Occurrence and Distribution of an Amphibian Pathogen in Eastern Missouri

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Introduction to the Distribution of Bd and The Global Threat to Amphibian Diversity

The emerging pathogenic chytrid fungus *Batrachochytrium dendrobatidis* (Bd) poses one of the greatest threats to amphibian biodiversity in the world today (Skerratt *et al.* 2007) and has been called the "worst infectious disease ever recorded among vertebrates in terms of the number of species impacted, and its propensity to drive them to extinction" (Gascon *et al.* 2007). Bd was first isolated and shown to cause chytridiomycosis, an amphibian disease linked to numerous declines and extinctions, in the late nineties (Berger *et al.* 1998; Longcore *et al.*1999). In the last ten years Bd has been identified on all continents capable of sustaining amphibian life (Berger *et al.* 1998; Weldon *et al.* 2004; Ron 2005; Lips *et al.* 2006; Bosch *et al.* 2007; Vredenburg *et al.* 2010; Bai *et al.* 2010), and since Bd is a generalist pathogen, it is known to infect a diverse array of amphibian hosts.

In some areas, such as Central America, Australia, and Spain, the pathogen is clearly emergent, spreading across the landscape in wave-like patterns, and decimating amphibian populations in its path (Lips *et al.* 2006; Vredenburg *et al.* 2010; Bosch *et al.* 2007). However, in other areas, such as Southern Africa, Eastern North America, and Japan, Bd occurs sparsely across the landscape and has not led to host population declines (Weldon *et al.* 2004; Longcore *et al.* 2007; Goka *et al.* 2009). In these areas, it is not known whether Bd is emergent or endemic, partly because so little is known about the geographic range of Bd prior to 1998 (but see Weldon *et al.* 2004; Longcore *et al.*

2007; and Goka *et al.* 2009 for arguments of endemicism in their respective regions). The regional differences in host responses to the disease may be due to variation in environmental conditions (Bosch *et al.* 2007), evolutionary histories, host susceptibility, or pathogen virulence (Daszak *et al.* 2004; Weldon *et al.* 2004). Regardless, it is essential to better understand host-pathogen dynamics in all ranges where Bd occurs in order to better understand the risk it poses to global amphibian biodiversity.

The origins of Bd are poorly understood. Because it was only recently identified as a causative agent of amphibian disease and declines, very little data exists describing its distribution and range prior to the 1990s, although a survey of museum specimens did detect an unexpectedly high prevalence of Bd infections in North American anurans and salamanders dating back to the 1960s (Ouellet *et al.* 2005). This same study did not find any historical evidence of Bd occurring in Japan or Europe; however, another survey reported finding Bd in African anuran populations as early as 1938 (Weldon *et al.* 2004). These analyses reveal that Bd may have been present in Africa or North America long before it emerged on a global scale in the 1990's. Genetic approaches to determining the origin of Bd have also been inconclusive, although early studies reported very low genetic diversity among Bd genomes worldwide, suggesting that the virulent Bd strain is a recently emerged clone (Morehouse *et al.* 2003; Morgan *et al.* 2007). Other studies have suggested that the diversity of fungal strains may be higher in introduced amphibians, such as the American bullfrog (*Rana catesbeiana)* (Goka *et al.* 2010).

Several hypotheses have suggested mechanisms for the recent global emergence of this pathogen. Humans are thought to play a large role in the spread of Bd, as fisherman or research scientists moving between ponds may unintentionally disperse

zoospores (St.-Hilaire *et al.* 2009). Often, a resistant vector amphibian species is also implicated in the spread of Bd. The African clawed frog (*Xenopus laevis*) and the American bullfrog (*Rana catesbeiana*) have both been shown to be resistant to Bd (Daszak *et al.* 2004; Weldon *et al.*), and these species have both been transported around the world as part of the live amphibian trade (Schloegel *et al.* 2009) and have all established viable populations in non-native ranges (see Bai *et al.* 2010 for the first report of Bd in China, which, incidentally, was found infecting a non-native population of American bullfrogs; see also Solis *et al.* 2010 for a report of Bd in Chile infecting a feral population of African clawed frogs).

These species are also good candidates as reservoir species, which may be needed to maintain Bd populations, especially in areas where Bd can wipe out local amphibian populations, thus eliminating potential hosts. For instance, in pools in the Sierra Nevada, the larval stage of the mountain yellow-legged frog (*Rana muscosa*) is a Bd reservoir, because larval anurans can sustain infections while remaining in pools throughout the year and potentially infecting adults (Briggs *et al.* 2005). Here, transmission to adult *R. muscosa* is dependent on the number of zoospores present in the aquatic environment, which if severe can lead to epidemics (Briggs *et al.* 2010). Since both bullfrogs and clawed frogs are also resistant to Bd (Daszak *et al.* 2004; Weldon *et al.*) and have long-lived larval stages, their presence could likewise help maintain high numbers of Bd zoospores in the ponds they inhabit. Their host populations could serve as a permanent reservoir for Bd, while periodic outbreaks and waves of disease transmission sweep through naïve and susceptible local amphibian populations, causing declines and in some cases extinctions.

As data has accumulated describing the impacts of Bd throughout the world, two hypotheses have been proposed as explanations for this rapid emergence. The 'novel pathogen hypothesis' posits that emergence of the pathogen is a direct result of anthropogenic actions (Berger *et al.* 1999; Daszak *et al.* 1999). Alternatively, the 'endemic pathogen hypothesis' suggests that emergence of the pathogen was facilitated by degrading environmental factors that made amphibian species more susceptible to pre-existing Bd infections (Daszak *et al.* 2000; Blaustein and Kiesecker 2002). It seems likely that a hybrid of these theories might best describe the observed global emergence of Bd, and that the relative importance of each theory might vary depending on the geographical area in question (Goka *et al.* 2010). For instance, Bd occurrence in North America seems to fit the 'endemic pathogen hypothesis' better: Bd has existed in the landscape for at least 50 years (Ouellet *et al.* 2005), has not caused amphibian declines (Longcore *et al.* 2007; Rothermel *et al.* 2009) except in the Sierra Nevada (Vredenburg *et al.* 2010), and diversity among strains seems highest for Bd found with the American bullfrog (Goka *et al.* 2010). Additionally, it has been suggested that climate change has increased the risk of Bd-related amphibian declines in montane regions (Bosch *et al.*2007), thus explaining the declines in the Sierra Nevada.

It is not known whether Bd will continue to emerge at its current alarming rate in the future. Ecological niche modeling has revealed that Bd could survive in most of the world's temperate and tropical terrestrial ecosystems (Ron 2005), but most reported amphibian declines have occurred in Central America (Lips *et al.* 2006) and Australia (Berger *et al.* 1998). Furthermore, in Central America, the full extent of Bd's invasion may not yet have run its course (Lips *et al.* 2006). In contrast to these tropical locations,

Bd has also caused multiple declines in high-latitude, high altitude environments such as the Sierra Nevada mountains of the Western United States and the Sierra de Guadarrama mountains of Central Spain (Vredenburg *et al.* 2010; Bosch *et al.* 2007). In light of the 'endemic pathogen hypothesis' it seems impossible to predict areas that might be susceptible to chytridiomycosis outbreaks in the future.

Despite the rapid emergence of Bd, the dispersal and transmission of the pathogen at a local scale is also surprisingly poorly understood. Bd is a generalist pathogen which infects the keratinized skin of amphibians, but outside of its host (as a free-living zoospore) Bd is thought to be a poor competitor with no known resting stage (Berger *et al.* 2005). Despite these limitations, it has been shown that Bd can survive in water as a free-living zoospore for up to six weeks (Johnson and Speare 2003). However, it seems likely that Bd relies on some other organism or natural process to facilitate its dispersal. One possibility is that dispersal of adult amphibians provides the mechanism by which Bd moves across the terrestrial landscape. However, alternatives, such as transmission by water birds flying between ponds, or through the transportation of moist soil, are also possible (Johnson and Speare 2005). Again, humans may play a direct role in transporting Bd as researchers and fisherman move between bodies of water (St.-Hilaire *et al.* 2009), if care is not taken to disinfect all equipment that comes in contact with water (see Schmidt *et al.* 2009). Efforts to detect Bd on non-amphibian hosts have been unsuccessful (Rowley *et al.* 2007). However, this does not preclude the possibility that Bd could disperse in association with some vector species unrecognized by science, or that Bd may even assume an airborne form capable of being wind-dispersed (see the discussion in Johnson and Speare 2005).

Perhaps because Bd's dispersal mechanism is not clearly known, local patterns of occurrence of Bd are also poorly understood. This is complicated by the fact that once Bd becomes established in a pond, it may or may not remain there; several studies have noted a strong temporal turnover in presence and prevalence of Bd (Briggs *et al.* 2010; Padgett-Flohr and Hopkins 2010). In areas where the pathogen is clearly emergent, it may seem like only a matter of time before Bd reaches all ponds in the area (note the wave-like spread of Bd through Central America in Lips *et al.* 2006), even when the mechanism for the spread of the disease is not understood. In other areas like the American Midwest, patterns of occurrence are even more poorly understood, even though Bd may have been historically present in biological communities here for hundreds of years (Ouellet *et al.* 2005). Since Bd is a poor competitor in the abiotic environment outside an amphibian host (Berger *et al.* 2005), the presence of a reservoir species, may also be important for the sustained survival of Bd in a pond (Briggs *et al.* 2005). Since Bd is an organism with certain niche requirements (see Ron 2005), it can be expected that other environmental gradients may also predict its occurrence. In the American Midwest, anuran species of the genus *Rana* are most likely to be good reservoir species, because these anurans seem unaffected by Bd, but their larvae (tadpoles) sustain infections and remain in ponds throughout the winter.

My research goal is to learn more about the ecology of Bd in the American Midwest. The first chapter of my thesis focuses on identifying patterns of the occurrence of Bd in Eastern Missouri. This research was conducted in the summer of 2009 by visiting twenty-nine ponds in Eastern Missouri. All data from this field season describe

Bd infection prevalence and intensity in larval anurans of the genus *Rana*. I use a variety of methods to analyze this data, including a statistical examination of how traditional disease parameters (host density, pathogen density, and host diversity) affect Bd occurrence, a GIS-based approach using pond isolation as a predictor for transmission of Bd, and ordinations and multivariate statistics to describe biotic differences between ponds in which Bd does and does not occur.

The second chapter of my thesis encompasses an observation component (comparing interspecific infection prevalence between *Rana clamitans* and *Hyla versicolor*) as well as an experimental component (quantifying the rate at which Bd spreads from a focal infected pond to peripheral mesocosms). My third and final chapter uses a mathematical model to show how the global emergence of Bd may have been facilitated by an association with an invasive, resistant amphibian species. Taken together, all of these results are intended to advance the knowledge of the environmental and biological factors contributing to the observed patterns of Bd occurrence in the Midwest, and also to suggest how this knowledge complements what is known about Bd and amphibian declines in other parts of the world.

Chapter 1: Patterns of Occurrence of Bd in *Rana sp.* in Eastern Missouri

A. Introduction to Bd in the American Midwest

Although very little is known about Bd's history in the American Midwest, an examination of anuran and salamander museum specimens has revealed that Bd was present in the Midwest at least as early as the 1960s (Ouellet *et al.* 2005). Even though Bd may be endemic to the Midwest, very little is known about its range and distribution, because prior to the amphibian declines of the late 1990s, Bd was not known to science. Even as Bd became a globally recognized threat to amphibians, its presence in the Midwest was neglected, presumably because it did not lead to population declines there like it did elsewhere. As a result, scientists still know very little about the environmental conditions in the Midwest most suitable for the existence of Bd.

There are many reasons why a large-scale observational study of the occurrence and prevalence of Bd in the Midwest would be a valuable addition to knowledge about chytrid fungus. First, it would allow us to determine how prevalent Bd is in the Midwest. More importantly, it would allow us to search for environmental gradients which might be able to predict the occurrence of Bd. This information could then be applied to areas in the world where the disease is emergent. It would also allow us to explore the hypothesis that Bd may be endemic to the Midwest. Finally, observing the natural patterns of occurrence of Bd would allow us to design experiments and test specific

hypotheses that could significantly advance the knowledge of this globally emergent disease.

B. Field and Laboratory Work

Field Work. In summer 2009, a team of researchers from Washington University's Tyson Research Center (Eureka, Missouri) visited 54 ponds throughout East-Central Missouri. We included 29 of these ponds in our survey of Bd. We travelled to 9 conservation areas and sampled 1-5 ponds in each area: Tyson Research Center (3 ponds), Shaw Nature Reserve (2 ponds), Daniel Boone Conservation Area (4 ponds), Longridge Conservation Area (1 pond), Meramec Conservation Area (5 ponds), Little Indian Creek Conservation Area (2 ponds), Pea Ridge Conservation Area (3 ponds), Huzzah Conservation Area (4 ponds) and Mark Twain National Forest, near Potosi (5 ponds). The geographical coordinates of all ponds were entered into a Google Earth Pro database for future reference. All ponds were circular with approximate diameters of twenty meters, no more than two meters deep, embedded in an oak-hickory forest canopy, and within 250 kilometers of St. Louis. Ponds were selected to be semi-permanent (hold water during most years) and to be free of fish predators. Ponds were sampled in the months of June and July between the hours of 10:00 a.m. and 5:00 p.m.. Care was taken to rinse all nets and waders with dilute bleach solution when moving between ponds in order to prevent accidental spreading of Bd, as recommended by Schmidt *et al.* 2009.

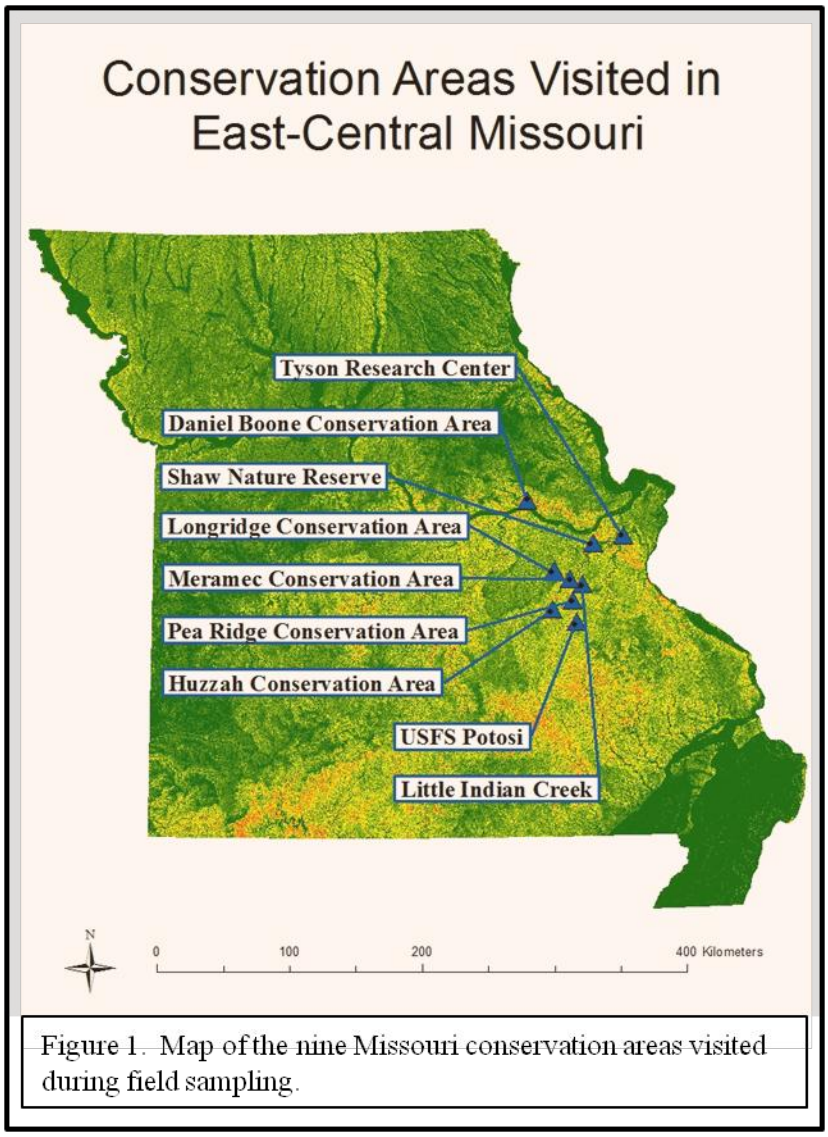

Figure 1. Map of the nine Missouri conservation areas visited during field sampling.

