

FINAL REPORT

Effects of Climate on Host-pathogen Interactions in Chytridiomycosis

SERDP Project RC-2638

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Corinne Richards-Zawacki
University of Pittsburgh

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List of Acronyms

AIC	Akaike's information criterion
AMP(s)	Antimicrobial peptide(s)
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
<i>Bd</i>	<i>Batrachochytrium dendrobatidis</i>
<i>Bsal</i>	<i>Batrachochytrium salamandrivorans</i>
CV	Coefficient of variation
DAPC	Discriminant analysis of principle components
DD	Data deficient (IUCN risk category)
DNA	Deoxyribonucleic acid
DoD	Department of Defense
ELISA	Enzyme linked immunosorbent assay
EN	Endangered (IUCN risk category)
EX	Extinct (IUCN risk category)
GAM	Generalized additive model
GAMM	Generalized additive mixed model
GLM	Generalized linear model
GLMM	Generalized linear mixed effects model
GPL	Global pandemic lineage (of <i>Bd</i>)
HadGEM2-ES	Met Office Hadley Centre Earth Systems Model
HYVE	<i>Hyla versicolor</i>
IC50	50% inhibitory concentration
IPM	Integral projection model
ITS	Internal transcribed spacer
IUCN	International Union for Conservation of Nature
LA	Louisiana
LC	Least concern (IUCN risk category)
LIBL	<i>Rana [Lithobates] blairi</i>
LICH	<i>Rana [Lithobates] chiricahuensis</i>
LICL	<i>Rana [Lithobates] clamitans</i>
LIPI	<i>Rana [Lithobates] pipiens</i>
LISP	<i>Rana [Lithobates] sphenoccephala</i>
LM	Linear model
LME	Linear mixed effects model
LRT	Likelihood ratio test
MALDI-TOF	Matrix assisted laser desorption/ionization time of flight mass spectrometry
MIC	Minimum inhibitory concentration
N	Number (as in sample size)
NM	New Mexico
OD	Optical density

OH	Ohio
(s)OTU	(sub) Operational taxonomic unit
PA	Pennsylvania
PERMANOVA	Permutational analysis of variance
PCR	Polymerase chain reaction
PCoA	Principal coordinates analysis of microbiomes
PHA	Phytohaemagglutinin
qPCR	Quantitative polymerase chain reaction
RCP	Representative concentration pathway
rRNA	Ribosomal ribonucleic acid
SE	Standard error
SNP	Single nucleotide polymorphism
SON	Statement of need
SVL	Snout-vent length
T _{max}	Thermal maximum
T _{min}	Thermal minimum
TN	Tennessee
U. S.	United States
VU	Vulnerable (IUCN risk category)
VT	Vermont
ZE	Zoospore equivalents

Keywords

Wildlife disease, climate change, ecoimmunology, immune defense, chytrid fungus, pathogenicity, epizootic

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Abstract

Introduction and Objectives

Clarifying the links between climate and host-pathogen interactions will provide valuable insights into the dynamics, ecology and evolution of infectious diseases and provide direction for effective management of threatened populations. Chytridiomycosis, a disease caused by *Batrachochytrium* fungi, has recently caused declines and extinctions of amphibian populations on several continents. Our central objective was to develop predictive models of chytridiomycosis, based on a clear understanding of how climate impacts the ecology and dynamics of this host-pathogen interaction, and use these models to investigate the potential of alternative disease management tactics.

Technical Approach

Our technical approach included (Objective I) field surveys to investigate how climate impacts the ecology of chytridiomycosis, (Objective II) field and lab experiments to assess the impacts of current and future climates on amphibian-*Batrachochytrium* dynamics, and (Objective III) the development of data-driven mechanistic models to evaluate how climate affects the host-pathogen interaction. Our models will permit us to work with wildlife management personnel to address (1) where and when outbreaks of chytridiomycosis are likely to threaten amphibians on DoD installations and (2) the effectiveness of alternative management strategies for sustaining threatened amphibian populations through times of increased disease risk.

Results

We detected *Batrachochytrium dendrobatidis* (*Bd*) at all our field sites and in nearly every amphibian species sampled but we did not detect *B. salamandrivorans* (*Bsal*). We found strong patterns of seasonality in *Bd* prevalence and load in amphibians as well as seasonal variation in these animals' defenses against *Bd*. The *Bd* we detected on field-captured amphibians is strikingly diverse and belongs to two sublineages of the global pandemic lineage (*Bd*-GPL). Our experimental results suggest that *Bd* may be able to adapt to new climates and that developing as larvae under climate stress can impair a host's ability to defend itself against *Bd*. Our modeling efforts suggest that management actions should focus on identifying reservoir host species and reducing *Bd* persistence in aquatic habitats. Our models also suggest that efforts aimed at reducing the potential of particular host species to act as pathogen reservoirs, and specifically efforts that cause the stochastic extinction of *Bd* from an aquatic breeding habitat, may be an effective way to prevent outbreaks of chytridiomycosis on DoD lands as the climate changes.

Benefits

Benefits of our work to the DoD include a clearer understanding of (1) the geographic, genetic, and host distribution of the *Bd* and *Bsal* pathogens, (2) drivers of seasonal disease dynamics, and (3) impacts of climate change on disease-relevant host-and pathogen physiology in the chytridiomycosis system. The models we have developed will also aid in determining which hosts are potential reservoirs for *Batrachochytrium* pathogens and where, when, and how management actions can be taken to reduce *Bd* persistence and chytridiomycosis risk for threatened and endangered amphibian species. Given the potential impact of climate change on disease, studies of this nature will be critical in developing strategies to promote the long-term health of threatened amphibians and other wildlife on Department of Defense (DoD) lands.

Executive Summary

Introduction

Discovering the mechanisms that link climate to patterns of infectious disease will be crucial in this time of climate change. Temperature appears to be a key factor, especially for ectothermic ('cold blooded') animals where ambient conditions constrain the physiological responses of both host and pathogen (Blanford & Thomas 1999). Fungal pathogens are especially sensitive to temperature and increasingly recognized as drivers of wildlife declines and extinctions (Casadevall 2005, Fisher *et al.* 2012, Fisher and Garner 2020). Recent examples of amphibian chytridiomycosis (Kilpatrick *et al.* 2010) and white nose syndrome in bats (Foley *et al.* 2011) underscore the need to understand the mechanisms by which climate affects fungal virulence. Resolving the links between climate and the outcomes of host-fungal interactions will provide valuable insights into the dynamics, ecology and evolution of infectious diseases and provide direction for effective management of threatened populations on DoD lands.

Declines and extinctions of as many as 500 amphibian species have been linked to fungi in the genus *Batrachochytrium* (Martel *et al.* 2014, Scheele *et al.* 2019). *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*) are water-borne chytrid fungi that infect amphibian skin causing the disease chytridiomycosis (Longcore *et al.* 1999, Pessier *et al.* 1999, Martel *et al.* 2013). *Bd* (first described in 1999) has caused declines in amphibians around the world (Longcore *et al.* 1999, Fisher *et al.* 2012, Fisher and Garner 2020). *Bsal* (first described in 2013) has caused declines of European salamanders (Martel *et al.* 2013, 2014). In the U.S., field studies have confirmed that *Bd* is widespread, yet it has been associated with die-offs only in certain areas and hosts (Carey *et al.* 1999, Ouellet *et al.* 2005, Vredenburg *et al.* 2010). For example, *Rana muscosa* was driven to near extinction by *Bd* in the Sierra Nevada Mountains (Briggs *et al.* 2010), and *Bufo boreas* has been impacted in the Rocky Mountains (Pilliod *et al.* 2010). At the start of this project, *Bsal* had not yet been detected in the U. S., though sampling had only recently begun. The U.S. is a hotspot for salamander diversity, so the introduction of *Bsal* to the U.S. would threaten global amphibian diversity.

Batrachochytrium fungi colonize skin cells of adult amphibians and *Bd* also colonizes the mouthparts of tadpoles. Infection occurs when a motile aquatic zoospore encysts in amphibian skin (Berger *et al.* 1998, Longcore *et al.* 1999). There the fungus grows and develops into a zoosporangium, in which new infectious zoospores develop (Berger *et al.* 2005). These new zoospores later emerge and land either onto the surface of the host to re-infect the individual or they move away from the host and into an aquatic environment (Berger *et al.* 1998, Longcore *et al.* 1999). *Batrachochytrium* can be transmitted by contact with infected hosts or via contaminated water or substrate (Rachowicz & Vredenburg 2004). *Bd* causes death by interfering with the essential ion transport functions of the skin (Voyles *et al.* 2007, 2009, 2012). The damage to the most superficial layers of epidermis is life-threatening because amphibians rely on their skin for osmoregulation and exchange of respiratory gases.

At the start of this project, a growing body of evidence already suggested that climate strongly affects the host-*Batrachochytrium* interaction. In culture, these fungi show reduced growth and mortality at high temperatures (Piotrowski *et al.* 2004, Woodhams *et al.* 2008, Martel *et al.* 2013), and survival of exposed amphibians is often greater at higher temperatures (Berger *et al.* 2004, Andre *et al.* 2008, Sonn *et al.* 2017). In nature, disease prevalence and severity tend to increase during cooler months and declines due to *Bd* have been largely limited to areas with at least seasonally cool climates (Berger *et al.* 2004, Kriger *et al.* 2007, Olson *et al.* 2013). These findings suggest that an increase in mean temperature, as is predicted with global climate change,

may be favorable to amphibians, decreasing the likelihood of outbreaks and declines. However, climate change is also expected to affect the variability of temperature and precipitation, setting the stage for different, and likely complex, host-pathogen dynamics under future climate scenarios (Schar *et al.* 2004, Pounds *et al.* 2006, Yeh *et al.* 2009).

While climate appears to play a key role in *Bd* epidemics, prior to this project it was unclear whether this was due to temperature effects on host immune function, the pathophysiology of *Bd*, or both (Raffel *et al.* 2011). Amphibians have a suite of immune defenses against fungal pathogens. Antimicrobial peptides (AMPs) secreted in skin mucus provide a barrier defense (Rollins-Smith *et al.* 2005, 2011). Mucus also contains lysozyme (Zhao *et al.* 2006, Savage *et al.* 2014), antibodies (Ramsey *et al.* 2010), and antifungal products of symbiotic bacteria. Thus, a variety of defenses can help to prevent infection in an individual host. Immunity in ectotherms is strongly affected by temperature (Rollins-Smith *et al.* 2011, Rollins-Smith 2020), though the degree to which climate-induced immunosuppression affects the likelihood or outcome of the host-*Batrachochytrium* interaction has been understudied. In addition to direct effects on the elements of the mature amphibian immune system, changes in climate may affect the development of immune defenses in earlier life stages in ways that weaken the host's ability to resist infection later in life. Recent metamorphs can be especially vulnerable to chytridiomycosis, in part because their immune systems are often immature at this life stage (Rachowicz & Vredenburg 2004, Tobler & Schmidt 2010, Walker *et al.* 2010).

In addition to affecting host defenses, temperature strongly influences the virulence characteristics of fungal pathogens. *Bd* strains differ markedly in virulence (Berger *et al.* 2005, Fisher *et al.* 2009, Farrer *et al.* 2011) and in their responses to a range of temperatures (Stevenson *et al.* 2013). Yet prior to this project, we knew little about how differential responses to temperature could influence the mechanisms of virulence among *Bd* isolates. The distribution and diversity of *Bd* lineages within the United States was also poorly known prior to the start of this project. Finally, it is likely that climate change will exert selective pressure on *Batrachochytrium*, with the potential to broaden the range of temperatures at which these fungi can spread and be pathogenic. Fungi are known to adapt to new thermal conditions (Feller & Gerday 2003) and thermotolerance of fungal pathogens is a topic of intense research (Caspeta *et al.* 2014). Clarifying the adaptive responses of *Batrachochytrium* to changes in temperature will be important when forecasting chytridiomycosis under future climate scenarios.

This project aimed to improve our understanding of the ecological factors that impact host and pathogen interactions in chytridiomycosis, an emerging disease that threatens the health of amphibians on DoD lands. We focused specifically on climate and undertook field and laboratory studies to clarify how climate affects the physiology and life history of both host and pathogen and how this impacts disease development and transmission. We integrated our empirical data on host-pathogen-climate relationships with modeling approaches to project changes in exposure pathways and disease severity under future climate conditions and to investigate the potential impacts of alternative wildlife management scenarios. These models are an important step toward developing adaptive management strategies to control fungal pathogens and sustain at-risk amphibian species on DoD lands.

Objectives

The objectives of this project were designed to meet three research needs of the SERDP program. In the following paragraphs we describe how we addressed each research need using a combination of field surveys, experiments, and modeling.

Ecology of Chytridiomycosis - To address *research need #1: ecology of potential pathogens and their vectors of concern on DoD lands as this relates to wildlife exposure*, we (1) conducted a non-invasive multi-site evaluation of seasonal patterns of *Bd* prevalence, pathogen loads, and anti-*Bd* immune defenses, (2) examined how variation among *Bd* strains (e.g., in thermotolerance, life cycle, and lineage) affects host/pathogen dynamics, and (3) screened amphibian populations for *Bsal*, which has yet to be detected in the U.S. but has caused die offs in Europe.

Impacts of Current and Future Climate Variation on Amphibian-Batrachochytrium Dynamics - To address *research need #2: plausible scenarios of future change that may impact exposure pathways for wildlife and disease severity*, we asked whether warmer and more variable weather, as predicted under global climate change, will increase the potential for transmission and disease development by investigating: (1) how the development and expression of host anti-fungal defenses, including innate, adaptive, and microbial skin defenses, are affected by changes in climate, (2) how climate will affect *Batrachochytrium* virulence and the potential for transmission, including the potential for pathogen niche evolution, and (3) how community structure, including shifts in host niches/ranges under future climate scenarios, might affect the dynamics and spread of chytridiomycosis.

Modeling the Distribution and Severity of Chytridiomycosis Risk on DoD Lands Under Alternative Climate and Management Scenarios - To address *research need #3: models that can project how exposure pathways and disease severity may change in the future and their implications for wildlife management*, we are developing data-driven, mechanistic models that use our empirically derived relationships between climate variables and vital rates for amphibians and the *Batrachochytrium* species/strains found at our field sites (from Objectives 1 and 2) to evaluate: (1) how changes in climate will affect the host/pathogen interaction, (2) where and when chytridiomycosis outbreaks are likely to threaten amphibians on DoD installations, and (3) the effectiveness of alternative management strategies for sustaining at-risk amphibian populations through times of increased disease risk.

Technical Approach

Objective 1. Ecology of Chytridiomycosis - To investigate links between pathogen presence, disease, and climate, we sampled amphibian communities for *Batrachochytrium* in five regions of the United States (Figure 1). We chose these locations because they represent a broad range of climates and contain our focal amphibian hosts in the leopard frog species complex. We chose leopard frogs because many species within this complex are susceptible to chytridiomycosis, several are declining in relation to historical numbers (e.g., *R. pipiens*, *R. sphenoccephala*, *R. yavapiensis*), some are threatened (*R. chiricahuensis*, *R. onca*), and one species is thought to be extinct (*R. fisheri*) (IUCN 2018). At each site we selected a set of wetlands where we tested for *Bd* and *Bsal* presence and intensity on amphibians (via skin swabs, see details below) with a quantitative real-time PCR assay (Hyatt *et al.* 2007). We sampled during the active seasons for amphibians to identify seasonal patterns of *Batrachochytrium* presence, prevalence, infection intensity and host immune response.

