists in the system, setting two truly different vital rate parameters equal to one another will produce biased estimates of both parameters, and may also cause bias in other parameters (Langtimm 2009). Another solution is to carefully evaluate which parameters are identifiable, as I did here, and to avoid interpretation of parameters that are not a true reflection of the biology of the system.

Our dynamic multispecies results illustrate that, for a plausible real-world system, many models of interest may yield misleading or imprecise results when states are unobservable. These problems are exacerbated by data sparseness in the form of missing data, small sample sizes, and rare or difficult-to-detect species of interest. Data sparseness in these various forms is known to be problematic for occupancy models in general (Mackenzie and Royle 2005), but difficulties are amplified when unobservable states and time variation in vital rate parameters occur. For these reasons, it will be difficult to estimate time variation in occupancy dynamics for species that are inherently rare, infrequently sampled, or for species that have declined to only a fraction of their former range. If temporal variation in species vital rates is truly of interest, the only way to confidently estimate these dynamics is to eliminate the unobservable state. In the amphibian-*Bd*

system that I describe, using water filtration to detect the pathogen in its free-living form (Kirshtein et al. 2007), sampling other non-target host species, or using sentinel animals would provide information on pathogen presence and detection that is independent of host detection. In other systems, additional sampling strategies that would eliminate the unobservable state may be appropriate. Another alternative is to model seasonal variation in vital rates as a function of temporal covariates, which can eliminate redundancy (Cole and Morgan 2010). Given the tradeoff between surveying more sites, or surveying fewer sites more intensively, I suggest that researchers minimize missing observations at a smaller collection of sites because I found that the inclusion of missing data created larger standard errors than small sample sizes.

In situations where the suggestions above are not possible (for instance, when historic data have already been collected as in the SRM case), researchers can use the numeric-analytic method and data cloning approaches, described here, to identify potential biases and parameter redundancy in their own systems. To my knowledge, I am the first to investigate how bias, precision, and parameter identifiability are influenced by state uncertainty in multistate occupancy models. While my study was motivated by a specific host-pathogen system, my work is widely applicable to other host-pathogen systems and to studies involving other interactions (e.g., competitive, predator, mutualistic, etc.). I encourage researchers to think carefully about the system being studied, and to strive to find an appropriate way to model dependencies in the data structure. Simulation and analyses of generated data specific to a system are valuable tools that can help researchers identify potential biases, elucidate realistic expectations for precision, and guide the interpretation of findings to maximize learning in complicated biological systems.

Table 1.1: Transition probabilities matrix φ_t in table form, illustrating transitions among four states for a 2-species dynamic occupancy model. Seasonal transition probabilities are expressed as combinations of vital rate parameters, colonization (γ) and extinction (ε) for both species (A and B) and are conditional upon the state at t.

State at t:	**State at $t+1$:**			
	A	**B**	**U**	**AB**
A	$(1-\varepsilon^{A})(1-\gamma^{BA})$	$\varepsilon^{A}\gamma^{BA}$	$\varepsilon^{A}(1-\gamma^{BA})$	$(1-\varepsilon^{A})\gamma^{BA}$
B	$\gamma^{AB}\varepsilon^{B}$	$(1-\gamma^{AB})(1-\varepsilon^{B})$	$(1-\gamma^{AB})\varepsilon^{B}$	$\gamma^{AB}(1-\varepsilon^{B})$
U	$\gamma^{A}(1-\gamma^{B})$	$(1-\gamma^{A})\gamma^{B}$	$(1-\gamma^{A})(1-\gamma^{B})$	$\gamma^{A}\gamma^{B}$
AB	$(1-\varepsilon^{AB})\varepsilon^{BA}$	$\varepsilon^{AB}(1-\varepsilon^{BA})$	$\varepsilon^{AB}\varepsilon^{BA}$	$(1-\varepsilon^{AB})(1-\varepsilon^{BA})$

Table 1.2: Detection probability matrix p_t in table form, illustrating state-dependent detection probabilities for the general case of a 2-species dynamic occupancy model with four states. In the system I explored, p^B and r^{Ba} are constrained to 0 to illustrate that species B can only be detected when species A is both present and detected (as is the case with amphibian swab sampling for pathogens).

True state at t:	**Observed state at $t+1$:**			
	A	**B**	**U**	**AB**
A	p^A	0	$(1-p^A)$	0
B	0	p^B	$(1-p^B)$	0
U	0	0	1	0
AB	$r^A(1-r^{BA})$	$(1-r^A)r^{Ba}$	$(1-r^A)(1-r^{Ba})$	$r^A r^{BA}$

Table 1.3: 'True' parameter values used to generate expected values data under a dynamic 2-species occupancy model for two host-pathogen scenarios: a common species with a moderate decline (modeled after boreal toads and Bd in the Southern Rocky Mountains), and a rare species with a severe decline. The true model assumes state-specific occurrence (ψ), colonization (γ) and extinction (ε) processes, and detection probabilities ($p^A, p^B, r^A, r^{BA}, r^{Ba}$), in a 4 state system with one unobservable state.

Parameter Value	**SRM boreal toad-*Bd***	**Rare and Susceptible**
$\psi_{t=1}^{A}$	0.90	0.20
$\psi_{t=1}^{BA}$	0.20	0.80
$\psi_{t=1}^{Ba}$	0.05	0.40
γ^{AB}	0.05	0.05
γ^{A}	0.70	0.10
γ^{BA}	0.20	0.80
γ^{B}	0.10	0.40
ε^{AB}	0.40	0.90
ε^{A}	0.05	0.60
ε^{BA}	0.10	0.05
ε^{B}	0.30	0.10
p^{A}	0.90	0.70
p^{B}	0.00	0.00
r^{A}	0.80	0.40
r^{BA}	0.60	0.70
r^{Ba}	0.00	0.00
Decline in host occurrence over 4-season study period	21% (from 0.90 to 0.71)	65% (from 0.20 to 0.07)

Table 1.4: Model structures used to investigate bias, precision, and parameter redundancy using the analytic-numeric approach for a host-pathogen system with an unobservable state. All combinations of these structures were used, resulting in 48 total models that were fit to each expected values dataset. I constrained the total number of models investigated by assuming that estimated detection probabilities must have the same model structure, and that vital rate parameters for a particular species (for example, γ^A and γ^{AB}) must have the same structure.

Model Parameters			
Occupancy $(\psi_{t=1}^{A}, \psi_{t=1}^{BA}, \psi_{t=1}^{Ba})$	**Colonization** (γ)	**Extinction** (ε)	**Detection** (p^A, r^A, r^{BA})
Constant	Constant	Constant	Constant
	Annual time variation in γ^A and γ^{AB}	Annual time variation in ε^A and ε^{AB}	Annual time variation in all detection parameters
	Annual time variation in γ^B and γ^{BA}	Annual time variation in ε^B and ε^{BA}	Within-season time variation in all detection parameters
	Annual time variation in all γ	Annual time variation in all ε	

Table 1.5: Metrics used to evaluate bias and precision of a dynamic single-species occupancy model using pathogen detection as a covariate, compared to 'true' parameter values.

	$\psi^{A}_{t=1}$	γ^{A}	γ^{AB}	ε^{A}	ε^{AB}	p^{A}	r^{A}
True Model Parameter Value	0.9	0.7	0.05	0.05	0.4	0.9	0.8
Estimated Parameter Value	0.91	0.35	0.33	0.08	0.42	0.87	0.87
Absolute Bias	0.01	0.35	0.28	0.03	0.03	0.03	0.07
Standard Error	0.04	0.09	0.09	0.02	0.08	0.02	0.03

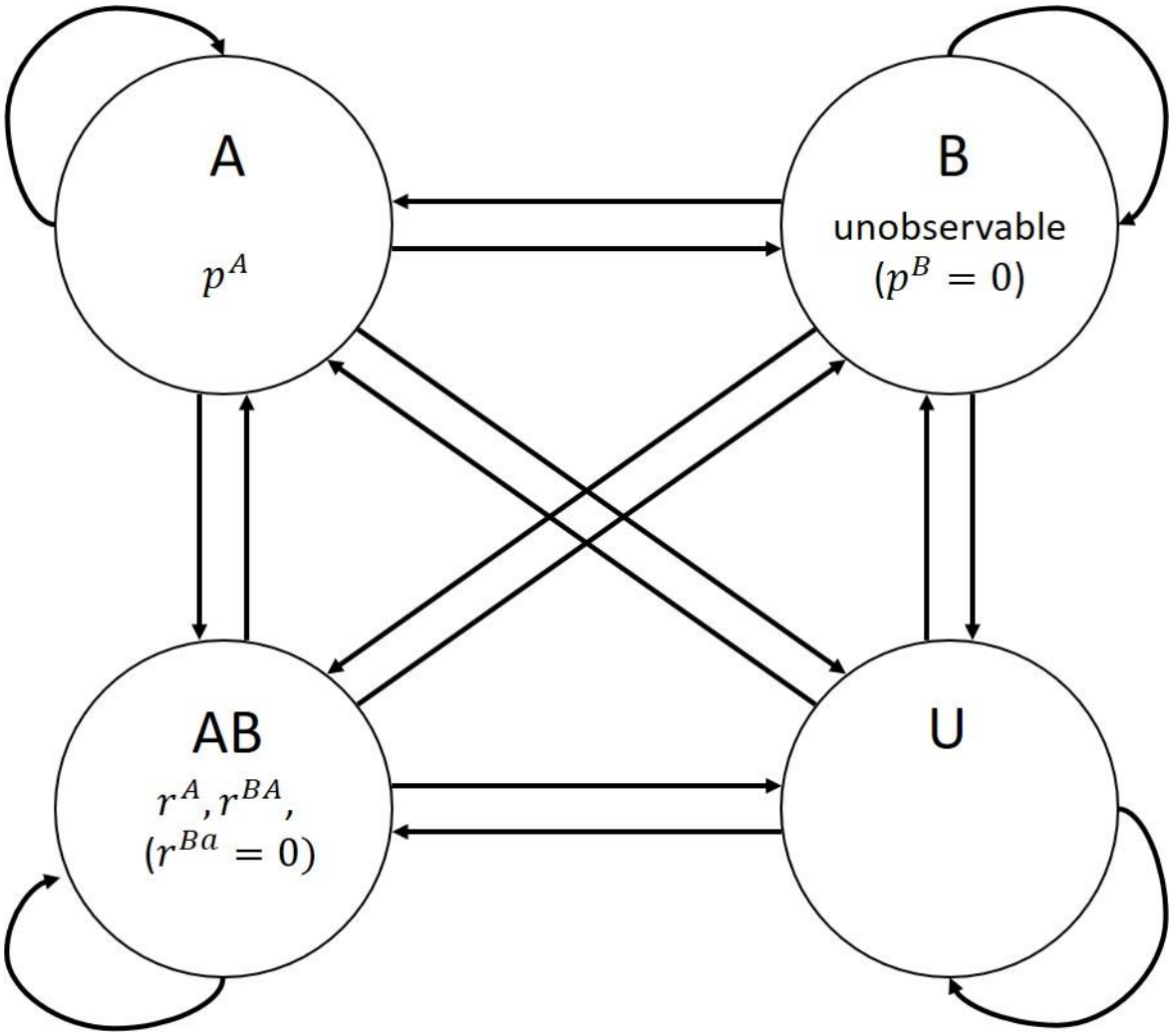


Figure 1.1: State diagram and detection probabilities for a 2-species dynamic occupancy model with one unobservable state (state B). Species of interest can occur separately (states A or B), together (state AB), or not at all (unoccupied, state U). Arrows represent the seasonal transitions among states, with transition probabilities defined in Table 1.1. Species detection probabilities for occupied states are listed within each circle. Unobservable events are signified by detection probabilities being fixed at 0, and happen when species B occurs alone (p^B) and when species A and B occur together, but species A is undetected (r^{Ba}).

CHAPTER 2: COMPLEX ECOLOGICAL RELATIONSHIPS INFLUENCE EXTINCTION RISK IN AN AMPHIBIAN-PATHOGEN SYSTEM

SUMMARY

Emerging infectious diseases are an increasingly common threat to wildlife, and emergence is often driven by changes in host susceptibility, pathogen infectivity, or the environment. Chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), is an emerging infectious disease that has been linked to amphibian declines around the world. Few studies exist that explore amphibian-*Bd* dynamics at the landscape scale, limiting our ability to identify factors influencing variation in population susceptibility and develop effective *in situ* disease management. Declines of boreal toads (*Anaxyrus boreas boreas*) in the southern Rocky Mountains are largely attributed to chytridiomycosis but there is variation in local extinction of boreal toads across this metapopulation. Using a large-scale historic dataset, I explored several potential factors influencing disease dynamics in the boreal toad-*Bd* system: genetic isolation of populations, amphibian community diversity, climate differences, and habitat permanence. I found evidence that boreal toad extinction risk was highest at low elevations where temperatures may be optimal for *Bd* growth and where large boreal toad populations facilitate density-dependent disease transmission. I illustrate a framework that will be useful to natural resource managers striving to make decisions in amphibian-*Bd* systems, and provide evidence that the physiological tolerances of *Bd* may interact with habitat features to shape amphibian declines.

INTRODUCTION

Emerging infectious diseases (EIDs) can increase species extinction rates, shape community dynamics, and fundamentally alter how ecosystems function (Whiles et al. 2013, Rothermel et al. 2016, Langwig et al. 2016). Mechanisms underlying disease emergence are often poorly understood because declines happen quickly, pathogens may be newly described, and static patterns of disease metrics (e.g., occurrence or prevalence) fail to yield strong inferences about the mechanisms driving these dynamic systems (Hastings 2001, Yackulic et al. 2015). Despite these difficulties, understanding the ecology of host-pathogen relationships and the factors that influence disease-related extinction events are central goals of ecologists and managers (Langwig et al. 2015).

Though most pathogens are not predicted to drive their hosts to extinction (Anderson and May 1991, De Castro and Bolker 2005), evidence of this phenomenon exists in many natural settings (Thorne and Williams 1988, Schloegel et al. 2006, Smith et al. 2006, Ryan et al. 2008). Moreover, population-level responses to pathogens can vary within a single species over small spatial and temporal scales (Hosseini et al. 2004, Scherer et al. 2008, Savage and Zamudio 2011). Variation in extinction risk may be linked to elements of host susceptibility, pathogen infectivity and virulence, environmental features, or interactions of these elements. Understanding the mechanisms that give rise to variation in disease dynamics underlies sound ecological insights and is the basis for conservation and management activities (Garner et al. 2016).

Chytridiomycosis has emerged as an infectious disease of amphibians and is caused by the aquatic fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*; Berger et al. 1998). Despite almost 20 years of research on this pathogen, questions remain about which factors shape local

host-*Bd* dynamics (Venesky et al. 2014b). The global impacts of chytridiomycosis (Skerratt et al. 2007), coupled with local extirpations and differential susceptibility of amphibian populations (Schloegel et al. 2006, Murphy et al. 2009), make identifying the factors that lead to extinction a conservation priority. Local genetic differences, host species community richness, variation in climate, and habitat differences across sites have all been suggested to influence amphibian-*Bd* dynamics (Searle et al. 2011, Heard et al. 2013, Addis et al. 2015, Clare et al. 2016a). Assessing the relative contributions of these factors to host-pathogen dynamics will improve our ability to successfully manage landscapes, species, and populations challenged by chytridiomycosis (Venesky et al. 2014b, Garner et al. 2016).

One way that genetic differences among local populations of a species can arise is when immigration and dispersal processes are disrupted by physical barriers or habitat fragmentation (Gibbs 2001). Amphibians may be especially prone to population isolation due to their limited dispersal abilities (Blaustein et al. 1994), high breeding site fidelity (Smith and Green 2005), and their vulnerability to barriers like roads (Fahrig et al. 1995, Marsh et al. 2005) and inhospitable terrain (Funk et al. 2005, Murphy et al. 2010a, Watts et al. 2015). Some studies have identified genetic correlates of chytridiomycosis susceptibility in amphibians (Savage and Zamudio 2011, Bataille et al. 2015, Addis et al. 2015) that may account for the differences in disease dynamics observed among species and populations (Knapp et al. 2016, Savage and Zamudio 2016).

The presence of an environmental or alternate host species "reservoir" could increase the potential of a pathogen to drive a host species to extinction (Rosà et al. 2003, Almberg et al. 2011). While evidence for a long-lived or resting stage of *Bd* is minimal (Johnson and Speare 2003; but see Di Rosa et al. 2007), multiple amphibian species often occupy the same breeding habitat and could serve as reservoir species that may alter local population extinction risk. Field

and laboratory studies in amphibian-*Bd* systems have found that host species community richness can either dilute or amplify disease risk depending on the composition and traits of co-occurring hosts (Reeder et al. 2012, Venesky et al. 2014a, Han et al. 2015).

Spatial and temporal differences in climate also influence patterns of disease (Selig et al. 2006, Lafferty 2009). Changes in moisture or temperature across these dimensions can interact with the physiological tolerances of free-living pathogens or vectors to shape pathogen distributions (Minakawa et al. 2002) and can influence host stress and associated immune responses (Raffel et al. 2006). *Bd* growth and survival are sensitive to cold and hot temperatures, and, in culture, optimal growth of *Bd* occurs between 15 and 25 degrees Celsius (Piotrowski et al. 2004). In the tropics, chytridiomycosis-related amphibian declines are most pronounced in colder months and at high elevations where temperatures are near the thermal optimum for *Bd* growth (Pounds et al. 2006, Gründler et al. 2012, Sapsford et al. 2013). Nevertheless, studies of whether cold climates at northern latitudes limit the effects of *Bd* on amphibians are equivocal (Muths et al. 2008, Savage et al. 2011, Knapp et al. 2011).

Differences in habitat quality or structure may also alter host-pathogen dynamics (Penczykowski et al. 2014) or effects of disease on individuals (Sauther et al. 2006). Some evidence suggests that warmer water and drier microhabitats may inhibit *Bd* growth (Puschendorf et al. 2011, Heard et al. 2013), such that amphibians breeding in semi-permanent wetlands experience a refuge from high *Bd* loads. However, these semi-permanent sites require tadpoles to metamorphose quickly and may lead to frequent reproductive failure even in the absence of disease (Karraker and Gibbs 2009, Zipkin et al. 2012).

I evaluated the competing roles of geographic isolation, host species community composition, climate, and habitat permanence in shaping amphibian disease dynamics using a

long-term dataset on boreal toads (*Anaxyrus boreas boreas*) and *Bd* in the Southern Rocky Mountains (SRM). I used a dynamic two-species occupancy model to account for the imperfect detection of both toads and *Bd* and to obtain unbiased estimates of host and pathogen dynamics (Richmond et al. 2010, Chapter 1). My objectives were to: 1) identify the major factors driving local variation in amphibian-*Bd* occurrence dynamics in a temperate amphibian-*Bd* system, and 2) quantify this variation, providing empirical estimates of boreal toad-*Bd* dynamics that will be useful for understanding amphibian extinction risk in the SRM and other temperate settings.

