THE NORTH AMERICAN BULLFROG (*LITHOBATES CATESBEIANUS):*
DISPERSAL AND DISEASE RESERVOIR POTENTIAL OF A PROBLEMATIC INVASIVE
SPECIES IN THE COLORADO FRONT RANGE


By


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The North American Bullfrog (*Lithobates catesbeianus*): Dispersal and Disease Reservoir

Potential of a Problematic Invasive Species in the Colorado Front Range

Thesis directed by Assistant Professor Valerie J. McKenzie

**THESIS ABSTRACT**

The anthropogenic introduction of species into new habitats is one of the leading factors driving declines in plant and animal populations across the globe. This is especially true of amphibians, as this group is currently considered the most threatened class of vertebrates on Earth. The North American bullfrog (*Lithobates catesbeianus*) is one such introduced species that has had devastating impacts on native amphibian communities in regions where it has been introduced. For my master's thesis research, I synthesized information from field surveys, laboratory experiments, and geographic information to investigate the problematic North American bullfrog, *L. catesbeianus,* in the Colorado Front Range.

Bullfrogs are considered an invasive species in the Colorado Front Range, and have been implicated in the decline of native amphibian species in this region. In my first chapter, I identified wetland-specific and landscape-level features that relate to the detection of bullfrog populations, and elucidated potential routes this species may use when moving across the Front Range landscape. In my second chapter, I clarified the role that bullfrogs may play in influencing the dynamics of the amphibian pathogen *Batrachochytrium dendrobatidis* (Bd) in this region. I developed specific criteria to determine if bullfrogs are more likely to act as a reservoir for Bd

relative to other native amphibian hosts. By pairing field surveys with laboratory experiments, I determined that bullfrog populations fulfill these criteria and appear to be a reservoir for Bd in the Colorado Front Range. However, I also identified other native amphibian species that may be important reservoirs of Bd as well. By using data collected at multiple scales, this study also provides a unique insight into potential mechanisms that may be driving the patterns of Bd prevalence across differing amphibian communities.

This research may facilitate the development of plans targeted at limiting North American bullfrog populations, and may help to inform management of the pathogen Bd in regions where this pathogen overlaps with bullfrog populations. Application of the results of this study can help to limit the spread of invasive species and disease, and may help mitigate the impact of these two important drivers of biodiversity loss.

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**CHAPTER ONE**

Investigating the dispersal routes used by an invasive amphibian, *Lithobates catesbeianus*, in human-dominated landscapes

Abstract

Clarifying how species move across and utilize human-modified landscapes is key to the conservation of declining populations, as well as to the management and control of invasive species. The North American bullfrog (*Lithobates catesbeianus*) is a globally distributed invasive amphibian that has been implicated in the decline of native amphibians across its invasive range and may also act as a transport vector for a number of deadly amphibian pathogens. Identifying the landscape-level features that facilitate or hinder this species as it moves across an ever-changing landscape is necessary to inform control efforts and limit this species' impact on already declining amphibian populations. We conducted surveys of 243 wetlands across the Colorado Front Range and used an information-theoretic approach to evaluate the contribution of wetland-specific characteristics and landscape-level factors in determining the detection of bullfrog populations and breeding bullfrog populations. Specifically, our goal was to determine whether features related to overland dispersal or to the connectivity of wetlands were better predictors of bullfrog occurrence. Our results indicated that landscape-level factors that may either hinder or facilitate overland movement, such as topographic complexity and the density of wetlands, were the best predictors of bullfrog occurrence at the scale of our analysis, rather than characteristics relating to the connectivity of wetlands to lotic waterway systems. We suggest that when considering the control or eradication of this species, efforts should be directed at

reducing hydroperiod of wetlands and should target regions with a high density of wetlands and/or low topographic relief.

Introduction

Anthropogenic activity can have differential impacts on species. While many species respond negatively to anthropogenic influence, others have increased in range as a result of human activities (McKinney and Lockwood 1999). Humans can facilitate this range expansion through at least two major processes: by directly introducing invasive species into new systems, and by modifying the habitat in such a way that favors invasion (McKinney and Lockwood 1999, Rahel 2002). While often relatively benign, invasive species can occasionally have catastrophic negative impacts on native species, and thus are considered one of the leading factors contributing to biodiversity loss globally (McKinney and Lockwood 1999, Sala 2000, Rahel 2002). Managing invasive populations is often of concern when considering the conservation of declining populations, emphasizing the importance of understanding how invasive species spread across the landscape to inform eradication efforts or to reduce their spread into new habitats.

Aquatic communities are among the most threatened and highly invaded systems on the planet (Dudgeon et al. 2006, Sala et al. 2000), and large-scale habitat alteration can further facilitate invasion into these systems (e.g. Havel et al. 2005, Johnson et al. 2008, Rahel 2002, 2007). Landscape-level human modification can increase connectivity of aquatic systems across the landscape, which is known to facilitate the dispersal and invasion of highly aquatic species such as fish (e.g. Hohausova et al. 2010, Rahel 2002, 2007). However, fragmentation of the landscape is also known to inhibit movement of more terrestrial invasive species (With 2002). In order to better understand how invasive species move across human-dominated landscapes,

studies of animal movement should attempt to capture the complexity of an animal's environment. Often times, straight-line distance measures may not capture the reality an animal faces as it moves across a landscape (Murphey et al. 2010). Including measures such as topography or barriers to dispersal may provide a more accurate picture of how species, especially those that can move either overland or via aquatic systems, disperse in human-dominated landscapes.

Amphibians present an interesting challenge with regard to studying dispersal because they can uniquely use both terrestrial and aquatic systems and are often physiologically constrained to specific habitat types. Additionally, amphibian populations often undergo patterns of localized extinction and re-colonization (Ray et al. 2002, Semlitsch 2008, Smith and Green 2005), making them especially sensitive to anthropogenic habitat alteration, which may disrupt or facilitate these processes (Ficetola and De Bernardi 2004). The North American bullfrog (*Lithobates catesbeianus,* hereafter referred to as bullfrog) is a nearly globally distributed invasive amphibian that is originally native to the Eastern United States and has been associated with declines in native amphibian populations across their invasive range (Adams 2000, Casper and Hendricks 2005). Studies examining how bullfrogs disperse between aquatic habitats remain limited, and have mostly focused on movement distances of individuals (e.g. Ingram and Raney 1943, Willis et al. 1956). Studies that address a more complex suite of landscape-level features that may promote this species' dispersal and establishment remain lacking, but are of importance given the widespread and potentially detrimental nature of invasive bullfrog populations.

In arid regions such as the western United States, two major types of aquatic habitat alteration can potentially influence the spread of bullfrogs, and include the alteration of the

hydroperiod of standing water bodies, as well as alteration to flowing water systems (Nilsson et al. 2005, Smith et al. 2002, Weiner et al. 2008) (Fig. 1). The goal of our study was to clarify which of these two types of habitat modification may be most important for facilitating the dispersal of bullfrogs. We concentrated our efforts on bullfrog populations in the Front Range region of Colorado, as this is the most densely populated region of the state, and consists of a heterogeneous patchwork of protected grasslands, urban development and agriculture (Fig. 2). Over the last 30 years, human population growth and suburbanization in the Colorado Front Range has exceeded most other parts of the US and increases of up to 51% are projected for the next 25 years (Fishman and Roberts 2001). The suburbanized landscapes have led to the creation of many artificially permanent water bodies, primarily in the form of retention ponds, ornamental community ponds, and hazard ponds on golf course properties (Hammerson 1998, Wright 1914, Wiener et al. 2008). Bullfrog tadpoles require two years to complete their development prior to metamorphosis, and thus require permanent wetlands for breeding and for developing tadpoles to over-winter (Wright 1914). The increase in number of permanent wetlands may facilitate bullfrog persistence by creating stepping-stones of hospitable habitat (Maret et al. 2006) that can aid in the dispersal of invasive species into new landscapes (Havel et al. 2005). Additionally, in Colorado, as in much of the west, flowing waterway systems have been highly modified (Nilsson et al. 2005, Wiener et al. 2008). Such modification is known to facilitate the movement of other aquatic invaders such as fish (Hohausova et al. 2010) and reduction in the variability of water flow is known to favor bullfrog presence (Fuller et al. 2011). Man-made ditches and stream diversions may facilitate bullfrog movement by increasing connectivity of wetlands with areas of hospitable habitat.

In our study, we combined wetland survey information, GIS landscape analysis, and a model selection approach to examine whether landscape features that may relate to overland dispersal of bullfrogs, waterway dispersal of bullfrogs, within-wetland site characteristics, or a combination of these factors are the strongest predictors of detection of invasive bullfrog presence and bullfrog breeding presence. We sought to include measures that would address the complexity of the landscape in the Colorado Front Range, with the goal of illuminating features that may facilitate or hinder the dispersal of bullfrogs into new landscapes. Such information can inform management and control strategies of invasive bullfrog populations, can inform niche modeling integral to predicting regions most vulnerable to bullfrog invasion, and can provide a broader understanding of how semi-aquatic invasive species move across human-modified terrestrial and aquatic habitats.

Methods

*Wetland Surveys*

To determine the distribution of bullfrog populations in wetlands across the Colorado Front Range, we combined information from wetland surveys of 243 wetlands from eight counties (Fig. 2). These wetlands occur in urban, grassland, forest, and agricultural areas and are found on both public and private lands. We haphazardly selected sites across different land use types to encompass a variety of land-use practices with varying degrees of wetland and lotic water body density, although practicality and accessibility were considered when selecting wetlands to include in this study. We sampled all wetlands during the months of May- August during the years 2007-2011. All wetlands surveyed were below 2,000 meters in elevation, as bullfrogs are known to inhabit mostly lowland habitats, and are rarely found above this elevation

(Hammerson 1998, Moyle 1973). A 3 - 4 person field crew sampled all wetlands utilizing the same sampling protocol in all years (as per Johnson et al. 2011). Upon arriving at a wetland, we conducted a visual encounter survey (VES) to establish the presence of adult and recently metamorphosed amphibians. During the VES, we walked the perimeter of each wetland and recorded the number and species of all amphibians seen or heard within 3 meters of shoreline and also noted the presence of other vertebrate activity. We conducted dip-net sweeps by pulling a 1.4-millimeter mesh size dip net rapidly through the water in a 1.5-meter line perpendicular to the shore. These sweeps were conducted every 15 meters around the circumference of the pond. We placed the contents of each sweep into a plastic tray and recorded the number and identity of all larval and adult amphibians captured. The only non-native amphibian we encountered in this study was the North American bullfrog (*Lithobates catesbeianus*), while the native amphibian species we encountered included: Western chorus frog (*Pseudacris triseriata*), Northern leopard frog (*Lithobates pipiens*), Woodhouse's toad (*Anaxyrus woodhousii*), and the tiger salamander (*Ambystoma tigrinum*). Whenever possible, we completed 2 - 4 seine net hauls, with a net of 0.8 meters x 2 meters by stretching the net between two people, and dragging it a distance of 3 - 8 meters. We recorded the number and identity of all amphibians captured in each seine net haul. After completion of sampling at each pond, we decontaminated all waders, nets and other equipment with a 10% bleach solution and sun-dried the gear in order to reduce the risk of spreading material and pathogens between wetlands.

*Hypothesis and model building*

We used an information theoretic approach (Burnham and Anderson 2002) to create and select among competing models to determine the within-wetland characteristics and landscape-level features that are most important for predicting the detection of bullfrogs at a wetland

(bullfrog occurrence), as well as the bullfrog breeding presence at a wetland (bullfrog breeding).

If we detected any bullfrogs, including adults, sub-adults, recently metamorphosed individuals or

larval stages, we considered the wetland a bullfrog occupied site. If we detected the presence of

larval bullfrogs or bullfrog egg masses at a wetland, it was considered a bullfrog-breeding site.

We created three categories of variables to determine the combination of wetland-specific

characteristics and landscape level factors important for predicting bullfrog occurrence and

bullfrog breeding across the landscape. Specifically, our three categories of variables included

the following: 1) within-wetland characteristics, which included variables that describe the

individual wetlands, 2) overland dispersal, which included variables hypothesized to either

facilitate or impede overland movement of bullfrogs, and 3) waterway connectivity, which

included variables that relate to the connectivity of a wetland with lotic systems (see Table 1 for

an outline of all models). The rationale and variables associated with each of the three categories

of variables is further elaborated upon below.

*Within-wetland characteristics*

We hypothesized that the within wetland characteristics area and hydroperiod may

influence bullfrog occupancy, as bullfrog tadpoles require permanent wetlands to complete their

lifecycle (Wright 1914), and because larger wetlands are more likely to contain amphibian

predators, such as fish, which may influence bullfrog presence (Adams et al. 2003). We used a

hand-held Garmin GPS model 60CSx (Garmin International, Olathe, Kansas) to determine the

coordinates of each wetland and to calculate surface area when sampling each wetland. We

determined the hydroperiod (categorized as either permanent or temporary) of each wetland by

pairing on the ground observations with Google Earth™ imaging. In the Front Range Region of

Colorado there are many Google Earth™ image layers available which allowed us to view

satellite images of each wetland in summer, fall and winter seasons. We categorized a wetland as temporary if it was dry during any season, or at any time during field sampling. We categorized all other wetlands as permanent wetlands.

*Overland Dispersal*

We quantified geographic characteristics within a 1-kilometer radial buffer of each wetland surveyed. While bullfrogs are known to move greater than 1-kilometer in distance (e.g. Willis et al. 1956), such long distance dispersal is not typical of most individuals, and thus a spatial extent of 1 kilometer is likely an appropriate scale when determining landscape features relevant to amphibians (Semlitsch 2008). If bullfrog dispersal is mostly via overland movement, we hypothesized that the average percentage of wetland area within a 1-km radial buffer zone may positively associate with bullfrog presence, as marshy, wet areas may facilitate the overland movement of this highly aquatic species. Reservoirs are known to act as source populations for a number of aquatic invaders (Johnson et al. 2008), and thus we hypothesized that wetlands nearer to lakes or reservoirs may more often associate with bullfrog presence. We also hypothesized that the average percentage of impervious surfaces and topographic complexity may represent barriers to amphibian overland movement (Fahrig et al. 1995, Murphy et al. 2010) and therefore associate negatively with bullfrog occupancy and breeding presence.