We conducted acoustic and visual encounter surveys during the time periods when amphibians are active at each of the respective field sites. Because amphibian susceptibility to chytridiomycosis is highly dependent on environmental conditions, we measured relevant

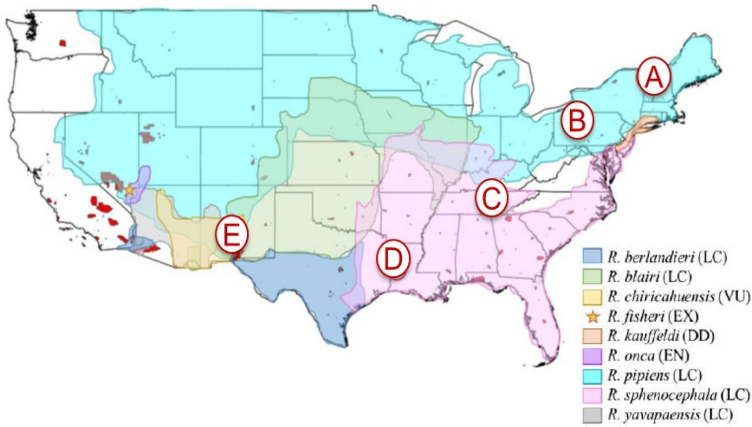


Figure 1. Map of the United States showing (1) distributions of species in the leopard frog species complex (shaded according to key), (2) locations DoD installations (red shading) and (3) field sites: (A) Camp Ethan Allen, VT; (B) Pymatuning Lab of Ecology, PA; (C) Arnold Air Force Base, TN; (D) Fort Polk, LA; (E) White Sands Missile Range, NM). Two letter abbreviations after species names indicate IUCN status (DD = data deficient, LC = least concern, VU = vulnerable, EN = endangered, EX = extinct).

variables (e.g., site elevation, habitat type, water temperature, turbidity and pH) and obtained climate and weather information from weather stations placed in or near each site. We also measured body and microhabitat temperatures for each amphibian captured. Specifically, once an amphibian was sighted, and before the amphibian was captured, we used non-contact infrared thermometers to measure the body and microhabitat temperatures. These devices are a non-invasive way to obtain accurate body temperature information (Rowley & Alford 2007).

During each sampling session, we captured amphibians (all species encountered), with strict adherence to field hygiene protocols to reduce pathogen transmission (Phillot *et al.* 2010). Once an amphibian was captured, we collected morphological data including, snout-to-vent length (SVL), mass, and sex, and noted life-stage and/or reproductive status (e.g., gravid females). We collected skin swab samples using a non-invasive, standardized protocol that involves rubbing a sterile swab across the ventral skin on the abdominal and inguinal surfaces, and on the digits of the hands and feet (Hyatt *et al.* 2007). Next, we extracted genomic DNA from the skin swab samples and determined the prevalence and intensity of infection of *Bd* and *Bsal* using an established quantitative real time Polymerase Chain Reaction (qPCR) assay (Boyle *et al.* 2004). For a subset of the DNA extracts that tested positive for *Bd* we conducted a custom genetic assay to identify the sample to strain (Byrne *et al.* 2017).

We isolated and cryopreserved *Batrachochytrium* pathogens encountered near our field sites using standard microbiological methods (Longcore *et al.* 1999; Cook *et al.* 2018). The process involved collecting a small tissue sample from an amphibian (skin from adults or mouthparts from tadpoles) and purifying using a streaking method on agar plates with antibiotics (Cook *et al.* 2018). Once we purified *Bd*, we maintained cultures either in liquid medium or on agar plates with a tryptone medium (Longcore *et al.* 1999). We cryo-archived all isolates for long term storage and to eliminate the risk of pathogen attenuation. This allowed us to recover (i.e., revive) isolates for *in vitro* experiments (described below) without problems associated with laboratory maintenance (Boyle *et al.* 2003). We also used a custom genetic assay (Byrne *et al.* 2017) to reveal the genetic background of all isolates we used in our experiments.

To examine the links between climate and host defenses, we sampled and quantified anti-*Batrachochytrium* host defenses. To do this, we collected mucus from leopard frogs across seasons and field sites and assayed their “mucosome”, which is defined as the combined microbial and host factors in the mucus, including secondary metabolites, AMPs, lysozymes, mucosal antibodies, and alkaloids. We collected frog mucus using skin rinses and tested for anti-

Batrachochytrium properties using *in vitro* assays (Cell-titer Glo 2.0) to measure *Bd* viability. After swabbing for *Bd* load, frogs were soaked in 15 mL sterile artificial pond water for one hour to collect mucosal secretions, and then washes were stored frozen at -20°C. We examined zoospore shedding rates from the same samples by qPCR. Our previous work has shown this sort of mucosome assay to (1) detect variation in immune function against *Bd* under varying environmental conditions, and (2) predict disease risk (Woodhams *et al.* 2014).

In addition to whole mucosome assays, we separately quantified antimicrobial peptide and skin microbe activity against *Bd*. We enriched mucus samples for hydrophobic peptides by passing the skin washes over C18 Sep-Paks and identified secreted AMPs using mass spectrometry. We measured the activity of peptides against *Bd* using *in vitro* growth inhibition assays (Rollins-Smith *et al.* 2006). We examined the microbial communities in mucus samples using Illumina MiSeq sequencing, targeting the 16S rRNA gene (bacteria) or ITS gene (fungi), and correlating skin community structure with mucosome function against *Batrachochytrium* (e.g., Becker *et al.* 2014, Loudon *et al.* 2014). We also tested bacteria and fungi isolated from leopard frog skin individually for their capacity to inhibit *Batrachochytrium* growth. Inhibitory microbes were identified by Sanger sequencing of the 16s rRNA gene (bacteria) or ITS gene (fungi) and added to a global database of amphibian skin microbiota (Woodhams *et al.* 2015).

Objective 2. Impacts of Current and Future Climates on Amphibian-Bd Dynamics -

We examined the effect of climate on amphibian immune defenses and *Batrachochytrium* virulence using lab and field experiments. To investigate how climate impacts the development of anti-*Batrachochytrium* defenses, we raised leopard frogs from eggs under different climate scenarios. To do this we placed mesocosms both outdoors (at our field sites) and in indoor environmental chambers (where we could simulate future temperatures). We also investigated how climate impacts *Batrachochytrium* virulence, and the potential for pathogen niche evolution using laboratory *in vitro* experiments conducted under a variety of climate scenarios. Here we report the findings from three outdoor mesocosm experiments to test 1) the effect of shortened hydroperiod on the development of pathogen defenses in five populations of three species, 2) for local adaptation of two populations to environmental conditions, and 3) how amphibian community structure affects *Bd* transmission dynamics and microbiome composition. We also report the findings from one indoor experiment in which we simulated an increase in temperature due to global warming on frogs from four populations of two species during larval development and measured the effects of this change in climate on fitness, focusing on immune development and susceptibility to chytridiomycosis. Finally, we report results from an artificial evolution experiment aimed at understanding how the *Bd* pathogen can respond, in terms of its range of thermal tolerance and virulence traits, to a warmer climate.

Objective 3. Modeling Disease Risk Under Alternative Climate and Management

Scenarios - We used two approaches to develop and parameterize data-driven, mechanistic models of the impacts of climate on disease dynamics at the individual and community levels. The first approach involves using the detailed experiments in Objectives 1 and 2 to determine the appropriate forms of each of the functions in the models, and to determine how temperature affects the model parameters. We have termed this approach *Experiment-based models*. Our work on this approach is still in progress, because due to shutdowns resulting from the COVID-19 pandemic, the samples and data from the experiments are still being processed and analyzed. In the meantime, we have made substantial progress on our second approach for modeling this system, which involves fitting Integral Projection Models (IPMs) to the patterns of prevalence

and *Bd* load through time from the field survey data from each of the five regions. We have termed this approach *Field survey-based models*.

Our *field survey-based models* fit flexible mechanistic models directly to patterns of infection from the field survey data for each of our field sites. This approach has the advantage of parameterizing the models for the populations under natural field conditions (including temperature variation), and in the presence of the entire amphibian host community. To do this, we used host-pathogen IPMs (Wilber *et al.* 2016) that explicitly account for *Bd* infection intensity and the distribution of *Bd* loads in the population of each host species. We have used these models to explore how individual-level heterogeneity in pathogen load, a nearly ubiquitous characteristic of host-parasite interactions that is rarely considered in models of microparasites, generally reduces pathogen invasion probability and dampens virulence-transmission trade-offs in host-parasite systems. We have also used this modeling approach to (1) disentangle the relative contribution of seasonality in host demography (e.g., seasonal reproduction and breeding aggregations) and temperature-mediated seasonality in host and pathogen vital rates (e.g., pathogen survival or host immunity) on seasonal patterns of *Bd* infection, and (2) ask how seasonal variation in host maintenance potential affects the long-term persistence of *Bd*.

Our next steps in the modeling process are to (1) incorporate the rest of the amphibian community into the IPMs to investigate how climate and amphibian community composition may interact to determine the potential for chytridiomycosis outbreaks; (2) use these community-level models as a framework for investigating the efficacy of alternative management strategies to mitigate the effects of chytridiomycosis on threatened amphibian species.

Results and Discussion

Objective 1. Ecology of Chytridiomycosis - We monitored wetlands at each of our field sites (Table 1), allowing for the comparison of amphibian community composition and pathogen dynamics over time. Over three years, we sampled over 14,000 amphibians across five sites for *Batrachochytrium* pathogens. This includes 30 frog and toad species (anurans) and 20 salamander species (urodeles). In addition, we sampled 2,303 leopard frogs (*Rana pipiens* in VT and PA, *R. sphenoccephala* in TN and LA, and *R. chiricahuensis* and *R. blairi* in NM) for both seasonal pathogen loads and measures of immune function.

Table 1. Number of monitored wetlands at each of our field sites, as well as the number of species and total amphibians sampled.

Field site	Wetlands monitored (N)	Species sampled (N)		Total amphibians (N)	Leopard frogs (N)
		Frogs & toads	Salamanders		
Vermont	6	8	4	963	132
Pennsylvania	7	7	10	5338	435
Tennessee	5	13	9	2772	591
Louisiana	5	14	7	2685	425
New Mexico	4	9	2	1115	720

Bd infection prevalence varies seasonally across the entire amphibian community in PA, TN, and LA. In general, *Bd* prevalence peaks in the winter and spring, and is lowest in the summer months. Community-wide infection prevalence is less seasonal in VT and NM, with prevalence remaining relatively high (~50%) and low (~10%), respectively, year-round. While prevalence was not seasonal across the entire amphibian community in NM, there was extreme seasonality in infection intensity, with short bouts of very high infection loads in the

winter months. This appeared to be primarily driven by leopard frog species in the amphibian community. Importantly, we found no evidence of *Bsal* at our field sites.

Bd infection prevalence was associated with several environmental variables. When accounting for seasonality, body temperature predicted infection prevalence, with prevalence decreasing at body temperatures below 5°C and above 25°C, mirroring the thermal performance limits for *Bd* growth. In addition, water temperatures one month previously predicted infection prevalence at the sampling site level, with temperatures lower than 15°C resulting in higher prevalence overall. There was also an interaction between canopy cover and water temperature, in which there was low *Bd* prevalence in amphibian communities when canopy cover was low and temperatures were either high (> 30°C) or low (< 5°C).

We were able to genotype the *Bd* from skin swabs from 359 infected amphibians. The *Bd* in all our samples fell within the Global Panzootic Lineage of *Bd* (*Bd*-GPL). We found two major clusters among our samples, corresponding to previously published subclades GPL-1 and GPL-2 (Schloegel *et al.* 2012). Generally, GPL-1 is described as a North American lineage and is proposed to be ancestral to GPL-2, which is globally distributed and is the lineage responsible for declines in Central America (James *et al.* 2015). In our data, this split between GPL-1 and GPL-2 does not seem to correspond to site, as both clades and many ‘mixed’ phenotypes are represented in samples collected from VT, PA, and LA, and the proportion of each lineage at each site does not change between sampling years.

We discovered clear patterns in the microbiomes on amphibian skin including differences among host species and seasons and shifts in the microbiome corresponding to *Bd* infection status. Bacterial communities changed across years and varied across sample locations for each season. The percent anti-*Bd* function of the microbiome differed between sample seasons across years, and with *Bd* infection, but did not correlate with *Bd* loads on individual amphibian hosts. The proportion of bacteria that can function to inhibit *Bd* growth was greatest when risk of *Bd* infection was highest.

When we analyzed the peptide defenses on the skin of leopard frogs we also found seasonal patterns, suggesting that similar to other aspects of immunity, these defenses may be entrained to seasonal temperature and day length cues. Across all locations, greater stored antimicrobial peptide (AMP) defenses were typically observed around the peak in *Bd* prevalence in leopard frogs. Specifically, northern (*R. pipiens*) and southern (*R. sphenocephala*) leopard frogs exhibited the peak in potential *Bd* defenses and infection risk during cooler seasons, while the endangered Chiricahua leopard frog (*R. chiricahuensis*) uniquely stored greater amounts of peptide defenses during warmer months when infection risk was lowest. Peptide defenses of young Chiricahua leopard frogs decreased with *Bd* infection. Similarly, infected frogs in TN had lower inhibitory capacities of secretions than uninfected frogs, despite similar peptide amounts.

Objective 2. Impacts of Current and Future Climates on Amphibian-Batrachochytrium Dynamics - Our experiments, which involved leopard frog larvae developing under an array of climate stressors (pond drying, elevated temperature, non-local climates, and altered amphibian community structure), showed that early development under conditions simulating climate change can have negative impacts on the fitness and ability to fight infection for amphibians later in life. In our pond drying experiment, only *R. sphenocephala* from LA showed accelerated development but animals from other leopard frog species (*R. pipiens*) and populations showed evidence of reduced fitness due to developmental stress, including smaller mass at metamorphosis and increased larval and juvenile mortality. In *R. sphenocephala* we saw carry-over effects of drying, including more severe infections when these animals were exposed to *Bd*

after metamorphosis. The general lack of developmental responses to drying, reduced survival in northern leopard frogs, and greater disease susceptibility in southern leopard frogs suggest that more variable hydroperiods may lead to population-level consequences.

In our reciprocal transplant experiment, in which *R. sphenoccephala* larvae from TN and LA were reared in both locations (i.e., both local and non-local climates), we saw evidence of costs associated with developing in foreign climates. The LA animals raised in TN were less likely to survive to metamorphosis and TN animals raised in LA were more likely to metamorphose with severe deformities (missing and non-functional limbs). After metamorphosis, growth rates were developmentally plastic, allowing animals to compensate for stressful developmental conditions and small sizes at metamorphosis. However, measures of immune function were less plastic and varied depending on population of origin, regardless of developmental and post-metamorphic environment. For frogs exposed to *Bd* post-metamorphosis, probability of survival was driven by *Bd* isolate, rather than population origin or developmental environment, with 97.5% surviving exposure to the TN isolate, and only 6.3% surviving exposure to the LA isolate. All frogs exposed to the LA isolate developed infections, and at much higher infection loads, demonstrating that *Bd* isolates from different regions can vary greatly in pathogenicity. TN froglets died at a faster rate and had higher infection loads than LA froglets when exposed to the LA isolate, suggesting there may be some degree of local adaptation to regional *Bd* strains.

In an experiment in which we reared leopard frog larvae in environmental chambers to simulate both current and future pond temperatures, we documented potential negative impacts of developing in future climates. Leopard frogs that developed in the future temperature treatment were smaller at metamorphosis. After metamorphosis, froglets that developed as larvae under simulated future temperatures tended to secrete lower quantities of skin peptides and fewer of the AMPs known to be effective against *Bd*. However, when we compared aspects of the adaptive immune system among frogs from our current and future temperature treatments, we saw more mixed results. Sometimes we found greater concentrations of immune cells in frogs from the current temperature treatment and other times we found greater concentrations in frogs from the future temperature treatment. Thus, the response of the adaptive immune system to the stress of development under future climate scenarios appears to be complex and will require further research to predict with any certainty. When exposed to *Bd* after metamorphosis, we rarely saw differences in infection or pathogen load among frogs from the two temperature treatments, though we did sometimes see differences in mortality. In particular, *R. pipiens* from VT that were reared under future temperatures had greater mortality after exposure to *Bd* than their current temperature counterparts. These mismatches between mortality and pathogen load suggest that developing under stressful climate conditions may affect the ability of amphibian hosts to tolerate *Bd* infection (i.e., pathogen tolerance).