Biodiversity was sampled at all ponds using two techniques: chimney sweeps and rare sweeps. Chimneys are tall plastic cylinders with two-foot diameters, which when inserted into the substrate of a pond form a spatially constrained water column, or "chimney." The chimney sweep sampling method involved establishing three chimneys in different areas of a pond, and removing all living organisms from the resulting spatially constrained water column with a dip net. The water column was declared empty when ten successive sweeps with the dip-net yielded no additional organisms. All amphibian species were identified and counted on site, and all macroinvertebrate species

were stored in a whirl pack of 70% ethanol pending future identification in the lab. Species abundance data was normalized according to pond size, allowing a direct comparison of the relative abundances of all resident species in all of the ponds sampled.

The use of the rare sweep method allowed us to include any rare species that might have been missed in the chimneys. This second method is particularly useful for sampling fast-moving species that are able to swim away from a descending chimney. One rare sweep was defined as pulling a long-handled dip-net across approximately six feet of the surface of the pond. We conducted ten rare sweeps at each pond being careful to vary the depth and location across which each sweep was pulled. If a rare sweep yielded a species which had not been found in a chimney, it was stored in a new whirl pack filled with 70% ethanol.

Figure 3. Chimney sampling at a pond (left) and processing *Rana* tadpoles (right).

Amphibian diversity sampled from these ponds included Eastern newts (*Notophthalmus viridescens*), spotted salamanders (*Ambystoma maculatum*), Southern leopard frogs (*Rana sphenocephala*), green frogs (*Rana clamitans*), American bullfrogs (*Rana catesbeiana*), pickerel frogs (*Rana pulustris*), gray tree frogs (*Hyla versicolor*), spring peepers (*Pseudacris crucifer*), Western chorus frogs (*Pseudacris triseriata*),

Blanchard's cricket frogs (*Acris crepitans*), and American toads (*Bufo americanus*). For detection of Bd, we chose to examine larval anurans of the genus *Rana*, because these species overwinter in ponds and are more likely to serve as Bd reservoirs. Removing larvae rather than adults has a smaller impact on the amphibians' population structure, and detecting Bd in oral discs from larval anurans is more reliable than detecting Bd in swabs or toe clips of adults (Hyatt *et al.* 2007).

Figure 3. Green frog, *Rana clamitans* tadpoles (left) and adult (right). Pictures from USGS.gov and visitingnature.org.

We collected an average of 27 tadpoles from each pond in order to ascertain the infection prevalence and intensity of Bd. If sufficient tadpoles were not obtained from chimney and rare sampling, then additional specimens were caught with dip nets. Of the total 793 tadpoles collected, 564 were green frogs (*Rana clamitans*), 162 were Southern leopard frogs (*Rana sphenocephala*), and 67 were pickerel frogs (*Rana palustris*). Since these three species have similar life-histories, we treat them as a single pooled group of potential hosts. Preference was given to larger tadpoles, since larger tadpoles are more likely to be infected with Bd (Smith *et al.* 2007). The snout-vent length was measured and recorded for each tadpole before processing. Tadpoles were euthanized on-site in a

small chamber with concentrated MS-222, and after the hearts of all tadpoles had visibly stopped beating, the oral discs were excised with a scalpel and stored in centrifuge tubes filled with 70% ethanol.

The blade of the scalpel was periodically wiped clean with alcohol wipes in order to kill any Bd zoospores. A negative control was recorded for each pond by dipping the scalpel blade in centrifuge tube of 70% ethanol. If the control tested positive for Bd, it would suggest that our field methods did not sufficiently address the possibility of contamination between tadpoles. After processing, the bodies of the tadpoles were buried no closer than 10 meters away from the pond. This entire process was conducted while wearing disposable latex gloves treated with a fungicide in order to prevent spreading zoospores between the individuals being handled.

Laboratory Work. I determined macroinvertebrate diversity by examining our biodiversity samples with a dissecting microscope. Macroinvertebrate diversity from the ponds that we sampled included 97 species is eleven orders: eighteen species of *Odonata* (dragonfly and damselfly larvae); fifteen species of *Coleoptera* (aquatic beetles and beetle larvae); twelve species of *Hemiptera* (aquatic true bugs); six species of *Diptera* (midge and mosquito larvae); seven species of *Gastropoda* (snails); two species of *Arhynchobdellida* (probiscisless leeches); two species of *rhynchobdellida* (jawless leeches); one species of *Bivalvia* (bivalves); one species of *Trichoptera* (caddisfly larvae); one species of *Emphremeroptera* (mayfly larvae); and one species of *Collembola* (springtail larvae).

The Bd samples were sent to the Storfer Laboratory at Washington State University, where technicians conducted quantitative real-time PCR assays in order to quantify the number of zoospores per tissue sample. All oral discs from a single pond were pooled together, and if the pool tested positive for Bd, the oral discs were tested individually in order to determine infection prevalence within a pond. Three separate 20 mg tissue samples were taken from each excised oral disc and replicated using a thermocycler, and fluorescent markers were used to identify zoospore equivalents. The average of the triplicate samples was used to identify mean infection intensity per individual.

C. General Results

Summary. With all ponds pooled together, Bd was detected in 78 of 793 larval anurans (infection prevalence of 9.8%). Eleven of the twenty-nine ponds contained larval anurans that tested positive for Bd, and six of nine conservation areas included ponds that contained larval anurans that tested positive for Bd (figure 4). All negative controls tested negative for Bd, suggesting that cross-contamination did not occur and result in false positives.

In two conservation areas, all ponds sampled tested positive for Bd; in three conservation areas, zero ponds sampled tested positive for Bd; and in four conservation areas, approximately half of the ponds sampled tested positive for Bd. However, it should be noted that other surveys have shown that Bd does exist in some ponds at Tyson Research Center and Shaw Nature Reserve, although this survey failed to detect Bd in

either of these areas (Strauss and Smith unpublished data). Note also that due to limitations of time and funding, only a small subset of ponds at each conservation area were sampled. The ponds sampled were chosen because they were similar enough to warrant direct comparisons.

Three infection metrics were obtained for each pond: 1) Bd occurrence (presence or absence), 2) Bd infection prevalence (proportion of larval amphibian hosts infected), and 3) mean Bd infection intensity (average number of zoospores per host). Infection prevalence within a pond ranged from 0 to .897 (mean .097, standard deviation .192) and was arcsine square root transformed prior to statistical analyses. Mean infection intensity of individuals (zoospore equivalents) ranged from 0 to 41.76 (mean 3.93, standard deviation 7.44 among infected individuals), and mean infection intensity for ponds ranged from 0 to 4.795 (mean .409, standard deviation 1.087) and was logarithmically transformed prior to statistical analyses.

The zoospore loads detected in individual tadpoles at our ponds were much lower than loads detected in ponds that experience amphibian declines. For instance, in the Sierra Nevada, individual *R. muscosa* adults may have zoospore loads approaching 10^4 or 10^5 (Briggs *et al.* 2010). Briggs *et al.* (2010) makes the distinction between ponds where Bd seems to have a persistent, endemic presence (with mean loads of 10^4 or 10^5 zoospores per individual) and ponds, often nearby, where Bd has a epizootic, emergent presence (with mean loads of 220 zoospores per individual) (Briggs *et al.* 2010). Our results describing Bd loads in Missouri are more consistent with ponds exhibiting a persistent, endemic presence of Bd. Note that this use of the word "endemic" does not

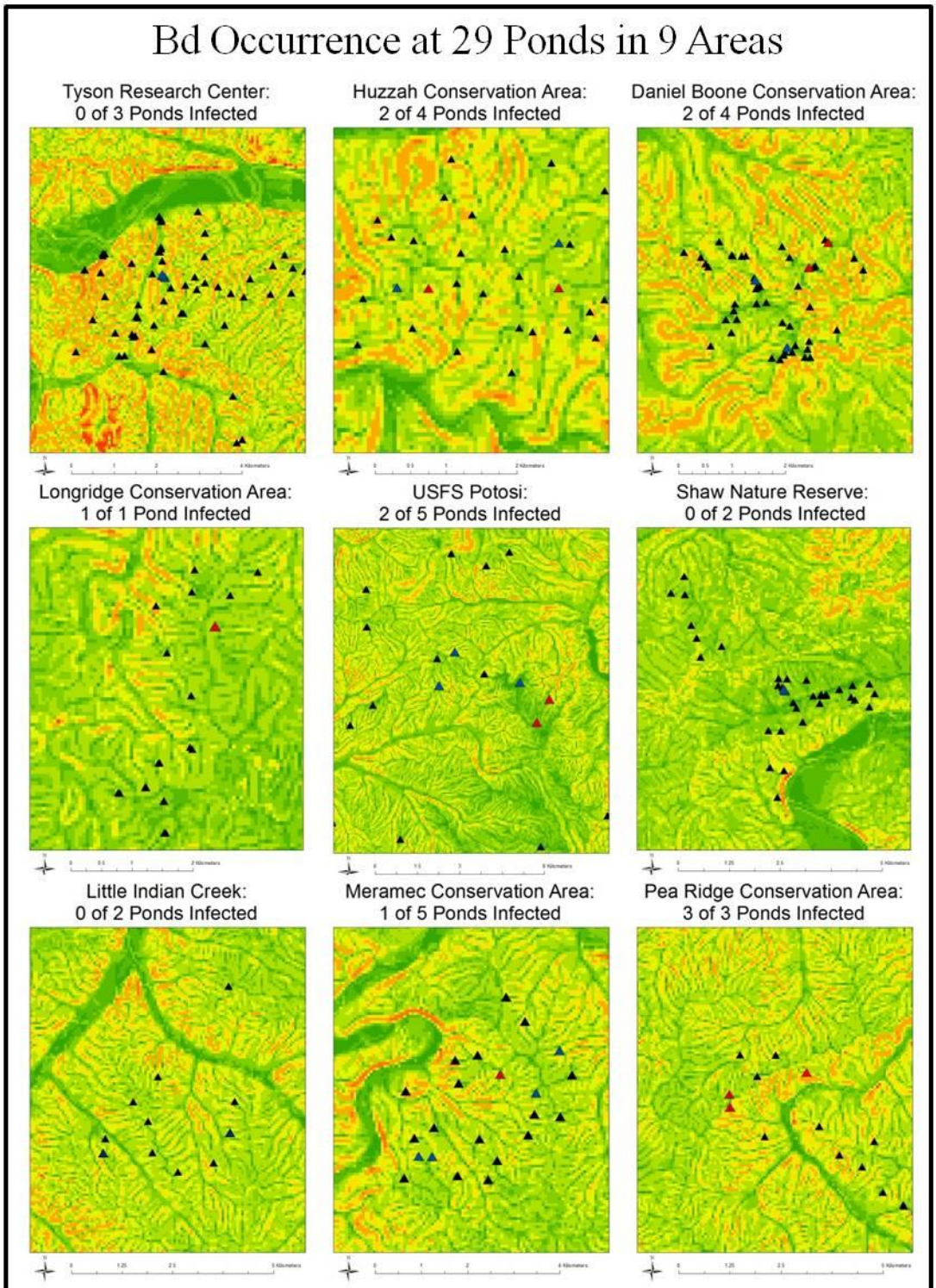

Figure 4. Geographical distribution and Bd test results for all sampled ponds within their respective conservation areas.

relate to the historical origin of Bd; rather, it is used to describe intra-pond dynamics of the pathogen. It is not surprising that Bd appears to have an endemic presence in the Midwest, because we know that the pathogen has not caused amphibian declines in this area.

D. Testing Traditional Disease Ecology Parameters

Introduction. Fundamental disease ecology theory states that disease prevalence should increase with pathogen density and host density (see Hochachka and Dhondt 1999 for an empirical example of density-dependent disease dynamics). These patterns are observed, because as the density of pathogens and hosts increases, the probability that a potential host comes in contact with a pathogen increases, and transmission rate between infected hosts also increases. Recent work in systems of multi-host pathogens (Lyme disease and West Nile Virus) has suggested that host diversity can also correlate with disease prevalence (Ostfeld and Keesing 2000; Ezenwa *et al.* 2006). If a competent reservoir host species is present, then infection prevalence may be higher; however, if a number of incompetent host species are presence, then infection rates can actually be lower due to a dilution effect (Ostfeld and Keesing 2000). The dilution effect is observed in Lyme disease and West Nile virus, because these diseases are transmitted explicitly via a vector, and the pathogen alone is unable to infect its host (Ostfeld and Keesing 2000; Ezenwa *et al.* 2006).

Bd is also a generalist pathogen, and in some cases it is thought that a reservoir species or host stage is needed in order to maintain infections within a pond (Briggs *et al.*

2005). However, a dilution effect has never been observed in amphibian communities infected with Bd. In the Midwest, a permanent Bd reservoir would need to exist in the pond throughout the year, including the winter. Many amphibian species lay their eggs in the spring and metamorph out in the fall; however, anurans of the genus *Rana* overwinter in ponds as larvae, and newts (*Notophthalmus visidescens*) overwinter in ponds as adults. These features make both of these species good candidates as Bd reservoirs. However, the potential for a dilution effect may be lower in the amphibian-Bd system, because amphibians are thought to become infected from free-living zoospores they encounter in the water column, where they can survive for up to six weeks (Johnson and Speare 2003). This makes transmission of chytridiomycosis fundamentally different from that of Lyme disease and West Nile virus.