To calculate the variables included in our overland dispersal category, we utilized the United States Geologic Survey (USGS) National Land Cover Dataset to quantify the percentage of wetland area and the average percentage of impervious surfaces within a 1-km buffer zone of each wetland. We used the USGS National Hydrography dataset to quantify the straight-line distance of each wetland surveyed to the nearest lake (defined as any water body > 10,000 meters$^2$ in area). Topographic complexity represents the maximum elevation change within each

1-km buffer, calculated from the National Elevation Dataset (USGS). All spatial analyses were conducted in ArcGIS 10 (ESRI).

*Waterway Connectivity*

If bullfrog dispersal occurs primarily via waterways, we hypothesized that the distance of a wetland to the nearest waterway and the amount of waterway present in the 1-km buffer zone may facilitate movement of bullfrogs, and thus we included these variables as predictors in our waterway connectivity category. To calculate these variables, we used the National Hydrography Dataset (1:24,000 USGS) layer to sum the lengths of all streams, rivers, ditches and canals within the 1-km buffer zone of each wetland and to calculate the straight line distance of each wetland to the nearest waterway of any type.

*Analysis*

We transformed predictor variables with a logarithm or square root transformation, and checked all predictor variables for collinearity. None was found (r < 0.4 in all cases), so all predictor variables were included in our analyses. We used generalized linear modeling (GLM) as we were predicting a binomial response. We fitted a GLM with all predictor variables from all categories, and checked the residuals of this global model for spatial autocorrelation utilizing a Monte Carlo (random) Moran's I, in which we completed 999 random simulations and compared the Moran's I value of our global model to the null Moran's I values produced by the random simulations. The results of this analysis suggested no spatial-autocorrelation in the residuals of the global model (Moran's I statistics= -0.0042, p= 0.933), and thus we utilized non-spatial models for the remainder of our analyses.

We created three categories of variables according to our hypotheses (within-wetland characteristics, overland dispersal, waterway connectivity), and created 33 candidate models to

predict bullfrog occurrence and bullfrog breeding presence. Sixteen of those did not include interaction terms. Those models consisted of: a single global model that included all predictor variables, 6 models that consisted of all combinations of the local, overland and waterway categories, 8 single variable models, and one null intercept model (Table 1). Due to the known association of bullfrog breeding populations with permanent wetlands, we also included the select interaction terms: hydroperiod x wetland %, hydroperiod x distance to lake, hydroperiod x topographic complexity, hydroperiod x distance to waterway and hydroperiod x amount of waterway into a set of models predicting bullfrog breeding and bullfrog occupancy. Seventeen of our models included these interaction terms in a factorial design.

Before proceeding with an analysis of all wetland sites, we examined whether the variable 'distance to nearest occupied site' was an important predictor variable. 'Distance to nearest occupied site' is a straight-line measure of each site to the next nearest site that is occupied with the species in question, which can sometimes be an important predictor of amphibian occupancy (e.g. Ficetola and De Bernardi 2004, Fuller et al. 2011, Knutson et al. 1999). Our sampling efforts were not complete in all areas across the scope of the region primarily due to access restrictions, so we avoided conflating 'distance to nearest occupied site' with the spatial gaps in our surveys by analyzing the region with the best sampling coverage. Therefore, we included the variable 'distance to nearest occupied site' as an a priori predictor in every model in an analysis of only wetlands in Boulder County (n=121), where sampling was more thorough across the landscape. We used logistic regression to determine the variables, or categories of variables, that best predict bullfrog presence and bullfrog breeding presence and ranked the models according to their second order Akaike Information Criterion ($AIC_c$) with the AICcmodavg package in R (Burnham and Anderson 2002, R Development Core Team 2008).

We used the $AIC_c$ due to our small sample size in comparison to the number of parameters used in our models (Burnham and Anderson 2002).

We ranked all models according to their $AIC_c$, and the model with the lowest $AIC_c$ was considered the best-supported model relative to all other models considered in our analysis. All models that were within 2 $\Delta AIC_c$ of the best-supported model were also considered well-supported predictors of bullfrog presence or bullfrog breeding presence relative to all other models included in our analysis (Burnham and Anderson 2002) (Table 2). We transformed the continuous probabilities predicted by each model to a binary variable by categorizing all predicted probabilities <0.5 as a 0, and all predicted probabilities >0.5 as a 1 for each wetland, and then calculated the Cohen's kappa value for all of the well-supported models (Table 2) (Fielding et al. 1997). As there were a number of well-supported models for each response variable, we used multi-model averaging to calculate the model averaged coefficients, standard errors, confidence intervals and Akaike weights (which provide a measure of the relative importance of the predictor variables included in the best-supported models) using the package MuMIn in R (Table 3). Individual predictor variables that had an Akaike weight > 0.8 or model averaged confidence intervals that did not include 0 were considered well supported by our data and are included in Table 3 (Burnham and Anderson 2002).

The variable 'distance to nearest occupied site' was not considered well supported by our data, and thus we removed this variable from all models and re-ran all analyses with the full Colorado wetland dataset (n=243). All results shown are from the analyses utilizing the full wetland dataset. Additionally, none of the interaction terms were considered well supported by our data when predicting either bullfrog occurrence or bullfrog breeding, so for simplicity we

removed all interaction terms from our analyses. All results shown are from the 16 candidate

models that did not include interaction terms (see Table 1 for a summary of these 16 models).

Results

During the years of 2007-2011 we sampled 243 wetlands across 8 counties in the Front

Range Region of Colorado (Fig. 2). Of those wetlands sampled, 198 were permanent wetlands

and 45 were temporary wetlands. See Fig. 3 for a breakdown of the proportion of permanent and

temporary wetlands that supported bullfrog occupancy and bullfrog breeding populations. Across

all wetlands, 169 (~70%) had the presence of some amphibian, while 122 (~72%) of amphibian

positive wetlands (~50%  of all wetlands) included the presence of bullfrogs. Fifty-five (~45%)

of the sites where bullfrogs were present supported breeding bullfrog populations. Native

Colorado amphibians were found at 82 (~34%) of the wetlands included in this study.

*Bullfrog occurrence*

Predictor variables in all three classes of variables were included in our well-supported

models predicting bullfrog occurrence in the Colorado Front Range. Both of the well-supported

models correctly predicted bullfrog occurrence at > 60% of the wetlands included in our study,

and also correctly categorized > 20% more wetlands than a baseline null model predicting

bullfrog occurrence across our full dataset (Cohen's Kappa >0.2) (Table 2). The wetland-specific

class of variables and the overland dispersal class of variables were included in all of our best-

supported models, while the waterway connectivity class of variables was included in only one

of our best-supported models (the global model). When considering wetland-specific

characteristics, bullfrog occurrence was negatively associated with both temporary wetlands and

with wetland area. The results of model-averaging show that both hydroperiod and area are well-

supported predictors of bullfrog occurrence at scale of our study, as hydroperiod has a confidence interval that did not include 0 and a cumulative AIC weight > 0.8 while area had an AIC weight >0.8 (Table 3). When considering landscape level variables, only variables relating to the overland dispersal of bullfrogs were well-supported predictors of bullfrog occurrence at the scale of our study. Areas with higher levels of topographic complexity and with higher levels of impervious surfaces within a 1-km radial buffer zone of the wetland were negatively associated with bullfrog occurrence. However, bullfrog occurrence was positively associated with wetlands surrounded by a high density of wetland area as well as the distance to the nearest lake (identified here as any water body > 10,000 $m^2$ in area).

*Bullfrog Breeding*

The within-wetland category of variables and the overland dispersal category of variables were included in our top selected models predicting bullfrog-breeding presence in Colorado Front Range wetlands. All of the well-supported models correctly predicted >75% of bullfrog breeding wetlands; however, none of these models showed a predictive power beyond a baseline null model predicting the absence of breeding (the most frequent outcome in our dataset) at all wetlands (Cohen's Kappa = 0) (Table 2). This may be due to the relative rarity of bullfrog breeding presence in our dataset, as Cohen's Kappa is sensitive to sample size and can fail when number of successes (presence of bullfrog breeding populations, n=55) is very low in comparison to the number of trials (number of wetlands sampled, n=243) (Fielding et al. 1997). After averaging across these top selected models, only the variable hydroperiod was considered a well-supported predictor of bullfrog breeding presence, with temporary wetlands negatively associated with bullfrog breeding populations.

Discussion

Aquatic freshwater systems are under intense pressure from human activities, especially in arid regions where these resources are limited (Dudgeon et al. 2006, Sala 2000, Wiener et al. 2008). As a result, aquatic systems are especially vulnerable to invasion, and understanding how anthropogenic activities influence invasion processes beyond the initial introduction phase is key to eradicating invasive populations or limiting their spread into new habitats (Dudgeon et al. 2006, Rahel 2007).

In our study we paired extensive survey information with geographic data and used an information-theoretic approach to determine the categories of variables that most often associate with bullfrog detection and bullfrog breeding in the Colorado Front Range. We focused on categories of variables rather than individual predictor variables in order to examine the relative support for different hypotheses regarding the dispersal patterns of invasive bullfrogs. The Cohen's Kappa values from our best-supported models (see Table 2) are similar to those found in other ecological systems predicting presence or absence of species (Manel et al. 2001), indicating that we can have some confidence in extending inferences from these findings. While our best-supported models predicting bullfrog detection do offer improvement beyond a null model, the moderate values of Cohen's Kappa suggest that there may be other unconsidered variables that are also important to bullfrog detection in the Colorado Front Range.

In all, of the variables considered in our analysis, our results indicate that bullfrog populations are more often associated with features relating to overland dispersal than to features relating to the connectivity of a wetland to lotic waterway systems. Additionally, within these categories of variables, we can offer some indication of the relative importance of the different variables considered in our analysis. Below we highlight the specific within-wetland

characteristics and landscape-level features that we included in our analysis that appear to more often relate to wetlands where we identified bullfrog occurrence and breeding presence in the Colorado Front Range.

*Within-wetland characteristics*

The within-wetland characteristic hydroperiod was included in all of our top-selected models, with the permanency of a wetland a consistent predictor of both bullfrog occupancy and bullfrog breeding populations in Colorado Front Range wetlands. Hydroperiod was the only well-supported predictor of breeding populations in our study system (Table 3). While the models predicting bullfrog breeding presence do not show predictive power beyond a null model, the patchy nature of amphibian breeding (e.g. Smith and Green 2005) as well as the overall low number of breeding sites found in our survey may make predicting amphibian breeding in our system especially difficult. However, our models do suggest that the permanence of a wetland is an important factor relating to bullfrog breeding presence, which is consistent with both the natural history of this species as well as with the results of other studies (Hammerson 1999, Maret et al 2006, Boone et al. 2008, Fuller et al. 2011, Johnson et al. 2011). Unlike native Colorado anurans, bullfrogs require permanent wetlands to complete larval development (Hammerson 1999, Willis et al. 1956). Permanent wetlands are historically rare across the Colorado Front Range landscape (Hammerson 1999,Wiener et al. 2008) and our results suggest that human modification may have facilitated the invasion of bullfrogs by increasing the number of permanent wetlands that fill a critical niche characteristic of this species. Our results are consistent with other studies that have suggested that alteration of hydroperiod, namely reducing the permanency of lentic systems, is likely to be useful when considering the control of bullfrog populations (e.g. Boone et al. 2008, Fuller et al. 2011, Maret et al. 2006, Johnson et al. 2010).

Reducing wetland hydroperiod will likely facilitate eradication of breeding populations, which appear to rely on these permanent wetlands. Additionally, it appears that in the Colorado Front Range mobile adult and sub-adult populations also utilize permanent wetlands. Reducing hydroperiod of these wetlands may also reduce more transient bullfrog populations consisting of individuals that are capable of long-distance dispersal and re-colonization.

*Overland dispersal*

To shed light on potential dispersal modes used by bullfrogs, our analyses included landscape-level features hypothesized to relate to either overland dispersal or to the connectivity of wetlands to waterway systems. It is not clear how these features relate to breeding bullfrog populations, as one of these landscape-level variables were considered well-supported predictors of bullfrog breeding populations. However, when considering bullfrog occurrence, models including the overland dispersal category of variables were consistently included in our top-selected models. We found that the distance of a wetland to the nearest lake, as well at the amount of wetland area within the 1-km buffer of a wetland, were positively associated with bullfrog presence (Fig. 4). Previous accounts have suggested that inadvertent introduction during lake fish stocking operations is one potential route of introduction of bullfrogs into the Colorado Front Range (Hammerson 1999) and man-made reservoirs and lakes have been identified as important introduction points for a number of other aquatic invaders (Havel et al. 2008, Johnson et al. 1998). In our study, we found a higher probability of observing bullfrogs at wetlands that are further from lakes. This is contrary to what might be expected if these lakes have acted as introduction points for bullfrogs in the past, suggesting that if any accidental introductions of bullfrogs into these lakes did occur, it was long enough ago or so infrequent that there no longer remains any signal of introduction into these systems. However, we did identify bullfrogs more

often in wetlands that are surrounded by a high density of wetland area- which suggest that wetland area can reduce the distance that bullfrogs need to move overland between sites, and supports the hypothesis that wetland areas can act as stepping-stones, facilitating dispersal across the landscape.

In addition to straight-line distance measures, we also attempted to include features that would realistically capture the landscape-level complexity faced by an amphibian as it moves across the landscape. We calculated the topographic complexity, which is a measure of the maximum elevation change within a 1-km radial buffer of a wetland, as well as the amount of impervious surfaces within the 1-km radial buffer of a wetland. We found that both of these variables were negatively related to bullfrog occurrence at a wetland, suggesting these characteristics may act as barriers for overland movement of bullfrogs, as has been seen with other amphibian species (e.g. Fahrig 1995, Murphy et al. 2010, Johnson et al. 2010) (Fig. 4). The impervious surfaces variable included in our analysis represents a measure of mostly artificial features such as parking lots, buildings and roadways. Wetlands surrounded by high levels of such surfaces may be unreachable by most amphibian species, including bullfrogs. However, other studies (e.g. Richter and Azous 2001) have suggested that urban wetlands are highly susceptible to bullfrog colonization. It is possible that the observed negative association with bullfrogs in these areas is a historical relict, as amphibian occurrence is also known to relate to wetland age (Birx-Raybuck et al. 2009). The age of the wetlands in our study was not known, and it is logical that developed areas in the Colorado Front Range may also hold more recently constructed wetlands, which bullfrogs may not yet have reached. Additional research should be conducted before determining the degree to which wetlands in urban areas can be considered potential bullfrog habitat.