We collected five *Bd* isolates from our DoD regions and investigated their responses to temperature in current and future thermal conditions (i.e., simulated to mimic temperatures we anticipate with climate change). To understand the differences among the five isolates in their thermal responses, we generated thermal performance curves that spanned the known *Bd* thermal range (4-27°C). We observed differences in many of the parameters that we measured, including viability and zoospore density. All isolates exhibited maximum viability at 21°C. However, we found that there were differences among the *Bd* isolates in their viability in both low and high temperature conditions. Contrary to predictions based on viability, two of the isolates we examined produced their maximum zoospore densities at low temperatures (4 and 12°C). We also saw differences in growth rates (*r*) and carrying capacities (*K*) across temperatures for each

isolate. Following 45 passages of *Bd* in culture under thermal conditions that either mimicked current or predicted future amphibian body temperatures, we often saw greater maximum viability and zoospore density in *Bd* lineages that had evolved under the ‘future’ thermal treatments. These results suggest that lineages evolved under warmer ‘future’ host conditions often outperform those evolved under ‘current’ host conditions, suggest that the risk that *Bd* poses for amphibians will not be attenuated by climate change because the pathogen has the capacity to adapt to novel thermal conditions.

Objective 3. Modeling Disease Risk Under Alternative Climate and Management

Scenarios - We developed a novel modeling approach, ‘reduced IPMs’, which are low dimension approximations of full host-parasite Integral Projection Models (IPMs), to facilitate the fitting of IPMs to population-level disease data of the sort we collected in our field surveys. The reduced IPMs are tractable models that allow for analytical calculations of population-level parameters, such as R_0 , the basic reproductive number of a pathogen, which determines when a pathogen can invade a host population (and which can inform pathogen control efforts). When we customized our reduced IPM to model *Bd* dynamics in *R. pipiens* and *R. sphenoccephala*, we found that demographic processes associated with changes in transmission risk, such as leaving and returning to aquatic habitats during breeding and hibernation, largely accounted for changes in *Bd* prevalence over the course of a season. Across all locations, our best fit models predicted a negative effect of temperature on *Bd* growth rate, such that *Bd* load on leopard frogs was predicted to be lowest during the warmest months and highest during the coldest months.

Our analysis of seasonal R_0 (pathogen reproductive number) predicted that post-metamorphic leopard frogs could maintain *Bd* in the population deterministically at the highest host densities we explored and when *Bd*-induced host mortality was zero. However, when *Bd*-induced mortality was minimal, but present, it was unlikely that leopard frogs in TN or PA could maintain *Bd* in the population even at high host densities. In contrast, our models predicted that leopard frogs in LA could still deterministically maintain *Bd* in the population when *Bd*-induced mortality was present. Given that *Bd* is unlikely to persist on leopard frogs alone at some of our field locations, these results highlight the importance of incorporating the rest of the amphibian host community into our models aimed at intervention strategies for controlling *Bd*. This is the next stage of our modeling work, which is currently underway.

Implications for Future Research and Benefits

We did not detect the *Bsal* pathogen on any of the amphibians we sampled for this project. This is good news, as the introduction of *Bsal* to naive amphibian communities in the U.S. could have dire consequences, especially for salamander species, the taxon that has been most adversely affected by this pathogen in Europe. Management actions aimed at keeping this pathogen out of DoD lands will be important. Coordination with working groups of the North American *Bsal* Task Force (<https://www.salamanderfungus.org/>) may be the most effective way for the DoD to develop and implement plans to prevent the invasion of this pathogen onto its lands.

We did, however, detect the *Bd* pathogen at every wetland and in almost every amphibian species we sampled for this project. The *Bd* we detected was not only ubiquitous, but also genetically diverse, suggesting that amphibians in the U. S. have coexisted with this pathogen for quite some time and that the pathogen has likely been moved from place to place frequently. Future studies comparing the virulence of different lineages, in combination with the genomic tools we have developed and employed here, will be valuable to the DoD in monitoring and mitigating the movement and spread of particularly lethal *Bd* variants. Our experimental

evolution study with *Bd* lineages from our study regions showed that *Bd* can adapt to new climates through shifts in thermal physiology. This suggests that we cannot count on global warming to limit the range of this historically temperature-sensitive and cool-adapted pathogen.

Our field surveys documented clear seasonal patterns in both *Bd* infection and the mucosal defenses that amphibian hosts employ to fight against this pathogen. Temperature, and to a lesser extent other environmental variables like pH and canopy cover, seem to be key determinants of seasonal infection dynamics. Management actions that provide access to warmer microhabitats, which allows for faster clearance of *Bd* infections, could be an option for helping amphibians through cooler months of the year, which often coincide with peaks in *Bd* prevalence and infection intensity in amphibian communities. However, we also documented for the first time that the mucosal defenses of amphibian hosts in the U.S. exhibit their own seasonal patterns, and in many cases these defenses are at their peak just before the seasonal peak in *Bd* infections. In places like NM, where endangered Chiricahua leopard frogs with reduced peptide defenses are particularly susceptible to developing heavy infections, management strategies should focus on reducing the risk of infection to the most vulnerable life stages (e.g., newly metamorphosed froglets) and at the time of year when infections are most prevalent (often the coolest months of the amphibian active period).

Our experiments demonstrated that larvae developing under stressful climate conditions, including pond drying, elevated temperatures, and non-local climates, often pay a cost in terms of fitness up to and shortly after metamorphosis. We also discovered that climate stress can impact the development of immune defenses important to the defense against *Bd*. We saw negative impacts of climate stressors experienced during the larval stage on mucosal defenses and susceptibility to *Bd* many months after metamorphosis. These results add to the growing concern that climate change will exacerbate the impact of chytridiomycosis, in that a greater frequency of pond drying and elevated water temperatures, as are expected under global climate change, will likely increase the intensity of *Bd* infection in recently metamorphosed amphibians. This suggests that management efforts focused on limiting the climate stress experienced during larval development may be effective in bolstering the ability of juvenile amphibians, the most susceptible life stage, to fight *Bd* infections. Our experiment in which frogs were exposed to both local and non-local *Bd* strains also suggested the potential for local adaptation to the local *Bd* strains. From a management perspective, this work indicates that the anthropogenic transfer and spread of *Bd* among amphibian populations (e.g., on equipment, or via the movement of animals) should be avoided, as non-local isolates may be more pathogenic than local isolates.

The reduced IPM models we developed, with input from our field and experimental studies, were successful in predicting the dynamics of *Bd* infections in amphibian hosts. When we used this approach to model the dynamics of *Bd* in two host species, we found that demographic processes associated with changes in transmission risk, such as leaving and returning to aquatic habitats during breeding and hibernation, largely accounted for changes in *Bd* prevalence over the course of a season. In other words, once we accounted for how temperature affected *Bd* load dynamics, we generally did not need to invoke any additional temperature dependence in transmission rates to describe the seasonal changes in *Bd* prevalence. Given that *Bd* is likely to persist on more than these two focal hosts at our field locations, our results highlight the importance of incorporating the rest of the amphibian host community into our models aimed at intervention strategies for controlling *Bd*. This is the next stage of our modeling work, which is currently underway.

Objective

Ecology of Chytridiomycosis

To address *research need #1* of SERDP's Resource Conservation and Climate Change (RC) Program Area's Fiscal Year 2016 Statement of Need (RCSON-16-01): *the ecology of potential pathogens and their vectors of concern on DoD lands as this relates to wildlife exposure*, we (1) conducted a non-invasive multi-site evaluation of seasonal patterns of *Bd* prevalence, pathogen loads, and anti-*Bd* immune defenses, (2) examined how variation among *Bd* strains (e.g., in thermotolerance, life cycle, and lineage) affects host/pathogen dynamics, and (3) screened amphibian populations for *Batrachochytrium salamandrivorans* (*Bsal*), which has yet to be detected in the United States but has caused die offs in Europe.

Impacts of Current and Future Climate Variation on Amphibian-*Batrachochytrium* Dynamics

To address *research need #2* of SERDP's RCSON-16-01: *plausible scenarios of future change that may impact exposure pathways for wildlife and disease severity*, we asked whether warmer and more variable weather, as predicted under global climate change, will increase the potential for transmission and disease development by investigating: (1) how the development and expression of host anti-fungal defenses, including innate, adaptive, and microbial skin defenses, are affected by changes in climate; (2) how climate will affect *Batrachochytrium* virulence and the potential for transmission, including the potential for pathogen niche evolution; and (3) how community structure, including shifts in host niches/ranges under future climate scenarios, might affect the dynamics and spread of chytridiomycosis.

Modeling the Distribution and Severity of Chytridiomycosis Risk on DoD Lands Under Alternative Climate and Management Scenarios.

To address *research need #3* of SERDP's RCSON-16-01: *models that can project how exposure pathways and disease severity may change in the future and their implications for wildlife management*, we are developing data-driven, mechanistic models that use our empirically derived relationships between climate variables and vital rates for amphibians and the *Batrachochytrium* species/strains found at our field sites (from Objectives 1 and 2) to evaluate: (1) how changes in climate will affect the host/pathogen interaction, (2) where and when chytridiomycosis outbreaks are likely to threaten amphibians on DoD installations, and (3) the effectiveness of alternative management strategies for sustaining at-risk amphibian populations through times of increased disease risk.

Background

Discovering the mechanisms that link climate to patterns of infectious disease will be crucial in this time of climate change (Dowell 2001, Raffel *et al.* 2013). Temperature appears to be a key factor, especially for ectotherms where ambient conditions constrain the physiological responses of both host and pathogen (Blanford & Thomas 1999). Fungal pathogens are especially sensitive to temperature and increasingly recognized as drivers of wildlife declines and extinctions (Casadevall 2005, Fisher *et al.* 2012). Recent examples of amphibian chytridiomycosis (Kilpatrick *et al.* 2010) and white nose syndrome in bats (Foley *et al.* 2011) underscore the need to understand the mechanisms by which climate affects fungal virulence. Resolving the links

between climate and the outcomes of host-fungal interactions will provide valuable insights into the dynamics, ecology and evolution of infectious diseases and provide direction for effective management of threatened populations on DoD lands.

Declines and extinctions of as many as 500 amphibian species have been linked to fungi in the genus *Batrachochytrium* (Skerratt *et al.* 2007, Martel *et al.* 2014, Scheele *et al.* 2019). *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*) are water-borne chytrid fungi that infect amphibian skin causing the disease chytridiomycosis (Longcore *et al.* 1999, Pessier *et al.* 1999, Martel *et al.* 2013). *Bd* (first described in 1999) has caused declines in amphibians around the world (Longcore *et al.* 1999, Fisher *et al.* 2012). *Bsal* (first described in 2013) has caused declines of European salamanders (Martel *et al.* 2013, 2014). In the United States, field studies have confirmed that *Bd* is widespread, yet it has been associated with die-offs only in certain areas and certain hosts (Carey *et al.* 1999, Ouellet *et al.* 2005, Vredenburg *et al.* 2010). For example, *Rana muscosa* was driven to near extinction by *Bd* in the Sierra Nevada Mountains (Briggs *et al.* 2010), and *Bufo boreas* has been impacted in the Rocky Mountains (Pilliod *et al.* 2010). At the start of this project, *Bsal* had not yet been detected in the United States, though sampling had only recently begun. The United States is a hotspot for salamander diversity, so the introduction of *Bsal* to the United States would threaten global amphibian diversity.

Batrachochytrium fungi colonize skin cells of adult amphibians and *Bd* also colonizes the mouthparts of tadpoles. Infection occurs when a motile aquatic zoospore encysts in amphibian skin (Berger *et al.* 1998, Longcore *et al.* 1999), forming a germination tube and sending its contents into cells of the epidermis (Greenspan *et al.* 2012, Van Rooij *et al.* 2012). Inside the cell the fungus grows in size and develops into a zoosporangium, in which new zoospores develop (Berger *et al.* 2005). As infected skin cells move toward the skin surface, the zoosporangium matures, a discharge papilla opens, and mature zoospores swim out, either onto the surface of the host to re-infect the individual, or away from the host and into an aquatic environment (Berger *et al.* 1998, Longcore *et al.* 1999). *Batrachochytrium* can be transmitted by contact with infected hosts or via contaminated water or substrate (Rachowicz & Vredenburg 2004).

Bd causes death by interfering with the essential ion transport functions of the skin, leading to a loss of electrolytes from circulation and ultimately asystolic cardiac arrest (Voyles *et al.* 2007, 2009, 2012). Unlike other fungal pathogens that move from the site of infection to other organs, *Bd* and *Bsal* appear to remain in the skin (or tadpole mouthparts). The damage to the most superficial layers of epidermis is life-threatening because amphibians rely on their skin for multiple physiological processes. Specifically, it is the primary site of osmoregulation and exchange of respiratory gases. Thus, these pathogens can cause a cascade of pathophysiological disruptions and death in infected amphibians (Voyles *et al.* 2007, 2009, Marcum *et al.* 2010).

At the start of this project, a growing body of evidence already suggested that climate strongly affects the host-*Batrachochytrium* interaction. In culture, *Bd* and *Bsal* show reduced growth and mortality at high temperatures (Piotrowski *et al.* 2004, Woodhams *et al.* 2008, Martel *et al.* 2013), and survival of exposed amphibians is often greater at higher temperatures (Berger *et al.* 2004, Andre *et al.* 2008, Sonn *et al.* 2017). In nature, disease prevalence and severity tend to increase during cooler months and declines due to *Bd* have been largely limited to areas with at least seasonally cool climates (Berger *et al.* 2004, Kriger *et al.* 2007, Olson *et al.* 2013). These findings suggest that an increase in mean temperature, as is predicted with global climate change, may be favorable to amphibians, decreasing the likelihood of outbreaks and declines. However, climate change is also expected to affect the variability of temperature and precipitation, setting

the stage for different, and likely complex, host-pathogen dynamics under future climate scenarios (Schar *et al.* 2004, Pounds *et al.* 2006, Yeh *et al.* 2009). For example, longer and colder winters in the United States due to the “Greenland block” effect (Gramling 2015) could delay breeding and shorten the period for larval development leaving newly metamorphosed amphibians with immature immune defenses. An increase in temperature variability is also predicted to impair amphibian defenses and increase the risk of *Bd* epidemics (Rohr & Raffel 2010, Raffel *et al.* 2013).

While climate appears to play a key role in *Bd* epidemics, prior to this project it was unclear whether this was due to temperature effects on host immune function, the pathophysiology of *Bd*, or both (Raffel *et al.* 2011). Amphibians have a suite of immune defenses against fungal pathogens. Antimicrobial peptides (AMPs) secreted in skin mucus provide the first line of defense (Rollins-Smith *et al.* 2005, 2011). Mucus also contains lysozyme (Zhao *et al.* 2006, Savage *et al.* 2014), antibodies (Ramsey *et al.* 2010), and antifungal products of symbiotic bacteria. As *Bd* modifies the infected cells, antigen presenting cells (macrophages, neutrophils and dendritic cells) should recognize the foreign invader and signal lymphocyte activation (Ramsey *et al.* 2010). Thus, a variety of defenses can help to prevent not only the first infection but also re-infection in an individual host. Immunity in ectotherms is strongly affected by temperature (Rollins-Smith *et al.* 2011), though the degree to which temperature-induced immunosuppression affects the likelihood or outcome has been understudied.