Bd dynamics may also not follow rules of density-dependent transmission. If disease prevalence within a pond is thought to be dependent on the density of infective zoospores, then mean infection loads of individuals should correlate with infection prevalence in the pond. However, analyses of host-pathogen dynamics in Sierra Nevada pools have shown that a high prevalence of infection is often maintained by relatively low zoospore counts (endemic versus emergent levels), and have also shown that complex Bd load dynamics were necessary to model the population-level outcomes of Bd infections (Briggs *et al.* 2010). Briggs *et al.* (2010) also found little correlation between host (amphibian) density and disease prevalence (Briggs *et al.* 2010), possibly because zoospores may remain infective in the water column for up to six weeks without requiring any direct amphibian-amphibian transmission (Johnson and Speare 2003). It is important to remember that these results from the Sierra Nevada may only be

characteristic of an area post-decline; the observed patterns may be the result of Bd first sweeping through an area and then equilibrating with its amphibian hosts, allowing Bd to persist in an endemic state (Briggs *et al.* 2010). Similar infection patterns were also observed in an Australian frog community post-decline (Retallick, McCallum and Speare 2004).

Although empirical data has been collected in areas post-decline, no observational studies have determined whether similar infection patterns exist in areas that have no record of chytridiomycosis-induced amphibian declines. One area well suited for this comparison is the American Midwest, where no data exists relating these fundamental disease ecology parameters (host diversity, host density, and infection intensity) with disease prevalence.

Methods. I tested for a dilution effect using two different hypothesized reservoir species: tadpoles of the genus *Rana* and newts (*N. viridescens*). Since I had used tissue samples from *Rana* tadpoles to diagnose Bd presence in a pond, all of my ponds included *Rana* tadpoles. In order to test a dilution effect, I hypothesized that a greater amphibian diversity (in addition to *Rana sp,*) would correlate with a lower disease prevalence. I was also curious to see whether additional amphibian diversity could predict disease occurrence within a pond or mean infection intensity within a pond. I used linear regressions to correlate additional amphibian diversity with disease prevalence and mean infection intensity, and I used a logistic regression to correlate additional amphibian diversity with disease occurrence.

In order to test the possibility that newts (*N. viridescens*), rather than *Rana sp.*, were a key reservoir species, I compared the disease dynamics between ponds with and without newts. I used a contingency table and Fisher's exact test to test for a relationship between newt presence and Bd presence, and I used t-tests to test for relationships between newt presence and infection prevalence and infection intensity.

I hypothesized that both infection intensity and host density would correlate with disease prevalence. In order to correlate mean infection intensity of individuals within a pond with disease prevalence within that pond, I plotted a simple linear regression. In order to test whether transmission of the disease was dependent on host density, I compared amphibian densities with Bd occurrence (using a logistic regression), prevalence (using a linear regression), and mean infection intensity (using a linear regression). I conducted these tests using pooled amphibian density as predictor variables (all individuals caught from chimneys), but I also did the same tests using only *Rana sp.* density and only newt density. The purpose of these tests was to ensure that one of my hypothesized reservoir species was not solely responsible for host-host transmission of Bd.

Each replicate used for all of these tests was a single pond, and each test considered twenty-nine replicates. All statistical analyses were conducted using SYSTAT.

Results and Discussion. The results of my statistical tests are below (table 1). I found no statistical evidence to support the hypothesis that amphibian communities infected with Bd experience a dilution effect. Amphibian diversity was unrelated to

disease prevalence (p= .404) as was newt presence (p=.244). The potential reservoir effect of *Rana sp.* or *N. viridescens* does not seem to be diluted by the presence of other

Tests of Traditional Disease Ecology Parameters

Hypothesis Tested	Predictor Variable	Response Variable (Statistical Test)		
		Bd Presence	Disease Prevalence	Infection Intensity
Dilution Effect (Reservoir Species)	Amphibian Density	p=.463 (log. reg.)	p=.404 (lin. reg.)	p=.236 (lin. reg.)
	N. viridescens presence	p=.412 (cont. tab.)	p=.244 (t-test)	p=.691 (t test)
Host Density	Amphibian Density	p=.983 (log. reg.)	p=.670 (lin. reg.)	p=.470 (lin. reg.)
	Rana sp. Density	p=.605 (log. reg.)	p=.628 (lin. reg.)	p=.809 (lin. reg.)
	N. viridescens Density	p=.983 (log. reg.)	p=.673 (lin. reg.)	p=.335 (lin. reg.)
Pathogen Density	Infection Intensity	n/a	p<.001 (lin. reg.)	n/a

Table 1. Testing traditional disease ecology parameters as predictors for Bd occurrence, disease prevalence, and mean infection intensity revealed that only mean infection intensity was able to predict disease prevalence; no variables were able to predict Bd presence or infection intensity.

amphibian species; this may be because *Rana sp.* and *N. viridescens* are not reservoir species, or it may simply stem from an unknown property of the way that Bd infects amphibians. As previously described, the dilution effect works best when the disease is vector transmitted (see Ostfeld and Keesing 2000; Ezenwa *et al.* 2006), whereas potentially infective Bd zoospores may live in the water column and not require host-host transmission (Johnson and Speare 2003).

This property of the amphibian-Bd system may also explain why disease prevalence was not predicted by total amphibian density (p= .670), *Rana sp.* density (p=.628), or *N. viridescens* density ((p=.673). This result is consistent with data collected in areas where Bd has caused massive amphibian die-offs such as the Sierra Nevada and New Zealand (Briggs *et al.* 2010; Retallick, McCallum and Speare 2004). However, rather than being a property of amphibian communities post-decline, it seems that this lack of host density dependence may be ubiquitous wherever Bd is not in an emergent state. Indeed, the model proposed by Briggs *et al.* (2010) suggests that host density dependence may only correlate with disease prevalence and infection intensity during the initial stages of an emergent Bd outbreak.

The most significant result I found was the correlation between mean infection intensity within a pond and infection prevalence within a pond (p<.001). This strong correlation (R^2=.7808; see figure 5) may not seem surprising; however, it is indicative that amphibian-Bd dynamics are very different between areas like the Sierra Nevada, where Bd recently emerged and became endemic (Briggs *et al.* 2010), and the Midwest, where Bd may have existed for hundreds of years in an endemic state (Ouellet *et al.* individuals may carry low Bd loads (Briggs *et al.* 2010; Retallick, McCallum and Speare

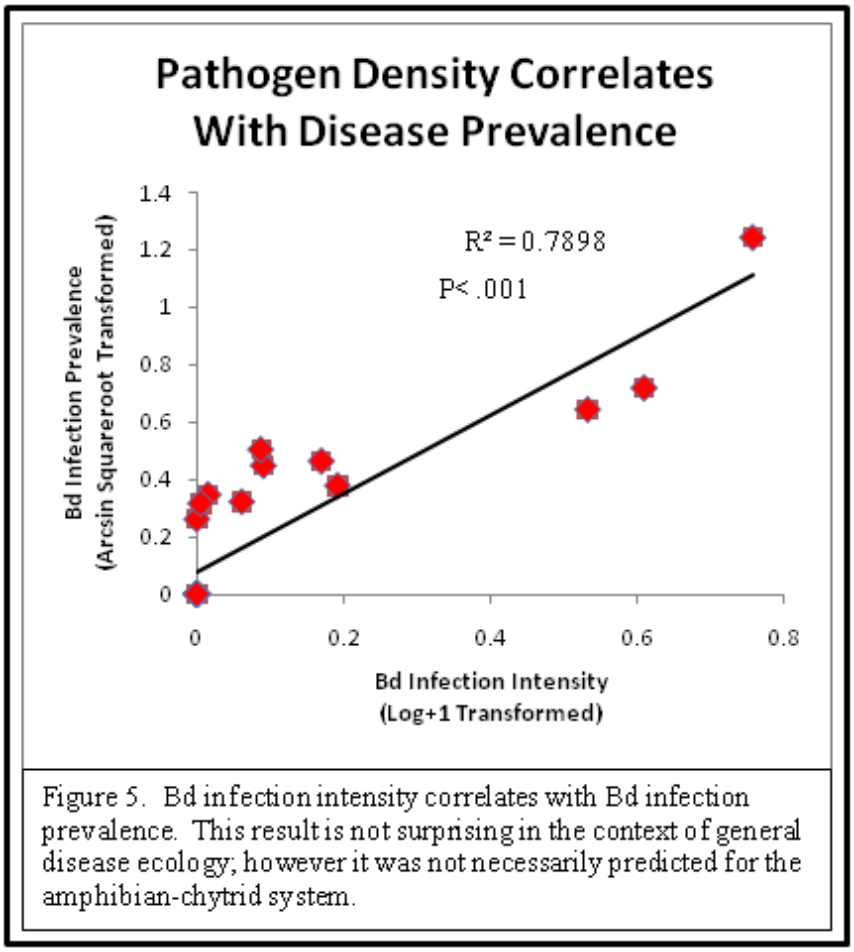

Figure 5. Bd infection intensity correlates with Bd infection prevalence. This result is not surprising in the context of general disease ecology; however it was not necessarily predicted for the amphibian-chytrid system.

2005). Remember that in the Sierra Nevada and New Zealand, a large proportion of 2004), which is inverse to the pattern I am observing. One explanation for this may be that the Bd loads I measured may have been too low to recognize a saturating effect on infection prevalence. Although the low Bd loads reported in the Sierra Nevada were slightly higher than my highest Bd loads from Missouri, the high Bd loads in the Sierra Nevada were several orders of magnitude larger. Therefore, as infection intensity increases, infection prevalence may also increase, but only until it becomes saturated, at which point higher infection intensity (perhaps orders of magnitude higher) will have no apparent effect on infection prevalence.

These results can be summarized by stating that no evidence was found that host diversity or host diversity affected disease prevalence, but that mean infection intensity might be able to predict disease prevalence when infection intensities are low. These observations support the hypothesis that amphibians become infected when they encounter zoospores in the water column (Johnson and Speare *et al.* 2003), and that zoospores may be shed from infected hosts back into the water via discharge papillae (Berger *et al.* 2005). Rigorous experimental tests would be required to corroborate this hypothesis, and have not yet been attempted.

E. Using GIS to Search for Physical Landscape Patterns of Bd Occurrence

Introduction. Very little is known about the large-scale patterns of occurrence of Bd in the Midwest, although Bd is known to occur throughout the Southeast and Northeast United States (Longcore *et al.* 2007; Rothermel *et al.* 2009). Additionally, it is not known how Bd moves between ponds, or even at what spatial scale transmission occurs. One possibility is that Bd moves between ponds in association with dispersing amphibians. This seems to be a logical hypothesis for areas like the Midwest, where Bd does not cause mortality at metamorphosis, thus allowing adult amphibians to potentially disperse Bd. Alternatively, since Bd has been shown to survive in feathers of birds, so it has been suggested that water birds may play a role in dispersal of this pathogen (Johnson and Speare 2005). Unfortunately, the spatial scales at which these or other potential dispersers could operate, vary significantly.

If these or any other explanations involving inter-pond transmission are correct and the correct spatial scale is analyzed, then it might be expected that Bd should be more likely to occur in connected ponds, and less likely to occur in isolated ponds; According to simple metapopulation theory (Tilman 1994), if Bd goes extinct in a patch, it is more likely to be recolonized if other nearby patches still contain Bd. Viewing Bd as a metapopulation at the scale of dispersing amphibians is supported by Padgett-Flohr and Hopkins (2010), who sampled 54 ponds within 16,000 acres and found that Bd presence in a pond may be temporally dynamic, but that spatial structuring patterns emerged after several years of data collection.

Clear geographical patterns of Bd occurrence are evident in areas where the pathogen is emergent (Lips *et al.* 2006; Vredenburg *et al.* 2010). For instance, in Central America, a clear wave-like pattern depicts where Bd has invaded and suggests how fast it will spread to additional locations (Lips *et al.* 2006). However, less spatial structuring is apparent in areas where Bd seems to have an endemic presence (Padgett-Flohr and Hopkins 2010). Padgett-Flohr and Hopkins (2010) found that within a particular year, Bd-positive ponds tend to cluster around "hot-spot" ponds, but that only "hot-spots" themselves were perennially infected. Hot spots were determined by biotic characteristics, such as the presence of a key host species, but without temporal data, it would be difficult to identify "hot-spot" ponds (Padgett-Flohr and Hopkins 2010). In the Midwest, where Bd may be endemic, isolated ponds may be Bd-positive if Bd colonized the pond sometime in the past and environmental conditions were sufficient to allow the pond to become a perennial "hot-spot." Therefore, if Bd were endemic to the Midwest, it

would not be surprising to find a low degree of spatial structuring of Bd occurrence. However, without any temporal data, ponds can only be hypothesized "hot spots."

In order to determine the degree of spatial structuring of Bd occurrence in Missouri ponds, one must first select a spatial scale and, implicitly, a mode of transmission. Because Padgett-Flohr and Hopkins(2010) found evidence of spatial structuring when examining ponds at a scale conducive to inter-pond transmission via dispersing amphibians, I will do the same. I have chosen to define pond isolation both in terms of geometric distance between ponds and in terms of the dispersal barrier posed to amphibians. This allows for the possibility that either amphibians or some other vector may transmit Bd at this similar spatial scale. Amphibians may respond to both changes in elevation and land cover as they move across a landscape (Richards-Zawacki 2009). Using variable slope and land cover to construct matrices for dispersing amphibians has been used to model patterns of gene flow (Richards-Zawacki 2009; Lee-Yaw *et al.* 2009) and amphibian population density (Pope *et al.* 2000), but has not yet been applied to model the spread and distribution of a pathogen over space.

Methods. I conducted spatial analyses using ArcGIS 10 and Spatial Analyst Tools. Coverages showing the location of the 29 ponds we visited in the field and all other nearby ponds were imported from Google Earth Pro into ArcMap. This map was compiled by Elizabeth Biro and Travis Mohrman of Tyson Research Center and imported with assistance from Karen DeMatteo of Washington University. Two additional coverage layers were downloaded from MSDIS (http://msdis.missouri.edu/): a "60-meter Digital Elevation Model of State of Missouri" and a "Land Use / Land Cover 2005 State

of Missouri." These layers were chosen, because both topography and land use affect an amphibian's ability to disperse across the landscape and potentially spread Bd.

I used the Surface-Slope Spatial Analyst Tool to derive slope from elevation. Then, I used Jenks natural breaks to classify the slope raster into ten new categories, with '1' signifying the most gradual slope (no dispersal barrier) and '10' signifying the steepest slope (greatest dispersal barrier). I also reclassified the land use coverage according to the difficulty each category would pose to a dispersing amphibian. Again, I used a scale of 1 to 10, with '1' signifying no dispersal barrier and '10' signifying the greatest dispersal barrier. I reclassified "open water," "woody dominated wetland," and "herbaceous dominated wetland" as '1s'; "deciduous forest," "evergreen forest," and "mixed forest" as '2s'; "deciduous woody/herbaceous" and "evergreen woody/herbaceous" as '3s' (these cover types were categorized by less than 60% forest cover with the remainder being open grassland); "cropland" and "grassland" as '5s'; and "impervious," "high density urban," "low density urban," and "barren/sparse vegetation" as '10s.' This designation of suitable and unsuitable land cover types is similar to the one used in Pope *et al.* 2000 for the dispersal of Northern leopard frogs (*Rana pipiens*).