METHODS

Study Species

Boreal toads are a species native to western North America that has experienced *Bd*-related declines throughout the SRM (Muths et al. 2003, Scherer et al. 2005), but some populations with *Bd* persist within this region (Carey et al. 2005). Boreal toad breeding sites in the SRM vary in their elevation, habitat type, and whether other amphibian species occur in sympatry with boreal toads. In addition, some sites have been isolated from others by rugged terrain or major interstate highways that have existed for many years. These sources of variation, coupled with long-term monitoring of both boreal toad and *Bd* detection, make this an ideal system to investigate factors that may shape disease-related amphibian extinctions.

Data Collection

Boreal toad surveys were conducted annually from 2001-2010 at 82 historic breeding wetlands (hereafter, sites) in mountainous regions of Colorado and Wyoming (Figure 2.1). Boreal toads breed in a variety of habitat types, including beaver ponds, wet meadows, oxbows, and lake or pond margins, and sites range in elevation from 2470-3680 meters. Between 0-10 (mean = 3) visual encounter surveys were conducted at sites each year during the boreal toad

breeding and developmental seasons. Boreal toad skin swab samples were collected opportunistically during the surveys and were analyzed using standard DNA extraction and qPCR protocols to detect *Bd* (Boyle et al. 2004, Bletz et al. 2015). In addition, researchers collected information on whether the breeding sites were semi-permanent (prone to seasonal drying) or permanent, and whether two other amphibian species native to the region (tiger salamanders [*Ambystoma tigrinum*] or boreal chorus frogs [*Pseudacris maculata*]) were detected during these visits.

Modeling Framework

I used a dynamic two-species occupancy model (Richmond et al. 2010, Miller et al. 2012a, Chapter 1) to estimate boreal toad (species A) and *Bd* (species B) occurrence dynamics simultaneously (Table 2.1). The model assumes that sites are in one of four mutually exclusive states in any year and allows for estimation of these state probabilities: sites can be occupied by boreal toads only (state A; $\psi^A(1-\psi^{BA})$), occupied by *Bd* only (state B; $(1-\psi^A)\psi^{Ba}$)), occupied by boreal toads and *Bd* (state AB; $\psi^A\psi^{BA}$), or unoccupied by either species (state U; $(1-\psi^A)(1-\psi^{Ba})$). Transitions among states can occur between years (Figure 2.2). Colonization (γ) and extinction (ε) parameters characterize these transitions and are estimated for each species conditional on whether the other species is present (Table 2.1). For instance, the probability of a site transitioning from state A (boreal toad only) in year t to state AB (boreal toad and *Bd*) in year $t+1$ is the product of the probabilities of boreal toad persistence in the absence of *Bd* in year t, and *Bd* colonization in the presence of boreal toads (i.e., $(1-\varepsilon^A)(\gamma^{BA})$;

Figure 2.2, transition 4). Colonization and extinction parameters can be time-specific provided all four states are observable.

Detection parameters (Table 2.1) describe the probability of detecting each species when they occur alone (p^A and p^B) or when they occur together (r^A and r^{BA}). Finally, the model allows for a different detection probability for *Bd* when toads are present but not detected (r^{Ba}). In my study, *Bd* is detected from skin swabs and therefore cannot be detected when boreal toads are absent or undetected ($p^B = r^{Ba} = 0$). This sampling limitation creates an unobservable state that has implications for parameter estimation. In Chapter 1, I examined the ramifications of this unobservable state for parameter estimation and found some parameters were unidentifiable when models contained combinations of time-specific vital rates. In time-constant models, colonization and extinction probabilities for *Bd* in the absence of the host (γ^B and ε^B) were estimated imprecisely. Accordingly, I explored only models with time-constant vital rates with the understanding that some estimates may be extremely imprecise.

Biological Hypotheses

Because I lacked genetic data at the broad spatial scale encompassed by the study, I focused on populations isolated by barriers to movement that may result in local adaptation and thus putative genetic differences. A major interstate highway established in 1956 runs east-west (Interstate 70) and intersects with the Continental Divide (which often exceeds 4000 meters in elevation): these features separate boreal toad populations into four regions (NE, NW, SW, and SE; Figure 2.1) whose connectivity may be limited by impervious surfaces and topography (Murphy et al. 2010b). I note that this coarse measure of isolation may not necessarily reflect real genetic differences in toads, but could also indicate lack of dispersal of infApprected animals, different strains of *Bd*, or habitat differences among regions.

I explored potential impacts of amphibian host community richness by treating the occurrence of other amphibians (tiger salamanders or boreal chorus frogs) as a site-level binary covariate (present or absent). I used elevation as a proxy for climatic differences, where high elevation sites are generally colder with higher snowpack than low elevation sites. Finally, to investigate the impacts of seasonal drying, I classified sites as semi-permanent (and subject to periodic drying) or permanent (no drying). I made *a priori* predictions about which vital rate parameters these factors would be most likely to influence and why (Table 2.2A).

In addition, I anticipated heterogeneity in the detection probability of both boreal toads and *Bd* across space and/or time. Accordingly, I generated *a priori* hypotheses and predictions for covariates influencing detection parameters as well (Table 2.2B).

Modeling Approach

I initially fit a simple model where all parameters were time-constant (14 total parameters); this model indicated that extinction probabilities for toads or *Bd*, in the absence of the other species, were zero ($\varepsilon^A = \varepsilon^B = 0.00$; 95% CIs: [0.00, 0.02] and [0.00, 0.35], respectively). Therefore, I modeled these extinction parameters as time-constant, rather than as a function of covariates, in subsequent models. I employed a step-down modeling strategy (Lebreton et al. 1992) whereby I first explored factors resulting in variation in detection probability using the most general vital rate structures and constant, state-specific initial occupancy probabilities (Appendix 2.1). I then used the best-supported detection structure to explore hypotheses about factors influencing host-pathogen dynamics. Specifically, I fit additive models to explore whether boreal toad detection probabilities in the presence (r^A) and absence (p^A) of *Bd* were constant or varied either linearly or quadratically with day-of-year (*DOY*). In

addition, I investigated whether *Bd* detection probability (r^{BA}) was constant or varied either linearly or quadratically with elevation (*elev*).

Factors thought to influence host-pathogen dynamics were weakly correlated: elevation and other amphibian presence (Spearman's rank correlation coefficient, r_s = -0.43), elevation and region ($r_s = 0.35$), and permanence and the presence of other amphibians ($r_s = 0.39$). To avoid overparameterization, given my data, I fit additive models for just one factor per model. The geographic isolation, or coarse region, of populations was expected to influence boreal toad vital rates in the presence of *Bd* only, while other factors were linked to *Bd* abundance and therefore could influence vital rate parameters related to either *Bd* or boreal toad dynamics (Table 2.2A). State-specific initial occupancy probabilities were modeled as constant across sites. Models were fit using the dynamic conditional-binomial 2-species occupancy model in Program MARK (White and Burnham 1999).

RESULTS

I found evidence of within-season variation in boreal toad detection probability, which was best modeled as a quadratic relationship with *DOY* (Figure 2.3A; Appendix 2.1). Detection of boreal toads at sites with *Bd* (r^A) was considerably lower than when *Bd* was absent (p^A), potentially due to differences in abundance. Detection of boreal toads was highest in the middle of the breeding season and declined toward the end of the season. The detection probability of *Bd* declined with elevation; linear and quadratic relationships received similar support (Figure 2.3, Appendix 2.1). I retained the simpler linear *Bd* detection structure for the subsequent vital rate analysis because it was better supported and its 95% confidence interval largely overlapped that of the quadratic structure (Figure 2.3B, Appendix 2.1).

The null model, indicating that vital rate parameters were not state-dependent (i.e., $\gamma^{A} = \gamma^{AB}$, $\gamma^{B} = \gamma^{BA}$, etc.), was the least well-supported of the 33 models investigated (model weight = 0.00, Table 2.3), suggesting that *Bd* alters the occurrence dynamics of boreal toads and that the presence of boreal toads influences the colonization and persistence of *Bd*. A model that assumed the state-specific vital rates were identical among all sites was also not supported, providing evidence that toad-*Bd* dynamics vary across the landscape (model weight = 0.01, Table 2.3). Models that included my hypothesized factors were all better supported than these null hypotheses. Elevation was the best-supported hypothesis: models with either linear or quadratic relationships between elevation and host and/or pathogen vital rates were included in the top 6 models (Table 2.3). Together, these 6 models received >60% of the cumulative model weight.

Models suggesting that the presence of other amphibian species or that habitat permanence influenced boreal toad-*Bd* dynamics had limited support (Table 2.3). The presence of other amphibian species was somewhat associated with a higher probability that boreal toads would recolonize a site occupied by *Bd* (Figure 2.4A). Colonization and extinction estimates from the best model containing the effect of habitat permanence (model weight = 0.04, Table 2.3) showed poor precision, though *Bd* extinction probability in the presence of toads was higher at semi-permanent sites, consistent with my *a priori* predictions (Figure 2.4B-C). Models suggesting that boreal toad-*Bd* dynamics differed among geographically isolated regions were not supported.

Consistent with my expectations, extinction probabilities for both boreal toads and *Bd* were strongly influenced by the presence of the other species (host or pathogen) and were highest at intermediate elevations within my study system (Figure 2.5A and 2.5B). In the absence of *Bd*, boreal toads always persisted ($\widehat{\varepsilon^{A}} = 0$), but toad populations with *Bd* were

vulnerable to extirpation at intermediate elevations where annual extinction probabilities were ~ 0.30 (Figure 2.5A). When boreal toads were present, the annual extinction probability of *Bd* was also highest at intermediate elevations (~0.30) and *Bd* was unlikely to go extinct at elevational extremes (Figure 2.5B). In the absence of boreal toads, *Bd* was likely to persist, though this parameter was difficult to estimate precisely ($\widehat{\varepsilon^B}$ = 0.02, 95% profile likelihood confidence interval from 0 to 0.15). Because I used only boreal toad swab samples in this study, I have very little information about *Bd* dynamics in the absence of toads such that the estimate of ε^B should be interpreted with caution (Chapter 1).

The colonization probabilities of boreal toads and of *Bd* were also influenced by elevation. When host and pathogen co-occurred, boreal toads were more likely to recolonize high elevation sites should they become locally extinct and *Bd* was more likely to colonize low and mid-elevation sites (Figure 2.5C and 2.5D). Boreal toads were very unlikely to recolonize low and mid-elevation sites after going locally extinct (Figure 2.5C). When *Bd* was absent, boreal toads were very likely to colonize unoccupied historic breeding sites at any elevation ($\widehat{\gamma^A}$ = 1.00, 95% profile likelihood confidence interval from 0.21 to 1.00), but this parameter was estimated imprecisely because boreal toads rarely go extinct in the absence of *Bd* (recall $\widehat{\varepsilon^A}$ = 0). In the absence of toads, *Bd* colonization was difficult to estimate well and should be interpreted with caution, again due to a lack of information about *Bd* in the absence of boreal toads (Figure 2.5D).

To summarize, *Bd* readily colonized low elevation sites and was unlikely to go extinct in those locations, while boreal toad sites at low elevations that went extinct were very unlikely to be recolonized by toads. At intermediate elevations, *Bd* colonization and local extinction both occurred to some degree. Boreal toads were also likely to go extinct at intermediate elevations, but had some chance of recolonizing sites after extinction events. Finally, *Bd* was unlikely to

successfully colonize high elevation sites, and but if the pathogen did become established it was unlikely to go extinct at these sites. At high elevation sites, boreal toads were unlikely to go locally extinct but if they did, the site could often be recolonized.

Based on these spatially varying dynamic patterns from the best-supported model, the proportion of sites where only boreal toads occur has declined steadily over time, and these declines were most pronounced at low elevations (Figure 2.6A). Simultaneously, the proportion of sites with *Bd* increased over the course of the study and was also highest at low elevations (Figure 2.6B). I confirmed that the elevation relationships generated by the best model were not merely a function of assuming constant initial occupancy states by running a post-hoc model where the initial conditional distribution of *Bd* (ψ^{BA} and ψ^{Ba}) varied as an additive function of elevation. This model was not as well-supported as the model with constant *Bd* occupancy probabilities (ΔAICc = 1.83), and produced nearly identical trajectories as those shown in Figure 2.6.

It is important to note that I could not model time variation in vital rate parameters (γ and ε), as the presence of an unobservable state yields biased estimates of most time-varying parameters (Chapter 1). Without time variation in vital rate parameters, the state distributions, i.e., the proportion of sites in each of the four mutually exclusive states, will reach an equilibrium (Green et al. 2011, Miller 2012), which may or may not reflect the biology of this system. Even with the simplifying assumption of time-constancy, I had difficulty identifying covariate relationships for $\varepsilon^A, \varepsilon^B, \gamma^A$, and γ^B due to data sparseness for various states. The only way to relax this assumption and to explore time-variation in host and pathogen vital rate parameters is to sample *Bd* when boreal toads are not detected, thus removing the unobservable state (Chapter 1).

DISCUSSION

Capturing the dynamics of both hosts and pathogens is important for understanding co-evolution, the potential for genetic resistance, and the factors that influence disease dynamics across landscapes and systems. I present the first landscape-scale analysis of an amphibian-*Bd* system where both host and pathogen dynamics are modeled explicitly and imperfect and variable detection of both species is considered. I present a framework for evaluating competing factors for local variation in host-pathogen dynamics and provide estimates of parameters in the boreal toad-*Bd* system. This framework will be applicable to other host-pathogen systems and will be immediately useful for guiding amphibian-pathogen monitoring and conservation efforts.

I found differences in boreal toad and *Bd* dynamics across elevations that resulted in less pronounced boreal toad declines at high elevations where *Bd* was also less likely to occur. High elevation sites in the SRM experience lower temperatures, shorter growing seasons, and higher daily temperature fluctuations than sites at lower elevations (Carey et al. 2005). Thus, the variation in dynamics that I observed across an elevational gradient may be related to: 1) temperature-dependent growth of *Bd*, 2) elevational influences on host densities, or 3) an interaction between these factors.

Experimental studies have identified upper and lower temperature thresholds between which *Bd* growth is optimal (Johnson et al. 2003, Piotrowski et al. 2004). Temperature has also been linked to chytridiomycosis outbreaks in natural settings (Pounds et al. 2006, Bosch et al. 2007). The colder temperatures at high elevations in temperate systems likely limit the pathogen's ability to invade and cause infection, resulting in lower *Bd* detection probabilities (Muths et al. 2008, Chestnut et al. 2014), reduced infection prevalence (Murphy et al. 2009), and decreased infection intensities (Kriger et al. 2007). My work corroborates the idea that

temperature may influence *Bd* growth, as I found lower *Bd* occupancy and detection at high elevations. Differences in *Bd* detection probability likely reflect heterogeneity in *Bd* abundance (Chapter 3).

An alternative, but not mutually exclusive, explanation for the elevational effects that I identified relates to amphibian life history characteristics. Like most amphibian species, high elevation boreal toad populations tend to have more frequent reproductive failure (Carey et al. 2005) and lower recruitment than low elevation populations. In addition, female boreal toads do not breed every year (Muths et al. 2010) and breeding frequency may be reduced at higher elevation sites (Muths et al. 2013). These processes can lead to lower population densities at high elevations. While it has been suggested that the high growth rate and reproductive potential of *Bd* sometimes drive hosts to extinction before density-dependent effects are realized (Vredenburg et al. 2010a, Fisher et al. 2012), the low density of boreal toads at high elevations may prevent *Bd* from causing mass mortality. Sub-optimal growth conditions for *Bd* may interact with low boreal toad densities at high elevations, lowering infection loads and/or decreasing transmission rates, providing a potential mechanism(s) for host population persistence in the face of disease (Briggs et al. 2010).

While I assume that the elevational relationships I identified were the result of temperature differences, additional variables such as canopy cover, precipitation, occurrence of aquatic predators may also be correlated with elevation and may be partially responsible for the differences I found (Becker et al. 2012, Lanier et al. 2016). In my system, elevation was negatively correlated with the presence of other amphibian species (r_s = -0.43) and the highest elevation sites lacked other amphibians. Experimental studies of multispecies amphibian host communities have found both positive and negative relationships between host diversity and the

risk of disease with *Bd* depending on species traits, life stages studied, and strength of competitive interactions (e.g., Searle et al. 2011, Venesky et al. 2014a, Han et al. 2015). Future work on this topic is needed and will require sampling efforts that target multiple species.

Management Implications

My ability to estimate most parameters related to *Bd* persistence, colonization, and occurrence in the absence of boreal toads was limited because swab samples were only collected when toads were both present and detected. Empirical estimates of these parameters would be valuable to management agencies as they consider mitigation strategies that target reducing the colonization and persistence of *Bd* (Gerber et al. in revision, Converse et al. 2017). Knowing how long *Bd* persists in the environment, or in reservoir hosts, after the extirpation of target amphibians can help guide amphibian reintroduction or translocation efforts and other management actions (Chapter 4). I stress that long-term monitoring initiatives in this and other host-pathogen systems with free-living hosts would benefit from employing methods to survey for pathogens outside of their target hosts.

In *Bd* systems, collecting skin swabs from co-occurring, but non-target, amphibian species and surveying for *Bd* zoospores using water filtration can provide additional information on the presence of the pathogen when the target host is not detected (Kirshtein et al. 2007, Chestnut et al. 2014). Expanding survey methods to include standardized surveys for non-target amphibians may also provide better information about host community structure and stability and disease prevalence in these other species. Samples collected using water filtration (i.e., environmental DNA) could document the presence of multiple amphibian and pathogen species simultaneously with a single water sample (Blooi et al. 2013).

Chytridiomycosis is one of the worst diseases to affect vertebrates in recorded history (Skerratt et al. 2007), but there are strikingly few examples of *in situ* management actions and their impacts on amphibian-Bd dynamics (Scheele et al. 2014, Garner et al. 2016). Empirical measures of host-pathogen dynamics can be valuable for population projections, structured decision-making, reintroduction efforts, and as a baseline for measuring impacts of management interventions (Gerber et al. in revision, Converse et al. 2017, Russell et al. 2017). My work identifies elevational variation in boreal toad extinction risk that results in high-elevation refugia for toads, provides empirical data as the basis for management decision-making, and provides valuable recommendations for the long-term monitoring of both amphibians and their pathogens.