In general, low temperatures are predicted to favor *Bd* because it is cold-tolerant (Woodhams *et al.* 2008, Voyles *et al.* 2012) and amphibian immune defenses are naturally delayed or diminished at low temperatures (Rollins-Smith *et al.* 2011, Rollins-Smith & Woodhams 2012). In contrast, higher temperatures, nearer to the maximum for *Bd* survival (28-30°C, Piotrowski *et al.* 2004, Stevenson *et al.* 2013), are predicted to favor the amphibians, enabling them to develop an effective immune response. In addition, thermal conditions influence the activity and production of antifungal metabolites by symbiotic microbes on amphibian skin (Woodhams *et al.* 2014). However, even if climate boosts the efficacy of host immune systems, recent work has shown that amphibian defenses tend to recover too slowly after unpredictable shifts in temperature, as are expected to occur more frequently with climate change, to provide an effective defense against fungal pathogens (Raffel *et al.* 2013).

In addition to direct effects on the elements of the mature amphibian immune system, changes in climate may affect the development of immune defenses in earlier life stages in ways that weaken the host’s ability to resist infection later in life. Recent metamorphs can be especially vulnerable to chytridiomycosis, in part because their immune systems are often immature at this life stage (Rachowicz & Vredenburg 2004, Tobler & Schmidt 2010, Walker *et al.* 2010). More rapid development in nutrient-limited conditions, as may occur more frequently in a warmer and less predictable climate, may result in metamorphs with less mature AMPs (Holden *et al.* 2015) and fewer lymphocytes (Rollins-Smith *et al.* 1988).

In addition to affecting host defenses, temperature strongly influences the virulence characteristics of fungal pathogens. *Bd* strains differ markedly in virulence (Berger *et al.* 2005, Fisher *et al.* 2009, Farrer *et al.* 2011) and also in their responses to a range of temperatures (Stevenson *et al.* 2013). Yet prior to this project, we knew little about how differential responses to temperature could influence the mechanisms of virulence among *Bd* isolates. Possible mechanisms for *Bd* virulence include protease-mediated degradation of host tissue (Joneson *et al.* 2011), lymphotoxic factors involved in evasion of host immunity (Fites *et al.* 2013, Piovia-Scott *et al.* 2014), and rapid life cycle turnover to increase propagule size (Langhammer *et al.*

2013, Voyles *et al.* 2014). However, it is not known how these virulence characteristics are affected by temperature, or how this interaction differs among *Batrachochytrium* species or strains. The distribution and diversity of *Bd* lineages within the United States was also poorly known prior to the start of this project.

It is likely that climate change will exert selective pressure on *Batrachochytrium*, with the potential to broaden the range of temperatures at which these fungi can spread and be pathogenic. Fungi are known to adapt to new temperature conditions (Feller & Gerday 2003) and thermotolerance of fungal pathogens is a topic of intense research (Caspeta *et al.* 2014). Clarifying the adaptive responses of *Batrachochytrium* to changes in temperature will be important when forecasting chytridiomycosis under future climate scenarios.

This project aimed to improve our understanding of the ecological factors that impact host and pathogen interactions in chytridiomycosis, an emerging disease that threatens the health of amphibians on DoD lands. We focused specifically on climate and undertook field and laboratory studies to clarify how climate affects the physiology and life history of both host and pathogen and how this impacts disease development and transmission. We integrated our empirical data on host-pathogen-climate relationships with modeling approaches to project changes in exposure pathways and disease severity under future climate conditions and to investigate the potential impacts of alternative wildlife management scenarios. These models will be an important step toward developing adaptive management strategies to control fungal pathogens and sustain at-risk amphibian species on DoD lands.

Materials and Methods

Objective 1. Ecology of Chytridiomycosis

To investigate links between pathogen presence, disease, and climate, we sampled amphibian communities for *Batrachochytrium* in five regions of the United States (Figure 1). We chose these locations because they represent a broad range of climates and contain our focal amphibian

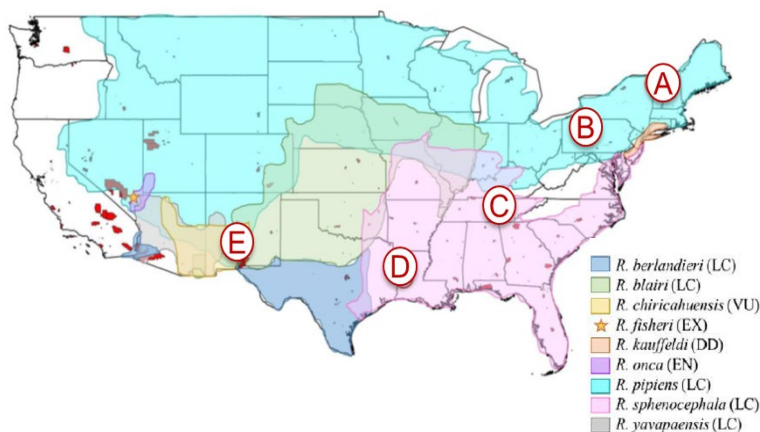


Figure 1. Map of the United States showing (1) distributions of species in the leopard frog species complex (shaded according to key), (2) locations DoD installations (red shading) and (3) field sites: (A) Camp Ethan Allen, VT; (B) Pymatuning Lab of Ecology, PA; (C) Arnold Air Force Base, TN; (D) Fort Polk, LA; (E) White Sands Missile Range, NM). Two letter abbreviations after species names indicate IUCN status (DD = data deficient, LC = least concern, VU = vulnerable, EN = endangered, EX = extinct).

hosts in the leopard frog species complex. We chose leopard frogs because many species within this complex are susceptible to chytridiomycosis (Davidson *et al.* 2003; Parris *et al.* 2006, Savage & Zamudio 2011), several are declining in relation to historical numbers (e.g., *R. pipiens*, *R. sphenoccephala*, *R. yavapaiensis*), some are threatened (*R. chiricahuensis*, *R. onca*), and one species is thought to be extinct (*R. fisheri*) (IUCN 2018). At each site we selected a set of wetlands where we tested for *Bd* and *Bsal* presence and intensity on amphibians (via skin swabs, see details below) with a quantitative real-time PCR assay (Hyatt *et al.*

2007). We sampled during the active seasons for amphibians (spring, summer, autumn, and winter where appropriate) to identify seasonal patterns of *Batrachochytrium* presence, prevalence, infection intensity and host immune response.

With this objective, we aimed to test the hypothesis that *Batrachochytrium* prevalence and intensity is highest when host immune defenses are weakest, and to determine optimal and suboptimal temperatures for host immune defense. Given that *Bd* is widely present in the United States, it is perhaps no surprise that we detected it in the amphibian communities at each of our study sites. Our surveys permitted us to assess in finer detail which amphibian species are affected, the prevalence of their infections, and their relative pathogen burdens across seasons. We predicted that *Bd* prevalence and pathogen burdens (fungal load; measured in genome equivalents using a qPCR assay, described below) would vary seasonally. For example, we expected that *Bd* burdens in *R. pipiens* and *R. sphenoccephala* would be highest in animals sampled early in spring as they emerge from overwintering conditions in Vermont (VT), Pennsylvania (PA), Tennessee (TN), and New Mexico (NM). In contrast, we predicted that prevalence and burdens may peak at a different time in Louisiana (LA), where many amphibians remain active year-round. Information on pathogen prevalence and burden across hosts, seasons, and our five field sites (which each represent distinct climates within the range of the leopard frog complex) informed the predictive models we developed in Objective 3. We also sampled for *Bsal* at our study sites in hopes that if detected, our efforts could help to provide an early warning as to which amphibian species may be vulnerable to die-offs due to this more recently discovered pathogen.

Our field sites for this project include wetlands on and near Camp Ethan Allan/Camp Johnson in VT, the Pymatuning Lab of Ecology in PA, Arnold Air Force Station in TN, White Sands Missile Range in NM, and Fort Polk in LA. In VT and PA, our focal leopard frog species is the northern leopard frog (*R. pipiens*). In TN and LA our focal leopard frog species is the southern leopard frog (*R. sphenoccephala*). In NM, three leopard frog species have been documented. These include the threatened Chiricahua leopard frog (*R. chiricahuensis*), the plains leopard frog (*R. blairi*) and the Northern leopard frog (*R. pipiens*).

Surveys for amphibians

We conducted acoustic and visual encounter surveys during the time periods when amphibians are active at each of the respective field sites (Spring, Summer, and Fall in VT, PA and NM, year-round in LA and TN). Both acoustic and visual encounter surveys are widely used approaches for assessing amphibian presence and abundances (Crump & Scott 1994). At each field site, we selected four or more distinct sampling areas (most often ponds or wetlands, >1 km apart if possible) that encompassed a wide diversity of the local amphibian fauna. We aimed to survey all sampling areas at least three times per season at a minimum, across all seasons, with the exception of winter in our northern locations (VT and PA). The order of site sampling was randomized within a sampling week. We attempted to collect 20 individuals/sampling area/species that were active during the time of sampling. Most of our surveys were nighttime surveys, but we also completed at least one daytime survey at least once for each sampling area in each season.

For our acoustic surveys, we listened for calling at each sampling area for 3 minutes for each visit. This was done prior to the start of the visual survey. For our visual surveys, we carefully searched in and around the pond, wetland or stream carefully for amphibians and used nitrile-gloved hands or nets to capture individuals. Once an amphibian was sighted, and before

the amphibian was captured, we used non-contact infrared thermometers to measure the body and microhabitat temperatures. These devices are a non-invasive way to obtain accurate body temperature information (Rowley & Alford 2007). We then captured the animal and collected samples for pathogen identification and detection (see below). Using the combined acoustic and visual survey data, we recorded presence/absence and estimated relative abundance for all species that we found in the sampling area. Because amphibian susceptibility to chytridiomycosis is highly dependent on environmental conditions, we measured relevant variables (e.g., site elevation, habitat type, water temperature, turbidity, and pH) and obtained climate and weather information from weather stations placed in or near each site.

To parameterize models of *Bd* dynamics, we first needed to determine how temperature affects individual *Bd* loads over time (which influence *Bd* transmission) in different amphibian species. To do this, we collected *Bd*-infected frogs of three species (*Rana pipiens*, *R. catesbeiana*, and *R. clamitans*) from two field sites in Pennsylvania. We then maintained frogs individually in the lab at either 11, 16, or 21°C for two weeks. We measured *Bd* loads upon collection and again after one and two weeks at treatment temperatures.

Sample collection for *Batrachochytrium*

During each sampling session, we captured amphibians (all species encountered) either by hand (using a fresh pair of gloves) or using nets or cover boards, with strict adherence to field hygiene protocols to reduce pathogen transmission (Phillot *et al.* 2010). Once an amphibian was captured, we collected morphological data including, snout-to-vent length (SVL), mass, and sex, and noted life-stage and/or reproductive status (e.g., gravid females). We collected skin swab samples using a non-invasive, standardized protocol that involves rubbing a sterile swab across the ventral skin on the abdominal and inguinal surfaces, and on the digits of the hands and feet (Hyatt *et al.* 2007). We transferred the swab sample to a sterile tube for transport to the laboratory for sample processing (described below). We then placed the samples in a -20 °C freezer until analysis. We collected one swab sample per individual.

Next, we extracted genomic DNA from the skin swab samples using standard methods (DNeasy Blood and Tissue kit, Qiagen, Inc., Valencia, CA, USA) and determined the presence, prevalence and intensity of infection of *Bd* and *Bsal* using an established quantitative real time Polymerase Chain Reaction (qPCR) assay (Boyle *et al.* 2004). We included negative and positive controls for *Bd* and *Bsal* in each qPCR run and generated quantitative estimates of infection intensity using a 7-fold set of dilution standards previously generated for each *Batrachochytrium* species (Pisces Molecular).

We shipped a subset of the DNA extracts that tested positive for *Bd* on dry ice to the University of California, Berkeley, where we conducted a custom genetic assay to identify the sample to strain (Byrne *et al.* 2017). These broad-scale genetic data allowed us to determine how localized *Bd* and *Bsal* strains are. Specifically, we assessed whether particular *Batrachochytrium* strains are more prevalent in certain geographic regions, associated with specific host species, or are more prevalent in some seasons or years. The power of this approach stems from our ability to genotype directly from field swabs without needing to generate pure fungal cultures.

We isolated and cryopreserved *Batrachochytrium* pathogens encountered near our field sites. We successfully isolated, purified, and cultured isolates from New Mexico and Vermont using standard microbiological methods (Longcore *et al.* 1999; Cook *et al.* 2018). The process involved collecting a small tissue sample from an amphibian (skin from adults or mouthparts from tadpoles) and purifying using a streaking method on agar plates with antibiotics (Cook *et*

al. 2018). Once we purified *Bd*, we maintained cultures either in liquid medium or on agar plates with a tryptone medium (Longcore *et al.* 1999). For representative isolates from Louisiana, Pennsylvania, and Tennessee, we sourced isolates that were collected and purified prior to the start of this project. We cryo-archived all isolates to eliminate the risk of pathogen attenuation, which can occur with laboratory maintenance (Langhammer *et al.* 2013). The process of cryopreservation involved suspending an actively growing culture in a cryoprotectant solution and slowly reducing the temperature to -80°C (Boyle *et al.* 2003). This method is effective for long-term storage of *Batrachochytrium* and allowed us to recover (i.e., revive) isolates for *in vitro* experiments (described below) without problems associated with laboratory maintenance (Boyle *et al.* 2003). We also used a custom genetic assay (described above) to reveal the genetic background of all isolates we used in our experiments.

Immunological studies

To examine the links between climate and host defenses, we sampled and quantified anti-*Batrachochytrium* host defenses. To do this, we collected mucus from leopard frogs across seasons and field sites and assayed their “mucosome”, which is defined as the combined microbial and host factors in the mucus, including secondary metabolites, AMPs, lysozymes, mucosal antibodies, and alkaloids. We collected frog mucus using skin rinses and tested for anti-*Batrachochytrium* properties using *in vitro* assays (Cell-titer Glo 2.0) to measure *Bd* viability. After swabbing for *Bd* load, frogs were soaked in 15 mL sterile artificial pond water for one hour to collect mucosal secretions, and then washes were stored frozen at -20°C. We examined zoospore shedding rates from the same samples by qPCR. Our previous work has shown this sort of mucosome assay to (1) detect variation in immune function against *Bd* under varying environmental conditions, and (2) predict disease risk (Woodhams *et al.* 2014).

Since the method is not yet published, we will provide details of the mucosome *Bd* viability assay here. First, frozen samples were lyophilized and rehydrated in 1000 µl of sterile MilliQ water, placed into a 96 deep well plate (USA Scientific #1896-1110), and frozen until viability assays could be performed. *Bd* zoospores (Section Line isolate) were harvested using 1% Tryptone media and counted using a hemocytometer to make a working stock of 2.0×10^6 zoospores/ml. Mucosomes were then thawed and 25 µl of the zoospore solution and 25 µl of each mucosome were added to a 96-well plate (Greiner CELLSTAR #655083), with each reaction set up in triplicate. Background controls were set up in duplicate and consisted of 25 µl of each mucosome and 25 µl of 1% Tryptone media. Controls, also set up in triplicate, were set up using the following recipes: Positive controls containing of 25 µl of zoospore solution and 25 µl of sterile MilliQ water (to account for the mucosome rehydration media), negative controls consisting of heat killed zoospores, and media controls using 25 µl of 1% Tryptone media and 25 µl of sterile MilliQ water. Plates were incubated for 1 h at room temperature, then in a dark room, 50 µl of CellTiter-Glo 2.0 Cell Viability solution (Promega #G9243) were added to each well. Plates were then sealed with foil and placed on a shaker for 2 minutes, then left to incubate at room temperature for another 13 minutes, causing cell lysis and the release of ATP. Finally, plates were read using the luminescent setting on a PolarStar Omega plate microplate reader (BMG Labtech, Ortenberg, Germany). To calculate the mucosal function, average background media values were subtracted from the positive and negative wells and average mucosome background values were subtracted from each mucosome test well to account for background ATP measured in the water and media solutions. Afterwards, negative control values were averaged and subtracted from each mucosome test and positive control to remove ATP

measurements of the dead zoospore solution. Finally, mucosome tests were divided by the corrected-positive controls to compute a viability ratio of how effective the mucosome was at killing *Bd* zoospores during the hour-incubation, termed mucosal killing capacity.