*Waterway connectivity*

We found little support for the hypothesis that connectivity to waterways is an important predictor of bullfrog presence, suggesting that bullfrogs do not often use these waterway corridors in our system as they disperse across the landscape. However, studies of other aquatic invaders have found that these waterway systems may act as important corridors of movement (e.g. Hohausova et al. 2010, Rahel 2002, 2007). In the Colorado Front Range, as in other arid regions, many of the waterways connecting wetlands are ephemeral in nature, and it is possible that waterway corridors might be more often associated with bullfrogs if they are permanent. We currently do not have information about the hydroperiod of the waterway systems we included in our analyses, but this information may be useful for elucidating if and when bullfrogs do utilize waterway corridors for movement. Further study is necessary to determine the full extent to which bullfrogs may utilize these lotic waterway systems. Additionally, we observed bullfrogs at ~57% of permanent wetlands, and breeding populations at ~26% of permanent wetlands (Fig. 3). Amphibian populations are known to have variable recruitment across years (i.e. Skelly 2003), making interpretation of these proportions difficult.  However, it is possible that this relatively high proportion of occupied permanent sites represents saturation of bullfrog populations across the landscape (i.e. bullfrog populations have established at most of the wetlands that are suitable). If this is the case, then the relationship of bullfrogs to landscape-level variables, such as waterway connectivity, may be diluted if bullfrogs are no longer dispersal limited.

*Implications and Management Suggestions*

In our study, only the landscape-level features relating to overland dispersal of bullfrogs were found to be well-supported predictors of bullfrog occurrence, suggesting that in the Colorado Front Range bullfrogs may be more likely to move via overland routes than via lotic

waterway systems. The moderate predictive power of our best-supported models (indicated by Cohen's Kappa, see Table 2) may also suggest that bullfrogs are no longer dispersal limited in our system and may be approaching saturation, as this is hypothesized to reduce the ability of landscape-level variables relating to dispersal routes to predict bullfrog occurrence. However, we did find a consistent relationship between bullfrog occurrence and characteristics relating to overland movement, emphasizing that these factors are likely important in influencing the distribution of bullfrog populations across the landscape. Studies of other aquatic invaders have highlighted the importance of waterway systems in facilitating their movement (Houhasova et al. 2010, Rahel 2002, 2007) and our results suggest that a single strategy may not be effective in controlling populations of different invasive species, even if all species are mostly aquatic. Rather, efforts aimed at managing populations of invasive animal species, especially those that can move either overland or via waterways, should consider both overland and waterway routes as potential corridors of movement.

Our study also highlights specific landscape-level features that should be considered when developing control or eradication strategies targeted at bullfrogs, or when developing niche models aimed at predicting future spread of this species into novel habitats. As has been suggested by other studies (e.g. Johnson et al. 2010, Fuller et al. 2011), we recommend that reducing hydroperiod of a wetland will likely be effective at eradicating both breeding bullfrog populations as well as limiting populations of transient adult or sub-adult populations. Specifically, efforts concentrating on reducing hydroperiod of wetlands located in areas with a high density of permanent wetlands per unit land area, or in regions with low levels of topographic relief, will likely be most effective at limiting or eradicating bullfrog populations in our system. Controlling such populations is especially important in the light of recent amphibian

declines, as amphibians are now considered the most threatened class of vertebrate on the planet, and habitat loss, invasive species, and disease are implicated as major factors contributing to these declines globally (Stuart et al. 2004). The North American bullfrog is a globally distributed invasive species that has been associated with declining amphibian populations across its invasive range (Casper and Hendricks 2000, Maret et al. 2006, Johnson et al. 2011), and this species has been implicated as a transport vector for a number of deadly amphibian pathogens (Schloegel et al. 2009, Schloegel et al. 2010). Clarifying the features that facilitate this species as it moves across an ever-changing landscape can aid in limiting the spread of this species into new regions and can limit its impact in the regions where it has already been established. Such actions may facilitate conservation of declining amphibian populations in Colorado, and can potentially facilitate management of this nearly globally distributed invasive species across its range.

**Table 1.1.** Summary of variables included in our 16 candidate models predicting bullfrog occupancy and bullfrog breeding utilizing all Colorado wetlands (n=243). These are the simple additive models that did not include any interaction terms

| Model Number | Model Type | Within Wetland Characteristics | | | Overland Dispersal | | | Waterway Connectivity | |
|---|---|---|---|---|---|---|---|---|---|
| | | Area | Hydroperiod | Wetland % | Distance nearest lake | Topographic Complexity | Impervious surfaces | Summed waterway amount | Distance to nearest waterway |
| 1 | Global | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 2 | Single Variable | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | Single Variable | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | Single Variable | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | Single Variable | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| 6 | Single Variable | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| 7 | Single Variable | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 8 | Single Variable | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| 9 | Single Variable | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| 10 | Single Variable | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| 11 | Wetland | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 12 | Overland | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| 13 | Waterway | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| 14 | Wetland+ Overland | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 |
| 15 | Wetland+ Waterway | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 |
| 16 | Overland+ Waterway | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |

**Table 1.2.** Best supported models predicting bullfrog occurrence and breeding presence in Colorado Front Range wetlands (n=243)

**All Colorado Wetlands**

| Response | Model[a] | K[a] | AIC$_c$[b] | ΔAIC$_c$[b] | AIC$_c$ Wt[b] | Proportion correctly predicted[c] | Cohen's Kappa[d] |
|---|---|---|---|---|---|---|---|
| Bullfrog Occurrence | Wetland + Overland | 7 | 318.02 | 0 | 0.51 | 0.62 | 0.23 |
| | Global | 9 | 318.46 | 0.44 | 0.41 | 0.68 | 0.36 |
| Bullfrog Breeding | Hydroperiod | 2 | 250.46 | 0 | 0.43 | 0.77 | 0 |
| | Wetland Characteristics | 3 | 251.21 | 0.74 | 0.29 | 0.77 | 0 |
| | Local + Overland | 7 | 251.95 | 1.49 | 0.2 | 0.77 | 0.03 |

[a] The categories of variables included in each model, see Table 1 for full description.

[b] K is the number of parameters included in each model examined, AIC$_c$ is the second order Akaike information criterion, ΔAIC$_c$ is the difference in AIC$_c$ units between the model with the lowest AIC$_c$ value and the model examined, AIC$_c$ Wt is the second order Akaike weight.

[c] The proportion of wetlands in the dataset that are correctly categorized as either bullfrog occupied wetlands or bullfrog breeding wetlands by the model examined.

[d] Cohen's Kappa is a measure of the proportion of wetlands correctly predicted by each model beyond chance expectation.

**Table 1.3.** Model averaged coefficients, standard errors, confidence intervals and cumulative Akaike weight for variables predicting bullfrog presence and bullfrog breeding presence at all Colorado Front Range wetlands (n=243)

| | | All Colorado Wetlands | | | | |
|---|---|---|---|---|---|---|
| *Response* | *Predictor* | *Model-Averaged Coefficient* | *Adjusted SE* | *Lower CI* | *Upper CI* | *Cumulative Akaike Weight* |
| Bullfrog Occurrence | Topographic complexity [2] | -0.0092 | 0.0040 | -0.0170 | -0.0014[*] | 1.00 |
| | Hydroperiod (temporary) [1] | -1.6200 | 0.4320 | -2.4600 | -0.7710[*] | 1.00 |
| | Wetland % [2] | 0.0278 | 0.0139 | 0.0006 | 0.0551[*] | 1.00 |
| | Area[1] | -0.0245 | 0.0745 | -0.1710 | 0.1220 | 1.00 |
| | Distance to Lake [2] | 0.0002 | 0.0002 | -0.0001 | 0.0005 | 1.00 |
| | Impervious Surfaces [2] | -0.0115 | 0.0105 | -0.0322 | 0.0091 | 1.00 |
| Bullfrog Breeding | Hydroperiod (temporary) [1] | -2.1100 | 0.7520 | -3.5800 | -0.6330[*] | 1.00 |

[1] Variables included in the wetland-specific category of variables
[2] Variables included in the overland dispersal category of variables
[*] Variables with a 95% confidence interval that do not include 0

**Fig. 1**



**Figure 1.1** (a) A permanent wetland occupied by bullfrogs, the surrounding habitat is typical of much of the land-use found across the Colorado Front Range, (b) A ditch system in our study area, also typical of those found across our study region (photo credit: VJ McKenzie (a), AC Peterson (b))
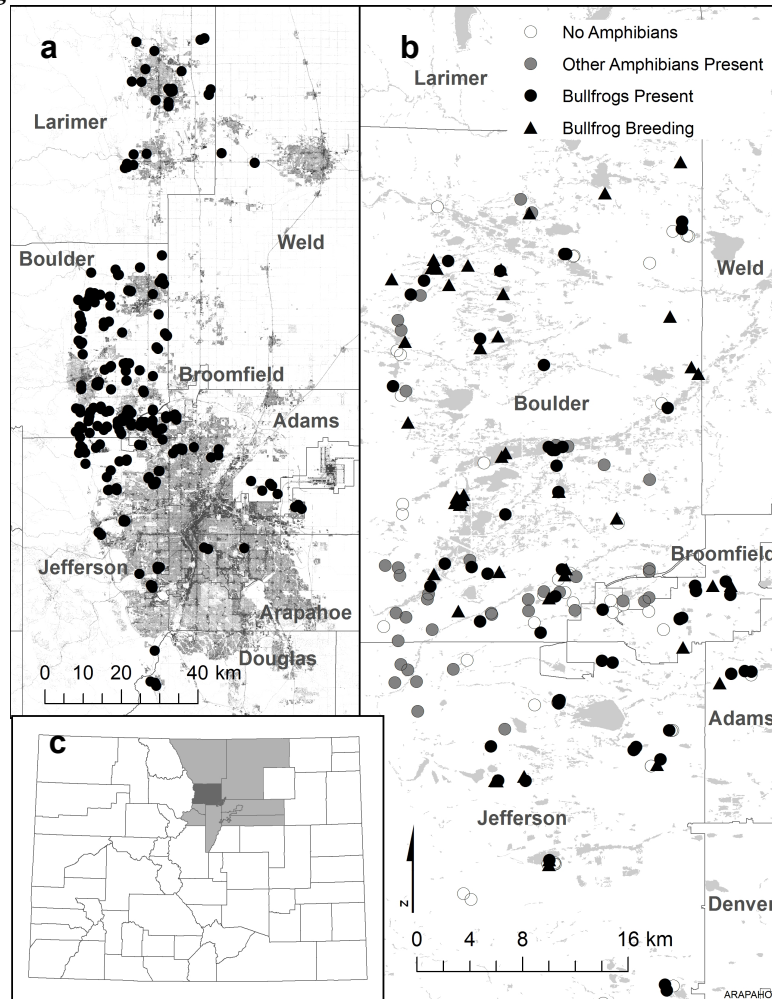
**Fig. 2**



**Figure 2.** (a) This map shows the distribution of all 243 wetlands (the black dots) surveyed from 2007-2011 in the Front Range of Colorado (light gray counties in (c) ), density of urban development is represented by the impervious surfaces layer (light – dark gray pixels). (b) This map represents sites sampled in Boulder County, CO (dark grey county in (c) ) from 2007-2011, including bullfrog breeding sites (black triangles), bullfrog present sites (black circles), other amphibians present (gray circles), and amphibians absent (white circles) with density of wetlands represented by the USGS Hydrography layer (gray polygons). (c) Inset of Colorado, showing the 8 counties sampled and represented in (a) (light grey counties) and Boulder County (dark grey county), which had the highest density of sampling and is represented in (b)