I used the Weighted Distance Spatial Analyst Tool to create a weighted overlay integrating slope and land use data. I weighted the slope raster at 75% and the land cover raster at 25%, because during periods of precipitation when amphibians are most likely to disperse, the difference between land cover types becomes less important (Dole 1965). An example of slope, land cover, and weighted overlay rasters are shown below (figure 6).

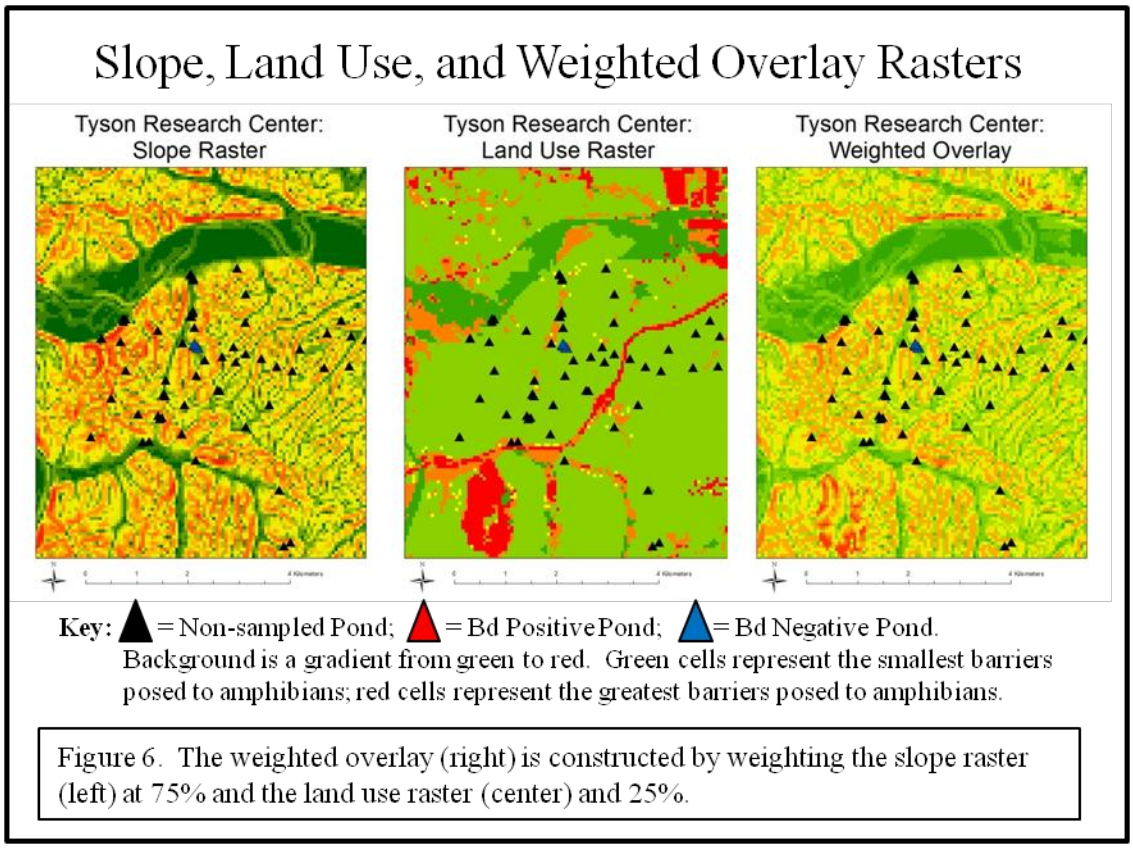

Figure 6. The weighted overlay (right) is constructed by weighting the slope raster (left) at 75% and the land use raster (center) and 25%.

I next exported the 29 ponds we had visited in the field into their individual layers so that they could be used as source points in least-cost-path analyses. I also exported groups of ponds from each conservation area to serve as their respective destination points. I used the Cost Distance, Cost Back Link, and Least Cost Path Spatial Analyst Tools to calculate least-cost-paths between each source pond and all destination ponds within 5000 units of path costs. In most areas, 5000 path cost units corresponded to 1,000 to 2,000 meters of unweighted distance. These spatial analyst tools assigned a value to each 60 by 60 meter cell, and then located the path that minimized the summed values of the all the cells that were passed. See below for an example (figure 7).

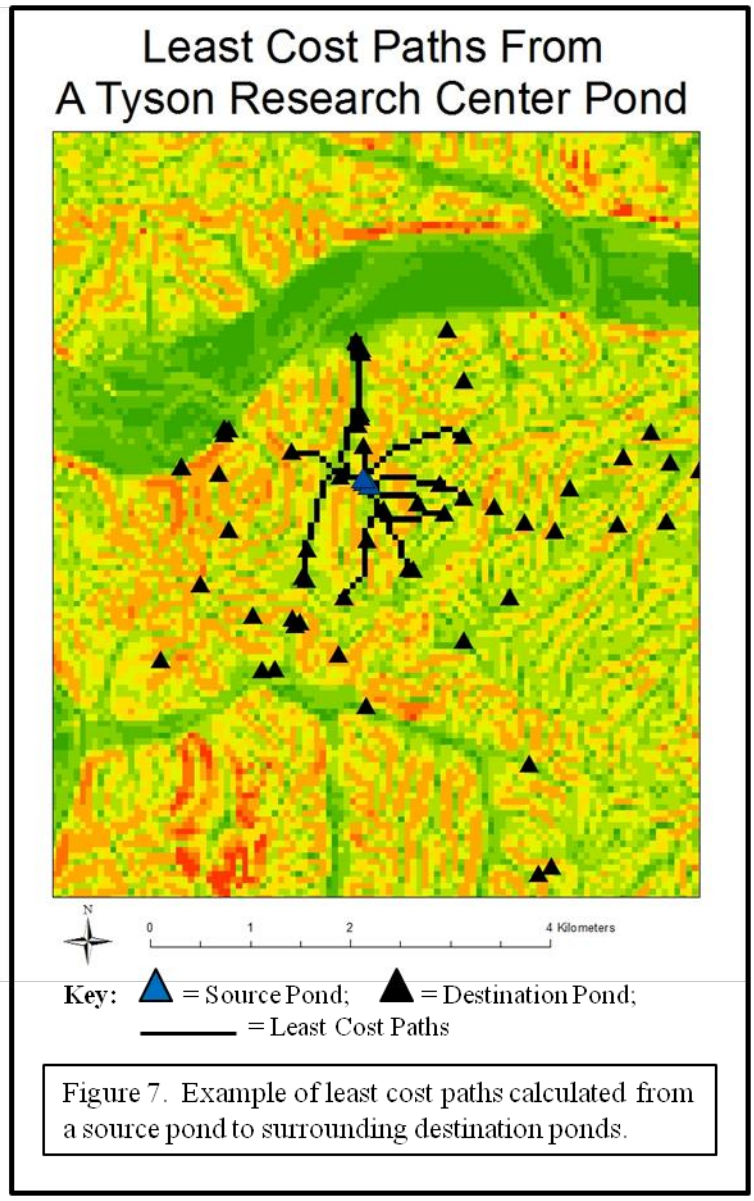

Figure 7. Example of least cost paths calculated from a source pond to surrounding destination ponds.

Two isolation metrics were exported into SYSTAT for statistical analysis: the cost the nearest neighbor pond (NNC) and the total number of neighboring ponds within 5,000 path cost units. These metrics were used as predictor variables and the Bd infection metrics were used as response variables. I used logistic regressions to test for spatial structuring in Bd occurrence and linear regressions to test for spatial structuring in disease prevalence and mean infection intensity.

In order to consider the possibility that Bd was transmitted between ponds at a similar spatial scale, but by a species that did not perceive slope or land use change, I also measured the unweighted nearest neighbor distance (NND) from each focal pond, as well as the total number of ponds within an unweighted 1,000 meters of each focal pond. These isolation metrics were also exported into SYSTAT and used to test for spatial structuring in Bd occurrence (using a logistic regression), disease prevalence (using a linear regression), and mean infection intensity (using a linear regression).

Results and Discussion. No statistical tests detected a significant relationship between weighted or unweighted pond isolation and Bd occurrence, prevalence, or intensity; Bd was equally likely to be found in connected and isolated ponds (table 2). This result indicates that if Bd is being dispersed at this spatial scale, then it is either an extremely good colonizer and able to reach all ponds (Tilman 1994), or that my definitions of pond isolation did not accurately reflect its mode of transmission (for instance if it were being dispersed by humans, in which case roads and urban areas could facilitate rather than hinder its dispersal – see St.-Hilaire *et al.* 2009). These results, while not dramatically informative, do establish that spatial patterns of Bd in the Midwest more closely resemble those found in areas where Bd has an endemic presence (as in Padgett-Flohr and Hopkins 2010) than those in areas where Bd has recently emerged (Lips *et al.* 2006).

These results may be consistent with those found in Padgett-Flohr and Hopkins (2010), if my isolated ponds that tested positive for Bd were perennial Bd "hot spots." This certainly warrants future research in order to determine the temporal turnover of Bd

at spatially isolated versus connected ponds. Because this spatial pattern of patchy Bd occurrence could also occur if Bd were an extremely good colonizing species (Tilman 1994), it may be important to consider whether environmental characteristics allow or disallow Bd to exist in certain ponds; Bd may be more limited by its niche than its dispersal. If this were the case, it should be possible to compare Bd and non-Bd ponds and identify environmental predictors of Bd occurrence.

Tests of Spatial distribution of Bd in Missouri

Predictor Variable	Response Variable (Statistical Test)		
	Bd Occurrence	Disease Prevalence	Infection Intensity
Nearest Neighbor Cost	p=.503 (log. reg.)	p=.943 (lin. reg.)	p=.622 (lin. reg.)
Number of Neighbors Within 5,000 Cost Units	p=.184 (log. reg.)	p=.900 (lin. reg.)	p=.780 (lin. reg.)
Nearest Neighbor Distance	p=.241 (log. reg.)	p=.739 (lin. reg.)	p=.863 (lin. reg.)
Number of Neighbors Within 1,000 Meters	p=.143 (log. reg.)	p=.443 (lin. reg.)	p=.626 (lin. reg.)

Table 2. No significant spatial structuring was detected in occurrence, prevalence, or intensity of Bd in a Missouri landscape. Bd ponds and non-Bd ponds were equally likely to be isolated or connected.

If Bd is limited by its dispersal, then my results suggest that the primary mode of transmission of Bd may not be adult amphibian dispersal. My GIS analyses were meant

to define pond isolation in terms of the dispersal barrier posed to amphibians; however, other potential vector species might perceive barriers and spatial scales differently. For instance, Bd has been shown to survive in bird feathers (Johnson and Speare 2005), and if birds are dispersers of Bd, then slope and land use might be less important than the identity of surrounding tree species. It is also possible that transmission occurs at a larger spatial scale than the one used in Padgett-Flohr and Hopkins (2010), and that no ponds in my analysis were isolated enough to allow me to detect an existing pattern. A further alternative is that anthropogenic movements have been the main source of Bd dispersal, and that Bd exists more commonly in ponds most frequented by researchers and fisherman (Morgan *et al.* 2007; St-Hilaire *et al.* 2009).

Because it was already known that Bd had an endemic presence in North America (Longcore *et al.* 2007; Rothermel *et al.* 2009), these results are not surprising. However, the factors which lead to Bd existing in some ponds and not others are still poorly understood. In order to draw more satisfying conclusions from this spatial analysis of Bd, additional years of data must be collected, and environmental data describing Bd and non-Bd ponds must be compared.

F. Using Ordination to Search for Biological Patterns of Bd Occurrence

Introduction. In Part C of this chapter, I showed that Bd was found to occur in approximately one third of the ponds that we visited in Missouri. However, no spatial patterns of Bd occurrence were detected, and Bd seemed equally likely to occur in isolated and connected ponds (Part D). This pattern might be observed if Bd were an

exceptionally good colonizing species, and if Bd were more limited by environmental conditions in local patches than by its dispersal range (metapopulation theory in Tilman 1994). This could lead to patterns of Bd occurrence that are more environmentally than spatially structured. Thus, certain environmental variables may be able to predict whether a pond is capable of supporting Bd or not.

Since Bd is an organism living in certain ponds in Missouri, it might be expected to respond to the same environmental gradients as other organisms living in ponds in Missouri. In order to compress the many environmental variables that might affect species distributions, a pond can be characterized by its biotic community. Non-metric multidimensional scaling (NM MDS) allows ponds to be ordinated in multi-dimensional space and facilitates an implicit comparison between the environmental factors leading to their particular biotic communities at those ponds.

Bd does not seem to have any significant population-level effects on amphibians in the Midwest, so it is unlikely that the pathogen would have a homogenizing effect on the regional community itself. However, it is possible that environmental gradients could lead to Bd occurring in a subset of local patches (ponds), along with the other host and non-host species which are also receptive to these gradients. To date, no one has attempted to use total species assemblage data to predict the occurrence of Bd. This method could be extremely useful for identifying predictive gradients for the distribution of Bd that may not have otherwise been intuitive or apparent.

Methods. I used species assemblage data gathered from chimney sweep and rare sweep sampling to ordinate the ponds in multi-dimensional space according to the

similarity of their species compositions (figure 8). This ordination was conducted using the program Paleontological Statistics (PAST). Ponds closer together have more similar compositions (α diversity), and ponds farther apart have very different compositions. A wide scatter of points reflects a high β diversity, as species turnover between ponds is large. Ponds were grouped according to whether they did or did not contain Bd, and 95% confidence ellipses were drawn around each set of ponds (Bd ponds in red, non-Bd ponds in green). Axes of the ordination plot are unitless and are derived from the similarity indices used to ordinate the ponds.

Bray-Curtis similarity was used to compare species abundances (data from chimney sweep sampling) and Raup-Crick similarity was used to compare species presences (data from all sampling methods). For each similarity metric, ponds were ordinated according to three different sets of species: all the species found regionally (108 species), all of the amphibian species found regionally (11 species), and all of the macroinvertebrate species found regionally (97 species).