In addition to whole mucosome assays, we separately quantified AMP, antibody, and skin microbe activity against *Bd*. We enriched mucus samples for hydrophobic peptides by passing the skin washes over C18 sep paks and identified secreted AMPs using mass spectrometry. We measured the activity of peptides against *Bd* using *in vitro* growth inhibition assays (Rollins-Smith *et al.* 2006).

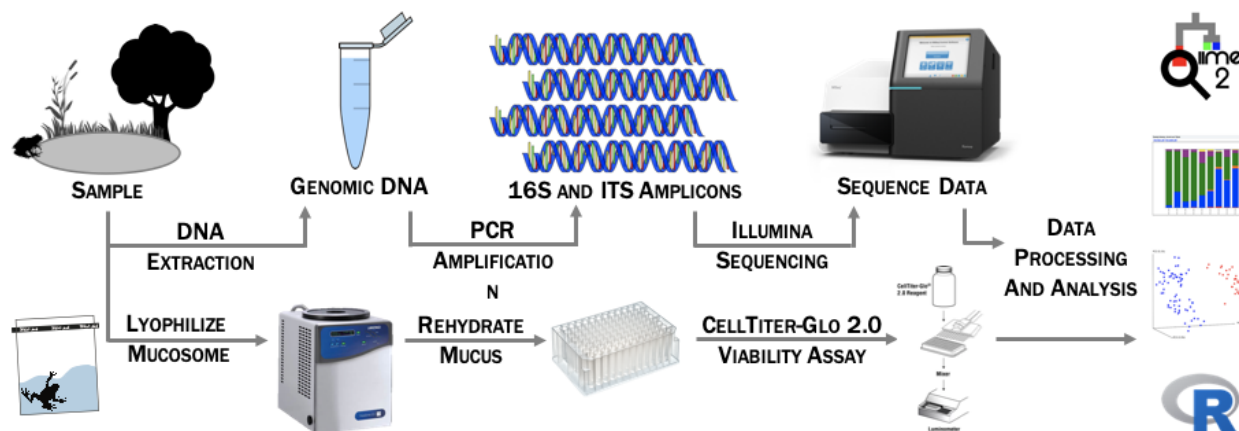


Figure 2. General sample processing workflow. First frogs were collected, swabbed for *Bd*, then given a 1 hour bath to collect mucosome and swabbed again for the microbiome. Swabs were extracted to collect the bacterial and fungal genomic DNA which was then PCR amplified for either 16S or ITS. PCR Amplicons were purified, normalized, and pooled for the sequencing library. The library was sequenced on an Illumina MiSeq 2 x 150 cycle V2 kit. Data was then processed in QIIME2 and modeled/visualized using R. Mucosomes were frozen and lyophilized to reduce the liquid volume and concentrate the mucosal components. Mucosomes were rehydrated in 1 ml of MilliQ water and used in a *Bd* viability assay (CellTiter-Glo 2.0) to measure the mucosal killing capacity for each frog. Data was then processed in excel and modeled/visualized using R (R Core Team 2020).

We examined the microbial communities in mucus samples using Illumina MiSeq sequencing, targeting the 16S rRNA gene (bacteria) or ITS gene (fungi), and correlating skin community structure with mucosome function against *Batrachochytrium* (e.g., Becker *et al.* 2014, Loudon *et al.* 2014; Figure 2). We also tested bacteria and fungi isolated from leopard frog skin individually for their capacity to inhibit *Batrachochytrium* growth. Inhibitory microbes were identified by Sanger sequencing of the 16s rRNA gene (bacteria) or ITS gene (fungi) and added to a global database of amphibian skin microbiota (Woodhams *et al.* 2015). We harvested cell-free secondary metabolites from the cultures and tested the metabolites for activity against *Batrachochytrium* (measured as reduced viability and growth) according to published methods (Woodhams *et al.* 2014, 2015). For each location, we processed only a subset of samples for mucosome and microbiome (Table 2). Another outcome of this work has been the identification and archiving of species with anti-*Batrachochytrium* properties that could be used for future bioaugmentation treatments to protect vulnerable amphibian species on DoD lands (Bletz *et al.* 2013).

For microbiome analysis, DNA was extracted from swabs using a DNeasy Blood & Tissue Kit (Qiagen, Inc., Valencia, CA, USA) following the manufacturer's protocol with slight modifications. An initial lysozyme (20 mg lysozyme/1 ml lysis buffer) incubation step was

Table 2. Number of microbiome samples for each species at the five DoD locations for each year. Sample counts are divided among the sample seasons for each year.

Location	Species	2017				2018			
		Winter	Spring	Summer	Fall	Winter	Spring	Summer	Fall
VT - All sites	<i>R. pipiens</i>		8		41		11	21	6
PA - Sites 3 & 4	<i>R. pipiens</i>		25	61	9		31	34	3
NM - All sites	<i>R. blairi</i>		6	35					
	<i>R. chiricahuensis</i>						20	38	24
TN – Sites 1 & 5	<i>R. spheonocephala</i>		30	28	22	17	16	20	12
LA – Sites 3 & 5	<i>R. spheonocephala</i>	1	45	24	24	15	21	16	9
Total			359				314		

conducted at 37°C for 1 hour (to lyse gram-positive bacteria). Next, 25 µl proteinase K and 200 µl buffer AL was added to each reaction and incubated at 70°C for 30 min before proceeding with the remaining kit instructions. Following DNA extractions, PCR was conducted in duplicate to amplify the V4 region of the 16 rRNA bacterial gene (F515 and R806 primers) following the Earth Microbiome protocol methods of (Caporaso *et al.* 2012) and then sequenced and processed following previously published methods (LeSage *et al.* 2021). Specifically, the raw Illumina 16S rRNA amplicon data was processed and quality filtered using QIIME 2 v2020.2 (Bolyen *et al.* 2018) and classified into sub-operational taxonomic units (sOTUs) using the Deblur workflow (Amir *et al.* 2017). Data were rarefied at 3190 reads per sample and then several metrics were conducted for analysis. Following significant results from the alpha (sOTUs richness and faith phylogenetic distance), beta (Bray-Curtis, Jaccard, Unweighted and Weighted UniFrac) diversity, and anti-*Bd* microbiome functions, we will further explore changes in the amphibian microbiome using differential abundance analyses using Aldex2 (Fernandes *et al.* 2013, Fernandes *et al.* 2014) and a linear decomposition model (LDM; Hu and Satten 2020). These analyses will help show transitional changes in the sOTU relative abundance across the different groups being analyzed.

Statistical Analysis

All statistical models in Objective 1 were run in R (R Core Team 2020). To compare *Bd* infection prevalence and *Bd* load across seasons and sampling sites we used the package *mgcv* (Wood 2017) to build generalized additive mixed models (GAMMs), including site as a random effect. We also used GAMMs to test for relationships between *Bd* prevalence and load and environmental variables (e.g., temperature, pH). The use of GAMMs allowed for non-linear predictors (via smoother functions). Models examining highly correlated variables were run separately and model comparison was performed (using Akaike’s information criterion, AIC). To test for correlations between *Bd* load and temperature in frogs that were collected with natural *Bd* infections and housed at different temperatures in the lab, we used generalized linear mixed models (GLMMs) in the package *lme4* (Bates *et al.* 2015), including individual as a random effect.

After sequencing the *Bd* DNA from swab samples, we used a discriminant analysis of principal components (DAPC) to evaluate the data for genetic clusters (Jombart *et al.* 2010). To model differences in the skin microbiomes sampled from field-caught leopard frogs among field sites we used a combination of alpha diversity metrics (including OTU richness and phylogenetic diversity) and beta diversity metrics (Bray-Curtis, Jaccard, Unweighted Unifrac, and Weighted

Unifrac matrices). Alpha diversity was modeled using GLMs and beta diversity was modeled using PERMANOVAs (*vegan* package, Oksanen *et al.* 2020) and then visualized shifts in microbial community structure using principal coordinate analyses (PCoAs). To test for a relationship between *Bd* load and the *Bd*-inhibitory capacity of leopard frog mucus we used GLMs. To compare the *Bd* killing capacity of skin mucus of leopard frogs sampled over time, across field sites, and across *Bd*-status categories, we used beta regression models (*glmmTMB* package, Magnusson *et al.* 2017).

To compare leopard frog total peptide secretions across seasons, life stages, *Bd*-infection statuses and temperatures, we used GAMs and generalized additive mixed models (GAMMs) with site as a random variable. When analyzing the presence and intensity of AMPs in secretions we used GLMs with the same set of predictors. And finally, to compare the MALDI spectra across these variables we used a PERMANOVA of Jaccard and Bray-Curtis distance matrices (*vegan* package, Oksanen *et al.* 2020). To compare the minimum inhibitory concentration (MIC) of skin mucus among leopard frogs sampled over time, across field sites, and across *Bd*-status categories, we used ANOVAs (*stats* package, R Core Team 2020).

Objective 2. Impacts of Current and Future Climates on Amphibian-*Batrachochytrium* Dynamics

We examined the effect of climate on amphibian immune defenses and *Batrachochytrium* virulence using lab and field experiments. To investigate how climate impacts the development of anti-*Batrachochytrium* defenses (including innate, adaptive, and microbial components), we raised leopard frogs from eggs under different climate scenarios. To do this we placed mesocosms both outdoors (at our field sites) and in indoor environmental chambers (where we can simulate future temperatures). We also investigated how climate impacts *Batrachochytrium* virulence, and the potential for pathogen niche evolution using laboratory *in vitro* experiments conducted under a variety of climate scenarios.

Here we report the findings from three outdoor mesocosm experiments to test (1) the effect of shortened hydroperiod on the development of pathogen defenses in five populations of three species, (2) for local adaptation of two populations to environmental conditions, and (3) how amphibian community structure affects *Bd* transmission dynamics and microbiome composition. We also report the findings from one indoor experiment where we simulated an increase in temperature due to global warming on frogs from four populations of two species during larval development and measured the effects of this change in climate on fitness, focusing on immune development and susceptibility to chytridiomycosis. Finally, we report results from an artificial evolution experiment aimed at understanding how the *Bd* pathogen is able to respond, in terms of its range of thermal tolerance and virulence traits, to a warmer climate.

Effects of hydroperiod on development of immune defenses

We hypothesized that populations of leopard frogs from warmer and/or drier climates are better able to adjust their development to cope with rapidly disappearing temporary water sources, a trait that may become increasingly important as the climate becomes warmer and more variable. To test this hypothesis, we conducted mesocosm experiments in VT, PA, TN, LA and NM with three pond duration treatments. In two treatments, we artificially reduced water levels in the mesocosms at different rates: one treatment simulated rapid drying of temporary ponds (as is predicted to occur under future climate conditions) and a second treatment simulated slower drying of temporary ponds. We also included a control treatment in which water levels were

maintained at a depth of 40 cm (i.e., no drying). For each treatment we used three replicate mesocosms containing 40 leopard frog tadpoles (VT and PA: *R. pipiens*, TN and LA: *R. sphenoccephala*, NM: *R. chirichauensis*). Water was removed from the fast-drying mesocosms at a constant rate each week such that the lowest water level (3 cm depth) was reached at the average larval period for each species. For the medium drying mesocosms, water was removed at half the rate of the fast-drying treatment.

Larvae in drying treatments were predicted to undergo more rapid larval development and to metamorphose sooner than is usual for their local habitat due to the risk of desiccation (O'Regan *et al.* 2014). After metamorphosis, we compared the immunological parameters of frogs that developed under the two drying treatments with those from the control, non-drying treatments. Surviving animals were also exposed to *Bd* to test their resistance or susceptibility to chytridiomycosis. We predicted that this experiment would demonstrate that a longer larval period is beneficial for immune development, thus suggesting that a management strategy to maintain adequate water levels in naturally occurring temporary ponds would reduce the risk of chytridiomycosis in metamorphic animals.

Local adaptation to climate

Both leopard frogs and their pathogens experience variation in climatic conditions across their geographic range. Populations may be locally adapted to survive in the conditions they experience during development, or they may be developmentally plastic, allowing for population persistence across a range of environmental conditions. Similarly, amphibians may be adapted to persist with local pathogen variants, rendering them less protected against non-local variants. The degree of local adaptation versus plasticity in amphibian developmental traits and immune function may help predict their ability to withstand future changing climatic conditions in the face of pathogens.

We investigated local adaptation in developmental traits in two populations of *R. sphenoccephala* (in TN and LA) using a reciprocal transplant mesocosm experiment. Then, we tested the relative importance of population origin versus developmental environment on immune defenses and disease outcome by exposing animals to either a local or non-local isolate of *Bd*. We collected adult male and female *R. sphenoccephala* from TN and LA, overwintered them in the laboratory, and induced them to lay eggs the following spring. Half of each egg mass (N = 2 from each population) was sent to TN and LA and allowed to develop to free-swimming (Gosner stage 25, Gosner 1960) outdoors. Tadpoles from each population were then placed in mesocosms (N = 30 per mesocom) at both locations, and their growth, development and survival were monitored periodically until metamorphosis.

After metamorphosis, we collected skin microbiome swabs and washes to examine host defense mechanisms of metamorphic animals. Animals were then housed in the laboratory at either room temperature for *Bd* exposure, or under simulated TN or LA summer temperatures in environmental chambers, to account for any effects of mismatches in temperature between pre- and post-metamorphic environments. We measured growth, survival, and pathogen load in animals exposed to one of two *Bd* strains (isolated from either TN or LA), and collected both skin microbiome swabs and washes after exposure. For animals in chambers, we tested the effects of population origin and pre- and post-developmental environments on catch-up growth and two measures of immune function: antimicrobial peptide (AMPs) recovery and the bacterial-killing ability of plasma.

Effects of amphibian community structure on pathogen defenses

In addition to changes in host defense and pathogen virulence, climate change may affect host-pathogen dynamics by changing the composition of biological communities. For example, climate change may promote the introduction of non-native host or pathogen species, loss of species, or changes in species abundance. We predicted that community composition would affect disease risk by modifying transmission dynamics, with *Bd*-reservoir hosts increasing disease risk and *Bd*-resistant hosts decreasing risk, as well as through trait-mediated indirect effects on host fitness and immune development. To examine the effects of host community composition on infection prevalence and host defenses, two experiments were conducted in the summers of 2019 and 2020 at our VT field site with *R. pipiens* raised in outdoor mesocosms with different combinations naturally co-occurring host species, *Hyla versicolor* and *R. clamitans*.

In 2019, 4 treatments were used to examine infection dynamics following the addition of *Bd*-GPL1 zoospores from laboratory culture, while one treatment (1) served as a *Bd*-free control to examine the effects of *Bd* infection on *R. pipiens* larvae in the absence of other species. *Bd*-exposed community treatments consisted of (2) 30 *R. pipiens* plus *Bd*-GPL1, (3) 20 *R. pipiens* and 10 *H. versicolor* tadpoles plus *Bd*-GPL1, (4) 20 *R. pipiens* and 10 *R. clamitans* tadpoles plus *Bd*-GPL1, and (5) 10 tadpoles of each species plus *Bd*-GPL1. Total tadpole density was held constant between treatments to minimize competitive effects arising from cohabitation (Relyea 2000) and density effects on *Bd* transmission. Tadpoles were fed a diet of 5g rabbit-chow per week to ensure adequate food resources to further reduce variability in competition intensity between community treatments. Tadpoles of all species were swabbed at 2-week intervals following *Bd* exposure and upon metamorphosis to survey infection prevalence and load. Body mass, snout-vent-length, and time to metamorphosis were recorded for each individual. In addition, a mucosome wash was collected from each *R. pipiens* metamorph for use in a *Bd* challenge assay to approximate mucosal function. Post-metamorphic individuals were relocated to the laboratory and survival was monitored for 8 weeks after metamorphosis to observe downstream effects of larval community treatments.