**Fig. 3**
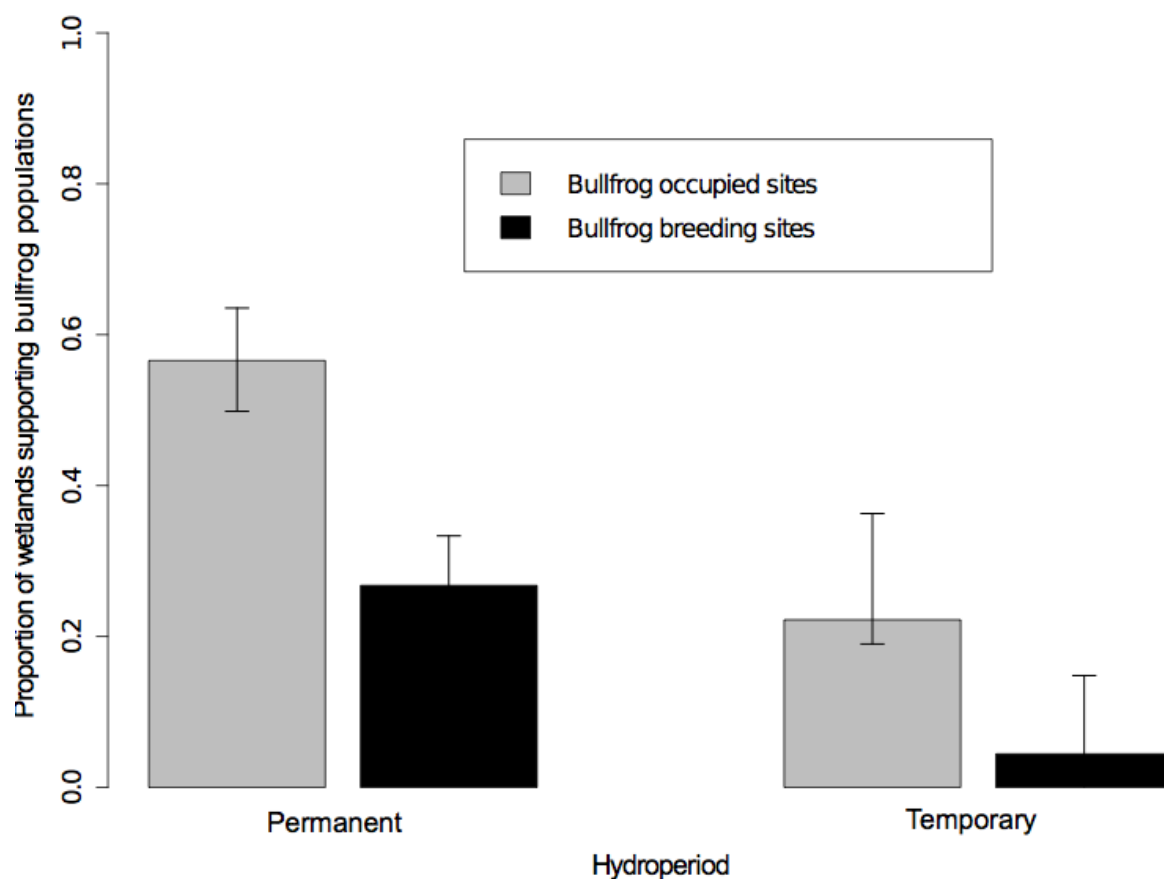


**Figure 3.** Proportion of permanent wetlands (n=198) and temporary wetlands (n=45) that support bullfrog populations and breeding bullfrog populations in the Colorado Front Range. Bullfrog occupied sites represent all sites where bullfrogs were present, while bullfrog breeding sites represent the subset of bullfrog occupied sites that supported breeding populations of bullfrogs
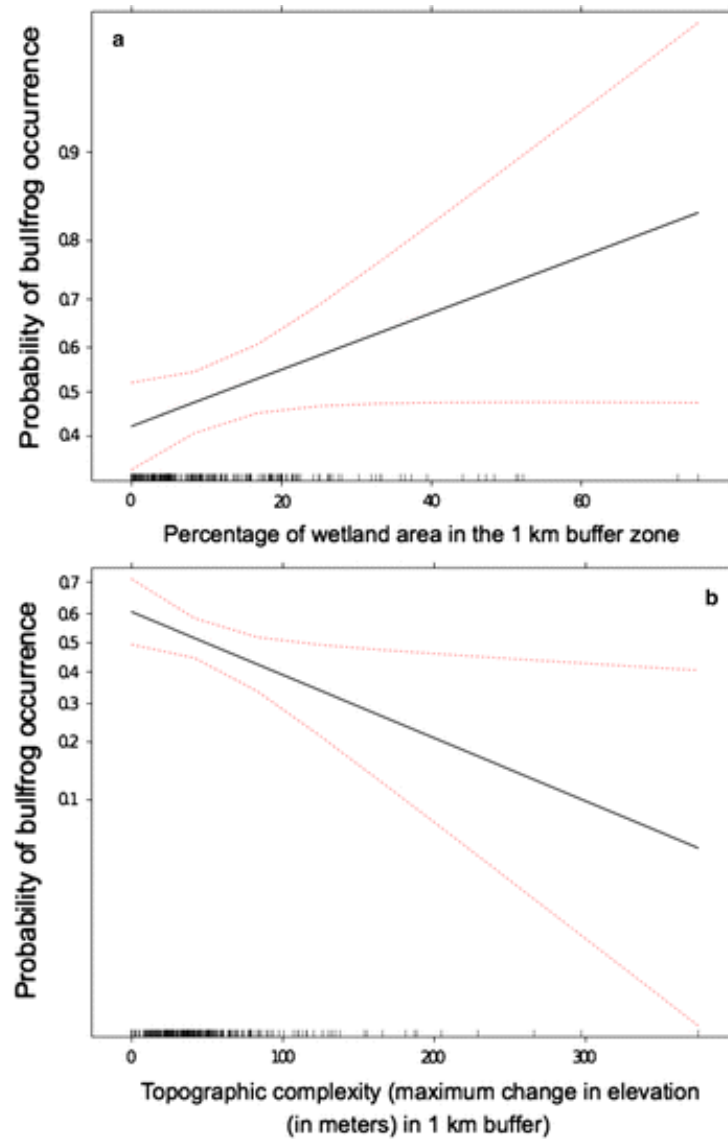
**Figure. 4** The probability of bullfrog occurrence increases with increasing amount of wetland area within the 1-km radial buffer of a wetland (a) and decreases with the level of topographic complexity (measured as the maximum change in elevation in meters in the 1 km buffer zone) (b) Fitted values (solid line) versus observed values (hash marks) obtained from the best supported model predicting bullfrog occurrence (wetland characteristics + overland dispersal) in (Table 1). Dashed lines represent 95% confidence intervals

**CHAPTER TWO**

Rethinking reservoirs: using a multi-scale approach to examine the gradient of disease reservoir potential across suite of hosts of the pathogen *Batrachochytrium dendrobatidis,* with special attention on *Lithobates catesbeianus* populations

Abstract

The introduction of species is known to contribute to the spread of disease when their pathogens or parasites are introduced with their hosts and spill over into naïve native host populations. Once established, introduced species may further influence disease if these species act as biotic reservoirs for pathogens in the landscape. A biotic reservoir of infection is a species or population that maintains a pathogen in the environment and acts as a source of infection for other species in the landscape. Identifying biotic reservoirs is challenging, but often key to the management of pathogens that infect multiple hosts. In this study, we developed criteria for identifying reservoirs of infection in multi-host systems, with special focus on developing criteria that are useful for systems in which spatio-temporal disease outbreak information is lacking. This approach allowed us to rank numerous species to determine the relative host ability of multiple species in a community. We then used these criteria to determine if introduced populations of invasive North American bullfrogs (*Lithobates catesbeianus*) are acting as a reservoir for the deadly amphibian pathogen *Batrachochytrium dendrobatidis* (Bd) in Colorado relative to other native amphibians in our community. We conducted a broad scale Bd survey to clarify patterns of Bd infection across different amphibian communities, and paired these field observations with a laboratory experiment designed to elucidate differences in the output of the Bd infective stage among different experimentally Bd infected Colorado species. From these

data, we identified *L. catesbeianus* as the most likely reservoir for Bd in our system, though our study also highlights other native amphibian species that may be important for the maintenance of Bd in the Colorado landscape. By investigating Bd dynamics at multiple scales, we are able to elucidate potential mechanisms driving differences in infection patterns across the landscape. This study provides insight into the general role of invasive species to act as reservoirs of infection, while also providing information that may facilitate the management of *L. catesbeianus* and the pathogen Bd.

Introduction

Emerging infectious diseases are an increasing threat to the health of both human and wildlife populations (Daszak et al. 2000, Jones et al. 2008). The human mediated global transport of plants and animals has contributed to the emergence and spread of disease by facilitating the introduction of species and their pathogens into new landscapes (Daszak et al. 2000, McKenzie and Peterson 2012). The subsequent spillover of multi-host pathogens from introduced species into naïve native host populations has resulted in devastating outbreaks of disease, as was the case with well-known diseases such as Rinderpest in African ungulates and with avian malaria in Hawaiian bird populations (Anderson and May 1986, Daszak et al. 2000, Dobson and Hudson 1986, Van Riper, et al. 1986). Recently, the global trade in North American bullfrogs (*Lithobates catesbeianus*) has been implicated in facilitating the transport of the nearly globally distributed and deadly amphibian fungal pathogen *Batrachochytrium dendrobatidis* (Bd) (Schloegel et al. 2012). This pathogen is transmitted directly between individuals though a flagellated free-swimming zoospore stage which infects the keratinized skin of amphibians. In some amphibian species, infection with Bd causes the disease chytridiomycosis, which results

from the disruption of cutaneous gas exchange and, in some cases, leads to cardiac failure and ultimately death (Longcore et al. 1999, Voyles et al. 2009). Bd infects nearly all amphibians and is responsible for massive declines and extinctions of amphibian populations across the globe (Skerett et al. 2007, Stuart et al. 2004).

It is well documented that introduced hosts and their pathogens can have devastating impacts on biodiversity in naïve host populations. However, more work needs to be done to clarify how invasive species influence dynamics of multi-host pathogens once these species have become established in native host communities (Kelly et al. 2009). Theory predicts that invasive species may act as competent reservoirs for pathogens. In this paper, we define a reservoir as a species or population that maintains a pathogen and acts as a source of infection for other species (Collinge and Ray 2006). Invasive species are hypothesized to acts as more competent disease reservoirs, as similar life-history traits that make a species a good invader, such as high fecundity, or a lower investment in defenses relative to native hosts, can relate to increased reservoir potential (Cronin et al. 2010).  This phenomenon has been demonstrated in plant systems (Malmstrom et al. 2005), as well as in some animal systems, such as with Eastern Equine Encephalitis Virus, Canine Distemper, and Ross River Virus (Cleveland and Dye 1995, Kelly et al. 2000, Russell 2002) in which non-native species, often domestic animals, act as reservoirs for a pathogen. Domestic populations may have very different ecological dynamics compared to wild invasive populations, yet there remain few studies investigating how feral non-native populations may influence dynamics of multi-host pathogens in sympatric native populations. Understanding such dynamics can lend important insight into aspects of invasion ecology and may also facilitate the management of multi-host pathogens.

Identifying reservoirs of infection in complex multi-host systems is challenging but is often key to the management of disease (Ashford 1997, Haydon et al. 2002). Identifying reservoir potential of different species has been fairly well established for vector-transmitted pathogens (i.e. Lyme disease, West Nile Virus) though many criteria focus on the vector host (Kilpatrick et al. 2006, LoGuidice et al. 2003). Attempts at identifying reservoirs in complex multi-host communities have been successful for some directly transmitted pathogens such as rabies and canine distemper in Africa, but notably criteria developed by these studies require spatio-temporal outbreak data (Cleveland and Dye 1995, Lembo et al. 2008). However, for many pathogens that infect wildlife but do not infect humans or domestic animals, such as Bd, disease outbreaks may go undetected, and there are rarely data to determine when and where outbreaks have occurred (but see Lips et al. 2006, Lips et al. 2008, Vredenburg et al. 2010). Thus, as with the case of Bd, widespread species that suffer little mortality from the pathogen have often been identified as biotic reservoirs (Schloegel et al. 2010, Woodhams et al. 2008), though the extent to which these species are acting as sources of infection for other species in the landscape is still unclear. Recently, efforts have been made to more fully clarify Bd reservoirs in systems that are currently undergoing disease-driven declines (e.g. Reeder et al. 2012). Our goal is to build on these studies to develop a framework for identifying reservoirs of directly transmitted pathogens in multi-host systems while also investigating the role that established invasive *L. catesbeianus* populations play in influencing dynamics of the globally distributed Bd pathogen. This framework will be especially useful for systems in which disease outbreaks go undetected or for systems in which spatial or temporal disease outbreak data are lacking.

In order to determine the Bd reservoir potential of multiple amphibian species, we sought to establish criteria that investigate pathogen dynamics across multiple scales. Previous work has

addressed identifying potential reservoir species with regards to prevalence and duration of

infection, as well as removal of potential reservoir hosts (Ashford 1997, Cleveland and Dye

1995, Haydon et al. 2009), though these criteria also often rely on having data on location and

times of disease outbreaks (Cleveland and Dye 1995, Haydon et al. 2009). Here, we have used

elements of these definitions as well as novel synthesis to provide criteria that may be more

conducive for identifying reservoirs of pathogens that may not be closely monitored at the

landscape scale. We established four qualities of a competent reservoir: 1) a reservoir should be

prevalent across the landscape and should live sympatrically with other hosts (i.e. increasing the

opportunity for cross-host transmission); 2) a reservoir should show relatively high prevalence of

infection in the landscape as compared to other species; 3) a reservoir should produce large

amounts of the infectious stage or for an extended period of time relative to other species and

finally, 4) removal of the potential reservoir should result in decreases in incidence of the

pathogen in other hosts. We have developed a conceptual diagram from these criteria, and list

specific traits related to these criteria that relate the reservoir potential of a species (Figure 1).

In our study, we specifically focused on non-native *L. catesbeianus*, as this host species

has been identified as an important transport vector for Bd and appears to have played an

important role in facilitating the global transport and potential hybridization of Bd lineages,

resulting in the most deadly and widespread Global Panzootic Lineage (GPL) Bd strain (Fisher et

al. 2012, Schloegel et al. 2009, Schloegel et al. 2012). *L catesbeianus* are nearly globally

distributed, with a range that includes some of the most amphibian-rich regions of the world

(Garner et al. 2006, Schloegel et al. 2010, Schloegel et al. 2012). *L. catesbeianus* are also

considered invasive in the Colorado Front Range region where we focus our study (Figure 2). In

this region of Colorado, the first records of *L. catesbeianus* populations date back to the early

1940's (Hammerson 1999). *L. catesbeianus* are now well established across this region (Peterson et al. 2013) making it an ideal system to investigate the influence that long established populations of non-native species have on pathogen dynamics. Additionally, the increase and expansion of *L. catesbeianus* populations in this region have been commensurate with declines in some native amphibian populations, as is the case with Northern Leopard Frog (*Lithobates pipiens*) populations (Hammerson 1982, Hammerson 1999, Johnson et al. 2011). Evidence suggests that both *L. catesbeianus* and Bd have played important roles in these declines, especially at high elevations (Carey 1993, Hammerson 1982, Johnson et al. 2011, Muths et al. 2003). There have not been documented Bd driven declines in low-elevation populations of amphibians in this region, though field observations have been lacking for much of this region during the period of decline (Johnson et al. 2011).

For this study we paired a broad-scale survey approach with a laboratory infection experiment to investigate the role of *L. catesbeianus* as a reservoir for Bd using our biotic reservoir criteria described above. In brief, the field survey sought to determine the abundance of amphibian species across the landscape, as well as the prevalence of Bd in different amphibian communities. We concentrated on quantifying Bd infection in native amphibians found co-occurring in the same wetland with *L. catesbeianus,* as well as from allopatric populations of native amphibians and *L. catesbeianus*. This approach allowed us to examine whether *L. catesbeianus* are acting as a source of infection for native amphibian species. In our lab experiment we isolated a local strain of Bd and used this to infect locally collected amphibians to determine the relative production of the infectious zoospore stage among amphibian species. With these multi-scale data, we are able to rank both *L. catesbeianus* as well as commonly encountered amphibian species in our system according to our set criteria to determine which

species may be acting as reservoir hosts for Bd in our system. By using a multi-scale approach, we are also able to disentangle potential mechanisms by which some species in our system may act as more competent hosts for Bd, and also highlight important mechanisms that may be driving the differences in infection prevalence across varying amphibian communities. This information is especially important given the devastating nature of Bd and the widespread distribution of invasive *L. catesbeianus* populations, and may facilitate mitigating the impact of these two factors on declining amphibian communities. Additionally, this approach can be broadly applied to other directly transmitted pathogens and can facilitate the identification of biotic reservoirs across a wide variety of systems.