Table 2.1: List of parameters estimated in the dynamic two-species occupancy model including initial state parameters (ψ), vital rate parameters (colonization, γ and local extinction ε), and detection probabilities (p and r). I fixed the detection of *Bd* (species B) to be 0 when boreal toads (species A) were absent (p^B) or present but undetected (r^{Ba}) to reflect that *Bd* can only be detected on boreal toad skin swabs in this system. While these parameters can vary through time (noted with subscript t below), the presence of an unobservable state in my study restricted us to assume that colonization and extinction parameters were constant through time.

Parameter	**Description**
$\psi_{t=1}^{A}$	Initial probability of occupancy for species A
$\psi_{t=1}^{BA}$	Initial probability of occupancy for species B, given species A is present
$\psi_{t=1}^{Ba}$	Initial probability of occupancy for species B, given species A is absent
γ_t^A	Probability of colonization by species A, given species B is absent
γ_t^{AB}	Probability of colonization by species A, given species B is present
γ_t^B	Probability of colonization by species B, given species A is absent
γ_t^{BA}	Probability of colonization by species B, given species A is present
ε_t^A	Probability of extinction of species A, given species B is absent
ε_t^{AB}	Probability of extinction of species A, given species B is present
ε_t^B	Probability of extinction of species B, given species A is absent
ε_t^{BA}	Probability of extinction of species B, given species A is present
p_t^A	Detection probability of species A, given species B is absent
p_t^B	Detection probability of species B, given species A is absent
r_t^A	Detection probability of species A, given species B is present
r_t^{BA}	Detection probability of species B, given species A is present and detected
r_t^{Ba}	Detection probability of species B, given species A is present and undetected

Table 2.2: Factors hypothesized to influence disease dynamics (A) and detection probability (B) in a boreal toad-*Bd* system. For each factor of interest, I hypothesize which parameters are affected, the expected effect (positive or negative), and the rationale for these predictions.

A. Colonization and Extinction Probabilities for Boreal Toads and *Bd*			
Factor (proxy)	*Covariate Type*	*Parameters Influenced (Hypothesized effect)*	*Rationale*
Genetic differences (isolated regions)	Categorical	$\gamma^{AB}, \varepsilon^{AB}$ (Differences expected, but direction unknown)	Geographically isolated boreal toad populations may adapt differently to disease.
Other amphibians present (single host species vs. multiple host species)	Binary (1 = other amphibians known to be present)	$\gamma^{AB}, \varepsilon^{AB}, \gamma^{B}, \gamma^{BA}, \varepsilon^{BA}$ (- , + , + , + , -)	Additional amphibian species may amplify or dilute impacts of disease on boreal toads, and amplification was hypothesized in this system.
Climate (elevation)	Continuous	$\gamma^{AB}, \varepsilon^{AB}, \gamma^{B}, \gamma^{BA}, \varepsilon^{BA}$ Negative (linear) or quadratic	Very cold or very warm temperatures may limit *Bd* growth.
Habitat type (semi-permanent vs. permanent)	Binary (1 = permanent)	$\gamma^{AB}, \varepsilon^{AB}, \gamma^{B}, \gamma^{BA}, \varepsilon^{BA}$ (- , + , + , + , -)	Semi-permanent wetlands are prone to desiccation and could lower *Bd* abundances.
B. Detection Probabilities for Boreal Toads and *Bd*			
Driver	*Covariate Type*	*Parameters Influenced (Predicted effect)*	*Rationale*
Elevation	Continuous	r^{BA} Linear (-) or quadratic	Very warm or very cool temperatures may limit *Bd* growth.
Day-of-Year	Continuous	p^{A}, r^{A} Linear (+) or quadratic	Boreal toad life stages present throughout the breeding season vary in their conspicuousness.

Table 2.3: Model selection table for boreal toad and *Bd* disease dynamics showing the 10 best-supported models. Covariates included elevation (*elev*), host amphibian species richness (*amph*), habitat permanence (*perm*), and the putative genetic isolation of populations (*gen*; none in table). Model selection information including, AICc, Δ AICc, model weights, number of parameters (K), and deviance are shown for each model. I explored only additive relationships between parameter pairs (γ^A and γ^{AB}, $\gamma^B$and γ^{BA}, etc.). Models names reflect the model structure for each vital rate parameter and include effects of elevation (*elev*), a quadratic effect of elevation (*elev*^{2}), the presence of multiple amphibian species (amph), habitat permanence (*perm*), and constant models (.). Genetic isolation was also explored but did not appear in the top 10 models. Each model in this analysis had the same detection structure (p^A/r^A (*DOY*^{2}) r^{BA} (*elev*); *DOY*^{2} represents a quadratic effect of day-of-year while *elev* represents a linear effect of site elevation), which was identified as the most parsimonious in the first part of the step-down procedure (Appendix 2.1).

Model Name	**AICc**	**Δ AICc**	**Model Weight**	**K**	**Deviance**
$\gamma^A(.)\,\gamma^{AB}(elev^2)\,\gamma^B(elev^2)\,\gamma^{BA}(elev^2)\,\varepsilon^A(.)\,\varepsilon^{AB}(elev^2)\,\varepsilon^B(.)\,\varepsilon^{BA}(elev^2)$	2228.07	0.00	0.28	25	2175.3
$\gamma^A(.)\,\gamma^{AB}(elev)\,\gamma^B(elev)\,\gamma^{BA}(elev)\,\varepsilon^A(.)\varepsilon^{AB}(elev)\,\varepsilon^B(.)\varepsilon^{BA}(elev)$	2229.27	1.20	0.15	21	2185.31
$\gamma^A(.)\,\gamma^{AB}(.)\,\gamma^B(elev^2)\,\gamma^{BA}(elev^2)\,\varepsilon^A(.)\varepsilon^{AB}(.)\,\varepsilon^B(.)\varepsilon^{BA}(elev^2)$	2231.05	2.97	0.06	21	2187.09
$\gamma^A(.)\,\gamma^{AB}(.)\,\gamma^B(elev)\,\gamma^{BA}(elev)\,\varepsilon^A(.)\varepsilon^{AB}(.)\,\varepsilon^B(.)\varepsilon^{BA}(elev)$	2231.42	3.35	0.05	19	2191.82
$\gamma^A(.)\,\gamma^{AB}(.)\,\gamma^B(elev)\,\gamma^{BA}(elev)\,\varepsilon^A(.)\varepsilon^{AB}(.)\,\varepsilon^B(.)\varepsilon^{BA}(.)$	2231.61	3.53	0.05	18	2194.17
$\gamma^A(.)\,\gamma^{AB}(.)\,\gamma^B(.)\,\gamma^{BA}(.)\,\varepsilon^A(.)\varepsilon^{AB}(.)\,\varepsilon^B(.)\varepsilon^{BA}(elev)$	2232.02	3.95	0.04	18	2194.58
$\gamma^A(.)\,\gamma^{AB}(.)\,\gamma^B(perm)\,\gamma^{BA}(perm)\,\varepsilon^A(.)\varepsilon^{AB}(.)\,\varepsilon^B(.)\varepsilon^{BA}(perm)$	2232.20	4.13	0.04	19	2192.6
$\gamma^A(.)\,\gamma^{AB}(.)\,\gamma^B(.)\,\gamma^{BA}(.)\,\varepsilon^A(.)\varepsilon^{AB}(.)\,\varepsilon^B(.)\varepsilon^{BA}(perm)$	2232.55	4.48	0.03	18	2195.11
$\gamma^A(.)\,\gamma^{AB}(amph)\,\gamma^B(.)\,\gamma^{BA}(.)\,\varepsilon^A(.)\varepsilon^{AB}(.)\,\varepsilon^B(.)\varepsilon^{BA}(.)$	2232.65	4.58	0.03	18	2195.21
$\gamma^A(.)\,\gamma^{AB}(.)\,\gamma^B(.)\,\gamma^{BA}(.)\,\varepsilon^A(.)\varepsilon^{AB}(.)\,\varepsilon^B(.)\varepsilon^{BA}(elev^2)$	2233.00	4.77	0.03	19	2193.40
$\gamma^A(.) \neq \gamma^{AB}(.), \gamma^B(.) \neq \gamma^{BA}(.), \varepsilon^A(.) \neq \varepsilon^{AB}(.), \varepsilon^B(.) \neq \varepsilon^{BA}(.)$	2235.26	7.03	0.01	17	2199.98
Null model: $\gamma^A(.) = \gamma^{AB}(.), \gamma^B(.) = \gamma^{BA}(.), \varepsilon^A(.) = \varepsilon^{AB}(.), \varepsilon^B(.) = \varepsilon^{BA}(.)$	2261.03	32.79	0.00	14	2232.15

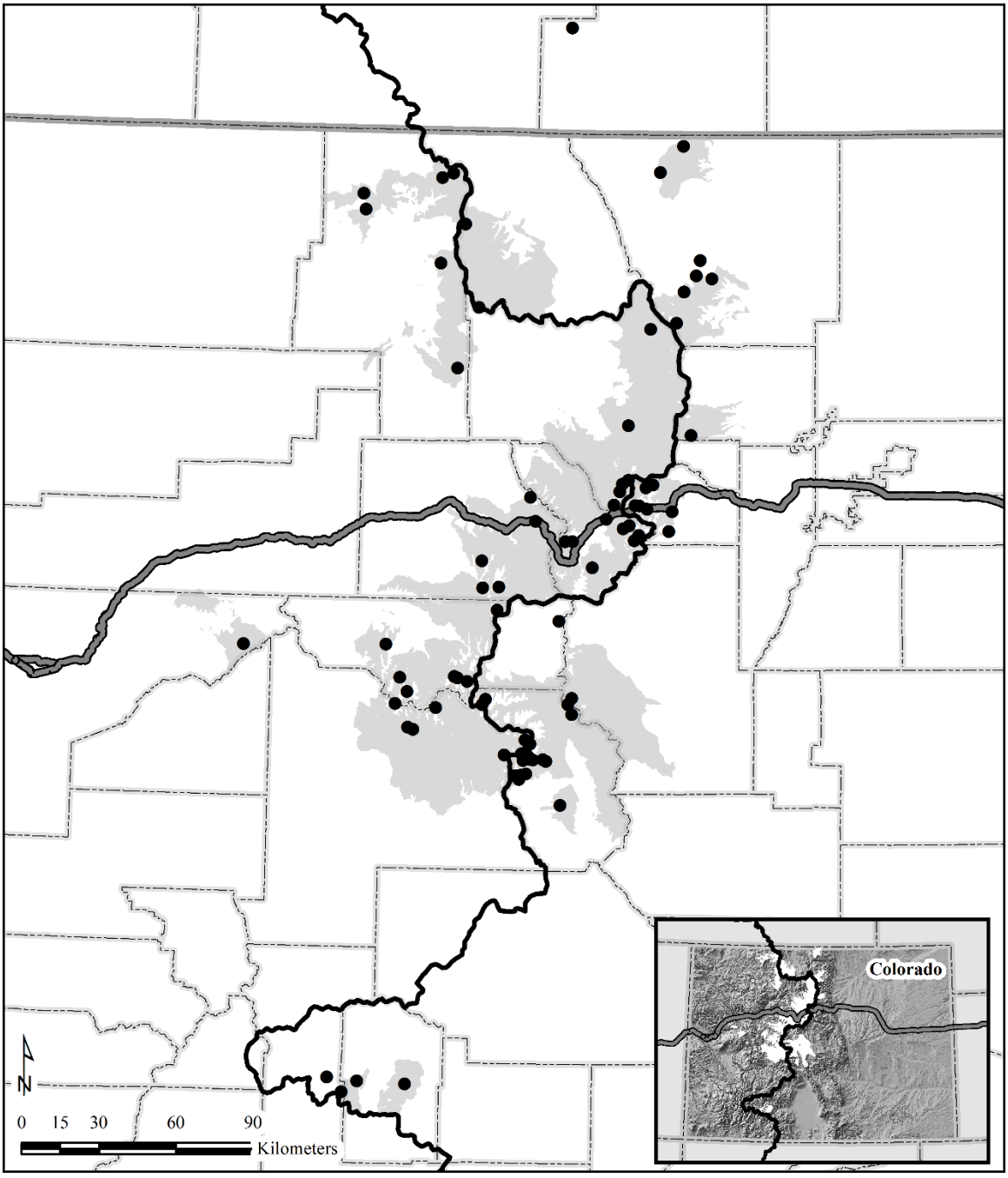
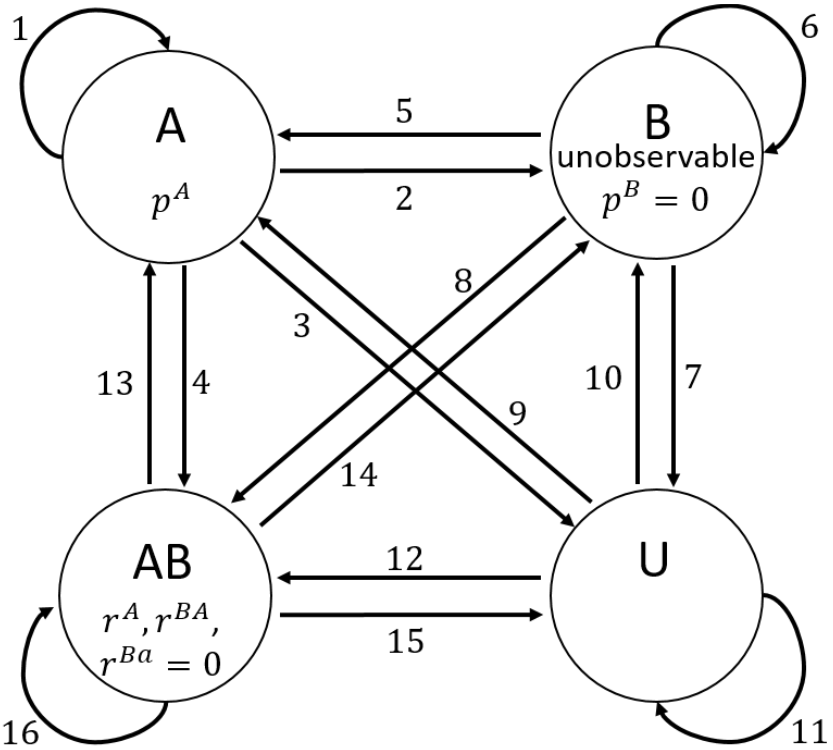


Figure 2.1: Map of 82 historic boreal toad breeding sites (black points) in Colorado and southern Wyoming with two potential geographic barriers (The Continental Divide [black line] and Interstate 70 [grey line]). The shaded region represents the known historic range of boreal toads in Colorado (Colorado Parks and Wildlife. 2017. CPW Boreal Toad Shapefile Download - Overall Range. Species Activity Mapping Project. http://www.arcgis.com/home/item.html?id=3723aadaf0eb41acaed0b95289e1b5f6. Site accessed March 27, 2017.).



State at t:	State at $t+1$:			
	A	**B**	**U**	**AB**
A	1: $(1-\varepsilon^A)(1-\gamma^{BA})$	2: $\varepsilon^A\gamma^{BA}$	3: $\varepsilon^A(1-\gamma^{BA})$	4: $(1-\varepsilon^A)\gamma^{BA}$
B	5: $\gamma^{AB}\varepsilon^B$	6: $(1-\gamma^{AB})(1-\varepsilon^B)$	7: $(1-\gamma^{AB})\varepsilon^B$	8: $\gamma^{AB}(1-\varepsilon^B)$
U	9: $\gamma^A(1-\gamma^B)$	10: $(1-\gamma^A)\gamma^B$	11: $(1-\gamma^A)(1-\gamma^B)$	12: $\gamma^A\gamma^B$
AB	13: $(1-\varepsilon^{AB})\varepsilon^{BA}$	14: $\varepsilon^{AB}(1-\varepsilon^{BA})$	15: $\varepsilon^{AB}\varepsilon^{BA}$	16: $(1-\varepsilon^{AB})(1-\varepsilon^{BA})$

Figure 2.2: State transition diagram and detection probabilities for a 2-species dynamic occupancy model. Boreal toads (species A) and *Bd* (species B) can occur separately (states A or B), together (state AB), or not at all (unoccupied, state U). Arrows represent the annual transitions among states, with transition probabilities consisting of products of state-specific colonization (γ) and extinction (ε) probabilities. Species detection probabilities for occupied states are listed within each circle. Unobservable states and events are signified by detection probabilities being fixed at 0; these occur when *Bd* exists in the absence of toads (species B occurs alone, p^B=0) and when toads and Bd occur together (state AB), but toads are not detected (r^{Ba}=0).

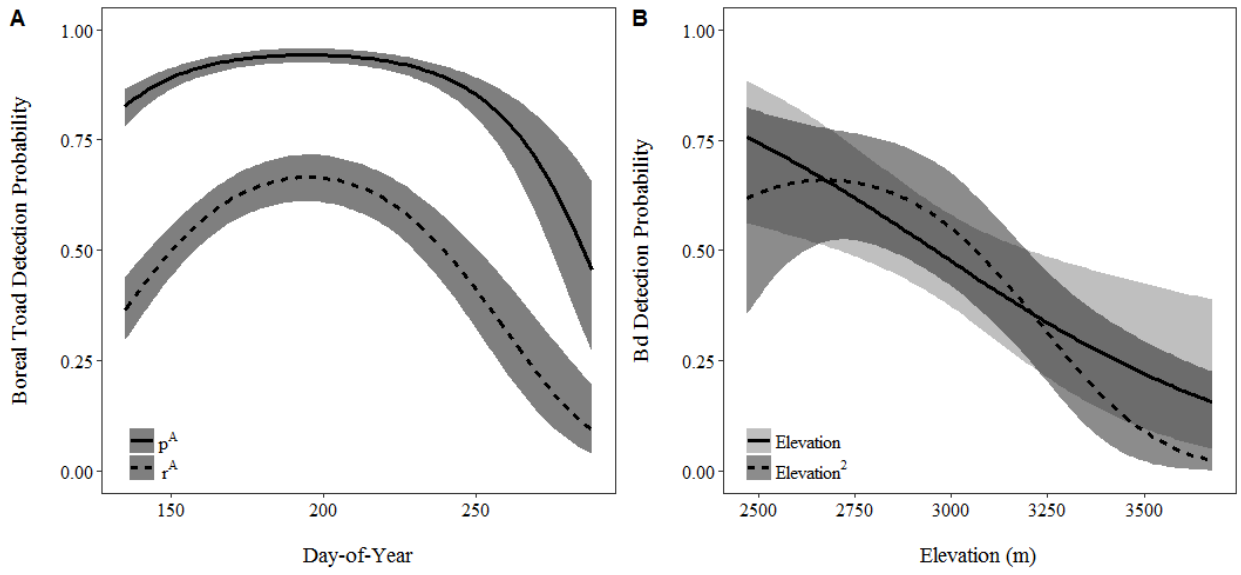


Figure 2.3: Best-supported detection probability relationships from the stepwise modeling procedure for boreal toads and *Bd*. Detection probability for boreal toads was related to day-of-year (A) and was lower when *Bd* was present (dashed line; r^A). Bd detection probability (B) varied with elevation in either a linear (solid line) or quadratic (dashed line) fashion. Estimates and 95% confidence intervals (shaded areas) are given for the best-supported detection structures given general structures for other parameters.