In 2020, community treatments were identical to those used in 2019, except that two *R. pipiens* tadpoles, co-housed with *Bd*-infected newts (*Notophthalmus viridescens*) prior to the start of the experiment, were added to each *Bd*-treated tank to seed infection. After determining that newt-exposure was not sufficient for transmission (qPCR assay), community treatments were applied in the absence of *Bd*. This allowed us to examine effects of community composition on the development of host defenses, independent of infection dynamics. Tadpoles of all species were swabbed from early free-swimming stages (Gosner 25) through metamorphosis (Gosner 46) at 2-week intervals to sample skin microbial communities. Morphometrics were collected as in 2019 and included an additional skin microbiome swab to analyze skin microbial communities at metamorphosis.

Effects of future climate on pathogen defenses

To investigate the impact of future climate scenarios on the development of immune defenses, we took a similar approach to the outdoor mesocosm study above, except that laboratory-spawned eggs of *R. pipiens* (from frogs collected from VT and PA) and *R. sphenoccephala* (from frogs collected from TN and LA) were housed in smaller mesocosms inside temperature and humidity controlled environmental chambers (Convion, Adaptis). These chambers were programmed to mimic either (1) the current daily pattern of pond temperature fluctuation during the normal season of larval development at the field site where the parent frogs were collected,

or (2) predicted patterns for this same season for each field site under a mid-range climate scenario for the year 2070 (HadGEM2-ES, RCP 6.0). Following metamorphosis, skin washes and mucosome challenge assays (described above) and *Bd* exposures (described above) were used to compare the effectiveness against *Batrachochytrium* of defenses of juvenile frogs that developed as larvae under current vs. future climate conditions.

Effects of climate on virulence traits

To examine how climate affects virulence and the potential for *Batrachochytrium* transmission, we obtained five *Bd* isolates for phenotypic profiling, including isolates from NM, LA, VT, TN and one from the state of Ohio (near our PA site). We characterized traits that are important for virulence and transmission potential, including culture viability, zoospore, densities, fecundity, growth rates, and carrying capacities.

To quantify these traits, we revived all *Bd* isolates from cryo-preservation and maintained them in tryptone media (Boyle *et al.* 2004). We harvested zoospores from each isolate by filtering out zoosporangia using 20 μ m pore filters (Voyles 2011). We then adjusted the zoospore densities from all isolates to reach a standardized dose before inoculating the zoospores into 96-well plates with multiple well and plate replicates. We incubated the plates at 17°C and monitored them daily using light microscopy (Voyles 2011).

Following the initial phenotyping assays at 17°C, we cultured the isolates at five temperatures across the known thermal breadth of *Bd* (4°C, 12°C, 17°C, 21°C, 25°C, 26°C, and 27°C) to quantify each isolate's responses to a range of temperatures including the thermal maximum (T_{max}) and optimal temperatures (T_{opt}). Because we used only zoospores to start the growth experiments, we were able to track and quantify several parts of the *Bd* life cycle as they occurred at different time points in these different temperature conditions. Specifically, by tracking cultures for multiple successive days, we were able to measure the change in population viability, using a colorimetric MTT assay, and also quantify time (days) to maximum zoospore densities. Using a hemocytometer, we calculated zoospore densities per ml, and culture fecundity. We also calculated the growth rates (r) and carrying capacities (K). We used a model-fitting approach (Voyles *et al.* 2017) to parameterize models of the growth rates of each *Bd* isolate and their differential responses to temperature.

To investigate the potential for evolution of *Bd* thermotolerance, we conducted serial transfer experiments (SPEs) in which we propagated isolates under current and future (to mimic climate change) temperature conditions. Specifically, we generated multiple lineages per isolate and propagated them under current and future temperature scenarios (e.g., exposure to higher thermal maximum and mean daily temperatures) and then measured *Bd* growth and reproductive traits (described above) to determine the impact of long-term propagation under these different thermal conditions.

After ~50 generations of serial passaging in the current and future temperature conditions, we measured time to zoospore release, maximum and cumulative zoospore densities, and the average and cumulative whole culture growth of each lineage in each respective thermal treatment. We measured zoospore densities by counting zoospores, using a hemocytometer, from random sample wells of the 96-well plate. To measure whole culture growth, we used the MTT viability assay (a colorimetric assay for assessing cell metabolic activity) and a BioTek absorbance reader. We compared these measurements among the *Bd* lineages, including the stable-control lineage, to determine what trait differences exist after experimental evolution to thermal climate change conditions.

Statistical Analysis

All statistical models in Objective 2 were run in R (R Core Team 2020). To compare dependent variables (e.g., rates of development, *Bd* load, anti-*Bd* function of skin secretions) among treatment groups in our experiments we used linear mixed models (LMMs, *lme4* package), ANOVAs (*stats* package) and GLMMs (*lme4* package). We used mixed models (LMMs or GLMMs) in cases where models included both fixed and random effects (such as mesocosm tank or individual) and generalized models (e.g., GLMMs) in cases where the data were a better fit to another type of distribution (e.g., Poisson) than the normal distribution and to run logistic regressions. To compare microbiomes among treatment groups we used GLMs for alpha diversity models and PERMANOVAs on unweighted UniFrac distances (*vegan* package) for beta diversity models. To compare survival among treatment groups we used Cox proportional hazards regression (*survival* package, Therneau 2020).

Objective 3. Modeling Disease Risk Under Alternative Climate and Management Scenarios

We used two approaches to develop and parameterize data-driven, mechanistic models of the impacts of climate on disease dynamics at the individual and community levels. The first approach involves using the detailed experiments in Objectives 1 and 2 to determine the appropriate forms of each of the functions in the models, and to determine how temperature affects the model parameters. We have termed this approach Experiment-based models. Our work on this approach is still in progress, because the samples and data from the experiments are still being processed and analyzed. In the meantime, we have made substantial progress on our second approach for modeling this system, which involves fitting Integral Projection Models to the patterns of prevalence and *Bd* load through time from the field survey data from each of the five regions. We have two manuscripts in review on our results from this second approach (Wilber *et al.* In Revision, Wilber *et al.* In Review). We have termed this approach Field survey-based models.

Experiment-based models – We are in the process of developing individual-level models of the *Bd* dynamics on individual frogs to provide a framework for interpreting and integrating the empirical findings in Objectives 1 and 2 and to determine the net effects of the various processes on disease outcome and infectiousness for each of our target species. The goal of the individual-level model is to explore how changing environmental conditions may alter the disease outcome at the individual level (e.g., shifting the outcome from sublethal infection in which adaptive immune responses can control *Bd* growth to rampant *Bd* growth and disease-induced mortality).

To parameterize individual-level models of *Bd* dynamics, we are using the data from the experiment described in Objective 1 in which *Bd*-infected frogs of three species (*R. pipiens*, *R. catesbeiana*, and *R. clamitans*) from two field sites in Pennsylvania were brought into the laboratory and held at a range of temperatures, to quantify the temperature-dependent individual-level *Bd* growth rate. The experiments in the Immunological studies in Objective 1 are providing estimates of temperature dependent *Bd* shedding rates (which contribute to pathogen transmission), and measurements of the anti-*Bd* properties of the mucosome and other components of the immune response. The Effects of climate on virulence traits experiments in Objective 2 are providing information on how the *Bd* genotypes at different locations impact the

disease dynamics. Once we have the data for all of the components of this model, we will carry out extensive sensitivity analysis to determine the effects of the parameters, functions, and model assumptions on the model predictions.

Field survey-based models – An alternative approach to using only the detailed laboratory experiments to parameterize the process-based host/pathogen model, is to fit flexible mechanistic models directly to patterns of infection (prevalence and *Bd* load) from the field survey data for each of our field sites. This approach has the advantage of parameterizing the models for the populations under their natural field conditions (including temperature variation), and in the presence of the entire amphibian host community. To accomplish this, we used host-pathogen Integral Projection Models (Wilber *et al.* 2016) that explicitly account for *Bd* infection intensity (*Bd* load) and the distribution of *Bd* loads in the population of each host species. We developed and fit our IPMs using custom code written in Python (v. 3.6). The code necessary to replicate all of our analyses is available at https://github.com/mqwilber/bd_seasonality".

Integral Projection Models (IPMs) are generally parameterized by fitting statistical (e.g., regression) models for each of the load-dependent (and potentially temperature-dependent) vital rate functions that describe the individual-level disease dynamics to longitudinal data that follow the load on marked individuals through time. In order to instead fit IPMs to our field survey data in which we did not uniquely mark individuals, we developed a novel approach that we have termed reduced dimension host-parasite Integral Projection Models, or “reduced IPMs” (Wilber *et al.*, In Revision). The reduced IPMs use a moment closure approximation to track the first moment (i.e., the mean) of the fungal load distribution across hosts in the population. We have a submitted a manuscript (Wilber *et al.*, In Revision) that describes this new modeling approach, and demonstrates that this approach can simplify fitting to field data, and also results in a tractable model that can be more easily analyzed than full load-dependent IPMs. In the submitted manuscript, we explore how individual-level heterogeneity in pathogen load – a nearly ubiquitous characteristic of host-parasite interactions that is rarely considered in models of microparasites – generally reduces pathogen invasion probability and dampens virulence-transmission trade-offs in host-parasite systems.

In our second submitted manuscript (Wilber *et al.*, In Review), we fit reduced IPMs to three years of our survey data (2017-2019) from four of our field locations (Vermont, Pennsylvania, Tennessee, and Louisiana). For this manuscript, we focused our analysis on the two leopard frog species, *R. pipiens* and *R. sphenoccephala*. We parameterized the model using local water temperature time series for each location, and incorporated *Bd* growth functions from our laboratory experiments. We used a Bayesian approach to fit each model to the field time series data on *Bd* prevalence and *Bd* load at the four locations, relying on information from the literature and our laboratory experiment to aid in parameterization. We used the model to disentangle the relative contribution of seasonality in host demography (e.g., seasonal reproduction and breeding aggregations) and temperature-mediated seasonality in host and pathogen vital rates (e.g., pathogen survival or host immunity) on seasonal patterns of *Bd* infection. We then used the model to ask how seasonal variation in host maintenance potential affects the long-term persistence of *Bd*.

Our next steps in the modeling process are to (1) incorporate the rest of the amphibian community into the IPMs to investigate how climate and amphibian community composition may interact to determine the potential for chytridiomycosis outbreaks; (2) use these community-

level models as a framework for investigating the efficacy of alternative management strategies to mitigate the effects of chytridiomycosis on threatened amphibian species.

Results and Discussion

Objective 1. Ecology of Chytridiomycosis

Task 1: Field surveys and sample collection

We monitored at least four long-term monitoring wetlands at each of our field sites (Table 3), allowing for the comparison of amphibian community composition and pathogen dynamics over time. Over three years, we conducted seasonal sampling of over 14,000 amphibians across five sites for *Batrachochytrium* pathogens. This sampling includes 30 frog and toad species (anurans) and 20 salamander species (urodeles). In addition, we have sampled 2,303 leopard frogs (*R. pipiens* in VT and PA, *R. sphenoccephala* in TN and LA, and *R. chiricahuensis* and *R. blairi* in NM) for both seasonal pathogen loads and measures of immune function (microbiome, mucosome, and antimicrobial peptides; see Task 3 below).

Table 3. Number of monitored wetlands at each of our field sites, as well as the number of species and total amphibians sampled.

Field site	Wetlands monitored (N)	Species sampled (N)		Total amphibians (N)	Leopard frogs (N)
		Frogs & toads	Salamanders		
Vermont	6	8	4	963	132
Pennsylvania	7	7	10	5338	435
Tennessee	5	13	9	2772	591
Louisiana	5	14	7	2685	425
New Mexico	4	9	2	1115	720

Task 2: Quantification and identification of pathogens - *Bd* infection prevalence varies seasonally across the entire amphibian community in PA, TN, and LA. In general, *Bd* prevalence peaks in the winter and spring, and is lowest in the summer months (Figure 3A). Community-wide infection prevalence is less seasonal in VT and NM, with prevalence remaining relatively high (~50%) and low (~10%), respectively, year-round (Figure 3A). Infection prevalence in *R. pipiens* and *R. sphenoccephala* (Figure 4A) also demonstrated this seasonal pattern, but to an even greater extent, with prevalence almost reaching 100% in the winter and spring and dropping down to ~30% or lower in the summer. While prevalence was not seasonal across the entire amphibian community in NM, there was extreme seasonality in infection intensity, with short bouts of very high infection loads in the winter months (Figure 3B). This appeared to be primarily driven by leopard frog species in the amphibian community (Figure 4B). Importantly, we found no evidence of *Bsal* at our field sites.

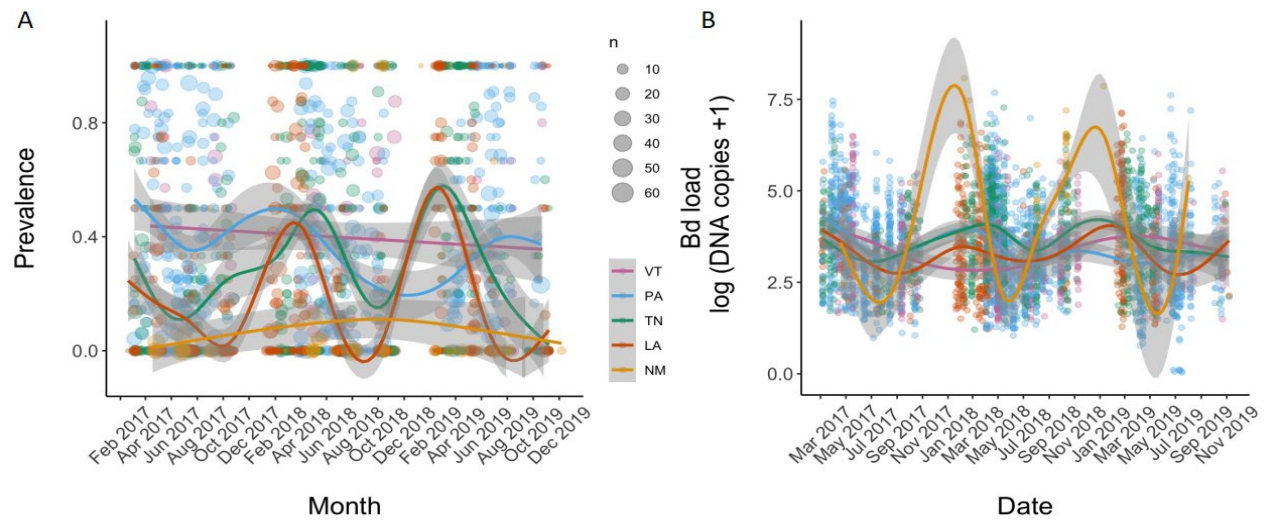


Figure 3. *Batrachochytrium dendrobatidis* (Bd) prevalence (A) and infection intensity (B) by sampling occasion at each of our field sites (VT, PA, TN, LA, and NM) from 2017-2019. A generalized additive model (GAM) smoother demonstrates trends by field site. For prevalence, circle size scales with number of individuals sampled, and the smoother is weighted by the number of animals caught per sampling occasion. Gray shading around a line indicates the 95% confidence interval.