Methods

*Field approach*

During the period of June-August 2011, we surveyed 99 wetlands across 6 counties in the Front Range region of Colorado (Figure 2) to obtain information about the abundance of different amphibian species across the landscape. We utilized past survey information to preferentially select a subset of these 99 wetlands to collect population-level Bd data from wetlands supporting large *L. catesbeianus* populations, large native amphibian populations, or sympatric populations of native amphibians and *L. catesbeianus* in the same wetland. We collected Bd samples from all amphibians encountered at each wetland, though only Bd data from wetlands with large enough amphibian populations to obtain population-level data are presented here. We obtained Bd population data from 11 wetlands where we detected only *L. catesbeianus* populations (LICA only), 14 wetlands where we detected sympatric populations of *L. catesbeianus* and at least one other native amphibian species (co-occur), and from 11 wetlands

where we detected at least one native amphibian species but no *L. catesbeianus* populations (native only). The remaining 63 wetlands included in our survey were haphazardly selected to represent common Colorado Front Range wetlands and were located in protected open space areas, on golf courses and in agricultural areas (Figure 2).

We collected observations on abiotic as well as biotic characteristics at each wetland. To determine the hydroperiod of a wetland (categorized as either permanent or temporary) we collected qualitative data about the connectivity of a wetland to ditch or stream systems as well as the depth of the wetland. We then paired these on- the- ground field observations with Google Earth™ imagining, which allowed us to view satellite images of each wetland in summer, fall and winter seasons. We categorized a wetland as temporary if it was dry during any season, or at any time during field sampling. We categorized all other wetlands as permanent wetlands.

To collect information about the biotic factors at each wetland, we used a combination of visual encounter surveys (VES), dip net sweeps and seine net sweeps to detect the presence of amphibian species at each wetland. The VES was conducted immediately upon arrival to a wetland, and was done by walking the perimeter of each wetland and noting the species and number of any amphibians seen or heard within 3 meters of the shoreline, including larval and adult stages. During the VES, we also calculated the coordinates, elevation and wetland area of each wetland using a handheld Garmin GPS 60CSx unit. Following the VES, we completed a total of 10 dip net sweeps at regular intervals around the shoreline by pulling a 1.4 mm mesh size net in a 1.5 meter line perpendicular to shoreline (as per Johnson et al. 2011). We placed all contents of the sweep into a plastic tray and recorded the number and species of all amphibians captured, as well as the number of all fish and crayfish captured. Whenever possible, we completed 3-4 seine net sweeps by pulling a 0.8 x 2 meter seine net through the water, and

recorded the distance of each sweep as well as the number and species of all amphibians captured and the number of all fish and crayfish captured in each seine haul. Adult or recently metamorphosed amphibians captured in the seine net and dip net sweeps, and met our life stage criteria (described below), were swabbed with a sterile cotton tipped swab 25 times on the ventral surface and 5 times on each foot (Hyatt et al. 2007). We swabbed tadpoles 25 times on their mouthparts, and then released all individuals back to the wetland (Hyatt et al. 2007).

At the subset of wetlands where we collected population-level Bd data, after completing the standardized seine net and dip net sweeps, we conducted additional seine net sweeps, dip net sweeps and hand-captures, to obtain at least 25 individuals of all species encountered at each wetland (when possible) for swabbing. To ensure that all individuals included in this study originated from the wetland sampled, we aimed to collect Bd samples from only late stage tadpoles or very recently metamorphosed individuals. For individuals of the species *L. catesbeianus* and *Ambystoma tigrinum* (tiger salamander), we concentrated sampling on either late stage larvae (Gosner stage 41-46) or recently metamorphosed individuals, as these are the life-stages most feasible to capture in large numbers and Bd detection on these life stages has been shown to be reliable (Adams et al. 2010, Hyatt et al. 2007, Padgett-Flohr and Longcore 2005). For individuals of the species *Pseudacris triseriata* (Western Chorus Frog) and *Anaxyrus woodhousii* (Woodhouse's Toad) we targeted only recently metamorphosed individuals for sampling as larvae of these species have small keratinized mouthparts, limiting areas of potential infection by *Bd* (and thus potentially detectability of the pathogen) (Adams et al. 2010, Hyatt et al. 2007). We obtained population-level Bd data from *Lithobates pipiens* (Northern Leopard frog) at only one wetland, and we collected swab samples from Gosner Stage 41 tadpoles at this wetland. After collection, we placed all swabs in a cooler and then froze them immediately upon

return to the University of Colorado, Boulder. To minimize contamination, all field personnel wore Nitrile gloves when handling amphibians, and we changed gloves between the handling and swabbing of each individual. Additionally, we sanitized all seine nets, dip nets, waders and other equipment with a 5-10% bleach solution after completion of sampling at each wetland and let all equipment sun-dry between sampling efforts.

We kept all swabs collected at each wetland frozen at - 20° C until DNA extraction. We extracted DNA from all swabs using PrepMan Ultra sample preparation reagent, diluted each sample 1/10, and tested all samples for Bd in duplicate using the qPCR protocol outlined in Boyle et al. 2004. In all qPCR analyses, we considered any samples with quantitative readings below our lowest standard (1.0 DNA copy) as 0.  We used TaqMan Exogenous Internal Positive Control to verify all negative samples represented true zeros, and were not a product of inhibition of the PCR process. About 8% of samples suggested inhibition, and these samples were diluted 1/100 and run a second time with TaqMan Exogenous Internal Positive Control, which showed that our dilution was sufficient to resolve sample inhibition issues.

*Bd isolation*

We isolated a local strain of Bd for use in the laboratory portion of this experiment by collecting fifteen (40-44 Gosner stage) *L. catesbeianus* tadpoles from a Boulder county wetland previously identified as having a high prevalence of *Bd* infection. We focused on collecting individuals that appeared to have mouthpart depigmentation to increase the probability of collecting a Bd infected individual. We returned these tadpoles to the University of Colorado, Boulder and placed them in individual containers and screened each individual for the pathogen Bd by swabbing them on their mouthparts and using the DNA extraction and qPCR methods described above and in Boyle et al. 2004. We euthanized infected individuals, removed their

mouthparts and placed them onto antibiotic-containing Tryptone plates in accordance with

Longcore et al. 1999. We placed one large successful colony of Bd sporangia in liquid Tryptone

+ Gelatin Hydrosylate broth with antibiotics and passaged the culture 1 time, then transferred the

culture to Tryptone + Gelatin Hyrdosylate broth without antibiotics. The culture was passaged 2-

3 more times before infecting the experimental animals.

*Lab experiment*

In order to determine the relative zoospore output of different amphibian species over

time, we collected recently metamorphosed and Gosner stage 42-44 *L. catesbeianus*, recently

metamorphosed Western chorus frogs (*P. triseriata)*, recently metamorphosed Woodhouse's

toads (*A. woodhousii)* and Tiger salamander (*A. tigrinum)* larvae from wetlands located in

Boulder county and brought them to the laboratory at the University of Colorado, Boulder. The

amphibians were placed in individual containers and kept in a temperature-controlled room at

20°C on a 12-hour light and dark cycle. To control for differences in Bd infection that may occur

between amphibian life stages, we concentrated on collecting only recently metamorphosed

individuals. No recently metamorphosed *A. tigrinum* individuals were found, and thus we used

late stage *A. tigrinum* larvae in this experiment.

Upon return to the lab we allowed individuals 3 days to acclimate, and then screened all

individuals for Bd and weighed and measured each individual. Individuals of each species were

split into two groups, one of which was infected with a low dose of Bd (~10,000 zoospores), and

the other group was infected with a high dose of Bd (~200,000 zoospores) (Kilpatrick et al.

2010).  We infected 14 *A. tigrinum*, 11 *P. triseriata*, 11 *L. catesbeianus*, and 14 *A. woodhousii*

with a low dose by placing each individual in a container with enough Holtfreter's solution to

cover their bodies, and then added ~10,000 zoospores (as counted with a hemocytometer) from

our Boulder County Bd strain. We infected 14 *A. tigrinum*, 10 *P. triseriata*, 11 *L. catesbeianus* and 14 *A. woodhousii* with a high dose of Bd zoospores by placing each individual in a container with enough Holtfreter's solution to cover their bodies and added ~200,000 zoospores of our Boulder Bd strain. We left all amphibians in their individual infection containers for 24 hours, and then removed them and placed them in individual housing with 20-150 ml of Holtfreter's solution, depending on species. Eight high infection and 5 low infection *A. tigrinum*, 6 low infection and 10 high infection *P. triseriata*, all low infection and 10 high infection *L. catesbeianus* and all low infection and 11 high infection *A. woodhousii* survived 3 days post-infection at which time they were swabbed to determine infection status. Mortality during the first stages of the infection was likely due to difficulty of maintaining very small recently metamorphosed (or in the case of the *A. tigrinum*, currently metamorphosing individuals) in the lab, as all dead individuals were swabbed post-mortem and showed low or no Bd infection. Of the surviving individuals, 100% of the low infection and 40% of the high infection *A. tigrinum*, 67% of the low infection and 30% of the high infection *P. triseriata,* and 27% of the low infection *L. catesbeianus* tested negative for Bd 3 and 6 days post infection. All of these individuals were re-infected with the same does of Bd and were screened again 3 and 6 days post infection. All of the *L. catesbeianus* were successfully infected the second time, though only 1 *A. tigrinum* and 1 *P. triseriata* were successfully infected a second time. Any individuals that remained uninfected after a second infection attempt were removed from the study. In all, 0 low treatment and 8 high treatment *A. tigrinum*, 6 low treatment and 6 high treatment *P. triseriata*, 11 low treatment and 10 high treatment *L. catesbeianus,* 14 low treatment and 11 high treatment *A. woodhousii* were included in this study.

Beginning 3 days post infection, we removed all species from their individual housing containers and placed each individual into a small plastic container with enough Holtfreter's solution to cover their bodies (either 100 ml, 50 ml, or 15 ml, depending on species) for 15 minutes. After 15 minutes we removed individuals from their soak container and immediately filtered the Holtfreter's solution through a Millex-HA 0.45 um filter (Reeder et al. 2012), to capture Bd zoospores released into the solution over the course of the 15-minute soak. We then wrapped each filter in parafilm and froze the filter until DNA extraction. This process was repeated once every 3 days for two weeks, after which we reduced the frequency of soaks to once every 4 days for a period of 2 weeks, and then reduced the soak frequency again to once every 5 days for two weeks. Individuals were weighed and measured at least two other times during the course of the experiment, and monitored on a daily basis for symptoms of chytridiomycosis. We terminated the project 62 days post-infection, and weighed and measured each individual at the end of the experiment. In total, all individuals that survived the entire experiment were soaked 15 times over the course of the 62-day experiment. We used PrepMan Ultra sample preparation reagent to extract DNA from all of the filters, diluted each sample 1/10, and ran each sample in duplicate using real time quantitative PCR to determine the number of DNA copies present on each filter (Reeder et al. 2012, Boyle et al. 2004). As with the field-collected samples, we considered any filter sample with quantitative readings below our lowest standard (1.0 DNA copy) as 0.  We used TaqMan Exogenous Internal Positive Control to verify that all negative samples represented true zeros, and were not a product of inhibition of the PCR process. None of the laboratory collected samples showed inhibition.

*Statistical Analyses*

Field Survey

To determine if the mean prevalence or mean infection load in *L. catesbeianus* and native amphibian populations differed across the different amphibian communities from which we collected population-level Bd data, we acrsin square root transformed the prevalence of Bd in native amphibian populations and in *L. catesbeianus* populations at each wetland, and used a one-way ANOVA to determine if the mean prevalence differed across the site types (Figure 3, a). The residuals of this analysis were normally distributed, so we used pairwise t-tests to examine all pairwise comparisons. The same analysis was done to determine if the mean load of infected individuals differed significantly between infected *L. catesbeianus* and native amphibians at wetlands where these were found sympatrically and allopatrically in the same wetland. We log transformed the quantitative load (# of DNA copies) of infected individuals and ran a one-way ANOVA and pairwise t-tests (Figure 3, b).

To determine if biotic and abiotic factors known to relate to Bd predicted the prevalence of Bd at the different site types, we fit a binomial generalized linear model predicting the prevalence of Bd in all amphibian populations for which we obtained population-level Bd data. We used a binomial generalized linear model (GLM) because our response variables are in the form of a proportion of the number of positive individuals out of the number of individuals tested for Bd. We checked all predictor variables for collinearity and none was found, so each global model was fit with the following predictor variables: the presence of *A. tigrinum*, presence of *L. catesbeianus*, presence of *A. woodhousii*, presence of *P. triseriata*, the hydroperiod of the wetland (temporary or permanent), a measure of density of all amphibians at a site (described in the following paragraph) and the elevation of the wetland. We also included the select interaction term between elevation and the presence of *L. catesbeianus* because *L. catesbeianus* are known to have a relationship with elevation in our system (Hammerson 1999). The global binomial

model predicting Bd prevalence in all amphibians suggested over dispersion (variance > mean) (Zuur et al. 2009), so we re-fit the global model with a quasibinomail distribution. We used the dredge function in the MuMIn package in R to create models of all predictor variables in a factorial design, and ranked the models according to their quasi-Akaike Information Criterion with a correction for small sample size ($QAIC_c$) (R Development Core Team 2008). All models within 3 QAICc were considered well supported by our data, and we averaged across all well-supported models to determine the relative importance of each predictor variable included in the well-supported models. Variables with confidence intervals that did not include 0 or had an Akaike weight >0.7 are included in Table 2 (Burnham and Anderson 2002).

Due to the difficulty of accurately measuring density of different species of amphibians at different life stages, we combined information from three different estimates of amphibian populations size: VES counts of adult amphibians/wetland area, measurements of larval density from seine net sweeps, and measurements of metamorphosed individuals and larval stages captured in the dip net sweeps. These three measures are on different scales, and to combine them into one measure that accurately captured the density of different life stages of amphibians encountered at each wetland, we assigned each wetland a rank for each of the three different density measures. For example, the density of amphibians captured in the seine varied between wetlands by an order of magnitude of 100, so we transformed the seine density values that were observed at each wetland to a rank between 1-100. We then gave the wetland with the largest density of amphibian captured in the seine a score of 100, and gave the wetland with the lowest density of amphibians captured in the seine a score of 1. This was done for the three different measures and the scores were summed to provide a single value to capture the total density of all amphibian life stages at a wetland. We included this density measure as a predictor variable in

our GLM and also used a Wilcoxon test to determine if the mean density measure of *L. catesbeianus* differed significantly at wetlands when this species co-occurred with native amphibians compared to wetlands where this species was the only species detected at a wetland (Figure 4).

We also translated the density measures for each species into a measure of biomass in order to account for the large difference in sizes of different amphibian species encountered in our study. To calculate the biomass of each species at each wetland, we multiplied the density rank for each population of each species by a body-scaling factor, which were drawn from observations of average mass of these species from our laboratory experiment (x10 for *L. catesbeianus*, x5 for *A. tigrinum*, x1 for *P. triseriata* and x1 for *A. woodhousii*). We then used a chi-square test determine if there was a difference in biomass of the different amphibian population across all wetlands, and tested for a correlation between biomass of amphibians at a wetland and the prevalence of Bd.