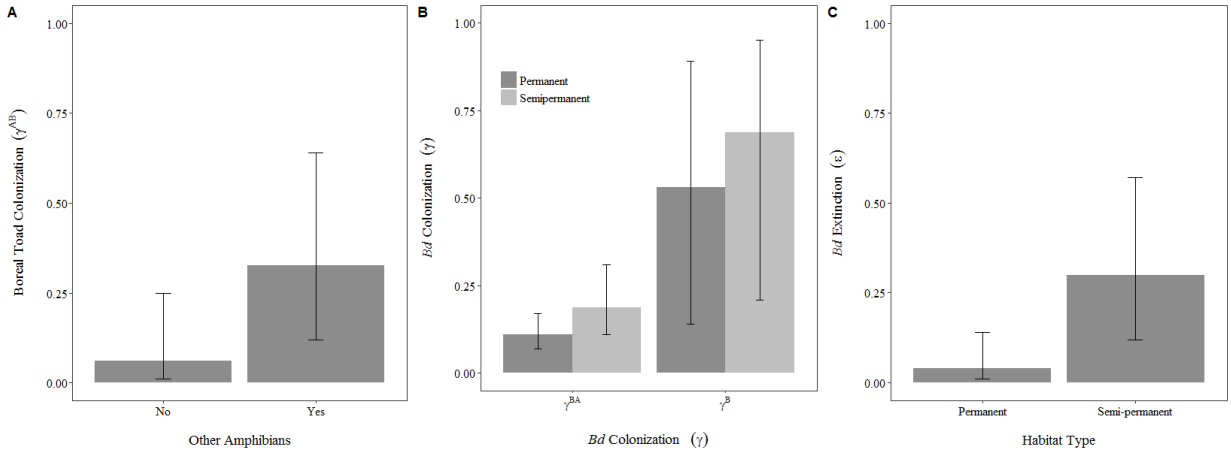


Figure 2.4: Estimates of boreal toad colonization and *Bd* extinction for Southern Rocky Mountain populations studied from 2001-2010. I present estimates of boreal toad colonization when *Bd* is present with and without other amphibian species (A) with 95% confidence intervals. I also present estimates of *Bd* colonization (B) and extinction (C) with 95% confidence intervals by habitat type when boreal toads are (dark grey) and are not (light grey) present. Each set of estimates comes from the best-supported model containing these factors, though none of these models were particularly well-supported.

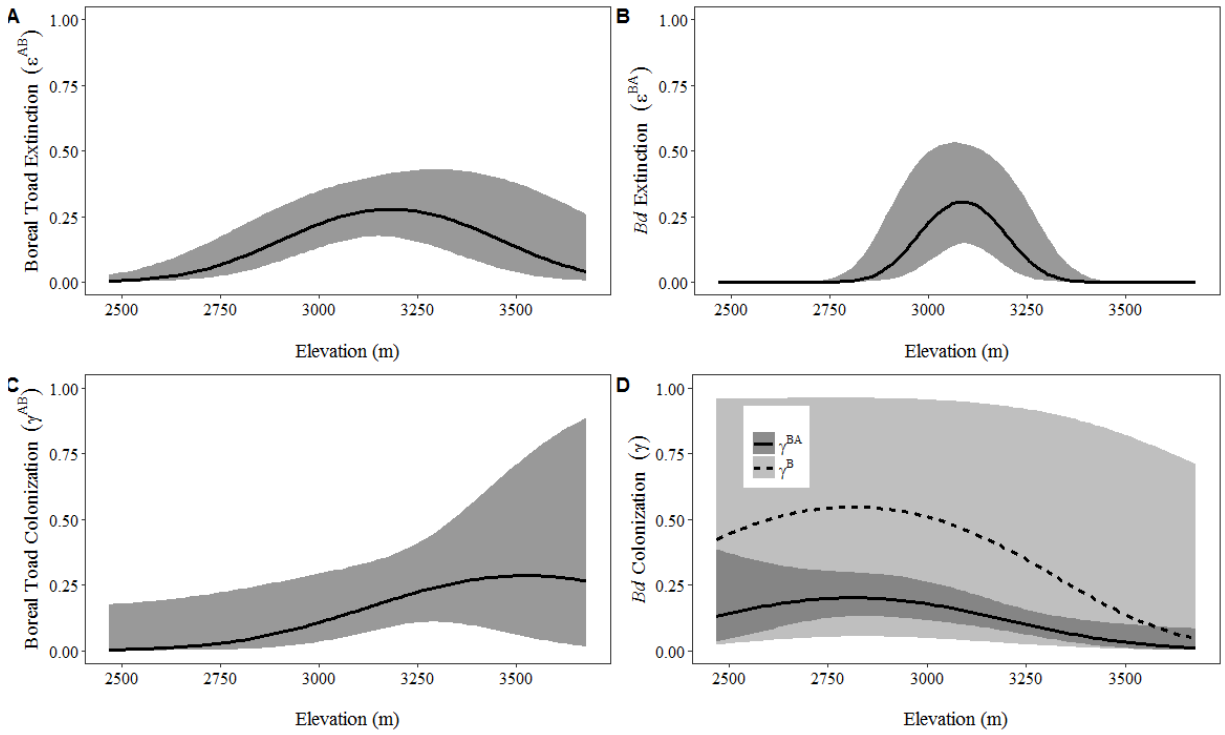


Figure 2.5: Effects of elevation on boreal toad and *Bd* dynamics for Southern Rocky Mountain populations studied from 2001-2010. Estimates and 95% confidence intervals are from the best-supported model of boreal toad-*Bd* dynamics. A quadratic effect of elevation influenced boreal toad (A) and *Bd* extinction (B) when host and pathogen co-occured. Elevation also influenced boreal toad colonization in the presence of *Bd* (C) and *Bd* colonization independent of boreal toad presence (D).

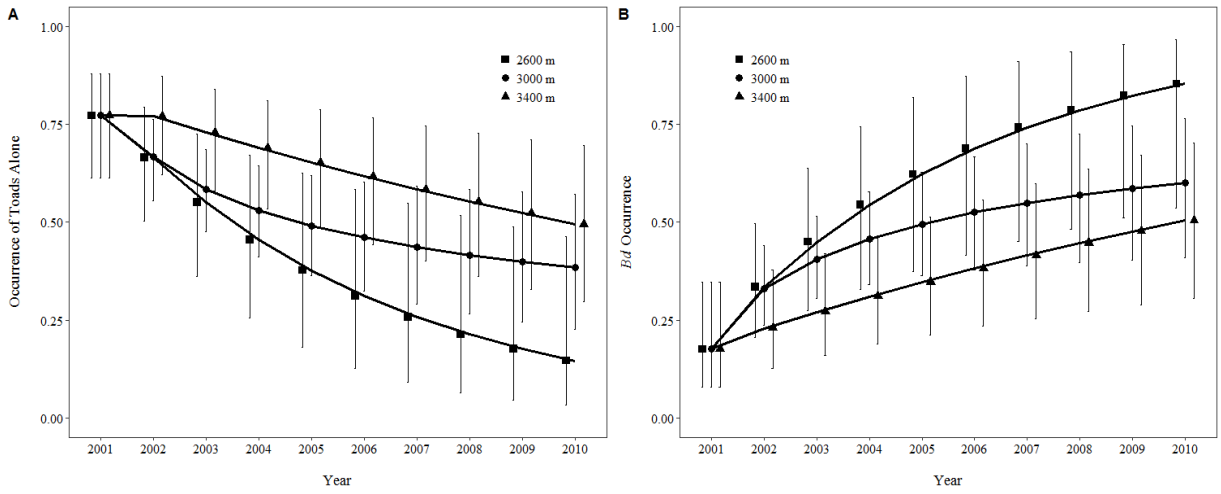


Figure 2.6: Derived annual estimates of boreal toad and *Bd* occurrence for southern Rocky Mountain populations studied from 2001-2010. Annual estimates of toad occurrence in the absence of *Bd* (i.e. the proportion of occupied toad sites without *Bd*) (A) and estimated annual proportion of sites with *Bd* (B). Estimates are given for three elevations of interest, representing low, mid and high elevation historic toad sites. Estimates and 95% confidence intervals are based on the best-supported model.

CHAPTER 3: DESIGN- AND MODEL-BASED RECOMMENDATIONS FOR DETECTING AND QUANTIFYING AN AMPHIBIAN PATHOGEN IN ENVIRONMENTAL SAMPLES

SUMMARY

Accurate pathogen detection is essential for developing management strategies to address emerging infectious diseases, an increasingly prominent threat to wildlife. Sampling for free-living pathogens outside of their hosts has benefits for inference and study efficiency, but is still uncommon. I used a laboratory experiment to evaluate the influences of pathogen concentration, water type, and qPCR inhibitors on the detection and quantification of *Batrachochytrium dendrobatidis* (*Bd*) using water filtration. I compared results pre- and post-inhibitor removal, and assessed inferential differences when single versus multiple samples were collected across space or time. I found that qPCR inhibition both *Bd* detection and quantification in natural water samples, resulting in biased inferences about *Bd* occurrence and abundance. Biases in occurrence could be mitigated by collecting multiple samples in space or time, but biases in *Bd* quantification were persistent. Differences in *Bd* concentration resulted in variation in detection probability, indicating that occupancy modeling could be used to explore factors influencing heterogeneity in *Bd* abundance among samples, sites, or over time. Our work will influence the design of studies involving amphibian disease dynamics and studies utilizing environmental DNA (eDNA) to understand species distributions.

INTRODUCTION

Emerging infectious diseases (EIDs) are a prominent threat to wildlife (Langwig et al. 2015) and are important drivers of local extinctions (Smith, Sax, and Lafferty 2006). In addition to affecting host species, disease-related declines can have cascading effects on community structure and ecosystem-level processes (Whiles et al. 2013, Hollings et al. 2014, Jachowski et al. 2014). Emerging infectious diseases in amphibian populations are on the rise, with ranavirus infections, saprolegniosis, *Ribeiroia* spp. infections, and chytridiomycosis contributing to mortality events (Daszak et al. 2003). Chytridiomycosis is caused by the fungal pathogens *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*). *Bd* is implicated in the declines of over 200 anuran species across the globe (Skerratt et al. 2007), and, though *Bsal* is a newly identified pathogen causing disease in urodelans, it has already been linked to fire salamander (*Salamandra salamandra*) extirpations in the Netherlands (Martel et al. 2013). Both pathogens are of concern to natural resource scientists and managers, and key uncertainties about pathogen transmission, distributions, and dynamics within amphibian host populations remain (Venesky et al. 2014b, Grant et al. 2016b).

Pathogen detection is central to understanding host-pathogen dynamics and to making informed management decisions (Voyles et al. 2014). Swabbing amphibian skin is the recommended (Hyatt et al. 2007) and most common method for detecting *Bd* and *Bsal*. Collecting skin swabs can be difficult at sites where host amphibian species are rare or extinct, but understanding the persistence and distributions of these pathogens in the environment in these places remains important for providing valuable ecological and conservation insights. For instance, sites without amphibians are preferred for amphibian reintroduction initiatives (Muths et al. 2014a) and *Bd* status of potential sites must be assessed to maximize the probability of

success of costly reintroductions. Relying on swabs can also make it difficult to answer basic ecological questions about pathogen persistence in the absence of amphibian hosts (Chapter 1) or to assess the spatial or temporal distribution of *Bd* or *Bsal* in water bodies.

Water filtration can be used to detect *Bd*'s infective stage (Berger et al. 2005) without relying on amphibian host presence and detection and it is an important technology for pathogen detection in both aquatic and terrestrial amphibian communities. Filtration has been used to detect zoospores in rainwater (Kolby et al. 2015), in baths of distilled water in which amphibians were soaked (Hyatt et al. 2007, Shin et al. 2014), and in aquatic habitats such as amphibian breeding ponds (Schmidt et al. 2013, Chestnut et al. 2014). Filtration can be used to survey potential amphibian reintroduction sites currently devoid of hosts, yielding information about pathogen distributions independent of host distributions. Additionally, filtration could make survey efforts more efficient by eliminating capture and handling of amphibians and by allowing multiple independent sample types (e.g., visual encounter surveys, amphibian swabs, and *Bd* filtration samples) to be collected during a single site visit. The relationship between *Bd* detection and *Bd* concentration is largely unknown because the filtration method has not been experimentally assessed at low concentrations or abundances of *Bd* that are likely characteristic of natural settings. For filtration to become a useful field method, its utility for both detecting and quantifying pathogen DNA must be assessed.

Many modern molecular methods (e.g., quantitative real-time polymerase chain reaction or qPCR) provide information about the occurrence and quantity of target DNA found in a sample. Quantities estimated from qPCR could be used to understand the relationship between infection load and disease risk for resident or reintroduced amphibians, but the validity of this index is not well-supported for *Bd* swabs (Clare et al. 2016b) and has never been assessed for

filtered water samples. Despite this lack of validation, quantitative estimates from qPCR have been used as both indices and true measures of *Bd* abundance (Miller et al. 2012b, Venesky et al. 2014a). Understanding the relationship between the estimated quantity of *Bd* and true *Bd* concentration is central to understanding infection thresholds (Vredenburg et al. 2010b), assessing impacts of management actions (Scheele et al. 2014), and targeting areas for reintroduction of declining amphibian species (Muths et al. 2014).

The presence of inhibitory agents (e.g., humic acid) in field samples can interfere with qPCR and cause errors (i.e., false negatives) which can bias biological inference. qPCR inhibition has been identified in amphibian swab samples (Kosch and Summers 2013) and in filtered water samples where shed DNA is captured (McKee et al. 2015). The presence of qPCR inhibitors likely influences both the detection and quantification of *Bd* DNA, but the extent of this influence has not been explored.

I designed an experiment to evaluate the effects of *Bd* concentration, water type (distilled and natural), and qPCR inhibition on detection and quantification of *Bd* captured using water filtration. I evaluated samples independently (single sample scenario) or in groups (multiple samples scenario) to mimic spatial and temporal replication in field studies. I chose concentrations of *Bd* that were low but biologically relevant to amphibians, as these concentrations will be most informative to those designing field studies, understanding disease dynamics, and developing conservation strategies. I assessed qPCR inhibition by comparing *Bd* detection and quantification in two water types (distilled and natural) and by analyzing samples with and without removing contaminants that can inhibit qPCR reactions. I discuss the implications of my work in the context of host-pathogen ecology, study design, and ecological

modeling, and provide information that will be useful to researchers and managers seeking to better understand and conserve amphibian communities.

MATERIALS AND METHODS

Experimental and Molecular Methodology

I cultured *Bd* strain JEL274, originally collected from a boreal toad (*Anaxyrus boreas boreas*) in Colorado, until zoospores were mature (Kirshtein et al. 2007). I used a hemocytometer and bright-field microscopy to determine the concentration of the harvested cultures and then diluted the zoospores with sterile deionized water to create solutions varying in *Bd* concentration.

I randomly assigned levels of two factors (concentration and water type) to 300 study units (250-mL glass jars) and investigated *Bd* detection via water filtration at 5 concentrations: 0, 0.05, 0.175, 1, and 50 zoospores/mL. The 0 zoospore/mL group served as a negative control, while the 0.05 zoospore/mL group was included to explore the lower limit of detection of *Bd* (Boyle et al. 2004, Kirshtein et al. 2007, Kerby et al. 2012). The highest concentration (50 zoospores/mL) was selected for its lethality to young-of-the-year boreal toadlets experimentally bathed in this concentration for 72 hours, whereas the intermediate levels represent concentrations that were sub-lethal to boreal toadlets and that likely exist in natural settings (Carey et al. 2006).

I considered two water types: distilled water and water from a natural, lotic source where *Bd* had never been detected. Natural water was selected to investigate how inhibitors influence pathogen detection, while distilled water was selected because it is commonly used to bathe amphibians prior to filtration (*sensu* Shin et al. 2014) and has been used in studies of molecular methodology (*sensu* Bletz et al. 2015). The natural water was autoclaved and allowed to sit for

20 days before the experiment to render any *Bd* DNA undetectable (Piotrowski et al. 2004, Thomsen et al. 2012). I included an equal number of jars (n = 36) for all groups except the control groups (n = 6).

I inoculated jars with known concentrations of *Bd* and let the jars rest for 18 hours prior to sampling. Upon sampling, I briefly agitated each jar and then drew one, 60-mL sample from a total volume of 200mL using a 0.22-µm Sterivex capsule filter with a male Luer-Lok (Millipore, Billerica, MA) connected to a sterile 60-mL syringe. After collection, I prepared the sample using lysis buffer according to the protocols in Chestnut et al. (2014). Samples were maintained at room temperature until DNA was extracted (within 17 weeks of collection).

I extracted DNA using Gentra Puregene Tissue Kits (Qiagen, Valencia, CA; Chestnut et al. 2014). I initially analyzed the extracts in triplicate wells using the qPCR assay outlined by Boyle et al. (2004) and updated by Kerby et al. (2012), but I found low *Bd* detection probabilities in the natural water that provided evidence of PCR inhibition (see Results). Subsequently, I used a post-extraction spin column purification kit (OneStep™ PCR Inhibitor Removal Kit, Zymo Research, Irvine, CA) on each sample and analyzed the resulting post-purification sample in triplicate with qPCR. Extraction and qPCR were conducted in separate lab spaces using dedicated supplies and workspaces. Aerosol-barrier pipette tips were used, and all laboratory equipment and benches were cleaned with bleach in between procedures.

The target region for amplification during qPCR was the internal transcribed spacer region one (ITS1), which is variable in copy number per zoospore among *Bd* strains (Longo et al. 2013). I estimated the number of ITS1 copies present in single JEL274 zoospore by comparing the amplification rates between a known quantity of JEL274 zoospores and a qPCR standard made from PCR amplicons of the ITS1 region. I used this information to convert estimated

number of copies from qPCR to estimated zoospore counts, and used these counts to quantify bias in estimates of *Bd* concentration. I investigated the correlation between qPCR copy number and *Bd* concentration because the *Bd* strain is unknown in most field studies.