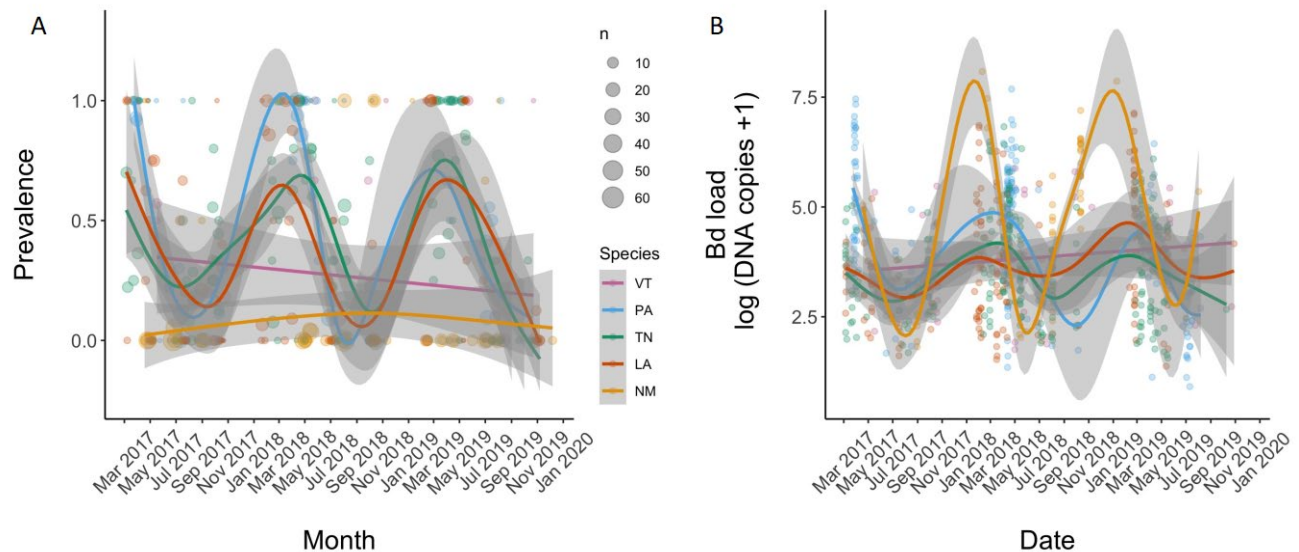


Figure 4. Seasonal prevalence (A) and intensity (B) of *Batrachochytrium dendrobatidis* (Bd) infection in leopard frogs only from 2017-2019: *Rana pipiens* in VT and PA, and *R. sphenoccephala* in TN and LA, and *R. blairi* and *R. chircahuensis* in NM. A generalized additive model (GAM) smoother demonstrates trends by field site. For prevalence, circle size scales with number of individuals sampled, and the smoother is weighted by the number of animals caught per sampling occasion. Gray shading around a line indicates the 95% confidence interval.

Bd infection prevalence was associated with several environmental variables, which were investigated using generalized additive models (GAMs) to allow for non-linear predictors (via smoother functions). Models examining highly correlated variables were run separately and

model comparison was performed (using Akaike's information criterion, AIC). When accounting for seasonality, body temperature non-linearly predicted infection prevalence, with prevalence decreasing at body temperatures below 5°C and above 25°C (GAM, body temperature (°C): $p < 0.001$; Figure 5A and B), mirroring the thermal performance limits for *Bd* growth (Piotrowski *et al.* 2004; Voyles *et al.* 2017). In addition, water temperatures one month previously predicted infection prevalence at the sampling site level, with temperatures lower than 15°C resulting in higher prevalence overall (GAM, 1 month lag temperature: VT: $p = 0.011$, PA: $p < 0.001$, TN: $p < 0.001$, LA: $p = 0.008$; Figure 5C). When looking at data across all sites, we also found an interaction between water pH and temperature, in which we saw lower *Bd* prevalence at high pH and high ($> 30^\circ\text{C}$) and low ($< 10^\circ\text{C}$) amphibian body temperatures (GAM, pH x average water temperature tensor smooth: $p < 0.001$; Figure 5D). Furthermore, there was also an interaction between canopy cover and water temperature, in which there was low *Bd* prevalence in

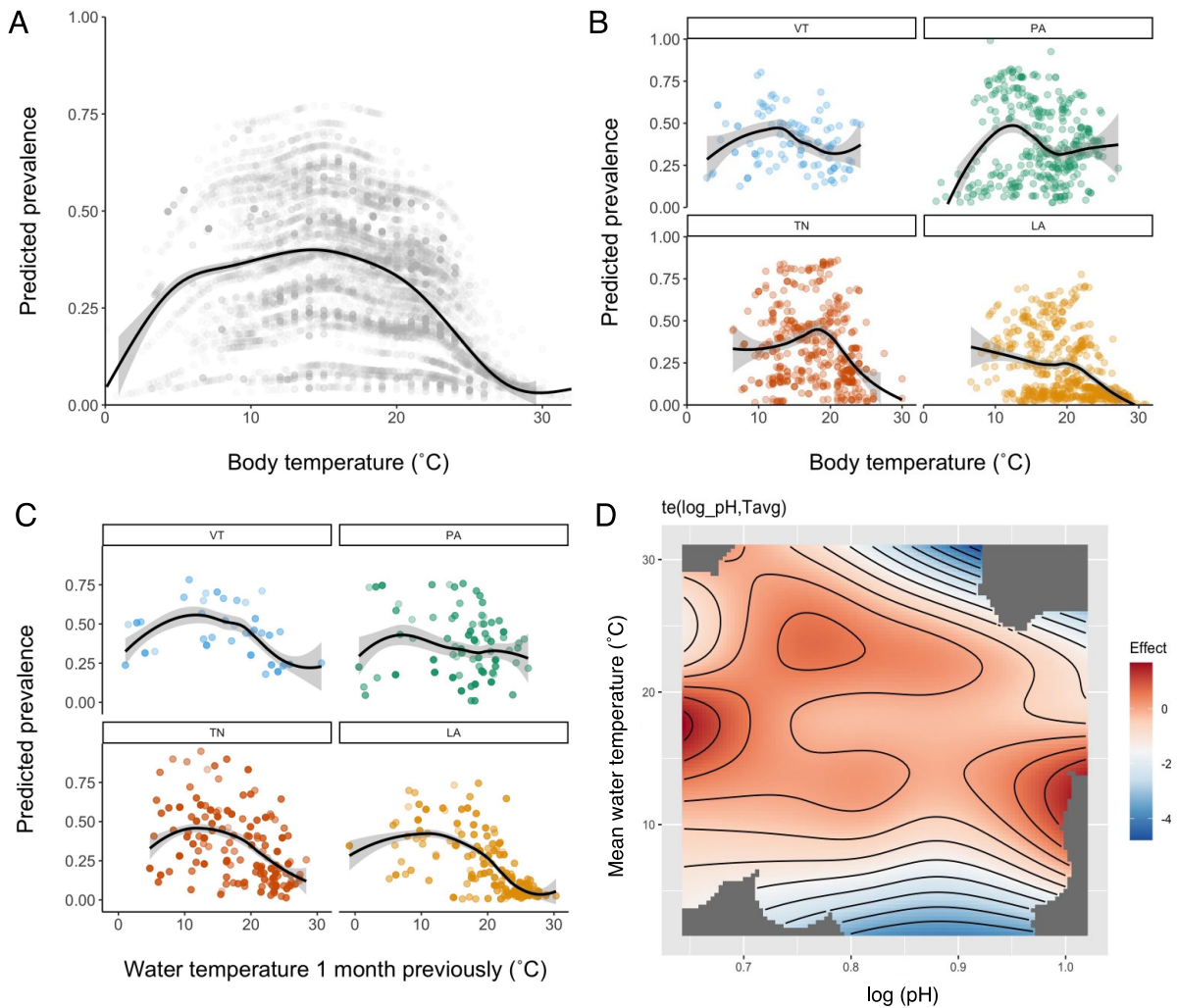


Figure 5. Predicted prevalence of *Bd* infection across all sites and species (A), and for each study site separately (B), as a function of amphibian body temperature. (C) Predicted prevalence of *Bd* infection across all sites and species as a function of water temperature 1 month previously. (D) Predicted prevalence of *Bd* infection across all sites and species as a function of the interaction between mean water temperature (°C) and pH, visualized with a contour plot. A generalized additive model (GAM) smoother demonstrates trends. In (A) through (C), gray shading around a line indicates the 95% confidence interval.

amphibian communities when canopy cover was low and temperatures were either high ($> 30^{\circ}\text{C}$) or low ($< 5^{\circ}\text{C}$) (GAM, canopy cover x average water temperature tensor smooth: $p < 0.001$). When we measured *Bd* loads on naturally infected frogs from PA after holding them at one of three different temperatures, we found that *Bd* loads decreased over time on frogs from all three species tested, but temperature effects on *Bd* clearance rate depended on frog species. On *R. pipiens*, *Bd* load decreased linearly over time, and the rate at which *R. pipiens* cleared *Bd* increased linearly with treatment temperature, such that frogs at 11°C cleared infection the slowest (Figure 6A, $F_{1, 11.8} = 11.8$, $p = 0.001$). For *R. catesbeiana* at 11°C , *Bd* clearance rate was initially low but later increased, whereas *Bd* clearance rate was initially high and then leveled off for *R. catesbeiana* at 16°C and 21°C (Figure 6B, $F_{1, 42} = 18.7$, $p < 0.001$). Finally, *R. clamitans* *Bd* load decreased nonlinearly over time (Figure 6C, $F_{1, 48} = 26.0$, $p < 0.001$), but temperature did not influence *Bd* clearance rate. These results, relating changes in *Bd* load over time to temperature for naturally infected frogs, were important for parameterizing integral projection models developed in Objective 3.

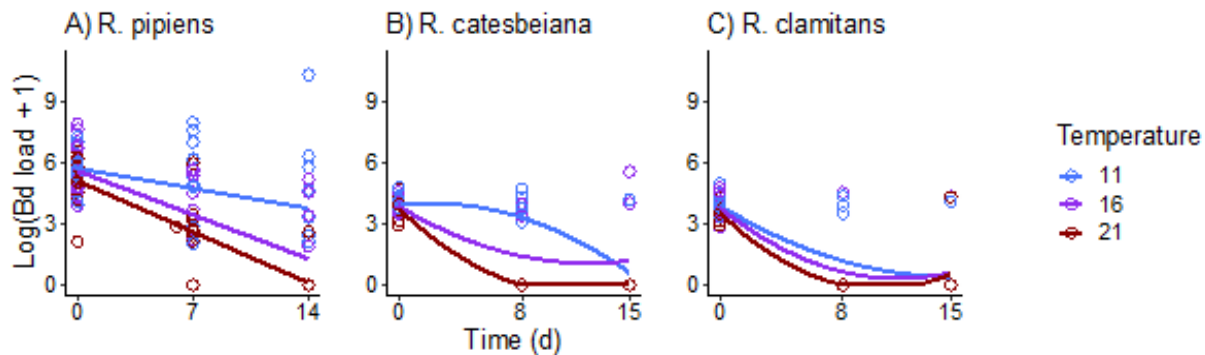


Figure 6. *Bd* loads of (A) *R. pipiens*, (B) *R. catesbeiana*, and (C) *R. clamitans* collected in the field (Day 0) and then maintained at one of three temperatures (11, 16, or 21°C) for two weeks.

We attempted to sequence *Bd* DNA from 522 skin swab samples collected from all five locations from 2017-2019. A total of 359 skin swabs returned enough data (at least 50 amplicon sequences) for further analyses. From these sequences we called genetic variants, trimmed variants for quality and linkage, and calculated a principal components analysis (PCA). We also ran a discriminant analysis of principal components (DAPC) to evaluate the data for genetic clusters (Jombart *et al.* 2010). The *Bd* in all of these samples fell within the Global Panzootic Lineage of *Bd* (*Bd*-GPL). Additionally, we found two major clusters among our samples, supporting previous work reporting the presence of two sublineages of *Bd*-GPL present in the US (Figure 7A). These clades correspond to previously published subclades GPL-1 and GPL-2 (Schloegel *et al.* 2012). Generally, GPL-1 is described as a North American lineage and is proposed to be ancestral to GPL-2, which is globally distributed and is the lineage responsible for declines in Central America (James *et al.* 2015). We assigned samples to the lineages GPL-1 or GPL-2 based on the DAPC analysis; samples with a posterior probability of $< 99\%$ for

assignment into one of these groups were called “mixed” (Figure 7A). In our data, this split between GPL-1 and GPL-2 does not seem to correspond to site, as both clades are represented in

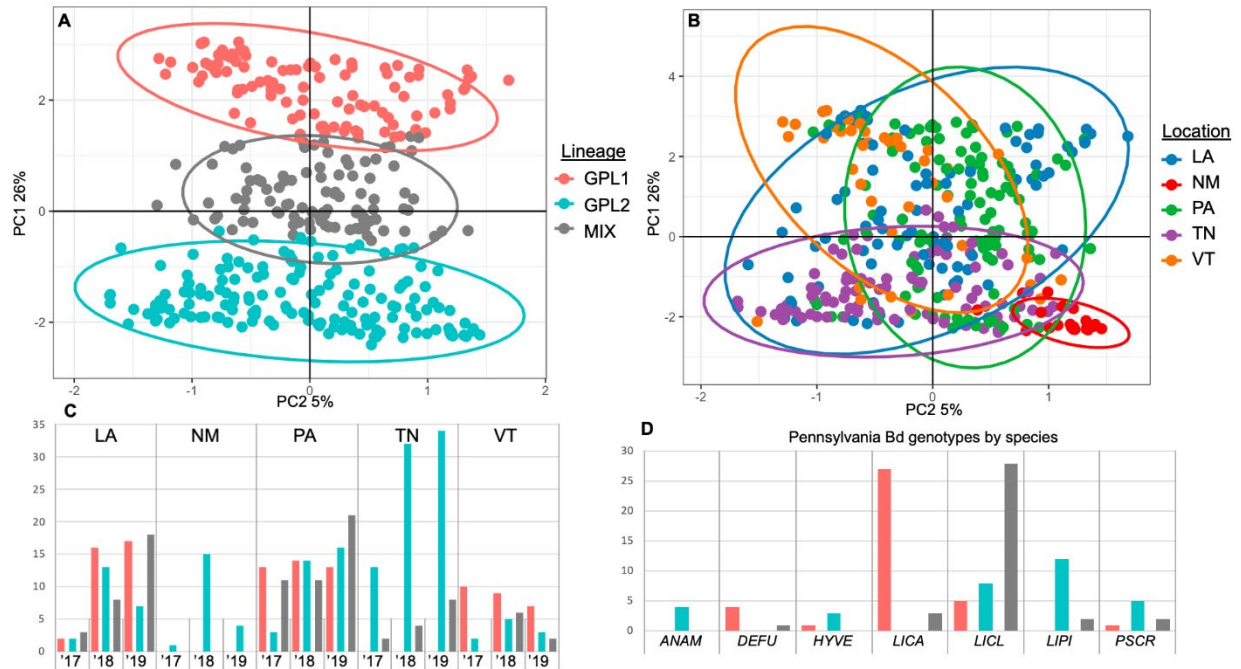


Figure 7. (A) PCA calculated from 66 unlinked single nucleotide polymorphisms (SNPs) from all 359 genotyped samples. Colors correspond to assigned lineage based on DAPC analysis. Samples with >99% posterior probability for assignment to either lineage are colored gray and called “mixed”. The 95% confidence interval ellipses are drawn around each group. (B) The same PCA in panel A colored by field site. (C) Bar chart of *Bd* genotypes at each field site in 2017, 2018, and 2019. Bars colored by genotype as in panel A. (D) Bar chart of *Bd* genotypes grouped by species for individuals captured in PA. Only species with 4 or more captures are shown. Bars colored by genotypes as in panel A. Species codes are as follows: ANAM: *Anaxyrus americanus*, DEFU: *Desmognathus fuscus*, HYVE: *Hyla versicolor*, LICA: *R. catesbeiana*, LICL: *R. clamitans*, LIPI: *R. pipiens*, PSCR: *Pseudacris crucifer*.

samples collected from VT, PA, and LA (Figure 7B), and the proportion of each lineage at each site does not change between sampling years (Figure 7C). In fact, there seems to be a unique genetic pattern for the *Bd* in each of the larger sites. In NM (N swabs = 20) all *Bd* is in the GPL-2 lineage and is genetically homogenous (Figure 7B). In VT (N swabs = 44) both GPL-1 and GPL-2 are present, however all eastern newts (*Notophthalmus viridescens*, N=15) were infected with GPL-1, even when other lineages were present at that locality. In TN (N swabs = 93) most *Bd* is within the GPL-2 lineage, however some *Bd* genotypes are intermediate between the two lineages. Interestingly, 71% (10/14) of the “mixed” samples are from bullfrogs (*R. catesbeiana*) while bullfrogs represent only 20% of the individuals captured in this location. In LA (N swabs = 86) there seems to be geographic partitioning of *Bd* lineages, with some localities having exclusively GPL-1 and others having GPL-2 or mixed samples. In PA (N swabs = 116) there is a higher proportion of mixed samples than any other locality. Further, at this site there is a strong correlation between species identity and *Bd* lineage (Figure 7D). Notably all the bullfrogs captured in PA (N=30) have either GPL-1 (N=27) or a mixed *Bd* genotype (N=3) despite GPL-2 being present at most sites.