Laboratory experiment

For the laboratory experiment, to determine which species output the most of the infectious zoospore stage over the 62-day time course of the experiment, we calculated the area under the curve of zoospore output over time for each individual included in our laboratory experiment. We used the Simpson's numeric integration of zoospore output over time of each individual of each species to calculate the area under the curve. We log transformed the integrated area under the curve for each individual, and used a simple one-way ANOVA to determine if the mean zoospore output over time differed significantly among the different species included in our laboratory experiment. We also calculated the mean zoospore output across all individuals within each of our two treatments (low and high infection) at each time

point (Figures 5). To account for the difference in sizes among the species we utilized in the laboratory experiment, we divided the zoospore output of each individual at each soak date by the individuals weight at that date, to provide a measure of zoospore output/gram. We then re-calculated the area under these curves using Simpson's numeric integration and used a one-way ANOVA on the log transformed integral value to determine if the zoospore output differed among species or treatments groups after controlling for differences in size among the different species. We also plotted the mean mass standardized zoospore output for each species over time in Figure 6.

Results

*Field Survey Results*

During our field survey that occurred during the months of June – August 2011 we detected *A. tigrinum* at 16 of the 99 (~16%) wetlands surveyed, *P. triseriata* at 29 of the 99 (~29%) wetlands surveyed, *A. woodhousii* at 25 of the 99 (~25%) wetlands surveyed, *L. pipiens* at 5 of the 99 (~5%) wetlands surveyed, and *L. catesbeianus* at 64 of the 99 (~64%) wetlands surveyed. Of these 99 wetlands, we collected population-level Bd prevalence data from 36 wetlands, at 11 of which we detected only *L. catesbeianus* populations, at 14 of which we detected populations of *L. catesbeianus* and at least one other native amphibian species, and at 11 of which we detected populations of only native amphibian species. See Table 1 for specific information about the number of infected sites, the number of individuals of each of the different species encountered, and the number of infected individuals at these three different site types. A one-way ANOVA on the acrsine square root transformed prevalence data showed that the mean proportion of individuals infected differed significantly across site types (p< 0.05, d.f.= 3,

F=12.631, Figure 3), and the results of the pairwise t-test comparisons showed that the mean

prevalence of Bd in *L. catesbeianus* populations was significantly higher than the mean

prevalence in native amphibian populations (*L. catesbeianus* at co-occur wetlands vs. native

amphibians at co-occur wetlands p= 0.004, *L. catesbeianus* at co-occur wetlands vs. native

amphibians alone, p=0.04, Figure 3). A one-way ANOVA on log transformed quantitative

infection load (in number of copies of DNA) also shows a significantly different mean infection

load of infected individuals across the different site types (p<0.05,d.f.=3, F=18.47, Figure 3).

The mean load of infected individuals was significantly higher in *L. catesbeianus* populations

when they were found alone as compared to the mean load of infected individuals in *L.*

*catesbeianus* populations found co-occurring with native amphibian populations and than native

amphibians alone  (p= 0.026, p=4.3e-7, Figure 3).  *L. catesbeianus* populations that were found

in the same wetland as native amphibians also had a significantly higher mean infection load

than native amphibians co-occurring with *L. catesbeianus* populations and native amphibian

populations when found alone (p= 0.002, p=0.03, Figure 3). Neither the average prevalence nor

the average infection load was significantly higher in native amphibian populations when these

species were found co-occurring in the same wetland with *L. catesbeianus* than when native

amphibian populations were found at wetlands without detected *L. catesbeianus* populations

(Figure 3).

    We found that the density of *L. catesbeianus* was significantly higher at wetland where

this species was detected alone compared to wetlands where this species was detected co-

occurring in the same wetland with native amphibian species (Wilcox test, W = 36.5, p-value =

0.02, Figure 4). The biomass of *L. catesbeianus* at the wetlands included in our study was

significantly larger in comparison to the biomass of other native amphibians at these wetlands

($\chi$= 5942.617, df = 105, p= < 2.2e-16). Finally, we also found that the total biomass of amphibians at a wetland was significantly correlated with the prevalence of Bd at a wetland (t = 2.184, df = 34, p-value = 0.03595, r=0.35).

To determine the biotic and abiotic factors that related to Bd prevalence at our wetlands, we averaged across all of the best ranked quasi-binomial GLM models (according to their $QAIC_c$ value), and found that the hydroperiod of a wetland, the presence of *A. woodhousii*, and the density of amphibians at a wetland were all supported predictors of prevalence of Bd in amphibian populations (Table 2). We found that temporary wetlands were negatively related to prevalence in all amphibian populations, as was the presence of the *A. woodhousii* at a wetland, while wetlands with a higher density of amphibian populations tended to have a higher likelihood of increased prevalence of Bd (Table 2).

*Laboratory Experiment*

There was not a significant difference in the mean zoospore output over time of individuals infected with ~10,000 zoospores or individuals infected with ~200,000 zoospores, as obtained by taking the Simpson's integral of zoospore output over time (simple one-way ANOVA, p>0.05) for any of the species included in this analysis. As such, high and low groups of each species were combined together for further analyses. *L. catesbeianus*, on average, produced significantly more zoospores over the course of the experiment than either *P. triseriata* or *A. woodhousii*, though they did not produce significantly more zoospores than *A. tigrinum* individuals over the course of the infection (one-way ANOVA p= 0.001643, F=5.756, d.f. 3,57, and pairwise t-test, Figure 4). To account for the difference in sizes among the species we utilized in the laboratory experiment, we divided the zoospore output of each individual at each soak date by each individual's mass, to provide a measure of zoospore output/gram. After

accounting for the difference in size among the species utilized in our laboratory experiment, *A. woodhousii* produced significantly more zoospores per gram during the course of the experiment than either *L. catesbeianus* or *A. tigrinum*, though not significantly more than *P. triseriata* (one-way ANOVA, p= 3.799e-05, F=9.412 on 3,57d.f., and pairwise t-test, Figure 5).

Discussion

Changes in the ecology of host populations are among the leading factors driving the emergence of wildlife epidemics (Hudson 2001) and the introduction of species is a major ecological disturbance driving the outbreak and spread of disease (Daszak et al. 2000, Wilcove et al. 1998). Introduced species are known to drive epidemics when their pathogens are also introduced into naïve native host populations (Daszak et al. 2000, McKenzie and Peterson 2012) and may have an even greater impact on disease dynamics in sympatric native communities if these introduced species also act as reservoirs for pathogens once they are established in the landscape (Kelly et al. 2009). In this study, we focused our investigation on non-native *L. catesbeianus* populations as well as native Colorado amphibian populations, to determine the relative ability of a suite of species to act as reservoir hosts for the devastating *Batrachochytrium dendrobatidis* (Bd) pathogen. The history of Bd in this region is not well understood, but there have been documented historical declines in some amphibian species in our study system, though no Bd monitoring was done during the period of these declines (Johnson et al. 2011). To determine if *L. catesbeianus* are acting as a reservoir of infection, we first developed a multi-scale framework for determining the reservoir potential of hosts in a complex multi-host system, as there is a lack of clear criteria for determining biotic reservoirs of infection for directly transmitted pathogens that are not driving clear disease outbreaks or declines in their hosts. We

then used a combination of field and laboratory studies to test a suite of native Colorado amphibians, and placed a special focus on non-native *L. catesbeianus* against our developed criteria to determine which species is the most likely a reservoir for the pathogen Bd in the environment (Figure 1, 6). By investigating Bd dynamics across multiple scales, from landscape-level patterns to individual species-level patterns, our study also provides unique insight into potential mechanisms that may be driving the patterns of Bd infection in different amphibian communities across the landscape.

*Maintenance of Bd by L. catesbeianus*

Using our established criteria (Figure 1), it appears that *L. catesbeianus* are the best amphibian reservoir for the pathogen Bd in our complex amphibian system (Figure 7). Drawing from both the field and laboratory studies, our results suggest that *L. catesbeianus* populations contribute more Bd to the landscape than any other amphibian species we encountered in our field survey. *L. catesbeianus* were the most commonly encountered amphibians species in our field study, and the prevalence of the pathogen Bd in these populations was significantly higher than in native amphibian populations (Figure 3). The average infection load of infected *L. catesbeianus* individuals was also significantly higher than the average infection load of native amphibian species (Figure 3). Additionally, in our laboratory experiment, *L. catesbeianus* individuals produced more of the infective zoospore stage over time than most of the other species included in our study (Figure 4). In all, these results suggest that *L. catesbeianus* populations are an important reservoir of Bd, and there is likely more of the Bd pathogen in the landscape due to the presence of this invasive species.

*L. catesbeianus as a source of infection*

Historical evidence suggests that *L. catesbeianus* populations have played a role in decline of *L. pipiens* populations in our system (Hammerson et al. 1986, Johnson et al. 2011) and it is possible that Bd may have mediated the displacement of *L. pipiens* by *L. catesbeianus* in the Colorado Front Range. In our study, we did not find a clear difference in the mean prevalence or mean infection load of native amphibian populations found in the same wetland as *L. catesbeianus* populations compared to native amphibian populations found allopatric with *L. catesbeianus* populations (Figure 3). Though our results do suggest there may be differences in responses among the native amphibian species we encountered. Infected *A. woodhousii* individuals (n=9) had a mean load of 1133 copies of DNA when they were found in the same wetland as *L. catesbeianus* populations, but the mean load of infected individuals found in wetlands allopatric with *L. catesbeianus* (n=2) was only 82 copies of DNA. For all other native species, the mean load and mean prevalence of Bd were nearly identical whether these populations were found in the same wetland as *L. catesbeianus* or allopatric with *L. catesbeianus* populations. However, the numbers of detected infected individuals of each species were too small to compare statistically between populations of each species living in the same wetland as *L. catesbeianus* and those allopatric with *L. catesbeianus*. For *A. woodhousii* individuals, we found that the presence of this species was a well-supported predictor of Bd prevalence, and was negatively related to Bd prevalence at the landscape scale (Table 2). This suggests that *A. woodhousii* may be incapable of inhabiting wetlands where there is a large amount of Bd present. This is further supported by our laboratory experiment, as *A. woodhousii* individuals showed clear evidence of chytridiomycosis driven mortality, and none of the individuals included in our experimental study survived longer than three weeks with Bd infection (Figure 5). Further studies should focus special attention on this species to better clarify if and when *L. catesbeianus*

are acting as a source of Bd infection for other amphibians across the landscape, with special attention to *A. woodhousii* populations.

*Mechanisms driving patterns of infection*

There are a number of life-history characteristics that may elucidate why the incidence of Bd infection in *L. catesbeianus* populations is much higher than in native amphibian populations. Relative to native Colorado amphibians, *L. catesbeianus* are more aquatic, have a much longer aquatic tadpole stage (Hammerson 1999) and also tend to occur most frequently in permanent wetlands (Peterson et al. 2013). Bd is an aquatic pathogen that cannot withstand desiccation (Longcore et al. 1999) and the pathogen may occur more frequently in permanent wetlands in our system because these sites offer more hospitable habitat to the pathogen (Table 2). The increase prevalence and load in *L. catesbeianus* compared to native amphibians may be due to the longer duration of time this species spends in the aquatic environment as compared to other Colorado amphibians, increasing the time for individuals to become infected with the pathogen before they leave a wetland.

The results of our laboratory experiment elucidate another important mechanism that may drive increased prevalence and load of Bd in *L. catesbeianus* populations: body size. While both *L. catesbeianus* and *A. tigrinum* individuals produce more of the infective Bd zoospore stage over time relative to the other species included in our study, recently metamorphosed individuals of *A. tigrinum* and *L. catesbeianus* are on average ~5 times and ~10 times larger, respectively, than similar life stages of other commonly encountered Colorado amphibians. After standardizing for this difference in size by dividing zoospore output of each individual by the individual's mass, *L. catesbeianus* produced fewer of the infective zoospore stage per gram relative to smaller species (Figure 6). This is likely due to the fact that Bd infects the keratinized

skin of individuals (Longcore et al. 1999), therefore species with larger body sizes may provide more area for the pathogen to colonize and ultimately this may allow for these host species to produce more of the infective zoospore stage.  Scaling these data to the landscape-level, we found that *L. catesbeianus* had significantly more biomass at the wetlands included in our study relative to any of the native amphibians, and the combined biomass of all species at a wetland was significantly positively correlated with the prevalence of Bd at a wetland. The larger body size of *L. catesbeianus* individuals in relation to native amphibians, combined with species-specific aquatic life-history characteristics, may explain the increased prevalence and overall reservoir potential of *L. catesbeianus* populations relative to the native amphibians found in our system.

Interestingly, in our study we found significantly lower mean Bd loads on *L. catesbeianus* individuals at wetlands where this species was sympatric with native amphibian species in the same wetland compared to wetlands where *L. catesbeianus* were the only species present (Figure 3, b). This result suggests that there may be a dilution effect occurring for *L. catesbeianus* in our system. Here we use a broad definition of a dilution effect, and refer to the condition that occurs when an increase in biodiversity (in this case measured as species richness) decreases disease risk (Keesing et al. 2006). Other studies have identified a potential dilution effect in Bd systems in a laboratory setting (Searle et al. 2011), though our study remains one of the first to demonstrate a potential dilution effect for Bd in the field. There are a number of potential mechanisms that may drive a dilution effect (Keesing et al. 2006), and one mechanism that may be mediating the decrease in Bd infection in *L. catesbeianus* populations when they are found sympatric with other species is a decrease in density of susceptible hosts when there are more hosts present. Bd is known to show density dependent characteristics in other systems

(Rachowitz and Briggs 2007), and in our study increased density was a moderately well supported predictor of increased Bd prevalence in amphibian populations. Additionally the results of our Wilcox test found that the overall density of *L. catesbeianus* populations was significantly lower in wetlands where this species was sympatric with native amphibians (Figure 4). Together, our results suggest that there may be a potential dilution of Bd in wetlands where *L. catesbeianus* are present with other hosts, and this dilution may be mediated through a decrease in density at these wetlands. However, we must caution interpretation of these results, as our sampling efforts were collected at one time point. It is important to monitor these wetlands for longer periods to determine that nature of this interaction over time. It is possible that, due to the single time-point nature of our sampling, we may have sampled wetlands that were undergoing transitions of amphibian communities, which could also result in lowered density of species and potentially only a dilution effect that will change if *L. catesbeianus* displace native amphibians at these co-occurring wetlands or vice versa.