Bd Occurrence and Detection

I investigated how two different sampling scenarios, "single sample" and "multiple samples", might influence bias in estimates of *Bd* occurrence. In the single sample scenario, single jars were used as sample units. This scenario corresponds to collecting a single water filter (i.e., field sample) at a wetland (Figure 3.1). For the multiple samples scenario, I redefined a study unit as a collection of three jars (i.e., field samples) within the same treatment group to emulate field protocols where multiple filters are collected at a single site over space or time (e.g., Chestnut *et al.* 2014; Pilliod *et al.* 2014; Figure 3.1). This reduced my sample size from 36 to 12 study units per treatment, but increased the number of opportunities for detection within each study unit.

Occupancy models use repeated surveys to estimate the probability that a study unit is occupied by the species of interest, while explicitly allowing for imperfect species detection. I used results from all 3 qPCR replicates (hereafter, "wells") per filter sample as repeat surveys to detect *Bd*. I modeled both *Bd* occurrence (ψ) and detection probability (p) as additive and interactive functions of *Bd* concentration and water type using standard occupancy models (MacKenzie et al. 2002) for the single sample scenario. For the multiple samples scenario, I used a multi-scale occupancy model (Nichols et al. 2008) to accommodate multiple filter samples per study unit and multiple qPCR wells per sample. In this case, I estimated the probability that a study unit (group of 3 jars) was occupied by *Bd* (ψ), the probability that *Bd* was present in an individual filter sample given that the study unit was occupied (θ), and the probability of

detecting *Bd* given that it was present on a filter (p). I modeled ψ, θ, and p as additive and interactive functions of *Bd* concentration and water type. In both sampling scenarios, I conditioned on samples that were inoculated with *Bd* and compared the estimated occupancy probabilities with the true values ($\psi_{true} = \theta_{true} = 1$) to quantify estimation biases using the observed data. Models were fit to both pre- and post-purification data.

Zoospore Quantification

I assessed the validity of copy number as an index of *Bd* concentration in the single and multiple samples scenarios by calculating the Spearman's rank-order correlation coefficient (r_s) between the mean qPCR copy number for a sample and the known concentration. I included nondetections (i.e., wells with qPCR copy number estimates of 0) and compared the correlation coefficients for both pre- and post-purification datasets to assess the impact of qPCR inhibition on quantity estimation.

Next, I converted qPCR copy number to zoospore concentration using strain-specific *Bd* information and used linear regression to evaluate if relative bias in the estimated concentration ($relative\ bias = \frac{Estimated[Bd] - Experimental[Bd]}{Experimental[Bd]}$) was related to water type or known *Bd* concentration. Positive relative bias values indicate an overestimation of concentration via qPCR, while negative values indicate underestimation. I used relative bias as the response variable for this regression analysis because I expected that bias and variance would vary substantially among concentrations.

Software and Multimodel Inference

I used an information-theoretic approach to rank candidate models using Akaike's Information Criterion corrected for small sample sizes (AICc; Burnham and Anderson 2002). Occupancy models were fit using the R package 'RMark' (White and Burnham 1999, Laake

2013). To account for model selection uncertainty, I report model-averaged parameter estimates that consider all models. Spearman's rank-order correlation and linear regression models for relative bias were conducted in R (R Development Core Team 2012).

RESULTS

Thirty-three percent (4/12) of negative control jars yielded false positive results when purification was not performed, compared to 8% post-purification (1/12 jars). The negative control jar that tested positive when inhibitors were removed did not test positive without removing inhibitors. I eliminated questionable detections in both datasets by imposing a threshold based on the highest copy number estimated in the negative controls (samples with fewer than 8.9 qPCR copies pre-purification and 63.7 copies post-purification were excluded). These samples were estimated to contain less than 1 zoospore.

The proportion of jars where *Bd* was detected in at least one qPCR well varied among concentrations and water types (Table 3.1). I detected *Bd* in at least one of three wells in 127 of 288 (44%) inoculated jars pre-purification, and in at least one of three wells in 185 of 288 (64%) inoculated jars post-purification.

Bd Occurrence and Detection

Of the 46 single sample occupancy models fit to the pre-purification data, only three were supported (Appendix 3.1A). All supported models included an interactive effect of water type and concentration on detection and, while detection increased with concentration in distilled water, it was unrelated to concentration in natural water (Figure 3.2A). The best-supported covariates for occupancy were an interactive effect of water type and concentration, an additive effect of these factors, and water type alone. Pre-purification estimates of occurrence in distilled and natural water were biased across concentrations, suggesting that *Bd* occurred in ≤25% of

natural water samples and ≤75% of distilled water samples at low concentrations though all jars were inoculated with *Bd* (Figure 3.2C, Table 3.1). Post-purification, a model where both detection and occupancy varied as an additive effect of concentration and water type received 0.85 of the model weight (Appendix 3.1B). Model-averaged estimates revealed that post-purification detection probability for natural water was higher and more closely related to concentration than pre-purification (Figure 3.2, top panel). Post-purification, model-averaged estimates of *Bd* occurrence increased with concentration in both water types but were still negatively biased at low concentrations (Figure 3.2D, Table 3.1). Interestingly, both detection and occupancy probabilities were estimated to be higher in natural water than in distilled water post-purification (Figure 3.2B and 3.2D).

When multiple samples were used and purification was not employed, several multi-scale occupancy models received support. Best-supported models generated unbiased estimates of *Bd* occurrence ($\hat{\psi}$ = 1.0) that were constant across treatment types (Table 3.1, Appendix 3.2A). However, estimates of *Bd* presence on individual filters (θ) varied with concentration and water type, were biased low, and were identical to ψ estimates from the single-sample occupancy analysis (Figure 3.2C). The best-supported models for the post-purification dataset also suggested a constant occurrence of *Bd* with estimates of 1.0 for all treatment groups (Table 3.1, Appendix 3.2B). The estimates of *Bd* availability were once again biased low, but were much higher in natural water than they were pre-purification (Figure 3.2D). Detection probability was influenced by concentration and water type in both datasets, and was identical to the detection probabilities estimated in the single-sample occupancy analyses (Figure 3.2A and 3.2B).

Zoospore Quantification

I removed five outliers from the pre-purification dataset and two from the post-purification dataset before assessing the quantification of *Bd* under the different sampling scenarios. The estimated mean copy number for these outliers was at least an order of magnitude higher than any other sample in the same treatment group. Different samples were identified as outliers in the pre- vs. post-purification datasets.

When single samples were considered, the pre-purification estimate of the correlation between qPCR copy number and known zoospore concentration was positive but low in natural water ($r_s = 0.13$, Appendix 3.3) and was much higher once purification was employed ($r_s = 0.79$, Appendix 3.3). The correlation between qPCR copy number and known *Bd* concentration was high for distilled water when single samples were used, regardless of whether purification had been applied ($r_s = 0.69$ pre-purification versus 0.72 post-purification, Appendix 3.3).

Combining multiple samples improved the correlation between qPCR copy number and known zoospore concentration in both water types, but not as much as did the application of the purification protocols in natural water (Figure 3.3 top panel, Appendix 3.3). The purification process resulted in a three-fold increase in the correlation between qPCR copy number and known zoospore concentration in natural water ($r_s = 0.31$ pre-purification versus $r_s = 0.90$ post-purification, Appendix 3.3) while no change was seen for distilled water ($r_s = 0.85$, Appendix 3.3) when multiple samples were used.

I found consistent negative bias in estimated pathogen concentration (i.e., the constant model was best-supported, Appendix 3.4A) using pre-purification data (Figure 3.3C). Post-purification analyses of relative bias supported an interaction between concentration and water type (Appendix 3.4B). Zoospore concentration was overestimated in natural water samples post-

purification, especially at the highest concentration, while bias in distilled water remained minimal and negative (Figure 3.3D). I present relative bias results for only the multiple samples scenario because the findings were identical to those from the single sample scenario.

DISCUSSION

As the amphibian pathogens *Bd* and *Bsal* become more widespread, amphibian declines are expected to become more common and severe (Yap et al. 2015). Researchers and managers need to understand the distribution and dynamics of these pathogens, even at sites where amphibians no longer occur, so that the success of management actions can be assessed. No previous study has experimentally investigated the consequences of imperfect detection and qPCR inhibition on inferences about *Bd* occurrence (Walker et al. 2007, Kirshtein et al. 2007). Further, the only assessment of quantification of *Bd* zoospores using water filtration focused on a concentration that was not biologically realistic (>1,000 times higher than my concentrations; Kirshtein *et al.* 2007). My work fills these knowledge gaps and yields findings that influence study design, molecular and statistical analyses, and associated biological inferences in amphibian-pathogen systems or environmental DNA (eDNA) studies.

When multiple samples were collected and qPCR inhibitors were reduced, I reliably detected *Bd* at low concentrations that are likely common in wild systems. A multiscale occupancy approach is a natural fit for pathogen detection data that are imperfect and generated in duplicate or triplicate (McClintock et al. 2010, Lachish et al. 2012) and such approaches can yield important insights about how covariates influence pathogen distributions. While I am not the first to recommend that conservation biologists and managers should collect multiple samples through time and space to maximize pathogen detection probabilities (Schmidt et al. 2013), I am

the first to show how qPCR inhibition and pathogen concentration influence estimates of pathogen occurrence and detection at multiple scales of interest.

I found that filter-level *Bd* occurrence (θ) is related to pathogen concentration, and I suggest exploiting this relationship to explore the factors influencing heterogeneity in *Bd* occurrence across temporal and spatial scales. For example, understanding the site characteristics that promote *Bd* occurrence may help managers identify which candidate reintroduction sites have little or no *Bd* and subsequently present the lowest risk of disease to reintroduced amphibians. In terrestrial amphibian communities, filtration may be a useful tool for detecting pathogens from fomites or amphibians bathed in water (Hyatt et al. 2007, Shin et al. 2014). In this case, using a framework like the one I present would allow the exploration of site- and individual-level covariates that influence the occurrence (ψ) and prevalence (θ) of *Bd* infections.

While three filters were sufficient to obtain unbiased estimates of site-level occupancy in my experiment, I expect more spatial and temporal heterogeneity in natural settings (Walker et al. 2007, Chestnut et al. 2014); additional samples will be required, especially at newly invaded sites with low concentrations of *Bd*. Pilot studies where multiple samples are collected and analyzed can help investigators anticipate plausible detection probabilities in their system and to optimize the number of samples needed to address study objectives. Collecting and analyzing multiple samples is expensive, but I have shown that the inferential gains of multiple samples are great. Further, strategies like pooling samples (Boyle et al. 2004) can reduce laboratory costs.

Previous studies mention a concern for false positives (Schmidt et al. 2013, Olson et al. 2013) and employ thresholds (Venesky et al. 2014a, Shin et al. 2014), indicating that false positives may be more common than is often acknowledged. Precautionary measures including changing gloves frequently, wearing room-dedicated laboratory coats, cleaning equipment with

bleach, and using aerosol-barrier pipette tips and a dedicated clean room for extraction and qPCR are all best practices that should be incorporated to reduce contamination (Goldberg et al. 2016). In addition, researchers should incorporate negative controls at each level of sample preparation to adequately assess if and when false positives occur (Goldberg et al. 2016). Incorporating an experimentally derived threshold to remove false positives (as I did here) will also remove true detections, resulting in decreased detection probabilities. Model-based methods to account for false positives also exist, and the best option to account for false positives will likely be context-dependent. For instance, if low-level contamination is seen in the negative controls, qPCR copy number could be used to classify detections as "certain" or "uncertain" (potentially a false positive) using a multiple detection state occupancy model (Miller et al. 2011).

I caution against using estimates of qPCR copy number as a direct measure of *Bd* abundance without further research, as copy number estimates were biased, especially at high concentrations in this study. I did find a high correlation between qPCR copy number and concentration treatment group when multiple samples were used, indicating that copy number may still be useful for differentiating between different types of sites that exist in natural settings (e.g., newly invaded [low *Bd* concentration], sub-lethal/endemic [intermediate *Bd* concentration], and lethal/epidemic [high *Bd* concentration]) using a multi-state occupancy approach (MacKenzie et al. 2009). Copy number may also be useful for modelling abundance-induced heterogeneity in detection among occupied sites in the field (Miller et al. 2012b). My findings mirror those of Clare et al. (2016), who found that, while qPCR copy number from swabs could allow differentiation between moribund and visually healthy individuals, it was not an accurate representation of true infection load. My study is the first of its kind to investigate how the

quantification of *Bd* isolated from the environment is influenced by qPCR inhibition and the collection of multiple samples.

Inhibition during qPCR strongly influenced *Bd* detection and quantification, and can cause negatively biased estimates of *Bd* occurrence and abundance especially when only a single sample is collected. When multiple samples were collected, purifying DNA led to a 3-fold increase in correlation between copy number and known *Bd* concentration in natural water. I recommend testing for qPCR inhibition in every sample using internal positive controls (IPC; Hyatt et al. 2007). When evidence of qPCR inhibition is found, I recommend that a process to reduce inhibition be used (e.g., OneStep™ PCR Inhibitor Removal Kit, Zymo Research, Irvine, CA). Other approaches for removing inhibitors should be examined in a rigorous experimental framework similar to what I present here before they are used with field samples. Studies that fail to identify and address qPCR inhibition may grossly underestimate pathogen distributions. Amphibian skin swabs also contain inhibitory agents (Kosch and Summers 2013, Blooi et al. 2013), and I expect that, without purification, those sample types are also subject to biased inferences of prevalence (Becker and Zamudio 2011) or individual infection loads (Stockwell et al. 2016).

Conclusions

Though improving and refining field and lab methods for the detection of amphibians and their pathogens is crucial, all sampling methods are imperfect. Thus, 'best practices' should include collecting multiple samples, using multiple detection methods, and accounting for imperfect detection using both laboratory and modelling techniques. Employing multiple detection methods on a single site visit creates gains in study efficiency; filter samples for eDNA can complement visual encounter surveys for amphibian detection (Pilliod et al. 2013) and filter

samples for *Bd* or *Bsal* can complement skin swabs of resident or sentinel individuals for pathogen detection (Schmidt et al. 2013). Performing multiple assays on a single filter sample offers opportunities to detect multiple hosts, pathogens, and other species simultaneously (Blooi et al. 2013).

Existing studies of amphibian-*Bd* or *Bsal* occurrence dynamics have been limited to studying the prevalence of these pathogens within one or several known host populations (e.g., Vredenburg et al. 2010b, Savage et al. 2011). Understanding the dynamics of amphibian hosts and their pathogens at the landscape scale requires the ability to sample each species independently (Chapter 1) and will yield insights about metapopulation dynamics, long-term species persistence, and the success of management actions. My findings are valuable to conservation biologists and managers as they strive to understand and manage complex amphibian-pathogen systems.

Table 3.1: Estimated proportion of sample units that were occupied by *Bd* using raw data and occupancy modeling approaches. I used both pre- and post-purification datasets for four *Bd* concentrations (zoospores/mL) in conjunction with these approaches. Using raw detection data, a sample was classified as occupied if *Bd* was detected in at least one of the three qPCR wells. Model-averaged estimates and unconditional standard errors (in parentheses) are given for occupancy approaches.

	Raw Data				*Single Sample Occupancy*				*Multiple Samples Occupancy*			
	Pre-purification		**Post-purification**		**Pre-purification**		**Post-purification**		**Pre-purification**		**Post-purification**	
[*Bd*]	*Distilled*	*Natural*	*Distilled*	*Natural*	*Distilled*	*Natural*	*Distilled*	*Natural*	*Distilled*	*Natural*	*Distilled*	*Natural*
0.05	0.47	0.11	0.06	0.56	0.72 (0.17)	0.16 (0.07)	0.10 (0.04)	0.54 (0.08)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)
0.175	0.53	0.25	0.31	0.81	0.66 (0.12)	0.23 (0.07)	0.31 (0.07)	0.82 (0.05)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)
1	0.69	0.28	0.58	0.95	0.73 (0.08)	0.27 (0.07)	0.60 (0.08)	0.94 (0.03)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)
50	0.94	0.25	0.92	0.97	0.86 (0.08)	0.31 (0.10)	0.90 (0.05)	0.99 (0.01)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)

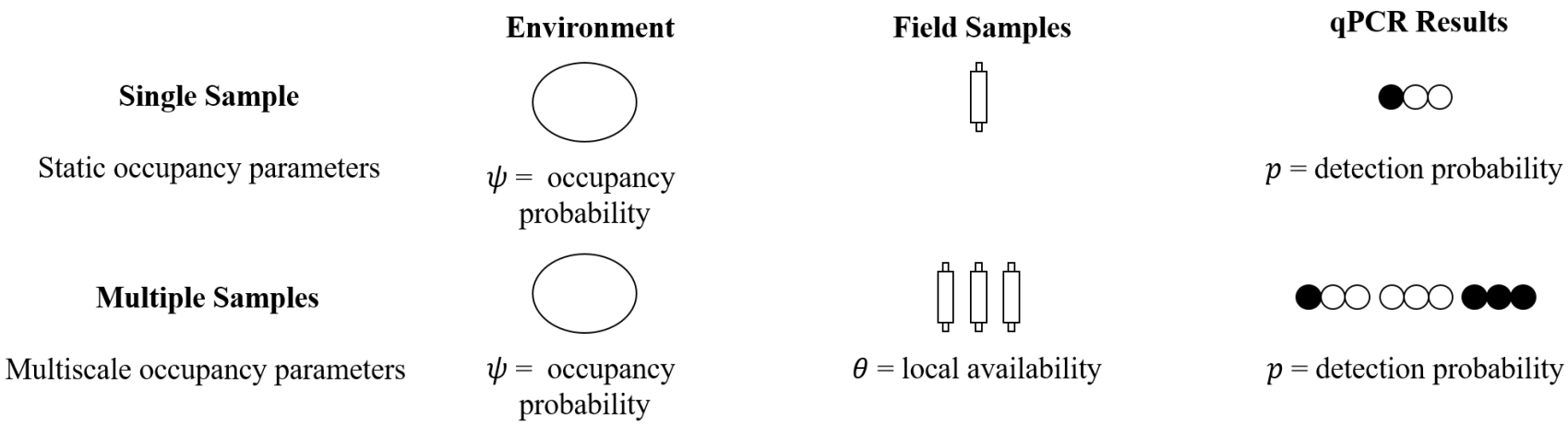


Figure 3.1: Single and multiple sample scenarios in an example field study of *Bd* occurrence in an aquatic environment. Single samples can be used to make inferences about *Bd* detection probability (p) and site-level occupancy (ψ). If multiple samples are collected, additional inferences can be made about heterogeneity in *Bd* occurrence across space or time (θ). Resulting replicate qPCR results from both sampling strategies can be analyzed in an occupancy framework that accounts for imperfect detection.