A non-parametric multiple analysis of variance (NP-MANOVA) was used to test for differences in location between Bd and non-Bd groups in ordination space. This test was conducted is PAST and used 10,000 permutations to generate p values, which can be interpreted as the probability that the two groups occur in similar biotic communities. However, a second test was needed to test explicitly for differences in dispersion between groups, especially when both groups have the same geometric center in ordination space. The program PERMDISP2 (Dr. Marti Jane Anderson) was used to test for differences in dispersion between groups and automatically corrected for the different sample sizes between my two groups (18 non-Bd ponds and only 11 Bd ponds). Bray-Curtis and

Raup-Crick similarity matrices generated in PAST were converted into dissimilarity matrices and imported into PERMDISP2 for analysis. Each test used 9,999 permutations

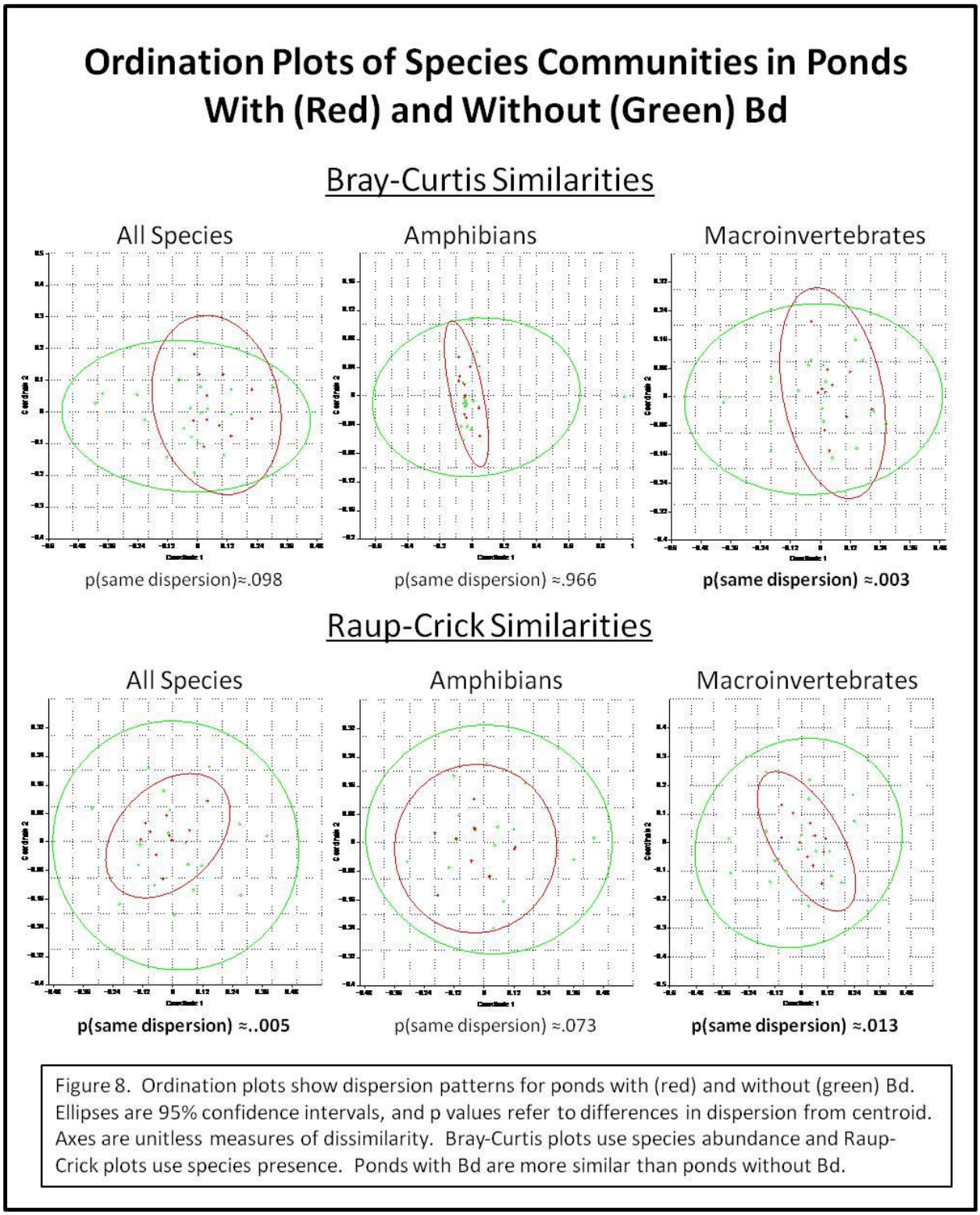

Figure 8. Ordination plots show dispersion patterns for ponds with (red) and without (green) Bd. Ellipses are 95% confidence intervals, and p values refer to differences in dispersion from centroid. Axes are unitless measures of dissimilarity. Bray-Curtis plots use species abundance and Raup-Crick plots use species presence. Ponds with Bd are more similar than ponds without Bd.

to generate p values, which can be interpreted as the probability that both groups can be described by the same dispersion pattern. PERMDDISP2 results include p values for dispersion from the centroid and dispersion from the spatial mean of the points.

Results & Discussion. The NPMANOVAs found no difference in location between Bd and non-Bd ponds, regardless of dissimilarity metric and subset of species used to ordinate ponds. However, several of the PERMDISPs did reveal significant differences in dispersion between Bd ponds and non-Bd ponds (table 3). When significant dispersion differences were detected, the Bd ponds always formed a spatial subset of the non-Bd ponds; in other words, β diversity was much lower for Bd ponds than for non-Bd ponds. This result can be visually corroborated by examining the ordination plots, where the red ellipse describing the species-defined location of the Bd ponds is contained within the green ellipse describing the location of the non-Bd ponds (figure 8). These ellipses have been standardized for the sample sizes of Bd and non-Bd ponds (11 versus 18 observations).

The difference in dispersion was always significant when only macroinvertebrate species were used to calculate the dissimilarity matrix (table 3). The difference in dispersion was also significant when all species were used to calculate the dissimilarity matrix, but only when the matrix was based on the Raup-Crick index (used to measure species' presence or absence rather than abundance). No difference in dispersion was detected using either similarity measurement if only amphibian species were considered. These results suggest that there is something different about Bd and non-Bd ponds. This

Results of Species Ordination Tests

Dissimilarity Metric Used	Species Used to Ordinate Plots	NPMANOVA Result	PERMDISP2 Centroid Result	PERMDISP2 Spatial Median Result
Bray-Curtis	All species	p=.174	p=.098	p=.167
	Amphibians	p=.295	p=.966	p=.975
	Macroinvertebrates	p=.139	**p=.003**	**p=.011**
Raup-Crick	All species	p=.382	**p=.005**	**p=.002**
	Amphibians	p=.250	p=.073	p=.132
	Macroinvertebrates	p=236	**p=.013**	**p=.006**

Table 3. Differences in species compositions of Bd and non-Bd ponds are measured statistically. NPMANOVA conducted in PAST tests for differences in location and PERMDISP2 tests for differences in dispersion. P values below .05 are bold.

result is surprising, because the twenty-nine ponds we visited were selected to be as similar to each other as possible. Two alternatives may explain this interesting observation: either Bd has caused these differences in biotic communities, or Bd is responsive to the same environmental factors which determine these biotic communities. If Bd were having an effect on the species communities present in ponds, then this effect would likely be mediated through amphibians, since amphibians are the only known group of organisms known to be affected by Bd (Berger *et al.* 2005). However, when amphibian species were used to ordinate ponds, the difference in dispersion patterns between Bd and non-Bd ponds disappeared. This result suggests that amphibian

communities do not seem to be structured according to the presence of Bd, and that Bd may be an effect, rather than a cause, of the variation between ponds.

Unfortunately this result does not bring us any closer to understanding the particular environmental factors that might separate Bd ponds from non-Bd ponds. In order to determine what these factors are, a large number of parameters must be gathered and rigorously tested with observational patterns of Bd occurrence.

G. Summary and Discussion of Bd in the Midwest

This study provides the first large-scale analysis of the distribution, severity, inter-pond dynamics, and intra-pond dynamics of Bd in the American Midwest. It has been known that Bd has existed in this landscape at least since the 1900s (Ouellet *et al.* 2005), yet no amphibian declines have been reported. A high proportion of amphibian species are known to experience low levels of Bd infections in Northeast and Southeast USA (Longcore *et al.* 2007; Rothermel *et al.* 2009), but it was not known if Bd had a similar endemic presence in the Midwest.

I found that Bd occurred in eleven of the twenty-nine ponds visited in East-Central Missouri (Part C). These ponds were selected because they were all semi-permanent, fishless, rainwater ponds in closed-canopy oak-hickory matrices, which offered good spatial replication over a large section of Missouri (see figure 1). Bd was detected in 78 of 793 tadpoles (9.8%), and infection prevalence within a pond ranged from 0% to 89.7%. The average Bd load carried by infected individuals was only 3.93 zoospore equivalents, and the highest Bd load carried by an individual was 41.76

zoospore equivalents. These numbers are significantly lower than areas where Bd has emerged and caused amphibian declines, such as the Sierra Nevada where Bd loads may exceed 10^4 or 10^5 zoospore equivalents (Briggs *et al.* 2010). This suggests that Bd has an endemic presence in the Midwest, similar to its presence in the Northeast and Southeast (Longcore *et al.* 2007; Rothermel *et al.*).

Briggs *et al.* (2010) has parameterized a model showing that Bd loads and inter-pond dynamics can regulate the population level effects of Bd. However, it was not known how inter-pond dynamics might be different in a region that has not experienced amphibian declines. No correlation with disease prevalence was found between host diversity or host density, but mean infection intensity of individuals within a pond did correlate strongly with disease prevalence in the same pond (Part D). These results are supportive of a model of transmission in which amphibians contact free-living zoospores in the aquatic environment (where they can survive for up to six weeks – Johnson and Speare 2003), and then infected amphibians discharge zoospores back into the water through discharge papillae (Berger *et al.* 2005). Thus, transmission is more dependent on the infection intensity of hosts rather than the density of hosts and the probability of a dilution effect is low due to the lack of an obligate vector species. Experimentation is warranted and would be needed to confirm this hypothesis.

Although Bd occurred in approximately one third of our ponds, it was unclear why it existed in some ponds and not others. A GIS-based spatial analysis revealed that isolated ponds and connected ponds were equally likely to contain Bd (Part E). This analysis was conducted at a scale intended to identify spatial patterns of Bd occurrence if Bd were being dispersed via adult amphibians and if Bd were dispersal limited. Our

survey was only conducted during a single summer, and spatial patterns may have become apparent if additional years' data were accumulated; Padgett-Flohr and Hopkins (2010) found that spatial patterns only became apparent after several years of sampling when perennial Bd "hot spots" were identified. In order to determine whether Bd is also spatially structured in our ponds, the temporal turnover of Bd in ponds must be determined.

An alternative way to identify the differences between non-Bd and Bd ponds is to ordinate ponds according to their species diversity (Part F). If Bd is not dispersal limited, then local conditions at ponds may have a greater bearing on whether Bd is able to survive in individual ponds. Non-metric multi dimensional scaling revealed that ponds with Bd were a subset of the ponds without Bd, as characterized by their macroinvertebrate diversity. This suggests that Bd could be responding to some underlying environmental gradients that allow or disallow it, along with numerous other species, to exist in only certain ponds. This intriguing result begs future research attempting to identify the putative environmental factors that may allow or disallow Bd from surviving in a pond.

Many questions have been answered with this observational research, yet many more questions have been raised. For instance, in the Midwest we still do not know how temporally variable Bd's presence is in isolated versus connected ponds. We do not know how Bd is being transmitted between ponds, or even at what spatial scale transmission occurs. Although I have uncovered a proximate difference between Bd and non-Bd ponds, we still do not know the ultimate, underlying cause of this pattern. Future research on Bd in the Midwest should focus and experimentally test some of the ideas

(such as the hypothesized transmission model and the possibility that environmental gradients allow Bd to only exist in certain ponds) that are presented by this observational work.

One theme emerging from the results of all of my research is that Bd appears to be present in an endemic state in the American Midwest. Infection intensity is always low for individuals and no spatial patterns of emergence are detectable (as in Lips *et al.* 2006). We know that Bd has existed here since the 1900s (Ouellet *et al.* 2005), but we don't know exactly when Bd appeared in the Midwest. Additionally, although the global diversity of Bd strains has been reported to be extremely low, indicative of a recently emerged clone, (Morehouse *et al.* 2003), greater diversity was found among Bd strains associated with the American bullfrog (*Rana catesbeiana*), a species endemic to the Midwest (Goka *et al.* 2009). The bullfrog has also been proposed as an international vector species for Bd (Daszak *et al.*2004) in part because of its presence in international trade markets (Schloegel et al 2009), a theory which has been also supported with empirical evidence (Garner *et al.* 2006; Bai *et al.* 2010). It seems possible that if Bd did originate in the American Midwest, that the bullfrog could have facilitated its global emergence. More rigorous genetic analyses would be needed to corroborate the hypothesis that Bd might be endemic, in the broad sense, to the Midwest.

We did not find many bullfrogs in the ponds we visited, because green frogs (*R. clamitans*) tend to outcompete bullfrogs in fishless ponds of the type we sampled (Werner and McPeek 1994). However, bullfrogs are certainly present in the same landscape that we sampled. Since Bd has recently emerged on a global scale, perhaps in part due to the introduction of American bullfrogs from the Midwest into other parts of

the world, understanding the patterns of Bd occurrence in the Midwest could help mitigate the risk posed to amphibians in other parts of the world. For instance, my observational research has suggested that there is a biological difference between ponds that do and do not contain Bd. If the environmental factors underlying this pattern can be identified, then measures may be taken to limit amphibian declines in areas where Bd has emerged.

Chapter 2: Interspecific Variation in Prevalence and Transmission of Bd

A. Specific Questions about Bd in the Midwest

Overview. The results of the observational research on Bd infecting *Rana sp.* anurans that I conducted in the summer of 2009 contributed a novel understanding to the factors that influence Bd's distribution in the American Midwest. For instance, Bd occurs patchily throughout the landscape, with no clear physical correlation with landscape characteristics or pond isolation. However, Bd does occur in a subset of the total available ponds, as characterized by the macroinvertebrate diversity found in those ponds. The difference between Bd and non-Bd communities may be reflective of underlying chemical gradients that allow some species, including Bd, to exist only in certain ponds.

The nature of this observational research left certain questions unexplored and also led to the framing of others. Two surprising results in particular stood out as topics warranting further investigation. This first result was that I was unable to find any relationship between amphibian population characteristics and disease prevalence, either in my tests of amphibian densities and presences (Chapter 1, Part D), or in my ordination of amphibian communities (Chapter 1, Part F). The second surprising result was that I was unable to find any data suggestive of the way Bd is transmitted between ponds (Chapter 1, Part E). In the summer of 2010, I investigated these topics more thoroughly

by testing for Bd in two different species of amphibians: the green frog (*Rana clamitans*), and the gray tree frog (*Hyla versicolor*). This change in methods allowed me to explore the possibility that the host-pathogen dynamics of Bd, a generalist pathogen, might depend on more than just a single host species.

Throughout my 2009 observational work, I only tested for Bd in larval anurans of the genus *Rana*, because these individuals seemed most likely to be infected with Bd. This is because *Rana* served as good candidate species for a Bd reservoir, due to their presence in ponds throughout the winter (see Briggs *et al.* 2005). However, we found nine other amphibian species in our pond sampling survey, and we had learned nothing about infection prevalence in these host species. One of these species, the gray tree frog (*H. versicolor*), is also very common in Eastern Missouri, but unlike, *R. clamitans*, *H. versicolor* does not overwinter in ponds. This life history trait makes *H. versicolor* a poorer candidate species as a Bd reservoir, but other experimental evidence has suggested that a *Hyla sp.* could be more susceptible to Bd infections than a *Rana sp.* (Blaustein *et al.* 2005). In order to determine how similar host-pathogen dynamics were between these two different host species, I compared infection prevalences between *Rana clamitans* and *Hyla versicolor*. I also used this opportunity to examine the effects these species have on each other when they co-occur in a pond where Bd is present.