*Other Bd biotic reservoirs in Colorado*

Based on our criteria, we are able to highlight *L. catesbeianus* as the most likely reservoir for Bd in our system, and we are also able to identify potential mechanisms driving their greater host potential and the patterns of Bd infection across the landscape. However, our results also highlight that, among the commonly encountered native Colorado amphibian species included in our study, there is a gradient of species abundance, infection prevalence, infection load, and zoospore production. Thus reservoir potential should be considered a gradient as well (Figure 1, 7). *A. tigrinum* populations also show a relatively high incidence of Bd infection across the landscape, and both A. *tigrinum* and *P. triseriata* are commonly encountered in our field study. Additionally, our lab experiment highlights intrinsic differences in host ability of the different

native amphibian species encountered in our study, as has been seen with other amphibian species (Gervasi et al. 2013). *A. tigrinum* individuals produced a similar amount of Bd zoospores as *L. catesbeianus* individuals over time, though both A. *tigrinum* and *P. triseriata* individuals showed some resistance to Bd infection. 46% of *A. tigrinum* individuals and 25% of *P. triseriata* individuals did not obtain Bd infection, even after being exposed to 2 doses of ~10,000 or ~200,000 zoospores, which was sufficient to induce infection in 100% of *L. catesbeianus* and *A. woodhousii* individuals. The potential resistance of *A. tigrinum* and *P. triseriata* to Bd infection may reduce the reservoir potential of these species in the field, though not to the extent that they should not be considered as potential reservoirs for Bd, as we still found infected individuals in the field and were able to induce infection in the laboratory. *A. tigrinum* and *P. triseriata* also frequently co-occur in the same wetland as other species, which may increase their ability to transmit infection to other species. Considering the zoospore output of these species, the prevalence of Bd in these populations, and their tendency to co-occur with other species, our results suggest that both *P. triseriata* and *A. tigrinum* may also be competent reservoirs for Bd (Figure 7), which is consistent with other studies investigating Bd dynamics in closely related species in other systems (e. g. Reeder et al. 2012, Davidson et al. 2003). This is potentially important given the limited elevation range of *L. catesbeianus* populations in Colorado. *L. catesbeianus* are rarely found in montane regions in Colorado (Hammerson 1999) though many of the documented Bd driven-declines in amphibian populations globally have occurred in high elevation amphibian populations, including in Colorado (Carey 1993, Muths et al. 2003). Both *A. tigrinum* and *P. triseriata* are found in montane regions in Colorado (Hammerson 1999, Johnson et al. 2011), and may play an important role in influencing Bd dynamics in these high elevation systems.

*Conclusions*

Identifying biotic reservoirs of infection is often key to mitigating the impact of infectious diseases in complex communities (Hudson et al. 2002), though this can be difficult for multi-host pathogens that are not driving clear outbreaks or declines of their hosts, or where monitoring of disease outbreaks is lacking. We feel our criteria may facilitate this process and can be useful when considering reservoir ability of multiple potential hosts across a broad range of species and pathogens. Additionally, by using a multi-scale approach to investigate reservoir potential of a complex suite of hosts, our study has the unique ability to highlight potential mechanisms for the differences in reservoir potential and also infection across species, populations, and ultimately the landscape.

Using our established criteria, we were able to determine that invasive *L. catesbeianus* appear to be the most likely species to act as a Bd reservoir in our Colorado Front Range system, as this species appears to contribute more Bd to the landscape than any other species. The role of *L. catesbeianus* as a source of infection for other species needs to be further investigated, as there appears to be differences in the response of different native amphibians in our system. Ideally, to fully clarify the role *L. catesbeianus* populations play in acting as a source of infection for Bd, this species should be removed from a large subset of wetlands (our final reservoir criteria) and the resulting effect on Bd dynamics in native amphibians should be monitored. This final criterion may be difficult to apply in many systems, including the system in which we have focused our study. However, even without meeting this final criterion, our study has elucidated important mechanisms that may be driving disease dynamics in our system, which may offer important insight into the management of Bd, and the role of non-native species to act as disease reservoirs in general.

The results of our study provide information that may be useful when considering the management of Bd in the landscape, which is especially important given the widespread and devastating nature of this pathogen. First, our results highlight that there may be a dilution of Bd in *L. catesbeianus* populations when they are found in the same wetland with other species. Importantly, it also appears that in our system this dilution effect of Bd is driven by a reduction in the density of hosts. This finding has important implications for management of Bd in our system, as well as many other systems where *L. catesbeianus* has become established. If the complete eradication of *L. catesbei*anus populations is not possible, manually reducing the density of *L. catesbeianus* populations, especially at wetlands where *L. catesbeianus* is the only species present, may be sufficient to reduce the overall prevalence of the pathogen Bd across the landscape. Additionally, we found in our study that reservoir potential should be considered a gradient, as many of the host species considered in our study have characteristics that may both increase and decrease their potential to act as reservoirs of infection (Figure 1, Figure 7). In our study, we found that *L. catesbeianus*, as well as the native amphibian species *A. tigrinum* and *P. triseriata* also have characteristics that may make these species good reservoirs for the pathogen Bd (Figure 7). This is especially important given that these species are found in high elevation systems in which *L. catesbeianus* are not present, and may be contributing to the Bd driven declines that have been documented in high elevation systems in Colorado (Muths et al. 2003).

Invasive species remain one of the leading threats to biodiversity globally (McKinney and Lockwood 1999), highlighting the need to investigate how such species influence a complex set of ecosystem functions, including pathogen dynamics. Broadly, our study can lend important insight into the general ability of invasive species to act as biotic reservoirs for infection in other systems. In our system, non-native *L. catesbeianus* populations appear to be the most likely

species to act as reservoirs for the pathogen Bd. Other studies investigating the role of invasive species as reservoirs have found that the same life history characteristics that make species good invaders, specifically the tendency for these species to invest in rapid growth rather than defenses, may increase the potential for these species to act as reservoirs of infection (Malmstrom et al. 2005, Cronin et al. 2010). Interestingly, *L. catesbeianus* do not follow this trend, as *L. catesbeianus* are slow growing relative to the native Colorado amphibian species we encountered in our study. A shorter phenology may explain the increased reservoir potential of species like *P. triseriata*, which have been found to be likely reservoirs for Bd in our system, as well as other systems (Reeder et al. 2012). *P. triseriata* have a very rapid growth rate and females of this species reproduce numerous times throughout a summer season (Hammerson 1999). The increased reservoir potential of *L. catesbeianus* may relate more to an evolutionary history with the pathogen Bd, as the most diverse isolates of the pathogen have been obtained from *L. catesbeianus* individuals suggesting this pathogen may have its origin in North American bullfrog populations (Fisher et al. 2009).

Infectious disease and invasive species are two of the leading factors driving biodiversity loss across the globe, and this is especially true for amphibian populations. Identifying how these anthropogenic influences drive disease dynamics is important for mitigating their impacts on global biodiversity loss. Our approach for identifying reservoirs of infection is valuable for the management of disease, and can be modified for a variety of pathogens and systems. Our study also provides valuable information that may shed light on one of the most devastating pathogens ever described by man, and may help mitigate the loss of rapidly declining amphibian populations.

**Table 2.1**. Site-level and population-level Bd prevalence from field survey of 36 wetlands. Wetlands that had only the presence of *L. catesbeianus* populations are *L. catesbeianus* pops. only; wetlands where we detected the presence of both *L. catesbeianus* and at least one other native amphibian are sympatric populations, and wetlands that supported populations of at least one native amphibian species but no *L. catesbeianus* populations are native pops. only.

| Prevalence of Bd | Communities present | | |
| --- | --- | --- | --- |
| | *L. catesbeianus* pops. only | Sympatric populations | Native pops. only |
| *Site level* | | | |
| # Wetlands sampled | 11 | 14 | 11 |
| # Wetlands positive | 11 | 10 | 7 |
| Site Level Prevalence | 100% | 71.40% | 63.60% |
| | | | |
| *L. catesbeianus* | | | |
| # L. catesbeianus sampled | 270 | 154 | - |
| # L. catesbeianus (Bd +) | 167 | 47 | - |
| L. catesbeianus prevalence | 62.50% | 30.50% | - |
| | | | |
| *A. tigrinum* | | | |
| # A. tigrinum sampled | - | 59 | 106 |
| # A. tigrinum (Bd +) | - | 5 | 29 |
| A. tigrinum prevalence | - | 8.50% | 27.40% |
| | | | |
| *A. woodhousii* | | | |
| # A. woodhousii sampled | - | 191 | 79 |
| # A. woodhousii (Bd +) | - | 9 | 2 |
| A. woodhousii prevalence | - | 4.70% | 2.50% |
| | | | |
| *P. triseriata* | | | |
| # P. triseriata sampled | - | 125 | 163 |
| # P. triseriata (Bd +) | - | 3 | 4 |
| P. triseriata prevalence | - | 2.40% | 2.50% |

**Table 2.2**. The model averaged values from the best-supported quasi-binomial models predicting site-level prevalence of Bd in amphibian populations.

| Outcome Variable | Variable | Coefficient | Unconditional SE | Lower CI | Upper CI | QAIC weight |
|---|---|---|---|---|---|---|
| Wetland level prevalence of Bd in amphibian populations | Hydroperiod (temporary) | -2.88 | 1.07 | -4.98 | -0.78 | 1 |
| | ANWO present | -2.01 | 0.72 | -3.43 | -0.60 | 1 |
| | Density | 0.03 | 0.02 | -0.02 | 0.07 | 0.75 |

**Figure 2.1**.



Reservoir Potential

Low — High

Resistant to infection
High mortality rate

Species abundant
High prevalence of Infection
Sympatric with other hosts
High pathogen output
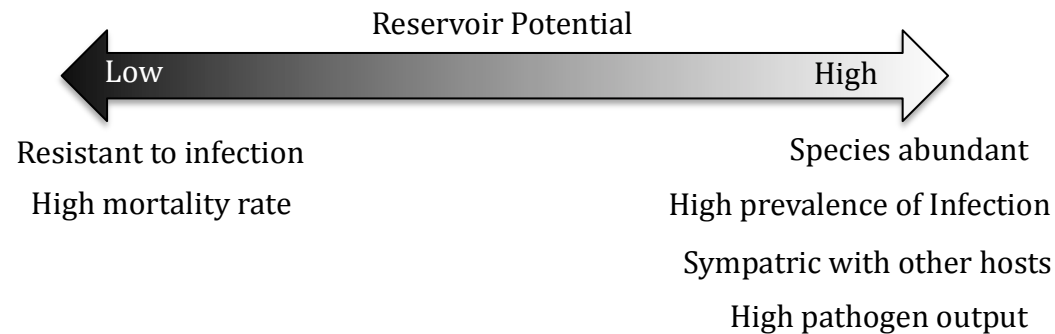
**Figure 2. 1.** Conceptual diagram of reservoir ability for a multi-host pathogen in a complex community. Certain traits are hypothesized to make a species a better or worse reservoir for a pathogen, and are based on the criteria established in this study.
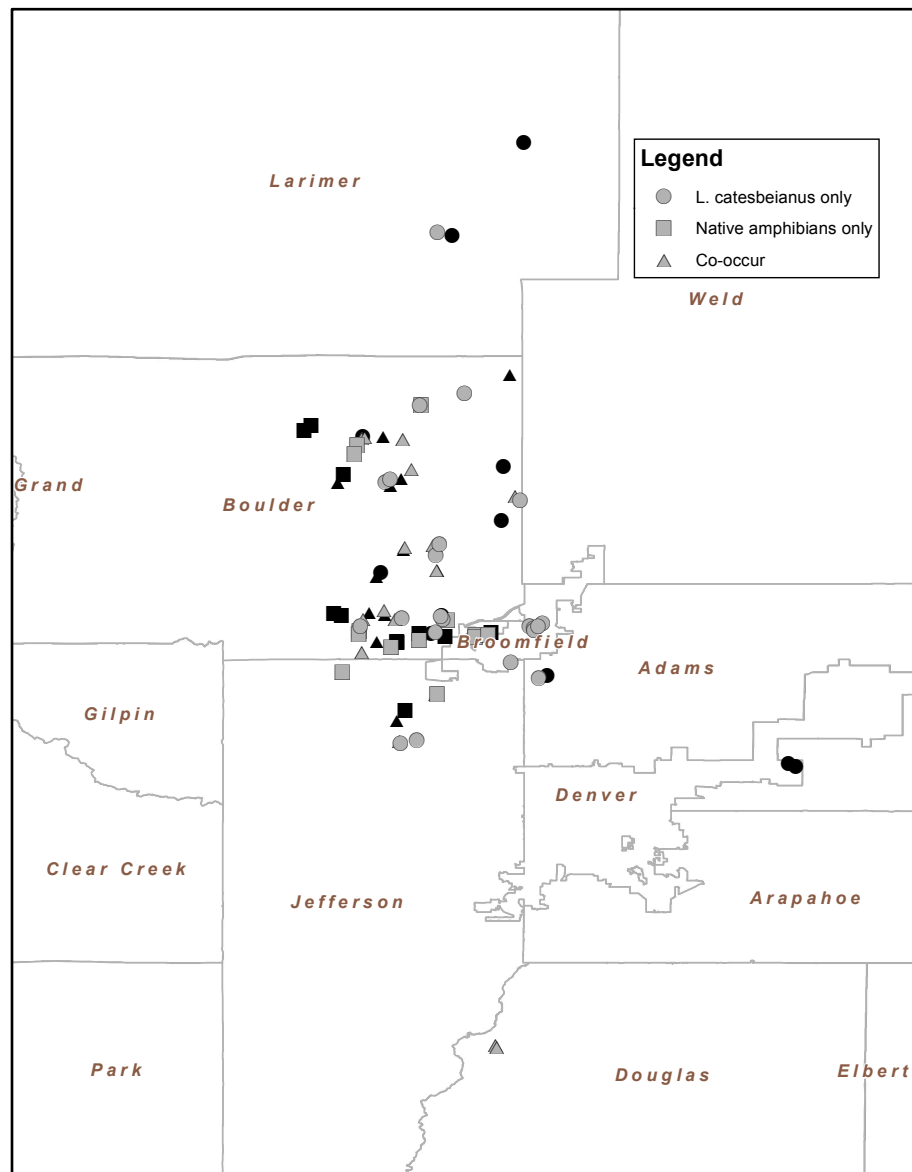
**Figure 2.2**.