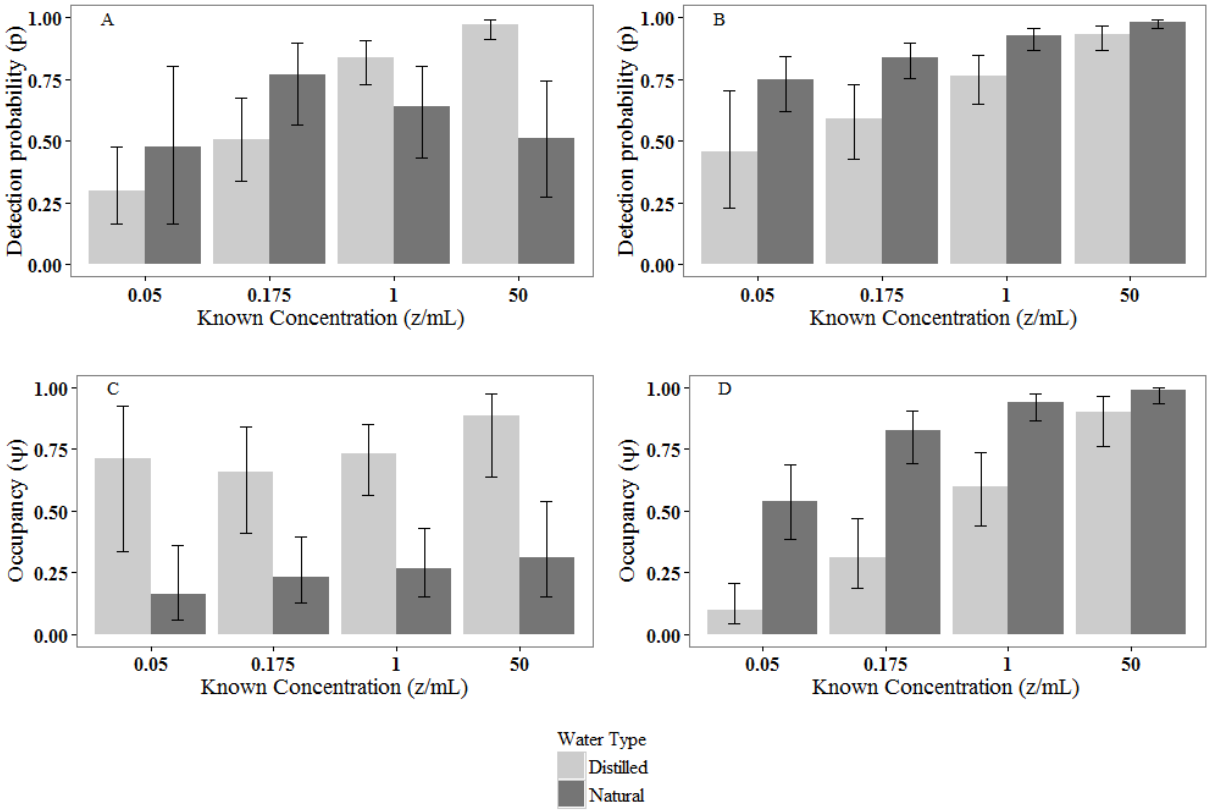


Figure 3.2: Model-averaged estimates of *Bd* detection and occupancy probability. *Bd* detection (p, A and B) and occupancy (ψ, C and D) are provided with 95% confidence intervals from standard single sample occupancy analyses when purification methods were (right column) and were not (left column) applied. Occupancy estimates presented here are identical to model-averaged *Bd* filter occurrence (θ) estimates from the multiscale occupancy analysis.

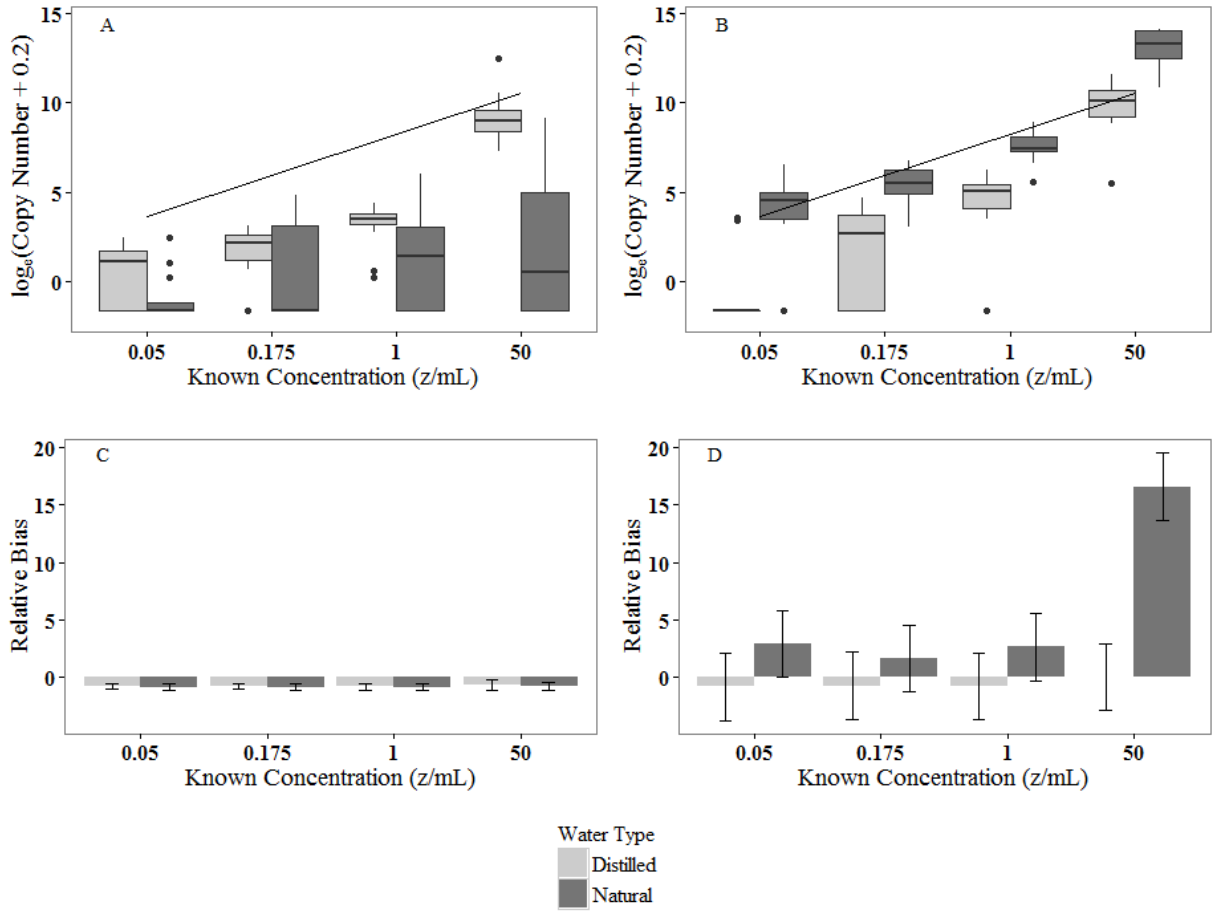


Figure 3.3: Relationship between qPCR copy number and known experimental concentration. This relationship was determined using multiple samples pre- (A) and post- purification (B). The 1:1 line in plots A and B illustrates the true relationship between qPCR copy number and known concentration. Model-averaged estimates and 95% confidence intervals for estimated relative bias in *Bd* concentration from linear regression models before (C) and after (D) purification when multiple samples were used.

CHAPTER 4: TESTING THE WATERS: USING ENVIRONMENTAL SAMPLING TO UNDERSTAND THE PERSISTENCE OF AN AMPHIBIAN PATHOGEN

SUMMARY

The persistence of pathogens, or how long the pathogen can survive outside of a host, is an important driver of host-pathogen dynamics. However, a reliance on host-based pathogen detection methods can complicate these insights. The amphibian pathogens *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*) are pathogens of global conservation concern. Despite having a free-living life stage, little is known about the distribution and persistence of these pathogens outside of the amphibian host. I combine historic amphibian monitoring data and contemporary host- and environment-based pathogen detection data to obtain estimates of *Bd* occurrence independent of amphibian distributions. We also assess inferential differences arising from using different decision criteria to classify samples as positive or negative, and evaluate differences in detection probability using water filters and amphibian swabs.

We found evidence of long-term *Bd* persistence for several years after the predominant amphibian species was last detected. The decision criterion used to classify samples as positive or negative was important; using a more liberal criterion yielded lower estimates of *Bd* occurrence than when a conservative criterion was used. The liberal and conservative criteria also suggested different covariates of importance when modeling *Bd* detection. Water filtration-based detection probabilities were lower than those from swabs, and swab-based detection probabilities varied seasonally, declining in the early fall. My work provides evidence of long-

term *Bd* persistence in the environment and also underscores the importance of environmental samples for understanding and mitigating disease-related threats to amphibian biodiversity.

INTRODUCTION

Pathogens that can persist in the absence of hosts often spread rapidly and are more likely to cause local extinctions than pathogens that are directly transmitted. For instance, the emerging infectious diseases of malaria, chronic wasting disease, and white-nose syndrome are all caused by persistent pathogens and are of considerable conservation concern (Woodworth et al. 2005, Sharp and Pastor 2011, Lorch et al. 2013). The mechanism of persistence depends on the pathogen's life history, but can be linked to vector populations (Minakawa et al. 2002, Gomez-Diaz et al. 2010), the presence of non-host reservoirs (Broza and Halpern 2001), or the pathogen's ability to survive for long periods of time in the absence of hosts (Breban et al. 2009, Almberg et al. 2011, Hoyt et al. 2016). Knowing whether, and how, pathogens persist outside of hosts is necessary for predicting the spread of disease (Rogers and Randolph 2000) and for understanding the risk of pathogen pollution or spillover into new host populations (Daszak et al. 2000). These predictions also have conservation implications and can be used to identify appropriate management actions for host species of concern.

Initial efforts to detect emerging pathogens often focus on host-based detection methods (e.g., histology, serology, fecal samples, skin swabs, etc.) and ignore the pathogen or vector's environmental niche. For pathogens with free-living or vectored stages, data gained from sampling the environment or vector directly, rather than the host, can provide valuable information about pathogen distributions. Findings can be used to complement theoretical, experimental, and *ex situ* studies and provide a more complete understanding of the ecology of newly emerging infectious diseases (Carver et al. 2010). The environmental life stages common

in fungi are often unsampled, and are therefore not well understood, despite consensus that these stages may be central drivers of host-pathogen ecology and ensuing disease dynamics (Briggs et al. 2010, Fisher et al. 2012, Lorch et al. 2013).

Emergence of the amphibian pathogens *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*) and the concomitant disease chytridiomycosis are of global conservation concern (Skerratt et al. 2007, Grant et al. 2017, Fisher 2017). These fungal pathogens have aquatic, free-living infective zoospores, and have been linked to declines of anurans (Fisher et al. 2009) and urodelans (Spitzen-van der Sluijs et al. 2013). Sampling amphibian hosts via histology, skin swabs, and water baths (Hyatt et al. 2007, Martel et al. 2013, Blooi et al. 2013, Shin et al. 2014, Dillon et al. 2017) are the most commonly used methods to detect *Bd* and *Bsal*, despite the fact that the infectious stage of these pathogens exists in the environment (Berger et al. 2005). In addition, *Bd* has been detected in non-amphibian hosts (Shapard et al. 2012, McMahon et al. 2013, Liew et al. 2017); the potential for non-amphibian hosts of *Bsal* has not yet been explored. A reliance solely on host-based pathogen detection methods created difficulties when estimating pathogen occurrence when amphibian hosts are undetected or locally extinct, and resulted in an incomplete view of pathogen distributions and dynamics (Chapter 1). The long-term environmental persistence of *Bd* and *Bsal* in the absence of amphibian hosts has never been addressed using data from natural settings, though a mechanism of long-term *Bd* persistence would explain many observed amphibian declines and have important implications for conservation efforts.

Surprisingly little is known about the persistence of *Bd* in the environment. Though zoospores remain motile for only 24 hours before encysting (Piotrowski et al. 2004), *Bd* can survive in water or sediment for up to 12 weeks in laboratory settings (Johnson and Speare 2003,

Johnson et al. 2005, Walker et al. 2007). In addition, experiments have shown that *Bd* can grow on sterile feathers, snakeskin, and other media, suggesting that non-amphibian hosts could play a role in transmitting *Bd* to novel environments (Longcore et al. 1999, Johnson et al. 2005, Garmyn et al. 2012). Filtration-based methods (Kirshtein et al. 2007, Hyman and Collins 2012) can be used to estimate the occurrence of *Bd* or *Bsal* independent of amphibian occurrence, but, to date, these methods have only been used at sites where amphibian populations are extant (Schmidt et al. 2013, Chestnut et al. 2014). An understanding of *Bd*'s and *Bsal*'s persistence and distribution in the absence of amphibian hosts would facilitate amphibian reintroduction efforts (Muths et al. 2014b), the identification of saprobic stages or non-amphibian hosts (Di Rosa et al. 2007, Stegen et al. 2017), and our ability to measure success of management actions focused on limiting *Bd'*s growth in the environment.

I sampled for *Bd* in the environment and on the skin of multiple species of amphibians to evaluate the long-term persistence of *Bd* in an area that has experienced amphibian declines. My objectives were to: 1) determine whether *Bd* persists in the environment after amphibian extinctions by modeling *Bd* occurrence as a function of the time since the last detection of a target amphibian species; 2) explore the factors that influence filter and swab-based pathogen detection probabilities; and 3) investigate whether my findings were sensitive to the criteria used to classify samples as *Bd* positive or negative. My work underscores the importance of developing and using environmental samples to understand the distribution of free-living and vectored pathogens and contributes new information about the long-term persistence of *Bd*.

MATERIALS AND METHODS

Study System

The boreal toad (*Anaxyrus boreas boreas*) is a native western North American amphibian species with populations that are currently in decline throughout the Southern Rocky Mountains (SRM; Gerber et al. in revision, Carey 1993, Muths et al. 2003). Boreal toads within the SRM usually inhabit wetlands, wet meadows, beaver ponds, oxbows, and pond margins found at elevations between 2,615-3,385 meters (Hammerson 1999). *Bd* was first detected in the SRM in 1998 and has been implicated in boreal toad declines throughout that region (Chapter 2, Corn 2003, Muths et al. 2003).

Data Collection

In 2014, investigators from Colorado State University and the Boreal Toad Conservation Team sampled 103 wetlands thought to be suitable for toad breeding throughout Colorado and southern Wyoming for *Bd* using water filtration and amphibian swabs. The sampled sites fell into two groups: sites that historically supported boreal toad populations (Group 1: 87 sites), and sites with suitable boreal toad breeding habitat, but where boreal toads have never been detected (Group 2: 16 sites). Sites were visited between 1 and 6 times each during the boreal toad breeding season. On a single site visit, I collected three water filter samples from wetland margins (Kirshtein et al. 2007, Chestnut et al. 2014). DNA was analyzed from filter samples using standard extraction procedures and real-time polymerase chain reaction (qPCR) protocols (Kirshtein et al. 2007, Kerby et al. 2012). A commercially available inhibitor removal kit (OneStep™ PCR Inhibitor Removal Kit, Zymo Research, Irvine, CA) was used before qPCR analysis to remove compounds that might inhibit the qPCR reaction (Chapter 3, McKee et al. 2015). I also made every attempt to capture and swab any amphibians encountered during site

visits. DNA from swab samples was extracted and analyzed using qPCR (Kerby et al. 2012, Bletz et al. 2015). Each filter and swab sample was analyzed in triplicate.

Sampling variation, difficulty in amplifying small amounts of DNA, and the presence of inhibitors can all result in inconsistency in results among PCR replicates (Chapter 3, Navidi et al. 1992). I created *Bd* detection histories for each site by condensing the triplicate qPCR results from each filter and swab into a detection (1) or non-detection (0) based on a "liberal" or "conservative" decision criterion. Using the liberal decision criterion, each sample (filter or swab) was considered positive if *Bd* DNA was amplified in at least one of the three replicates (*sensu* Hyatt et al. 2007, Schmidt et al. 2013). Under the conservative decision criterion, samples were considered positive if *Bd* DNA was amplified in at least two of the three qPCR replicates (*sensu* Kerby et al. 2012). The conservative criterion is currently employed by the Boreal Toad Conservation Team to classify environmental DNA samples and amphibian swabs as positive for the target species (host or pathogen; H. Crockett, personal communication).

Biological Hypotheses

I used a single-season, single-species occupancy framework (MacKenzie et al. 2002) to estimate the probability of *Bd* occurrence ('occupancy') at a wetland (ψ), which is defined as the sampling unit in my study. Conditional on occurrence, I also estimated the probability of *Bd* detection using filter samples (p_{filter}) and swab samples (p_{swab}).

I hypothesized that *Bd* occurrence would be negatively related to the amount of time since boreal toads were last detected, suggesting that *Bd* may go locally extinct following host amphibian extinction. I used historic boreal toad monitoring data collected from 2001-2014 by the Boreal Toad Conservation Team to determine the amount of time since the last boreal toad detection ('time since toads', or *TST*) at each site. While *TST* is not identical to the time since

local boreal toad extinction, I expect *TST* to be highly correlated with the time since severe boreal toad declines, because, if they are present, the detection probability of boreal toads is typically very high during the breeding season (Chapter 2, Pilliod et al. 2010). When *TST* was known (i.e., for Group 1), values ranged from 0 for sites where toads were detected in 2014 to 13 for sites where toads were last detected in 2001. I included an additive effect of *group* and a *group* by *TST* interaction (*group***TST*) so that the *TST* covariate was only used to model *Bd* occurrence for sites where *TST* was known (Group 1), estimating a mean *Bd* occurrence for sites in Group 2 that was independent of Group 1.

Host community composition can influence amphibian-*Bd* dynamics and the presence of less susceptible amphibian species may offer a mechanism for *Bd* persistence after declines and/or extirpation of a susceptible species (Reeder et al. 2012, Venesky et al. 2014a, Scheele et al. 2016). In the SRM, boreal toads sometimes co-occur with tiger salamanders (*Ambystoma tigrinum*), boreal chorus frogs (*Pseudacris maculata*), and/or wood frogs (*Lithobates sylvaticus*). Therefore, I investigated whether *Bd* was more likely to occur at sites where other amphibian species historically occurred (*amph* = 1) than where they did not (*amph* = 0). The *amph* covariate reflects whether other amphibian species were ever detected at each site. I hypothesized that an interaction between *TST* and *amph* (*TST***amph*) would be supported, and that *Bd* occurrence would decline more steeply with *TST* if alternate reservoir hosts were not present (Dobson 2004). Past work has shown that *Bd* occurrence in this system may decrease with elevation, likely due to the cold temperatures and short growing season at high elevation sites (Chapter 2), and I evaluated this hypothesis by exploring models where *Bd* occurrence was either linearly (*elev*) or quadratically ($elev^2$) related to elevation.