My GIS analyses suggested that isolated and connected ponds were equally likely to contain Bd. Then, my ordination revealed that only a subset of ponds, as characterized by their macroinvertebrate diversity, seemed capable of supporting Bd. These results suggest that even if Bd is physically able to reach all ponds, the pond it reaches may or may not be environmentally suitable for the pathogen. Although we are beginning to

understand where Bd is most likely to appear, we still know very little about the mechanism by which Bd is moving between ponds. I attempted to quantify the rate of spread of Bd from a focal infected pond by arraying mesocosms at various distances around the pond and testing larval anurans, that had developed in these mesocosms, for Bd. This experimental design assumes that the mesocosms themselves are capable of sustaining Bd, and that Bd is being transmitted at a local scale between ponds.

The species infected in the focal ponds were *R. clamitans*, but the only amphibian species that laid eggs in the mesocosms were *H. versicolor*. *R. clamitans* metamorphs were found in mesocosms at the closest and intermediate distances from the focal pond, although none were found in the most isolated mesocosms. If Bd were being transmitted via adult amphibian dispersal, I would expect the closer mesocosms to yield higher infection prevalence among developing *H. versicolor* tadpoles. However, if Bd were being transmitted via a more widely dispersing species, wind dispersal, or some unknown vector, I might not expect to find a relationship between isolation distance and infection prevalence.

Together, the results of these two smaller projects in 2010 helped me expand my knowledge of Bd to encompass how multiple host species interact to transmit and potentially spread Bd. This knowledge has also helped me refine the interpretation of my large-scale observational studies in 2009.

B. Comparing Disease Dynamics between *Hyla versicolor* and *Rana clamitans.*

Introduction. Little is known about the role Bd plays in amphibian communities. It has been shown that Bd can mediate predator-prey interactions (Parris and Beaudoin 2004) and competitive interactions (Parris and Cornelius 2004) in larval anuran communities, and it has been shown that some species may serve as potential reservoirs for the pathogen (for instance, the American bullfrog; see Daszak *et al.* 2004). Additionally, it is known that species react differently to exposure to Bd when in a laboratory setting (Blaustein *et al.* 2005), however little observational data exists describing infection prevalences between two species inhabiting the same pond. Little field data also exists to document the importance of a reservoir species for maintaining Bd presence in a community of amphibians.

Since Bd is a generalist pathogen, studying disease dynamics requires a community-level approach rather than targeting a single host-pathogen interaction. In the Central American and Australian tropics, Bd has been implicated in declines of numerous co-occurring species (Lips *et al.* 2006; Woodhams and Alford 2005), so understanding interspecific transmission must be necessary to explain the extent of the amphibian epidemic. In these warm climates, a reservoir species for Bd may be a species that is able to sustain an infection without dying (Woodhams and Alford 2005). In colder climates, where ponds freeze during the winter, a more important characteristic of a reservoir species is the ability to remain infected throughout the winter. For instance, the mountain yellow-legged frog (*Rana muscosa*), is a reservoir species in the Sierra Nevada mountains, because even though its adult stage suffers high rates of mortality, its larval stage remains in ponds below the ice throughout the winter (Briggs *et al.* 2005).

Since no amphibians are experiencing Bd-related declines in the Midwest, this provides a good environment in which to examine how life history traits might relate to infection risk, without risking mass mortality. The species *Hyla versicolor* (gray tree frog) and *Rana clamitans* (green frog) are good comparative study species, because they are both very common but vary in important life history traits. *H. versicolor* lay eggs in ponds in May which metamorph by July, and adults spend most of their life away from the water (except during breeding time). *H. versicolor* might be a poor reservoir species for Bd, because they spend little time in the aquatic environment where Bd is thought to be transmitted (Berger *et al.* 2005). On the other hand, once *R. clamitans* lay eggs, they often do not metamorph until over a year later, and even then adults reside in and around ponds. This means that a population of *R. clamitans* resides in the pond continuously, even when the pond surface is frozen in the winter, which is a trait consistent with a potential reservoir species (Briggs *et al.* 2005).

Figure 9. Green frog (Rana clamitans) adult (top left) and tadpoles (bottom left); Gray free frog (Hyla versicolor) adult (top right) and tadpoles (bottom right). Images from USGS.gov , visitingnature.org., and flickr.com.

I hypothesized that infection prevalence in *R. clamitans* tadpoles would be consistently higher than infection prevalence in *H. versicolor* tadpoles, since *R. clamitans* tadpoles have lived in an aquatic habitat and potentially been exposed to zoospores longer than *H. versicolor*. I further hypothesized that due to a reservoir effect, the presence of *R. clamitans* would increase the prevalence of infection among *H. versicolor*, but that the presence of *H. versicolor* would have no effect on the prevalence of infection among *R. clamitans*.

Methods. I visited a replicated set of twelve ponds at Tyson Research Center to obtain and test *H. versicolor* and *R. clamitans* tadpoles. All ponds had been dug by a

group of Tyson researchers during 2007 and 2008 and were approximately five meters in diameter and one meter deep. All ponds were surrounded by an overcanopy of oak-hickory forest and were isolated from the nearest body of water by at least 500 meters. The ponds were stocked with *H. versicolor* and *Acris crepitans* (Blanchard's cricket frog) egg masses in 2008, and have subsequently been colonized by other amphibian species (including *R. clamitans*).

In order to attain samples to be tested for Bd, tadpoles were captured from the ponds, anesthetized using MS-222, and their oral discs were excised and stored in centrifuge tubes with 70% ethanol. From every pond with either *H. versicolor* or *R. clamitans* tadpoles, eight to ten tadpoles were sampled, with priority being given to larger individuals. In ponds with both species, eight to ten tadpoles of each species were taken. The scalpel used to dissect oral discs was dipped in ethanol and flame-sterilized between excisions, and all tadpole bodies were buried in the woods no closer than ten meters away from the pond. Great care was taken to rinse all waders, boots, and nets with dilute bleach solution when moving between ponds, so as to limit the anthropogenic spread of Bd (Schmidt *et al.* 2009).

I performed all assays for the detection of Bd at Dr. Allan Larson's laboratory at Washington University in St. Louis. DNA was extracted from tissue samples from the field using Viogene tissue extraction kits. Each excised oral disc was cut in half, and one half was subjected to the Viogene DNA extraction protocol, which involved digestion by protease K in a lysis buffer in a hot water bath, suspension in ethanol and WS buffer, and centrifugation through spin columns with elute solution. The unused half of the oral disc was returned to its centrifuge tube with ethanol and saved for future use. Proper sterile

technique was used throughout this process, and the scalpel blade used to cut oral discs was dipped in ethanol and flame-sterilized between uses.

The DNA resulting from this extraction process included both amphibian and, potentially, Bd DNA. In order to isolate the fungal DNA, I conducted PCR assays specific for a highly conserved region of the *Batrachochytrium dendrobatidis* genome. I used ITS-1 Chytr3 and 5.8S Chytr primers as described in Boyle *et al.* (2004); however, I used these primers for the standard, not quantitative, PCR assay. This assay allowed me to calculate infection prevalence but not intensity. All of my PCRs were accompanied by two negative controls (one negative extraction control and one negative PCR control) and one positive control. The positive control was obtained from a pond at Tyson Research Center known to have a very high prevalence of Bd.

I loaded my PCR products onto 1.5% agarose gel and conducted a standard PCR assay, using a DNA ladder to identify all bands indicative of the presence of Bd. I constructed three contingency tables in SYSTAT to test whether infection prevalence varied between *H. versicolor* and *R. clamitans*, whether the presence of *H. versicolor* affected infection prevalence in *R. clamitans*, and whether the presence of *R. clamitans* affected infection prevalence in *H. versicolor*.

Results & Discussion. I sampled a total of 148 tadpoles, including 103 *H. versicolor* and 43 *R. clamitans*. All positive controls in the gel electrophoreses tested positive and all negative controls tested negative. *H. versicolor* were found at 11 of 12 ponds and *R. clamitans* were found at 3 of 12 ponds. One of the two species was found at all ponds, and two ponds contained both species. In total, 37 tadpoles tested positive

for Bd and 111 tested negative. The distribution of Bd results according to the species tested and the species communities are listed below in three contingency tables (table 4). A Fisher's Exact Test was used to calculate p values for each contingency table, because all tables included at least one value less than 10. P values can be interpreted as the probability that the proportion of infected individuals was equivalent between groups.

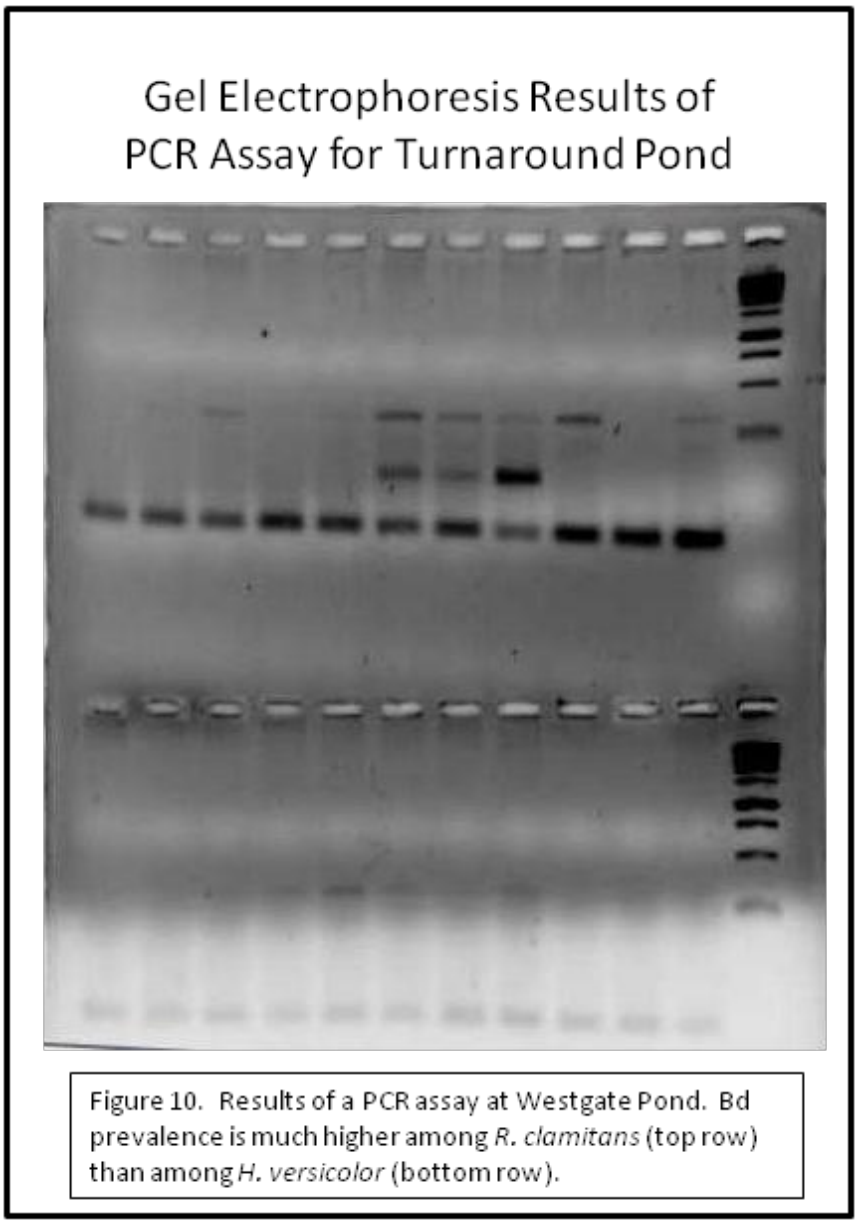

Figure 10. Results of a PCR assay at Westgate Pond. Bd prevalence is much higher among *R. clamitans* (top row) than among *H. versicolor* (bottom row).

When all tadpoles were pooled together, *R. clamitans* were infected more frequently than *H. versicolor* ($p<.0001$). The presence of *H. versicolor* had no significant effect on the infection prevalence of co-occurring *R. clamitans*; however, the presence of *R. clamitans* did significantly increase the infection prevalence of co-occurring *H. versicolor.* For instance, in a pond with both species six of nine *R. clamitans* were infected (top row of wells), but only three of eight *H. versicolor* were infected (bottom row of wells; figure 10). These results are consistent with the hypothesis that *R. clamitans* may be a reservoir species for Bd in the American Midwest.

The most likely reason that *R. clamitans* was able to behave as a reservoir for Bd is that *R. clamitans* tadpoles had overwintered in ponds and therefore were potentially exposed to Bd for a greater total number of days. Furthermore, if these individuals had become infected during the previous summer and retained their infections throughout the winter, they may have been able to transmit Bd to the *H. versicolor* tadpoles which had recently hatched in the pond. This would explain why *H. versicolor* tadpoles had a significantly higher infection prevalence in the presence of *R. clamitans.* The fact that the infection prevalence for *R. clamitans* was unaffected by the presence of *H. versicolor* supports the hypothesis that *H. versicolor* is a poor reservoir species. In order to further support these hypotheses and to determine whether the quality of a reservoir species is related to the length of time its tadpoles spend in ponds, similar pair-wise comparisons should be made between other local amphibian species. This would help to disentangle the effect of having a long larval period from other potentially confounding life history traits.

Contingency Tables

1: Interspecific Variation in Infection Prevalence

Bd Test Result	Number of *H. versicolor*	Number of *R. clamitans*	(row sum)	**Fisher's** exact test
Positive	5	32	37	p<.0001
Negative	98	13	111	
(column sum)	103	45	148	

2: Affect of *R. clamitans* Presence on *H. versicolor* Infections

Bd Test Results	*H. versicolor* with *R. clamitans*	*H. versicolor* without *R. clamitans*	(row sum)	**Fisher's** exact test
Positive	3	2	5	p=.0334
Negative	15	83	98	
(column sum)	18	85	103	

3: Affect of *H. versicolor* Presence on *R. clamitans* Infections

Bd Test Result	*R. clamitans* with *H. versicolor*	*R. clamitans* without *H. versicolor*	(row sum)	**Fisher's** exact test
Positive	25	7	32	p=.332
Negative	10	3	13	
(column sum)	35	10	45	

Table 4. Contingency table and accompanying Fisher's Exact Test results. Overall, *R. clamitans* are more likely to be infected than *H. versicolor*, and H versicolor are more likely to be infected when they occur with *R. clamitans.*

These results highlight the importance of studying the dynamics of multiple host species in the case of a generalist pathogen, and especially studying the community-level properties of disease transmission when multiple host species are present. In this case, *H. versicolor* would experience low infection prevalence if it were not for the presence of a reservoir species. This has a potential impact for areas where Bd is causing rapid and alarming amphibian declines; conservationists may be able to target high risk areas by identifying potential reservoir species and taking extra precautions in order to prevent Bd from invading areas where they are present.