**Figure 2.2** Map of all wetlands included in the survey of amphibian occurrence and Bd sampling. All 99 wetlands at which we sampled for amphibians are included above. Wetlands at which we collected population level Bd data are in black, all other wetlands are in grey.
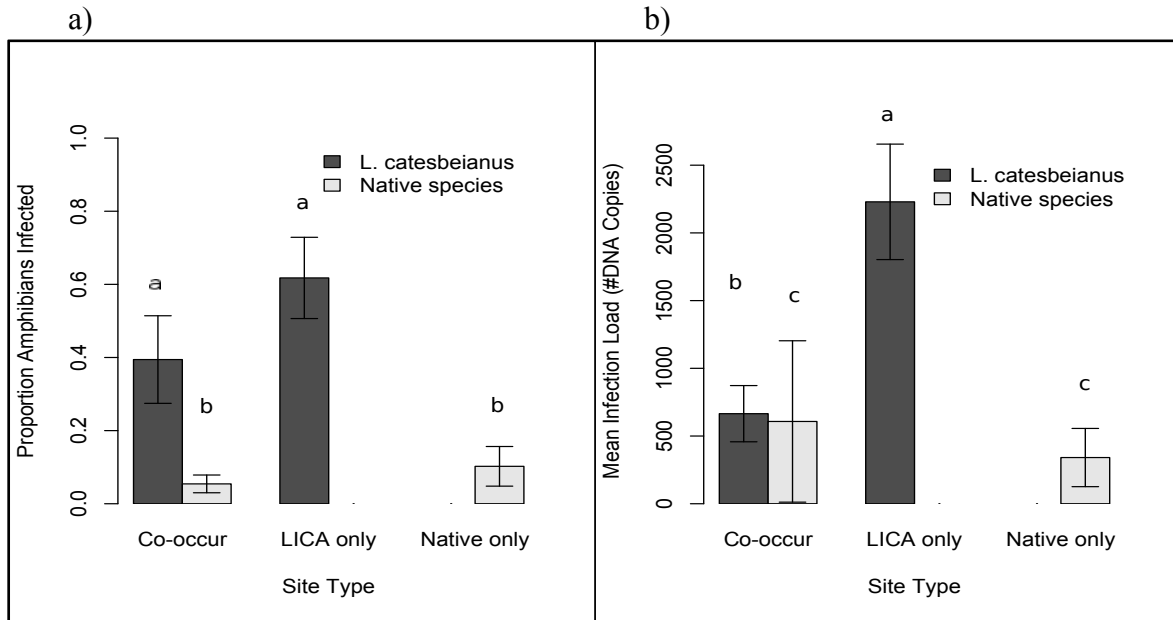
**Figure 2.3**



**Figure 2.3**. a) Average proportion of Bd infected individuals and b) average load of Bd infected native amphibians and *L. catesbeianus* in populations that were sampled for Bd. Co-occur sites are wetlands where we detected sympatric populations of *L. catesbeianus* and at least one native amphibian species, LICA only sites are wetlands where we detected only populations of *L. catesbeianus* no native amphibians, and native only sites are wetlands with the presence of at least one native amphibian species, but no detected *L. catesbeianus* populations.
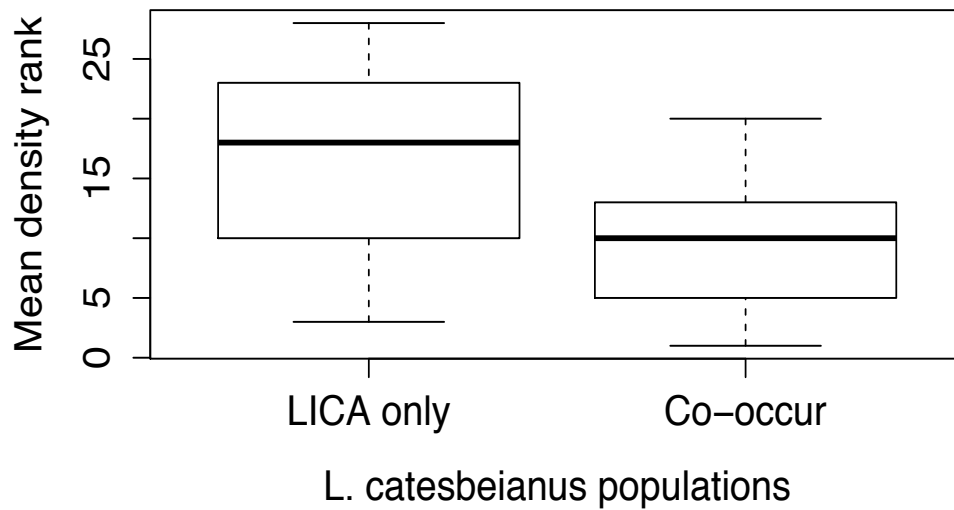
**Figure 2.4.**



**Figure 2.4.** Mean density rank score of *L. catesbeianus* populations at sites where they were the only species detected in a wetland (LICA only) and when they are found sympatric in the same wetland with at least one other native amphibian species (co-occur). The mean density rank score was significantly higher when *L. catesbeianus* were the only detected species at a wetland than when they were found in the same wetland as another amphibian species (Wilcox test, W = 36.5, p = 0.02)
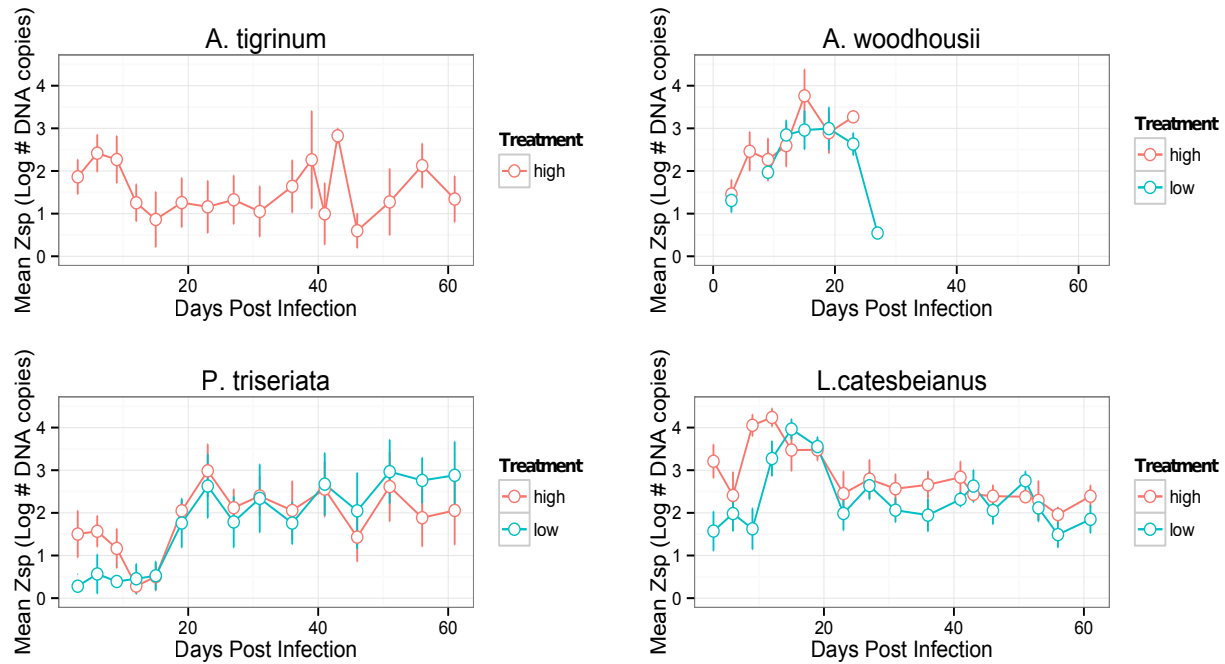
**Figure 2.5**.



**Figure 2.5.** Plots of mean zoospore output over time of individuals within each treatment group (high and low) for the four different species included in our laboratory experiment. No high or low groups were significantly different for any species. *L. catesbeianus,* on average, produced significantly more zoospores over time than either *A. woodhousii* or *P. triseriata*, though they did not produce more zoospores, on average, than *A. tigrinum* individuals.
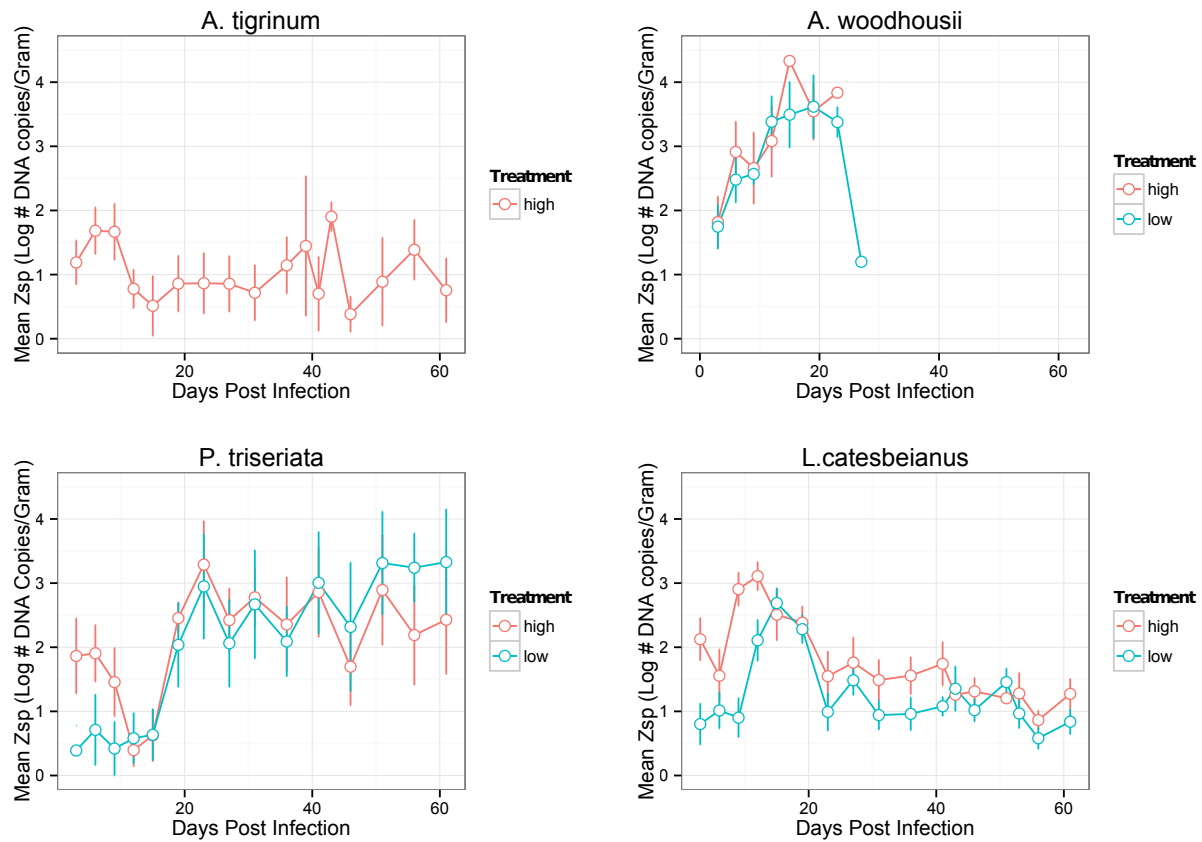
**Figure 2.6**.



**Figure 2.6**. Mean zoospore output over time across individuals within each treatment group (high and low) of the four different species included in our laboratory experiment after dividing the zoospore output on each soak date by the mass of each individual on that date. After accounting for size, *A. woodhousii* produced, on average, significantly more zoospores per gram of body mass than did *A. tigrinum* or *L. catesbeianus*, though not significantly more than *P. triseriata* individuals.
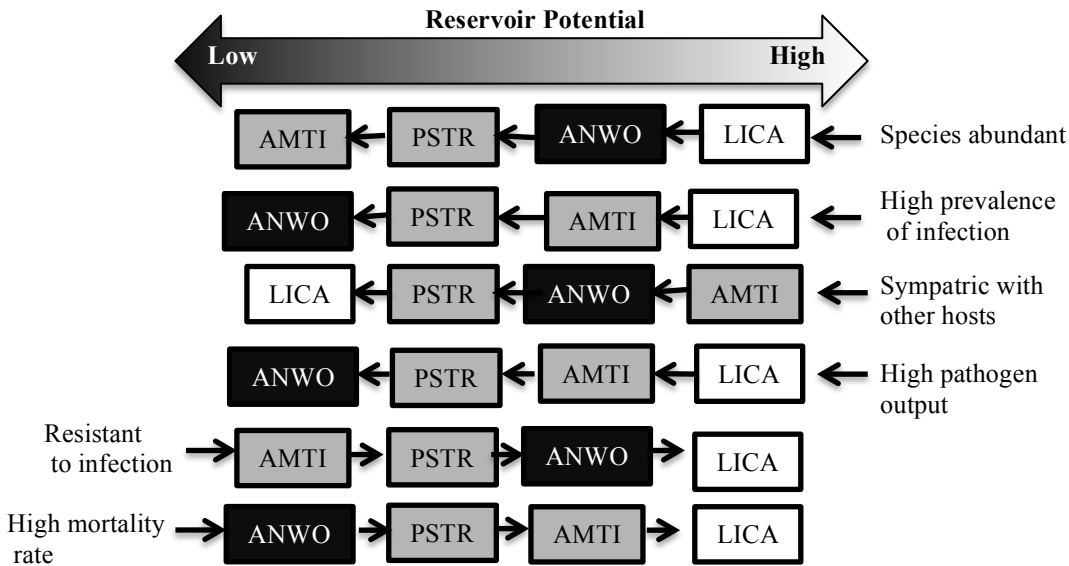
**Figure 2.7**.



**Figure 2.7** Diagram of reservoir ability for the different amphibian species encountered in our field surveys and included in our laboratory study. Each species is ranked relative to the other species for its value for each trait relating to reservoir ability. AMTI= *Ambystoma tigrinum*, PSTR= *Pseudacris triseriata*, ANWO= *Anaxyrus woodhousii*, LICA=*Lithobates catesbeianus*. *L. catesbeianus* is ranked as having the highest reservoir ability of the species included in this study, as it ranked highest for most of the traits relating to reservoir ability (as drawn from our criteria).

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