In addition to hypotheses associated with *Bd* occurrence, I also tested for differences in *Bd* detection at occupied sites. Differences in *Bd* abundance or *Bd* load can lead to detection differences in occupancy studies (Chapter 3, Royle and Nichols 2003). Laboratory experiments have shown that *Bd* growth is strongly influenced by temperature (Piotrowski et al. 2004) so I hypothesized that different temperature regimes across elevations or across the amphibian breeding season (*DOY*) would influence *Bd* detection probability using either sampling method. In addition, I expected that the total volume of water filtered would be positively related to *Bd* detection using filters, though this has not been supported by previous work (Chestnut et al. 2014). Susceptibility to chytridiomycosis is variable among amphibian populations and may be linked to differences in skin microbiome (Woodhams et al. 2007) or life history characteristics (Reeder et al. 2012, Venesky et al. 2014a). I hypothesized that swabs collected from boreal toads would have higher *Bd* detection probabilities than swabs collected from other amphibian species, reflecting a higher expected prevalence, and potentially, higher infection loads for toads. Accordingly, I evaluated effects of several covariates (elevation [*elev* and $elev^2$], day-of-year [*DOY* and DOY^2], filter volume [*volume*], and amphibian species swabbed [*sp*]) on detection parameters and hypothesized that *Bd* detection probabilities would be higher for swabs than filters at wetlands with *Bd.*

Modeling Framework

I fit the occupancy models in Program MARK (White and Burnham 1999) and compared models using Akaike's Information Criterion corrected for small sample sizes (AICc; Burnham and Anderson 2002). I employed a step-down modeling strategy (Lebreton et al. 1992) to identify best-supported hypotheses for each parameter. First, using the most general covariate structure that the data could support for *Bd* occurrence [$\psi($ *elev* + $elev^2$ + *amph* + *group* +

*group*TST + TST*amph*)] and a time-specific structure for *Bd* detection probability using swabs [$p_{swab}(t)$], I evaluated support for my competing hypotheses about variation in p_{filter}, which I expected to be both lower and less variable than p_{swab}. I evaluated 14 model structures for p_{filter}: *constant, elev, elev*^{2}, *DOY, DOY*^{2}, *volume,* and additive combinations of these variables. Retaining the most parsimonious structure for p_{filter}, I next fit a set of 14 covariate models to p_{swab}. These models were identical to those fit for p_{filter}, except that the amphibian species swabbed (*sp*) was assessed instead of the filter volume (*vol*). The most parsimonious structure for p_{swab} was retained for my investigation of factors influencing *Bd* occurrence (ψ).

When evaluating *Bd* occurrence, I used a common hierarchical modeling framework (*sensu* Doherty et al. 2002, Dugger et al. 2016) rather than running all possible combinations of models to reduce the possibility of spurious results (Doherty et al. 2012). I began with the general covariate structure for ψ and first removed interaction terms (*TST*amph* and *group*TST*), followed by main effects (*group, TST*, *amph, elev*^{2}, and *elev*), retaining the model with the lowest AICc at each step. I completed the entire model building and selection procedure for two datasets: one that used the liberal criterion for determining whether a sample was positive, and another that used the conservative criterion.

RESULTS

A total of 307 filters were collected from 103 sites and amphibian swab samples (n=296) were collected from 61 of the 103 sites. Of the swab samples collected, 253 were from boreal toads and 43 were from other amphibian species (*Ambystoma tigrinum* [4 samples], *Pseudacris maculata* [26 samples], and *Lithobates sylvaticus* [13 samples]). Using the conservative decision criterion instead of the liberal criterion resulted in 6 fewer sites with *Bd* detections from filter samples and 19 fewer sites with *Bd* detections using swabs. Naïve *Bd* occurrence ($\psi_{naïve} =$

$\frac{\#\ sites\ with\ \text{Bd}\ detections}{\#\ sites\ sampled}$) when detections from both sampling methods were combined decreased from 0.50 to 0.31 when the conservative decision criterion was used.

Detection Probability: Variable Selection and Estimates

The best-supported structures for p_{filter} and p_{swab} differed based on the decision criterion used (for details, see Chapter 4 Appendices). Using the liberal criterion, the best-supported structure for p_{filter} was a constant detection probability ($\hat{p}_{filter} = 0.12$; Figure 4.1A), while p_{filter} varied positively with *DOY* when the conservative criterion was used (from 0.05 in the early season to 0.32 in the late season; Figure 4.1A). Detection probability of *Bd* using swabs (also termed prevalence) was best modeled as a quadratic function of *DOY* using the liberal decision criterion, and ranged from as high as 0.57 in the middle of the breeding season to as low as 0.34 in the late breeding season (Figure 4.1B). Using the conservative criterion, p_{swab} was lower than with the liberal criterion, varied as an additive function of *DOY* and $elev^2$, and was highest in the early season and at intermediate elevations (Figure 4.1B). These best-supported detection structures were used to model *Bd* occurrence.

Bd Occurrence

I found support for the hypothesis that *Bd* occurrence was negatively related to *TST* regardless of the decision criterion used (weight > 0. 45 for each criterion; Table 4.1), but substantial uncertainty was associated with these estimates (Figure 4.1C). While the choice of decision criterion did not influence the model ranking or the direction of the effect (Table 4.1), estimates of *Bd* occurrence were much higher using the liberal decision criterion, especially when boreal toads had been detected within the past 5 years (Figure 4.1C). An effect of group was not supported (Table 4.1), indicating that *Bd* occupancy at wetlands where boreal toads had never been detected (Group 2) was similar to occupancy at historic boreal toad breeding sites

currently supporting toads ($\hat{\psi}_{Group2,Liberal} = 0.84$ and $\hat{\psi}_{Group2,Conservative} = 0.56$). Regardless of the decision criterion used, the second best-supported model assumed a constant probability of *Bd* occurrence (weight = 0.30 [liberal] and 0.27 [conservative]) and, together, the top two models received >75% of the total weight (Table 4.1). Other hypotheses including effects of elevation, the presence of other amphibians, and group differences were not well-supported; while elevation appeared to be somewhat supported, it was identified as a pretending variable because it differed from the top model by a single parameter and had a ΔAICc value of ~2 (Arnold 2010).

DISCUSSION

The ability of pathogens to persist in vectors or reservoirs can drastically alter outcomes of disease dynamics and predictions of host extinction risk (Godfray et al. 1999). Despite this, the survival of *Bd* and *Bsal* outside of amphibian hosts has not been explored in natural settings. Currently, a lack of understanding about the mechanisms influencing the distribution and environmental persistence of *Bd* and *Bsal* limit our ability to successfully mitigate chytridiomycosis in nature (Garmyn et al. 2012, Garner et al. 2016, Grant et al. 2017). I investigated the environmental distribution and persistence of *Bd* in a landscape where some amphibian populations were extirpated. I found that *Bd* occurrence slowly decreased after local target amphibian populations declined, regardless of whether other amphibian hosts were present. My work provides evidence of long-term *Bd* persistence and underscores the importance of learning more about this pathogen's distribution outside of amphibian hosts.

Researchers evaluating samples for the presence of *Bd* use a variety of decision rules to decide whether molecular samples are positive or negative and generally do not asses the ramifications of these choices. My case study shows that, while the choice of decision criterion did not influence the relative support for covariates of *Bd* occurrence, it did influence occupancy

estimates. In addition, inference regarding factors that influence *Bd* detection and subsequent detection probability estimates were sensitive to the criterion used. I did not find evidence of contamination or false positives in my negative controls, so the liberal criterion may be most appropriate in instances like my case study. Using the conservative criterion, which is the current method employed by the Boreal Toad Conservation Team, results in underestimates of *Bd* occurrence and prevalence (p_{swab}) and evidence for temporal heterogeneity in p_{filter} that may not be accurate. Because these estimates are sometimes used to influence future study designs (i.e., the timing and number of samples to collect), this decision is especially critical. Using best practices including assessing inhibition and incorporating positive and negative controls at each step in sample preparation (Goldberg et al. 2016) will help researchers understand the risk of false positives versus false negatives and guide decisions related to sample classification. The objectives of data collection may also influence the classification process; for instance, if early detection of the emerging pathogen *Bsal* is the goal, the more sensitive liberal criterion may be preferred.

Theoretical models and laboratory experiments have suggested that *Bd* and *Bsal* may persist in reservoir host populations or in the environment (Johnson et al. 2005, Di Rosa et al. 2007, Mitchell et al. 2008, Stegen et al. 2017), but evidence from natural settings has been minimal. Previous studies that sampled *Bd* in the aquatic environment focused only on sites where amphibian populations were extant during sampling (Schmidt et al. 2013, Chestnut et al. 2014). My work is the first to take place in a landscape where local extirpations have occurred and shows that *Bd* may persist for many years after amphibian species declines. The mechanism of persistence remains unknown, but deserves future study as conservation actions may differ depending on whether persistence is due to saprobic or dormant fungal life stages (Di Rosa et al.

2007), non-amphibian hosts (Shapard et al. 2012), or recolonization by transient amphibians or other mobile organisms (Garmyn et al. 2012).

I found little evidence that elevation influenced *Bd* occurrence in my study, though other studies have shown a negative relationship between *Bd* occurrence and elevation (Chapter 2, Chestnut et al. 2014). I did find evidence of an elevational effect on swab detection probability, which likely reflects increased *Bd* prevalence or infection loads at elevations representing moderate temperatures in the SRM system. I stress that the study presented here is a snapshot of *Bd* dynamics, and that future long-term studies that explore *Bd* colonization and extinction probabilities using environmental *Bd* samples are necessary to more fully understand *Bd*'s niche, especially in the absence of amphibian hosts (Yackulic et al. 2015). My work shows that future work on *Bd* dynamics should incorporate a mosaic of sites where amphibians are both extant and extirpated. Similarly, the presence of other amphibian hosts did not influence *Bd* occurrence in my study, but I was restricted to a coarse measure of this variable. Because reservoir hosts and environmental reservoirs can both promote pathogen persistence (Dobson 2004, Briggs et al. 2010), future work should continue to explore this potentially important variable.

I found evidence of temporal heterogeneity in *Bd* detection probability using filters, but only using the conservative criterion. Estimates of filtration-based detection probability in this system were substantially lower than those reported previously (Schmidt et al. 2013, Chestnut et al. 2014), but this was expected as I was sampling at sites where amphibians may not have been present. Detection probability using filters was lower than p_{swab}, but it was similar to that reported for low concentration *Bd* samples in an experimental setting (Chapter 3) and for other low density aquatic macroorganisms sampled using water filtration (i.e., eDNA; Moyer et al. 2014, Wilcox et al. 2016). If water filtration were the sole method used to sample *Bd*, my work

suggests that approximately 20 filters would be necessary to be 95% certain of detecting *Bd* when it is present at sites in my study area. However, when amphibians are not present or if the environmental distribution of *Bd* is of interest, sampling with filtration is effective and can be used to detect multiple species of amphibians and pathogens potentially resulting in efficiency gains overall (Blooi et al. 2013). Incorporating water filtration into monitoring initiatives and manipulative field experiments provides a means to track changes in *Bd*'s persistence through time, to identify reintroduction sites, and to measure the impacts of management interventions.

Low detection probabilities of *Bd* in the late summer and early fall from swab samples may be a function of lower *Bd* loads, decreased prevalence, the characteristics or behaviors of amphibians at the sites, or combinations of these. Seasonality of *Bd* infections has been identified in many amphibian systems, and has largely been hypothesized to be related to seasonal temperature patterns (Kriger and Hero 2007, Petersen et al. 2016). Swab-based detection was sensitive to the decision criterion used; elevation was identified as an important covariate only when the conservative criterion was used and detection estimates were lower under this criterion. Underestimating the prevalence of *Bd* infections (p_{swab}) may hinder the timely response of managers to amphibian declines, and I reiterate the importance of using adequate controls and standards to understand which criterion is most appropriate in a setting. Substantially fewer amphibian swab samples than filter samples need to be collected to achieve the same level of certainty about *Bd* occurrence and the exact number will depend on the time of year and elevation that samples are collected. I suggest collecting amphibian swab samples, either from target, non-target, or sentinel animals, whenever possible. However, in cases where amphibians are not present, I illustrate the importance of collecting environmental (filter) samples to detect *Bd.*

Conclusions

Understanding pathogen distributions, both in the presence and absence of target host species, is essential for predicting, mitigating, and preventing the emergence of infectious diseases of wildlife. Current *Bd* and *Bsal* distribution models, detection databases, and eco-epidemiological models rely solely on the detection of these pathogens on known hosts, using swab samples (Olson et al. 2013, Richgels et al. 2016). Predictions based on these data do not reflect the fact that *Bd* may persist in the environment in the absence of amphibians and have a different distribution in the environment than in amphibian hosts. Pathogen detection methods that sample free-living zoospores are necessary to understand the distributions, tolerances, and ecology of *Bd* and *Bsal*, to understand risk to resident amphibian populations, and to identify conservation solutions (Grant et al. 2017). My work provides evidence of the long-term persistence of *Bd* in the absence of target amphibians and I present sampling and analytical frameworks that can be used to understand *Bd* outbreaks and for understanding and predicting the spread of *Bsal*.

Table 4.1: Model selection results for *Bd* occupancy probability (ψ) at 103 sites in the SRM using best-supported detection structures. Model selection information including number of parameters (K), ΔAICc, model weights, and deviance are shown for each candidate model for two decision criteria used to classify samples as positive: *Liberal* (≥1 of 3 qPCR replicates positive) *and Conservative Criterion* (≥2 of 3 qPCR replicates positive). Model names reflect the occupancy model structure and include linear (*elev*) and quadratic (*elev*^{2}) effects of elevation, the time since the last boreal toad detection (*TST*), whether other amphibian species were historically present at the site (*amph*), a group effect indicating whether *TST* was known or unknown (*g*), and constant models (.). All models were fit using the best-supported decision probability structures for filter (p_{filter}) and swab samples (p_{swab}).

A. Liberal Decision Criterion

Model	K	ΔAICc	Model Weight	Deviance
p_{filter}(.) p_{swab}(*DOY+DOY*^{2}) ψ(*g*TST*)	6	0.00	0.47	555.33
p_{filter}(.) p_{swab}(*DOY+DOY*^{2}) ψ(.)	5	0.91	0.30	558.49
p_{filter}(.) p_{swab}(*DOY+DOY*^{2}) ψ(*elev + g*TST*)	7	2.30	0.15	555.32
p_{filter}(.) p_{swab}(*DOY+DOY*^{2}) ψ(*elev + elev*^{2} *+ g*TST*)	8	4.56	0.05	555.23
p_{filter}(.) p_{swab}(*DOY+DOY*^{2}) ψ(*elev + elev*^{2} *+ g*TST + amph*)	9	5.98	0.02	554.25
p_{filter}(.) p_{swab}(*DOY+DOY*^{2}) ψ(*g + elev + elev*^{2} *+ g*TST + amph*)	10	9.68	0.00	555.49
p_{filter}(.) p_{swab}(*DOY+DOY*^{2}) ψ(*g + elev + elev*^{2} *+ amph*)	9	10.79	0.00	559.06
p_{filter}(.) p_{swab}(*DOY+DOY*^{2}) ψ(*g + elev + elev*^{2} *+ g*TST + amph + TST*amph*)	11	12.19	0.00	555.49

B. Conservative Decision Criterion

Model	K	ΔAICc	Model Weight	Deviance
p_{filter}(*DOY*) p_{swab}(*DOY + elev + elev*^{2}) ψ(*g*TST*)	8	0.00	0.48	376.04
p_{filter}(*DOY*) p_{swab}(*DOY + elev + elev*^{2}) ψ(.)	7	1.13	0.27	379.52
p_{filter}(*DOY*) p_{swab}(*DOY + elev + elev*^{2}) ψ(*elev + g*TST*)	9	2.40	0.14	376.04
p_{filter}(*DOY*) p_{swab}(*DOY + elev + elev*^{2}) ψ(*elev + elev*^{2} *+ g*TST*)	10	3.73	0.07	374.91
p_{filter}(*DOY*) p_{swab}(*DOY + elev + elev*^{2}) ψ(*elev + elev*^{2} *+ g*TST + amph*)	11	6.12	0.02	374.79
p_{filter}(*DOY*) p_{swab}(*DOY + elev + elev*^{2}) ψ(*g + elev + elev*^{2} *+ g*TST + amph*)	12	8.65	0.01	374.75
p_{filter}(*DOY*) p_{swab}(*DOY + elev + elev*^{2}) ψ(*g + elev + elev*^{2} *+ amph*)	11	8.98	0.01	377.65
p_{filter}(*DOY*) p_{swab}(*DOY + elev + elev*^{2}) ψ(*g+elev+elev*^{2}*+g*TST+amph+ TST*amph*)	13	11.23	0.00	374.71

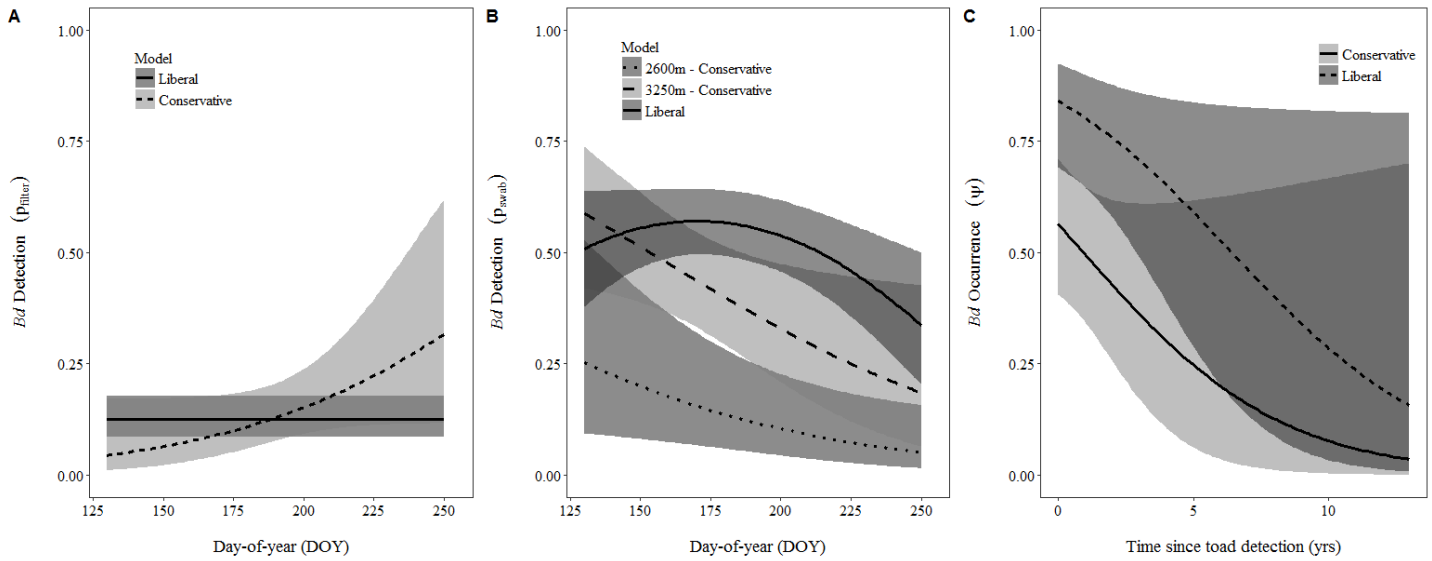


Figure 4.1: Best-supported factors influencing *Bd* detection (using filters [A] and swabs [B]) and *Bd* occurrence (C) in the SRM. Relationships are shown with 95% confidence intervals from the best-supported models using Liberal (≥1 of 3 qPCR replicates positive; solid line and dark shading) and Conservative (≥2 of 3 qPCR replicates positive; dotted or dashed lines, light shading) decision criteria. Different relationships for detection were supported depending on the decision criterion used, and the same covariate (*TST*) was supported for *Bd* occurrence.