C. Quantifying the Spread of Bd from a Focal Infected Pond

Introduction. Even though Bd has been identified on all six amphibian-inhabited continents in the past ten years, we are no closer to understanding how it moves from place to place. Recent evidence has implicated the American bullfrog (*Rana catesbeiana*) and the African clawed frog (*Xenopus laevis*) in the regional spread of Bd (Garner *et al.* 2006; Bai *et al.* 2010; Solis *et al.* 2010), but we are no closer to understanding the local movement of Bd between ponds. Bd may move in association with resistant amphibians as they disperse (such as the American bullfrog or African clawed frog), or it may rely on other means. It has been suggested that Bd may be transmitted between ponds by birds carrying zoospores in their feathers or animals tracking moist dirt (Johnson and Speare 2005). Although modern protocol emphasizes the washing of waders and nets with diluted bleach solution when moving between ponds (Schmidt *et al.* 2009), it is possible that research scientists or fishermen are responsible

for dispersing Bd between the ponds that they visit (St. Hilaire *et al.* 2009). Although purely speculative, it seems possible that Bd might even have a wind-dispersed life stage that is not yet known to science (see a discussion in Johnson and Speare 2005).

My GIS-based analysis did not detect any spatial structuring in the occurrence of Bd (Chapter 1, Part E). That is, isolated ponds were as likely to contain Bd as connected ponds. This result was surprising, because it suggested that the dispersal of Bd might not be limited by distance. In order to more rigorously test this observation, I designed an experiment to attempt to quantify the spread of Bd from a focal infected pond into surrounding mesocosms. This experiment made no explicit assumptions about the mechanism by which Bd was potentially spreading, but it lent itself to the hypothesis that Bd could be dispersed via adult amphibian dispersal.

Methods. In May 2010 I visited four ponds at Shaw Nature Reserve, forty miles West of St. Louis. Using methods as described in Chapter 2, Part C, I tested larval *R. clamitans* at each of these ponds for Bd. Tissue samples were taken in the field and DNA extractions, PCRs, and gel electrophoreses were conducted in the laboratory. I determined that one pond (hereafter Barn Pond) had a particularly high prevalence of Bd infection among tadpoles (fourteen of seventeen *R. clamitans* were infected, or 82%). Barn pond is a rainwater pond approximately one meter deep and ten meters in diameter, and lies under a canopy of oak-hickory forest. Old fields with sparse trees surround the pond. No larval *H. versicolor* were found in Barn Pond, although this species is known to inhabit the area.

In early June I established three groups of mesocosm tanks at 5, 50, and 100 meters North of Barn Pond in the old field. Each group consisted of seven tanks spaced at one meter intervals and situated in an arc. Each tank was one meter in diameter and was filled with approximately twenty gallons of water. Throughout June, July, and August macroinvertebrate species colonized the mesocosms and *H. versicolor* used the mesocosms to lay their eggs. During this period, nothing was done to interfere with the natural dispersal of species between Barn Pond and the surrounding mesocosms. In late August I selected the largest *H. versicolor* tadpoles from the mesocosms (just prior to their metamorphosis) and tested these individuals for Bd according to the procedure described in Chapter 2, Part C. Eight to ten tadpoles were removed from each mesocosm in which they had developed, and a total of 156 individuals were tested for Bd.

Results & Discussion. Of the 156 tadpoles, I collected 47 from the 5 meter mesocosms, 39 from the 50 meter mesocosms, and 29 from the 100 meter mesocosms. However, zero tadpoles tested positive for Bd. I designed this experiment to be most sensitive to the hypothesis that dispersing amphibians (*R. clamitans*) were capable of spreading Bd. I selected a focal pond with a high prevalence of infection, and I even found dispersing *R. clamitans* metamorphs in mesocosms at the 5 and 50 meter distances in late August. I tested the *H. versicolor* for Bd at the latest possible time, so that they would have a greater probability of being infected from a dispersing *R. clamitans*. Despite all of this, no *H. versicolor* were infected.

This result may be due to a number of reasons. One possibility is that Bd was not able to survive in the mesocosms. This seems plausible given my result from Chapter 1,

Part F which suggested that Bd is only able to persist in a subset of the total available ponds, presumably due to unknown environmental factors. Other possibilities are that *H. versicolor* were not exposed to the potentially infected *R. clamitans* for long enough, that the *R. clamitans* all cleared their infections before leaving Barn Pond, or that H. versicolor simply could not host Bd zoospores in this area. Unfortunately, I was unable to find any *H. versicolor* in Barn Pond itself, so I was unable to determine whether *H. versicolor* became infected as a positive control.

Yet another possibility is that Bd does not move between ponds via adult amphibian dispersal at all. Since very favorable conditions did not lead to the dispersal of Bd in this experiment, some unknown factor may be required to facilitate the spread of Bd. Although alternative hypotheses, for instance Bd being dispersed on the feathers of birds as they fly between ponds (Johnson and Speare 2005), may sound less likely, we do not possess enough data to discount them. In fact, given the lack of empirical evidence for any mode of transmission, all hypotheses seem equally likely. Transmission remains one of the most unexplored and potentially rewarding topics in Bd research.

D. Summary and Discussion of Community-Level Disease Dynamics

Bd seems to be more likely to infect tadpoles of *Rana clamitans*, a species with a long-lived larval stage, than *Hyla versicolor*, a species with a shorter larval stage. This may be because tadpoles of *R. clamitans* spend more time in the aquatic environment, potentially exposing themselves to Bd. A second effect of this longer larval stage is that tadpoles of R. clamitans may serve as Bd reservoirs since they are present in ponds

continuously (Briggs *et al.* 2005). This occurs because newly hatched tadpoles overlap with year-old tadpoles before they metamorph out of the pond. The reservoir effect is shown by Fisher's exact test (table 4, section 2), revealing that *H. versicolor* are more likely to be infected with Bd when they occur with *R. clamitans*. On the other hand, the presence of *H. versicolor* had no impact on the disease prevalence of *R. clamitans*, which might have been expected in light of a dilution effect. However, as per the discussion in Chapter 1, Part G, a dilution effect seems unlikely in the amphibian-Bd system due to the nature of transmission and the lack of an obligate vector species.

Although *R. clamitans* tadpoles seemed to increase the disease prevalence among *H. versicolor* tadpoles, I was unable to find any evidence that dispersing *R. clamitans* metamorphs were capable of increasing disease prevalence among *H. versicolor* tadpoles. This suggests that *R. clamitans* may be a better reservoir species than vector species. This could be for a variety of reasons (discussed above), the most intriguing being that environmental conditions in the mesocosms did not allow Bd to survive in them. Results of my ordinations in Chapter 1, Part F along with my GIS analysis in Chapter 1, Part E suggest that local environmental conditions may be more important in determining where Bd occurs than constraints on the pathogen's dispersal ability.

One of the main differences between Barn Pond and the mesocosms surrounding it was that Barn Pond contained the hypothesized reservoir species *R. clamitans* and the mesocosms did not. However, while it appears that the presence of a reservoir species may increase the probability of disease occurring in other amphibians, it is not enough to guarantee that Bd will survive in that pond (Chapter 1, Part C revealed that of 29 ponds with tadpoles of the genus *Rana*, only 11 tested positive for Bd). Many other differences

exist between Barn Pond and the mesocosms, including size, substrate, depth, basin shape, turbidity, age, and macroinvertebrate diversity. Any one of these or many other differences may have played a role in preventing Bd from infecting *H. versicolor* in the mesocosms.

It appears that Bd does not affect all amphibians equally, and that within an infected pond, amphibian-Bd dynamics may depend primarily on a single host species. In this case, the most important host species was an anuran with a long-lived larval stage. This pattern has been observed before: amphibian species that spend more time in an aquatic environment seem to be at greater risk from Bd (Kriger and Hero 2007). This danger is especially important to recognize in areas where Bd is causing amphibian declines, because some species may be in greater need of protection. If conservationists keep this in mind, then fewer amphibian species may be lost to this emerging disease.

Chapter 3: The Enemy Alliance Hypothesis: A Theoretical Mechanism for the Emergence of a Generalist Pathogen

Abstract.

Invasive species and emergent diseases pose two of the greatest threats to global biodiversity today, but the interaction of the two is rarely considered. I introduce 'The Enemy Alliance Hypothesis' to explain cases of species invasions and disease emergence when the nonindigenous species is also a resistant host of a generalist pathogen. The pathogen facilitates invasion via pathogen-mediated apparent competition and the host facilitates emergence via its self-dispersal. I provide the theoretical framework to explore the role that pathogens may play in species invasions, and show that the most important parameter regulating invasion success is the hosts' relative susceptibilities to the pathogen. I discuss several examples of such enemy alliances that may have contributed to the invasion of nonindigenous species or the emergence of disease. For illustrative purposes, I discuss the role that introduced American bullfrogs (*Rana catesbeiana*) may be playing in the global emergence of amphibian chytridiomycosis.

(Keywords: invasive species, pathogen spillover, apparent competition, chytridiomycosis)

Introduction.

Invasive species and emergent disease pose two of the greatest threats to global biodiversity today (Mack *et al.* 2000; Daszak *et al.* 2000), and as the world's populations

continue to be homogenized by intercontinental travel and trade, this threat can only be expected to grow (Mooney and Cleland 2001). Although the fields of disease ecology and invasion ecology have evolved separately, they share many principles, and in many cases, the micro-organisms causing emergent diseases are invasive species themselves. Since pathogens are generally much smaller than other nonindigenous organisms, they are arbitrarily classified as emergent diseases instead of invasive species. However, the only fundamental difference between invasive species and emergent pathogens is that an emergent pathogen requires competent hosts in order to spread, while an invasive species requires a mechanism to facilitate its invasion.

It is becoming increasingly apparent that pathogens may play significant and diverse roles in species invasions (Prenter *et al.* 2004). One of the most commonly invoked hypotheses explaining species invasions is the 'Enemy Release Hypothesis' (Wolf 2002), whereby nonindigenous species have higher fitness in their non-native range by virtue of leaving their enemies behind (see Keane and Crawley 2002; Mitchell and Power 2003). Here, I invoke a related concept for the invasion success of nonindigenous species—the 'Enemy Alliance Hypothesis' (hereafter abbreviated EAH). The EAH may occur when nonindigenous species do not leave all of their enemies behind, but instead brings at least some of those enemies with them to their non-native range. Those enemies (especially parasites and pathogens), then, have the potential to interact with other (native) species in the invaded range. If, for example, the nonindigenous species has gained some immunity to the parasite/pathogen (or the pathogen has reduced virulence), and native species have less such immunity, it is possible that the nonindigeneous species can gain advantage over native species in their

invaded habitat by virtue of apparent competition (sensu Holt 1977) mediated through their parasities/pathogens.

There are many empirical examples of pathogen-mediated apparent competition occurring in nature which result in a non-native species being able to expand its range or invade native communities (Settle and Wilson 1990; Holdich and Reeve 1991; Schall 1992; Tomkins *et al.* 2001; Tomkins *et al.* 2003; Malmstrom *et al.* 2005; reviewed in Hudson and Greenman 1998). However, no theoretical framework has explained pathogen-mediated apparent competition in the context of invasion ecology (but see Holt *et al.* 2003). Empirical examples may be due to one of two related phenomenon: pathogen spillover (Power and Mitchell 2004) and pathogen spillback (Kelly *et al.* 2009).

Pathogen spillover occurs when a nonindigenous host (often a domestic animal) acts as a reservoir for a nonindigenous pathogen which can then spill over and infect native species. Often, the nonindigenous host and pathogen have shared an evolutionary history together in their native range. Pathogen-mediated apparent competition may occur because the nonindigenous host has evolved resistance to the pathogen in comparison with the naïve and susceptible native host. Pathogen spillback occurs when a nonindigenous host acts as an additional reservoir for a native pathogen. The additional reservoir increases infection prevalence in native hosts, leading to apparent competition. In this case, the nonindigenous host and pathogen have not shared an evolutionary history, and resistance to the pathogen must be due to other physiological reasons.

Both pathogen spillover and pathogen spillback are consistent with the EAH; however, in the case of pathogen spillover, the EAH is an example of invasional meltdown (Simberloff and Von Holle 1999; Simberloff 2006), in which the indirect

effect of one nonnative species (the pathogen) facilitates the invasion of a second nonnative species (the host), and the invasion of the host likewise facilitates the invasion (or emergence) of the pathogen. For the remainder of this paper, the EAH will apply to alliances specifically between two non-native species.

I use a pathogen-resource module (Holt 1977; Holt *et al.* 1994; Holt *et al.* 2003) to demonstrate that how pathogen-mediated apparent competition can be a significant force in invasion biology through the EAH. Specifically, I use standard mathematical and graphical arguments to show the existence of enemy alliances, and explore the parameter space that allows this interaction to occur. Finally, I discuss several potential enemy alliances mediating the invasion of nonindigenous species and facilitating the emergence of diseases in nature.

The Model

My model involves four coupled differential equations describing the change in numbers native hosts (N), invasive hosts (I), pathogens (P), and resources (R). The equations and parameters are listed below.

eq. 1 $$\frac{dN}{dt} = N((\mathrm{R} - \mathrm{I}\alpha_{\mathrm{i}})\alpha_{\mathrm{n}}\beta_{\mathrm{n}} - \mathrm{d}_{\mathrm{n}} - e^{P\mu_n})$$

eq. 2 $$\frac{dI}{dt} = I((\mathrm{R} - \mathrm{N}\alpha_{\mathrm{n}})\alpha_{\mathrm{i}}\beta_{\mathrm{i}} - \mathrm{d}_{\mathrm{i}} - e^{P\mu_i})$$

eq. 3 $$\frac{dP}{dt} = P\left(N\lambda_n + I\lambda_i - d_p\right)$$

eq. 4 $$\frac{dR}{dT} = S - (N\alpha_{\mathrm{n}} + \mathrm{I}\alpha_{\mathrm{i}})$$

Parameter	Meaning
α	species-specific attack rate upon a resource
β	species-specific conversion of a resource into fecundity
d	species-specific death rate
μ	host-specific susceptibility to the pathogen
γ	host-specific growth rate of the pathogen
S	supply rate of a resource

Positive growth rate of hosts are determined by the product of the resource level (R), the host's attack rate upon the resource (α_n) or (α_i), and the host's conversion rate from resource to fertility (β_n) or (β_i). Together, ($\alpha\beta$) determines how efficiently the host is able to reproduce when only R is limiting and is inversely proportional to the host's R*, which represents the minimum amount of resources needed to sustain a positive growth rate (e.g., Tilman 1982, Holt *et al.* 1994). The availability of R is also influenced by exploitative competition between the native and invasive hosts, such that the amount of resources actually available to the native host is $(R - I\alpha_i)$ and $(R - N\alpha_n)$ for the invasive host. Growth rates are limited by background death rates (d) and death due to pathogens ($e^{P\mu}$), where P is the number of pathogens and μ is the host's susceptibility to the pathogen. The pathogen term is exponential, because even under the conditions of high resource availability, a host is unable to sustain a positive growth rate when pathogens are extremely abundant.