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APPENDICES

CHAPTER 1 APPENDICES

Chapter 1, Appendices 1.1–1.3 are available at <www.ecography.org/appendix/ecog-02849>.

CHAPTER 2 APPENDIX

Appendix 2.1: Model results for the 10 best-supported detection structures for boreal toad (species A) and *Bd* (species B) detection data from 2001-2010, using a dynamic two-species occupancy model. Model names, ΔAICc, model weights, number of parameters (K), and deviance are shown for each model. Parameters include the detection probability of boreal toads when *Bd* is present (r^A) or absent (p^A), and the detection probability of *Bd* when boreal toads are present and detected (r^{BA}). I investigated detection structures where boreal toad detection probability was constant, varied linearly (*DOY*) or quadratically (DOY^2) with day-of-year. I evaluated support for models where *Bd* detection probability was constant or varied with elevation, linearly (*elev*) or quadratically ($elev^2$). All models had state-specific initial occupancy probabilities and general structures for each covariate on vital rate parameters.

Model	Δ AICc	Model Weight	K	Deviance
$p^A/r^A(DOY^2)\ r^{BA}(elev)$ w/most general elevation structure on vital rates	0	0.46	25	2175.46
$p^A/r^A(DOY^2)\ r^{BA}(elev^2)$ w/most general elevation structure on vital rates	0.53	0.35	26	2173.76
$p^A/r^A(DOY^2)\ r^{BA}(constant)$ w/most general elevation structure on vital rates	3.82	0.07	24	2181.5
$p^A/r^A(DOY^2)\ r^{BA}(elev^2)$ w/most general amphibian richness structure on vital rates	4.54	0.05	22	2186.63
$p^A/r^A(DOY^2)\ r^{BA}(elev)$ w/most general amphibian richness structure on vital rates	4.93	0.04	21	2189.21
$p^A/r^A(DOY^2)\ r^{BA}(elev^2)$ w/most general amphibian richness structure on vital rates	7.19	0.01	22	2189.27
$p^A/r^A(DOY^2)\ r^{BA}(elev)$ w/most general amphibian richness structure on vital rates	7.91	0.01	21	2192.19
$p^A/r^A(DOY^2)\ r^{BA}(elev^2)$ w/most general genetic barriers structure on vital rates	8.95	0.01	30	2173.17
$p^A/r^A\ (DOY^2)\ r^{BA}(elev)$ w/most general genetic barriers structure on vital rates	9.29	0.00	29	2175.77
$p^A/r^A\ (DOY^2)\ r^{BA}(constant)$ w/most general amphibian richness structure on vital rates	14.14	0.00	20	2200.6

CHAPTER 3 APPENDICES

Appendix 3.1: Model selection results for pre-purification (A) and post-purification (B) models applied to *Bd* detection-nondetection data for each inoculated jar (single sample scenario). I examined additive (+) and interactive (*) effects of water type (WT) and concentration (treated as a categorical, Conc.f [factor], or continuous, Conc.c [continuous], variable) for both detection (p) and occupancy (ψ) parameters. Number of parameters (K), Akaike's Information Criterion adjusted for small sample sizes (AICc), ΔAICc, model weight, and deviance are given for each model.

A. Pre-Purification Single Sample Occupancy

Model	K	AICc	ΔAICc	Model Weight	Deviance
ψ(WT*conc.f) p(WT*conc.f)	16	685.82	0.00	0.48	651.81
ψ(WT+conc.f) p(WT*conc.f)	13	686.67	0.85	0.31	659.34
ψ(WT) p(WT*conc.f)	10	687.53	1.71	0.20	666.73

B. Post-Purification Single Sample Occupancy

Model	K	AICc	ΔAICc	Model Weight	Deviance
ψ(WT+conc.f) p(WT+conc.f)	10	644.59	0.00	0.85	623.80
ψ(WT+conc.f) p(WT*conc.f)	13	649.52	4.93	0.07	622.19
ψ(WT*conc.f) p(WT+conc.f)	13	649.66	5.06	0.07	622.33

Appendix 3.2: Model selection results for multi-scale occupancy models fit to pre-purification (A) and post-purification (B) *Bd* detection-nondetection data using the multiple samples scenario. Model parameters include *Bd* occupancy of the sample unit (ψ), *Bd* filter-level occurrence (θ), and detection probability (p). I examined additive (+) and interactive (*) effects of concentration (Conc.f [factor] and Conc.c [continuous]) and water type (WT) in both detection, occupancy, and local availability parameters. Number of parameters (K), Akaike's Information Criterion corrected for small sample size (AICc), ΔAICc, model weights, and deviance are given for each model.

A. Pre-Purification Multiple Sample Occupancy

Model	K	AICc	ΔAICc	Model Weight	Deviance
ψ(constant) θ(WT) p(WT*conc.f)	11	691.87	0.00	0.37	666.73
ψ(constant) θ(WT+conc.f) p(WT*conc.f)	14	692.53	0.65	0.27	659.34
ψ(constant) θ(WT*conc.f) p(WT*conc.f)	17	693.65	1.78	0.15	651.81
ψ(WT) θ(WT) p(WT*conc.f)	12	694.49	2.62	0.10	666.73
ψ(WT) θ(WT+conc.f) p(WT*conc.f)	15	695.34	3.47	0.06	659.34

B. Post-Purification Multiple Sample Occupancy

Model	K	AICc	ΔAICc	Model Weight	Deviance
ψ(constant) θ(WT+conc.f) p(WT+conc.f)	11.00	648.94	0.00	0.72	623.80
ψ(WT) θ(WT+conc.f) p(WT+conc.f)	12.00	651.56	2.62	0.19	623.80

Appendix 3.3: Spearman's rank-order correlation coefficients (r_s) for the relationship between qPCR copy number and experimental *Bd* concentration in distilled and natural water samples. Correlation was calculated under the single and multiple samples scenarios, both with and without DNA purification.

	Pre-purification	
	Distilled	Natural
Single Sample	0.69	0.13
Multiple Samples	0.85	0.31
	Post-purification	
	Distilled	Natural
Single Sample	0.72	0.79
Multiple Samples	0.85	0.90

Appendix 3.4: Model selection results for the best-supported linear regression models applied to data on relative bias in estimates of *Bd* concentration from my experiment. I examined additive (+) and interactive (*) effects of inoculated concentration (Conc.f [factor] and Conc.c [continuous]), water type (WT). Number of parameters (K), Akaike's Information Criterion for small sample sizes (AICc), ΔAICc, Akaike weights, and deviance are given for each model.

A. Pre-Purification Multiple Samples Relative Bias

Model	K	AICc	ΔAICc	Model Weight	Deviance
Constant	2	212.83	0.00	0.38	208.70
WT	3	213.37	0.54	0.29	207.11
Conc.f	5	214.81	1.98	0.14	204.15
WT+Conc.f	6	215.42	2.59	0.10	202.48
WT*Conc.f	9	215.69	2.86	0.09	195.60

B. Post-Purification Multiple Samples Relative Bias

Model	K	AICc	ΔAICc	Model Weight	Deviance
WT*Conc.f	9	598.74	0.00	1.00	578.65
WT+Conc.f	6	620.60	21.87	0.00	607.66
WT	3	640.72	41.99	0.00	634.46
Conc.f	5	645.36	46.62	0.00	634.70
Constant	2	659.71	60.97	0.00	655.58

CHAPTER 4 APPENDICES

Introduction

The best-supported structures for detection differed depending on the decision criterion used. Using the liberal criterion, a constant probability of p_{filter} was best-supported (weight = 0.31, Appendix 4.1A). When the conservative criterion was used, there was substantial uncertainty about the best-supported structure; a positive relationship between p_{filter} and *DOY* was supported (weight = 0.25, Appendix 4.1B). I retained a constant detection structure for p_{filter} for the liberal criterion, and *DOY* for the conservative criterion.

Using the best-supported structures for p_{filter} and a general model structure for *Bd* occurrence, I found evidence of a relationship between p_{swab} and *DOY* with both decision rules, though the form of the relationship differed and elevation was also important using the conservative criterion (Appendix 4.2). Using the liberal criterion, a quadratic effect of *DOY* was well-supported (weight = 0.31) and was retained for the next modeling step (Appendix 4.2A). Using the conservative criterion an additive effect of *DOY* and elevation was supported (weight = 0.27) such that detection was highest early in the season and at intermediate elevations (Appendix 4.2B). I retained these best-supported structures to model *Bd* occurrence.

Appendix 4.1: Model selection results for competing hypotheses about factors influencing *Bd* detection probabilities using water filtration (p_{filter}). Other model parameters include general structures for swab detection ($p_{swab}(t)$) and Bd occurrence (ψ). Given information includes: number of parameters (K), ΔAICc, model weights, and deviance. Results are shown for two decision criteria used to classify samples as positive: *Liberal* and *Conservative*. Models include effects of filter volume (*vol*), linear (*elev*) and quadratic (*elev*^{2}) effects of elevation, linear (*DOY*) and quadratic (*DOY*^{2}) effects of day-of-year, and constant models (.).

A. Liberal Decision Criterion - p_{filter}

Model	K	ΔAICc	Model Weight	Deviance
$p_{filter}(.)\ p_{swab}(t)\ \psi$(general)	20	0.00	0.31	550.09
$p_{filter}(vol)\ p_{swab}(t)\ \psi$(general)	21	0.87	0.20	547.80
$p_{filter}(DOY)\ \ p_{swab}(t)\ \psi$(general)	21	1.19	0.17	548.11
$p_{filter}(vol + DOY)\ p_{swab}(t)\ \psi$(general)	22	2.76	0.08	546.44
$p_{filter}(elev)\ p_{swab}(t)\ \psi$(general)	21	3.09	0.07	550.01
$p_{filter}(vol+elev)\ p_{swab}(t)\ \psi$(general)	22	4.10	0.04	547.78
$p_{filter}(DOY+DOY^2)\ p_{swab}(t)\ \psi$(general)	22	4.20	0.04	547.89
$p_{filter}(DOY+elev)\ \ p_{swab}(t)\ \psi$(general)	22	4.39	0.03	548.07
$p_{filter}(vol + DOY + DOY^2)\ p_{swab}(t)\ \psi$(general)	23	5.86	0.02	546.22
$p_{filter}(elev + elev^2)\ \ p_{swab}(t)\ \psi$(general)	22	6.23	0.01	549.91
$p_{filter}(vol + elev + elev^2)\ \ p_{swab}(t)\ \psi$(general)	23	7.27	0.01	547.63
$p_{filter}(DOY + DOY^2 + elev)\ \ p_{swab}(t)\ \psi$(general)	23	7.52	0.01	547.88
$p_{filter}(DOY + elev + elev^2)\ p_{swab}(t)\ \psi$(general)	23	7.63	0.01	547.99
$p_{filter}(DOY + DOY^{2\ +}\ elev + elev^2)\ p_{swab}(t)\ \psi$(general)	24	10.83	0.00	547.77

B. Conservative Decision Criterion - p_{filter}

Model	K	ΔAICc	Model Weight	Deviance
$p_{filter}(DOY)\ \ p_{swab}(t)\ \psi$(general)	21	0.00	0.25	382.32
$p_{filter}(.)\ p_{swab}(t)\ \psi$(general)	20	0.32	0.22	385.79
$p_{filter}(DOY+elev)\ \ p_{swab}(t)\ \psi$(general)	22	1.33	0.13	380.41
$p_{filter}(elev)\ p_{swab}(t)\ \psi$(general)	21	1.65	0.11	383.97
$p_{filter}(vol + DOY)\ p_{swab}(t)\ \psi$(general)	22	3.19	0.05	382.26
$p_{filter}(vol)\ p_{swab}(t)\ \psi$(general)	21	3.20	0.05	385.52
$p_{filter}(DOY+DOY^2)\ p_{swab}(t)\ \psi$(general)	22	3.22	0.05	382.29
$p_{filter}(vol+elev)\ p_{swab}(t)\ \psi$(general)	22	4.20	0.03	383.27
$p_{filter}(DOY + DOY^2 + elev)\ \ p_{swab}(t)\ \psi$(general)	23	4.23	0.03	379.98
$p_{filter}(DOY + elev + elev^2)\ p_{swab}(t)\ \psi$(general)	23	4.52	0.03	380.27
$p_{filter}(elev + elev^2)\ \ p_{swab}(t)\ \psi$(general)	22	4.66	0.02	383.74
$p_{filter}(vol + DOY + DOY^2)\ p_{swab}(t)\ \psi$(general)	23	6.49	0.01	382.23
$p_{filter}(vol + elev + elev^2)\ \ p_{swab}(t)\ \psi$(general)	23	7.17	0.01	382.92
$p_{filter}(DOY + DOY^{2\ +}\ elev + elev^2)\ p_{swab}(t)\ \psi$(general)	24	7.53	0.01	379.87

Appendix 4.2: Model selection results for competing hypotheses for factors influencing *Bd* detection probabilities using amphibian swabs (p_{swab}). Other model parameters include a best-supported structure for filter detection (p_{filter}) and a general structure for *Bd* occurrence (ψ). Given information includes: number of parameters (K), ΔAICc, model weights, and deviance. Results are shown for two decision criteria used to classify samples as positive: *Liberal* and *Conservative*. Covariates include species swabbed (*sp*), linear (*elev*) and quadratic ($elev^2$) effects of elevation, linear (*DOY*) and quadratic (DOY^2) effects of day-of-year, and constant models (.).

A. Liberal Decision Criterion

Model	K	ΔAICc	Model Weight	Deviance
$p_{filter}(.)\ p_{swab}(DOY^2)\ \psi$(general)	11	0.00	0.31	553.18
$p_{filter}(.)\ p_{swab}(sp+DOY^2)\ \psi$(general)	12	0.30	0.26	550.91
$p_{filter}(.)\ p_{swab}(DOY^2+elev)\ \psi$(general)	12	2.15	0.10	552.76
$p_{filter}(.)\ p_{swab}(sp)\ \psi$(general)	10	2.60	0.08	558.28
$p_{filter}(.)\ p_{swab}(DOY)\ \psi$(general)	10	3.42	0.06	559.11
$p_{filter}(.)\ p_{swab}(sp+DOY)\ \psi$(general)	11	3.84	0.04	557.02
$p_{filter}(.)\ p_{swab}(.)\psi$(general)	9	3.96	0.04	562.10
$p_{filter}(.)\ p_{swab}(DOY^2+elev^2)\ \psi$(general)	13	4.77	0.03	552.76
$p_{filter}(.)\ p_{swab}(sp+elev)\ \psi$(general)	11	4.94	0.03	558.11
$p_{filter}(.)\ p_{swab}(DOY+elev)\ \psi$(general)	11	5.84	0.02	559.01
$p_{filter}(.)\ p_{swab}(elev)\ \psi$(general)	10	6.09	0.01	561.77
$p_{filter}(.)\ p_{swab}(sp+elev^2)\ \psi$(general)	12	7.31	0.01	557.92
$p_{filter}(.)\ p_{swab}(DOY+elev^2)\ \psi$(general)	12	8.38	0.00	558.99
$p_{filter}(.)\ p_{swab}(elev^2)\ \psi$(general)	11	8.54	0.00	561.72

B. Conservative Decision Criterion

Model	K	ΔAICc	Model Weight	Deviance
$p_{filter}(DOY)\ p_{swab}(DOY+elev^2)\ \psi$(general)	13	0.00	0.27	374.71
$p_{filter}(DOY)\ p_{swab}(DOY^2+elev^2)\ \psi$(general)	14	1.10	0.16	373.13
$p_{filter}(DOY)\ p_{swab}(DOY^2+elev)\ \psi$(general)	13	2.20	0.09	376.91
$p_{filter}(DOY)\ p_{swab}(DOY)\ \psi$(general)	11	2.50	0.08	382.40
$p_{filter}(DOY)\ p_{swab}(DOY+elev)\ \psi$(general)	12	2.55	0.08	379.89
$p_{filter}(DOY)\ p_{swab}(elev^2)\ \psi$(general)	12	2.66	0.07	379.99
$p_{filter}(DOY)\ p_{swab}(DOY^2)\ \psi$(general)	12	2.81	0.07	380.15
$p_{filter}(DOY)\ p_{swab}(sp+DOY)\ \psi$(general)	12	3.53	0.05	380.86
$p_{filter}(DOY)\ p_{swab}(sp+DOY+DOY^2)\ \psi$(general)	13	3.81	0.04	378.53
$p_{filter}(DOY)\ p_{swab}(elev)\ \psi$(general)	11	4.12	0.03	384.02
$p_{filter}(DOY)\ p_{swab}(sp+elev^2)\ \psi$(general)	13	5.02	0.02	379.73
$p_{filter}(DOY)\ p_{swab}(sp+elev)\ \psi$(general)	12	5.28	0.02	382.62
$p_{filter}(DOY)\ p_{swab}(sp)\ \psi$(general)	11	5.82	0.01	385.72
$p_{filter}(DOY)\ p_{swab}(.)\psi$(general)	10	9.13	0.00	391.54