For the EAH to work, the native host must be able to utilize resources more efficiently and outcompete the invasive host (i.e., it has a lower R*) in the absence of a pathogen. Specifically, $\alpha_n\beta_n > \alpha_i\beta_i$. However, native hosts must also be more susceptible to the pathogen than invasive hosts, so $\mu_n > \mu_i$. Under these circumstances, when a pathogen is present, the negative effect of $e^{P\mu_n}$ may lower the native host's R* enough that the invasive host can invade via pathogen-mediated apparent competition.

Resources only affect the rate of change of pathogens indirectly through host abundances, and pathogens only are able to exist within a host, so there is no free-living pathogen form and the disease is transmitted instantaneously between hosts.

Transmission between hosts is density dependent, because I assume that hosts encounter each other at a rate proportional to their total abundance. Strict density dependence in the case of multiple host species also implies that both host species will have an equal proportion of infections; this generalist pathogen has no bias between hosts if they are equally abundant.

By setting eq. 1 and eq. 2 equal to zero I obtain the following solutions for P which describe the zero net growth isoclines specific to the native and invasive host in terms of number of pathogens:

eq. 5 $$P = \frac{1}{\mu_n} \ln\left((\mathrm{R} - \mathrm{I}\alpha_\mathrm{i})\alpha_\mathrm{n}\beta_\mathrm{n} - \mathrm{d_n}\right)$$

eq. 6 $$P = \frac{1}{\mu_i} \ln\left((\mathrm{R} - \mathrm{N}\alpha_\mathrm{n})\alpha_\mathrm{i}\beta_\mathrm{i} - \mathrm{d_i}\right)$$

These equations are accompanied by impact vectors (senzu Holt 1977) which describe the impact that the hosts have on pathogens (in the numerator) and pathogens (in the denominator):

eq. 7 $$V_n = \frac{\lambda_n P}{-\alpha_\mathrm{n}\mathrm{R}}$$

eq. 8 $$V_i = \frac{\lambda_i P}{-\alpha_\mathrm{i}\mathrm{R}}$$

Impact vectors can be used to determine when competing species coexist or drive each other extinct (Holt 1977; Holt *et al.* 1994), but in the context of the EAH, coexistence can still lead to invasion and emergence.

By assuming that background death rates are much lower than death rates due to the pathogen, and that resource supply rates are large enough to prevent resources from being limiting, the solution for P becomes simplified and takes the form $P = a\ln(bx)$:

eq. 9 $$P = \frac{1}{\mu}\ln(R\alpha\beta)$$

This form accommodates exploration of conditions which result from varying the parameters μ (pathogen susceptibility) and αβ (efficiency of resource use) between different host species.

By varying the parameters αβ (efficiency of utilizing the resource) and μ (susceptibility to the pathogen), I show that parameter space exists where pathogen-mediated indirect competition allows a host with a lower αβ to outcompete a host with a higher αβ (figure 1). In this figure, the ZNGIs in all three tiles are mathematically graphed according to functions of the form $y = a\ln(bx)$ and are depicted in the same x-y coordinate space.

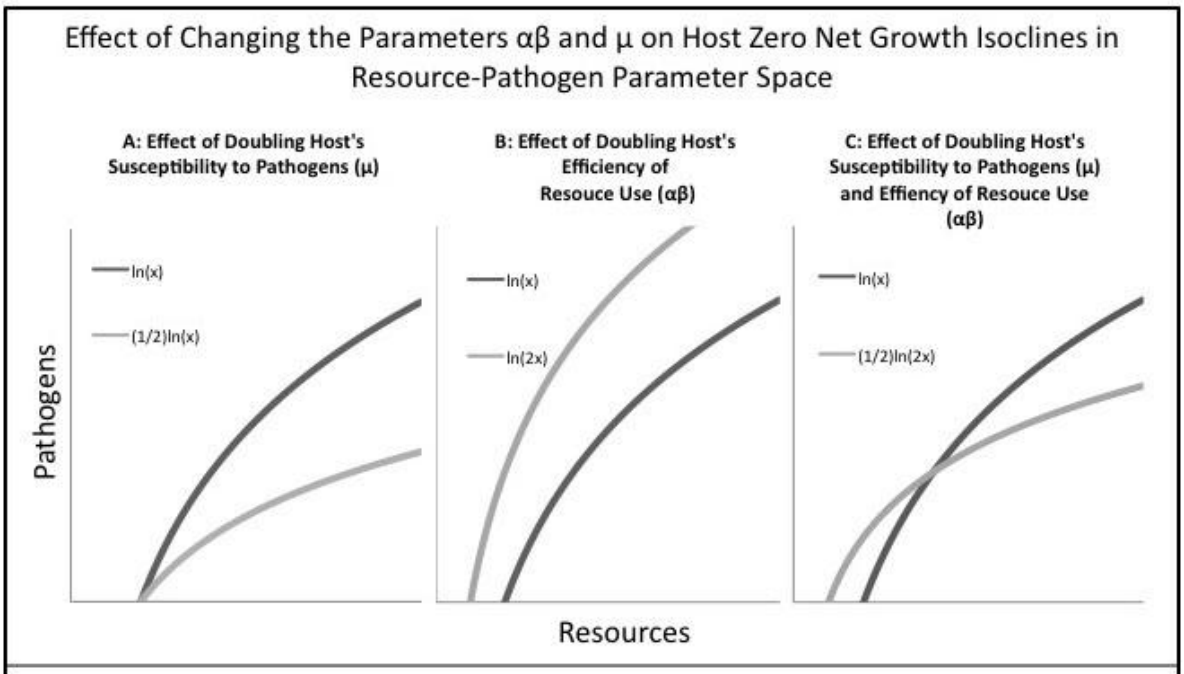

Figure 1. Changing the parameters αβ and μ has qualitative effects on host ZNGIs. In A, doubling μ results in a vertical compression of the ZNGI (light line), allowing the dark line host to dominate. In B, doubling αβ results in a leftward shift of the ZNGI(light line) , allowing the light line host to dominate. In C, doubling αβ and μ (light line) results in a potential coexistence between hosts.

Tile (A) shows the effect of doubling μ for one host. The dark line ZNGI (μ=1) is always above the light line ZNGI (μ=2), so the host with a lower μ will always outcompete the host with a higher μ in the presence of a limiting pathogen, given that a recourse is not limiting. Tile (B) shows the effect of doubling αβ for one host. The light line ZNGI (αβ=2) is always above the dark line ZNGI (αβ=1), so the host with the higher αβ will always outcompete the host with the lower αβ in the presence of a limiting resource, given that a pathogen is not limiting. Tile (C) shows the effect of doubling both μ and αβ for one host. Here, there is a potential equilibrium point where the dark and light ZNGIs cross, and dominance between hosts will depend on the particular point in parameter space where the system lies. This situation models an enemy alliance, where the native host (light ZNGI) has a higher αβ and a higher μ and the invasive host (dark ZNGI) has a lower αβ but also a lower μ.

Tiles (A) and (B) reveal that a host species is the superior competitor (or successful invader if it is nonindigenous) when it has a higher αβ or a lower μ. These results should not be surprising. However, tile C contributes the interesting result that a host is always able to invade when it has a lower μ, even if it also has a proportionally lower αβ. This result suggests that the most important parameter regulating species invasions under these conditions is μ, the host's susceptibility to the pathogen. By plotting the impact vectors (equations 7 and 8) as dotted lines intersecting the ZNGI equilibrium from tile (C), the pathogen-resource space that is susceptible to an enemy alliance becomes apparent (figure 2). In this example, the native host is a better host for the pathogen than the invasive host ($\lambda_n > \lambda_i$), although a similar diagram would result if $\lambda_n < \lambda_i$ or $\lambda_n = \lambda_i$.

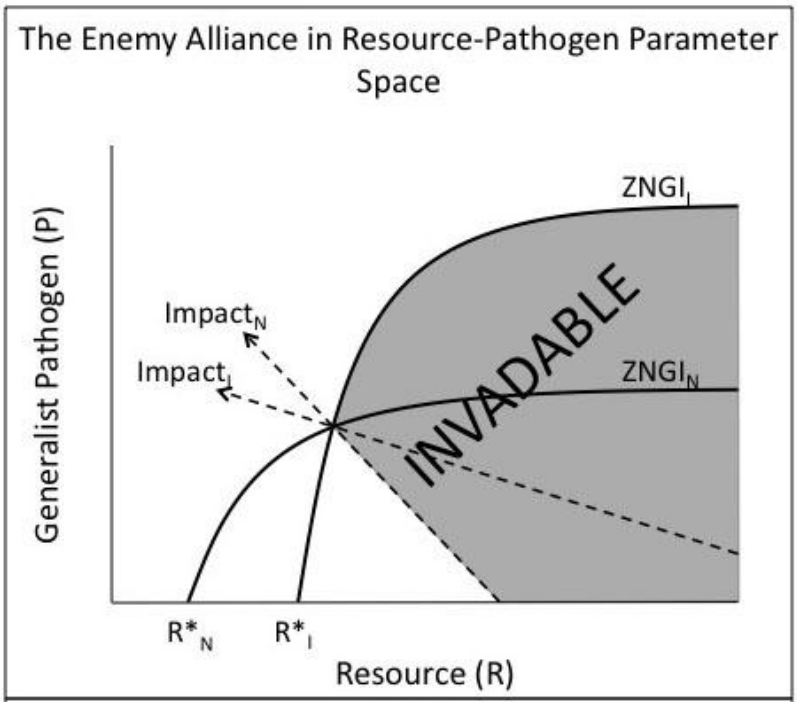

Figure 2. Parameter space showing indirect pathogen-mediated competition between an invasive, tolerant host (I) and a native, naïve host (N). In the presence of the generalist pathogen, the invasive host is able invade over more parameter space.

In figure 2, because the host most limited by the pathogen also makes the greatest contribution to its growth rate, the triangular parameter space under the ZNGIs bounded by the impact vectors indicates where both hosts can coexist. That is, the area is invadable but the invasive host will not drive the native host to extinction. In the area above this triangle but below the ZNGI of the invasive host, the invasive host will both invade and drive the native host to extinction. If $\lambda_n < \lambda_i$, then this same triangular parameter space would result in either the native or invasive host outcompeting the other, and that the outcome of competition would depend on a random walk. Hence, invasion will occur approximately fifty percent of the time. If $\lambda_n = \lambda_i$, there is no such parameter space, and the only invadable space is between the impact vector and the invasive host's ZNGI.

Similar results can be obtained if only α or β alone were manipulated, or if d were manipulated in an inverse direction. Incorporating direct exploitative competition for resources has a similar effect to increasing the death rate when resource levels are low. However, as resource levels increase, both hosts' ZNGIs converge to the same value of P. For this reason, the effect of direct exploitative competition can be ignored when resources are not limiting, and the only competition is mediated by the pathogen. However, changes in μstill always trump changes made to any other parameters.

Discussion.

The EAH can be used to explain both cases of species invasions and disease emergence. While it alone may not determine whether invasion or emergence are successful, it may increase the rate of a biological invasion or the likelihood of disease

emergence. Empirical examples from a variety of host-pathogen systems validate this claim.

One excellent recent example of an enemy alliance is that of the emerging parapox virus in squirrels in the United Kingdom (Tomkins *et al.* 2003). The range of the native red squirrel (*Sciurus vulgaris*) is being invaded by the exotic gray squirrel (*Sciurus carolinensus*), which is native to the United States. The invasive host is a reservoir and carrier of the virus, but is not affected by it directly (it has a low μ). The native host has a higher μ and is likely declining due to the increased transmission rate between red and gray squirrels (Tomkins *et al.* 2003). The parapox virus did not appear to be emergent until invasion of the gray squirrel became a concern.

Another example from the United Kingdom describes a nematode (*Heterakis gallinarum*) mediating competition between native wild grey partridges (*Perdix perdix*) and introduced common pheasants (*Phasianus colchicus*) (Tomkins *et al.*2001). The pheasants were introduced from Eastern Europe, and it is likely that they brought the nematode with them. The native host has a higher μ which results in pathogen-mediated apparent competition leading to a range expansion of the introduced species.

California vineyards, originally home to the congeneric grape leafhopper (*Erythroneura elangantula*) were first invaded by the variegated leafhopper (*Erythroneura variabilis*) in 1980, and the success of this invasion has likely been due to a shared parasitoid (*Anagrus epos*) (Settle and Wilson 1990). The parasitoid is native to the California vineyards and has historically been a cause of death in the native leafhoppers; therefore, this is a case of pathogen spillback, not pathogen spillover. Exploitative competition for resources could not explain the invasion, but the nonnative

host was shown to be less susceptible to the pathogen, and hence, was able to outcompete and invade the native host via pathogen-mediated apparent competition (Settle and Wilson 1990).

An enemy alliance may also be responsible for the recent global-scale emergence of *Batrachochytrium dendrobatidis* (Bd), the causative agent of amphibian chytridiomycosis. Bd was isolated and identified in the late nineties (Longcore *et al.* 1999; Berger *et al.* 1998) and has since been implicated in amphibian declines in Australia (Berger *et al.* 1998), Central America (Lips *et al.* 2004), Europe (Bosch *et al.* 2007), and the Western United States (Vredenberg *et al.* 2010). Bd is thought to have originated in South Africa (Weldon *et al.* 2004), but other evidence suggests that it may be endemic to the American Midwest (Strauss and Smith, unpublished). Since Bd is a microscopic fungi with limited motility in its free-living form (Berger *et al.* 2005), the facilitating mechanism behind its sudden global emergence is unknown.

It is possible that an enemy alliance between Bd and the American bullfrog (*Rana catesbeiana*) may have allowed Bd to rapidly spread throughout the world, thus also benefiting bullfrogs living in their nonnative ranges. The bullfrog, which is endemic to the American Midwest, is an invasive species in many other parts of the world, and frogs infected by Bd may be commonly introduced to the global food market (Schloegel *et al.* 2009). The bullfrog has been shown to be resistant to Bd (Daszak *et al.* 2004) and has recently been found to be a carrier of Bd in Japan (Goka *et al.* 2009), China (Bai *et al.* 2010), and elsewhere in the world (Garner *et al.* 2005). The full extent of the emergence of this disease may not yet have been realized.

These four examples demonstrate that enemy alliances exist in nature and that hosts can span a great diversity of organisms (mammals, birds, insects, and amphibians) as can the pathogens (viruses, worms, parasitoids, and fungi). Enemy alliances may provide a greater benefit to the invading species (the variegated leafhopper) or the emerging disease (Bd); or, both enemies may benefit equally (parapox and the grey squirrel). The Enemy Alliance Hypothesis model presented in this paper can be used as a theoretical framework necessary to understand future invasions and emergence that could not otherwise have been predicted.

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