Ours was the first broad-scale comparison of contemporary *Bd* genetic variation within the U.S. Some previous studies conducted either highly localized genetic analyses of *Bd* (e.g., Morgan *et al.* 2007), or broad global analyses that included few samples from the U.S. (Byrne *et*

al. 2019, O’Hanlon *et al.* 2018, Rosenblum *et al.* 2013). Our is the first to sequence *Bd* at fine spatial scales, and replicate this sequencing across diverse localities within the U.S. The large amount of *Bd* genetic diversity we were able to document in this study, and especially the mix of *Bd* lineages seen both within and among wetlands, was surprising. At some sites we see interesting patterns developing where certain amphibian hosts or individual wetlands are found in association with certain *Bd* lineages, but more data is needed to further investigate this trend. Overall, our results suggest that *Bd* has likely been coexisting with amphibian hosts in the U.S. for some time and that strains are easily moved from one place to another, likely by human activity or non-amphibian species (e.g., birds; Garmyn *et al.* 2012) as amphibians are not particularly vagile. Further study is needed to understand the history of *Bd*’s introduction and spread within the U.S. and to better understand the origin of the “mixed” *Bd* lineages we detected at many of our field sites. Future studies comparing the virulence of particular lineages, in combination with the genomic tools we have developed and employed here, will be valuable to the DoD in monitoring and mitigating the movement and spread of particularly lethal pathogen variants.

Task 3: Microbiome/Mucosome analyses

We processed samples from the 2017 through 2018 field seasons (Table 1) and discovered clear patterns in the microbiomes on amphibian skin including differences among host species and seasons, and also shifts in the microbiome corresponding to *Bd* infection status (Figures 8 - 10). Future analyses will continue to investigate mucus function against *Bd*, fungal and bacterial communities found on leopard frog skin, and the relationship between skin defense peptides, microbiomes, and *Bd* infection dynamics.

Alpha diversity metrics indicated higher sOTU richness and phylogenetic diversity in VT compared to the other field sites and slight differences across the two years. There did not appear to be differences in alpha diversity between the frog species (Figure 8). *Bd* infection status and

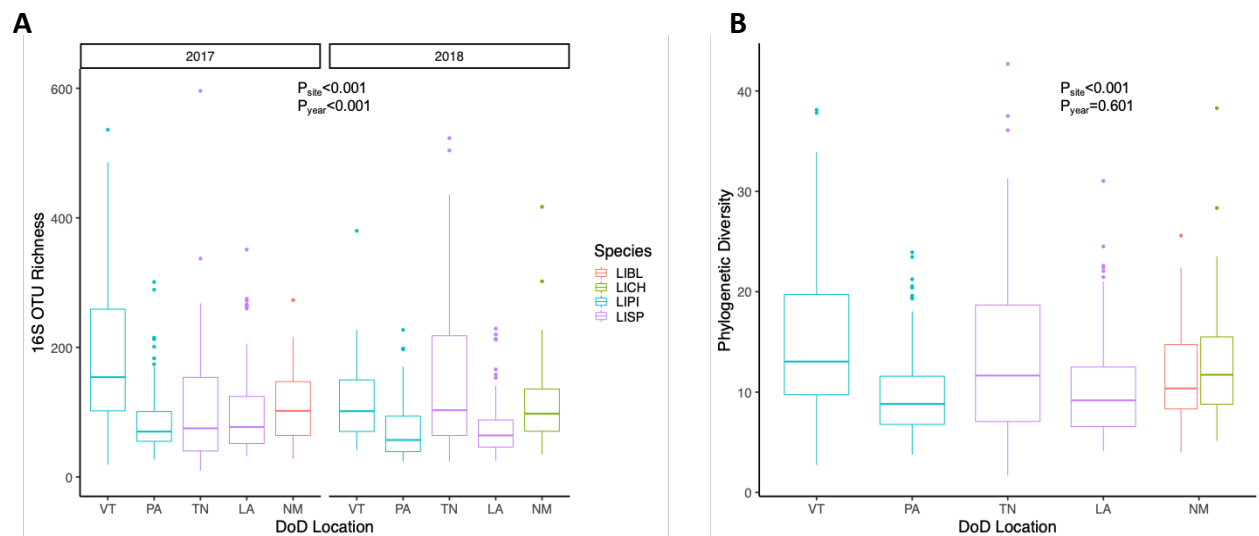


Figure 8. Box plots showing overall microbiome alpha diversity metrics (A) sOTU Richness and (B) Phylogenetic Diversity) for leopard frogs across the two sampled years and each field site. LIBL: *R. blairi*, LICH: *R. chiricahuensis*, LIPI: *R. pipiens*, LISP: *R. sphenoccephala*. For each box plot the center line indicates the median and the upper and lower extents of the box represent the 75th and 25th quartiles, respectively. The whiskers extend no further than 1.5 times the inter-quartile range.

Bd loads did not affect sOTU richness nor phylogenetic diversity. For microbiome beta diversity, bacterial communities changed across years in sOTU composition but showed less change phylogenetically (R^2 higher for non-phylogenetic metrics: Bray Curtis: $R^2 = 0.083$, $p < 0.001$; Weighted UniFrac: $R^2 = 0.026$, $p < 0.001$; Figure 9). Communities also varied across sample locations for each season (Bray Curtis: $R^2 = 0.135$, $p < 0.001$; Figure 10A). The percent anti-*Bd* function differed between sample seasons across years ($\chi^2 = 39.181$, $p < 0.001$), and with *Bd* infection ($\chi^2 = 4.619$, $p = 0.032$), but did not correlate with *Bd* loads ($\chi^2 = 1.135$, $p = 0.287$).

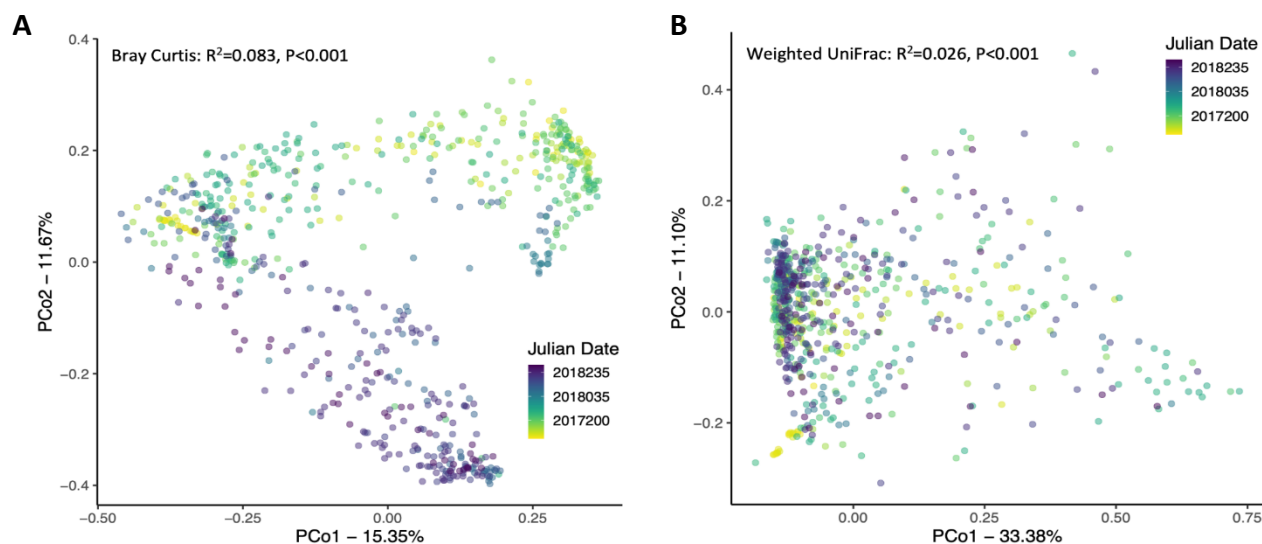


Figure 9. Principal Coordinate Analysis (PCoA plot) of microbial community structure for leopard frogs sampled in 2017 and 2018. Bacterial community members shifted through time but phylogenetic distribution of membership was better maintained [R^2 higher for (A) Bray-Curtis than (B) Weighted UniFrac]. Amphibian skin microbial communities were highly dominated by Proteobacteria, which may explain why the Weighted phylogenetic metric showed less obvious shifts in community composition through time.

Leopard frogs, in general, responded to higher *Bd* loads with more *Bd*-inhibitory mucus. But the data for all frogs show that there is no difference in mucosome function between *Bd*- and *Bd*+ frogs. Mucus function varied by species and was driven mostly by the PA *R. pipiens* samples, where frogs had higher mucosal function and higher percent anti-*Bd* bacteria when infected (Figure 10B). Overall, bacterial communities were similar across the field sites, with many shared microbial taxa (Figure 10C). Bacterial communities shifted seasonally and there were some taxa that were more abundant in, or unique to, to certain locations. Some taxa are known to be dominant and can function to inhibit *Bd* growth. For example, several *Janthinobacterium* taxa are known to inhibit *Bd* growth, but these bacteria do not grow very well at warmer temperatures. We found them to be in higher abundance in our two more northern locations, PA and VT, possibly helping protect those populations from *Bd*. In addition to culture-independent analyses, we isolated 104 bacteria and 16 fungi from leopard frog skin. Nineteen bacteria and one fungus inhibited growth of both *Bd* isolates tested (VT Rcla225, and Section Line). Many microbes inhibited the growth of one of the two *Bd* isolates (Figure 11).

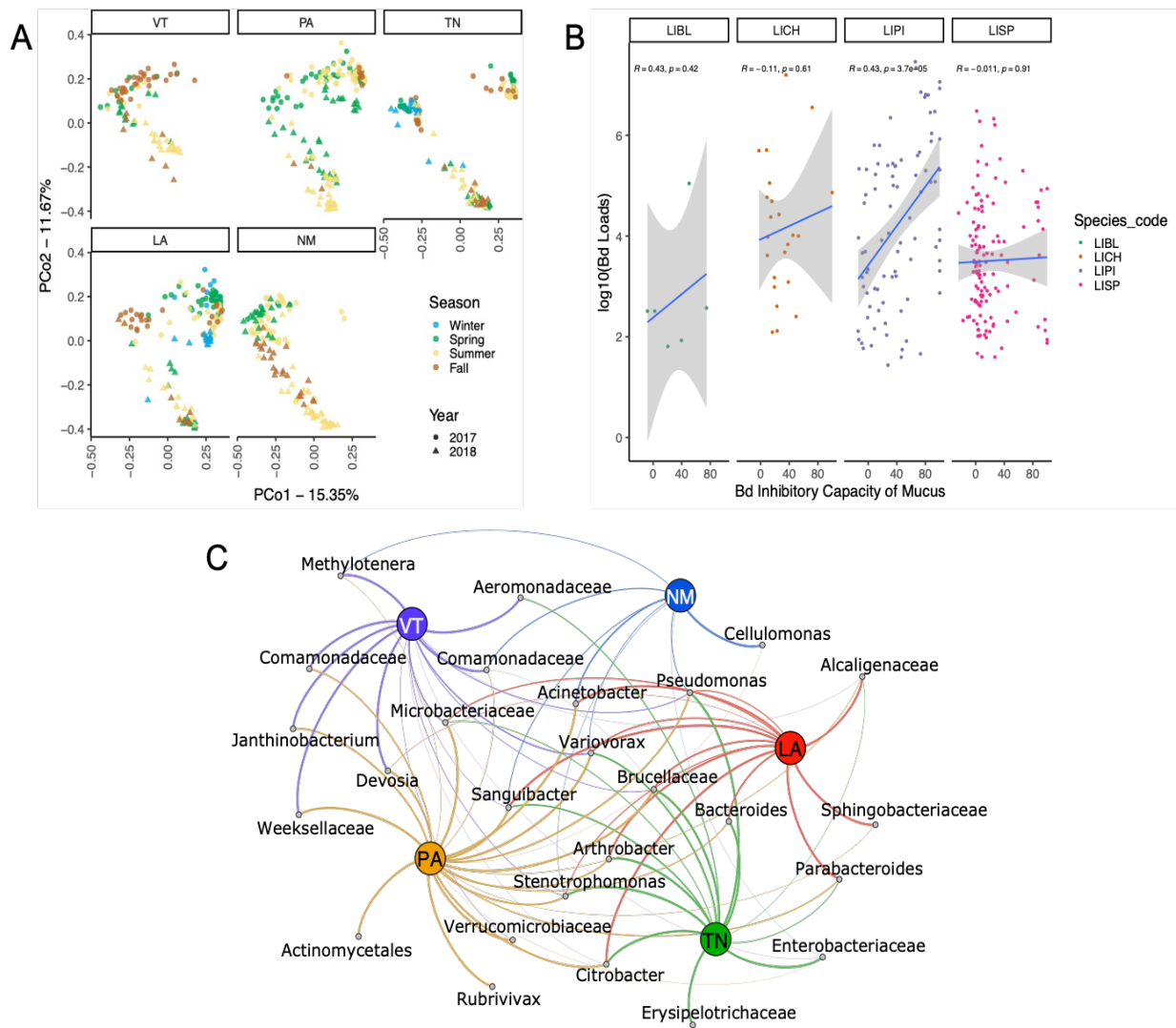


Figure 10. Microbiome results from the five DoD SERDP sites. A) PCoA plots highlighting the seasonal changes in the 2017 and 2018 microbiomes (Bray Curtis: $R^2=0.135$, $P<0.001$). Each point represents the microbial community of an individual frog sampled. Shapes indicate the year and color indicates the season. B) Inhibitory capacity of the mucosome against *Bd* correlates with the natural *Bd* infection across sites. Shaded areas indicate 95% confidence intervals. C) For the bipartite network, the large nodes indicate site and the small gray nodes indicate bacterial taxa at the family level. All bacteria families with relative abundances greater than 100 reads are represented by the gray nodes – the edges or connections between these nodes are then weighted by relative abundance; a thicker line indicates higher abundance. LIBL: *R. blairi*, LICH: *R. chiricahuensis*, LIPI: *R. pipiens*, LISP: *R. spheocephala*.

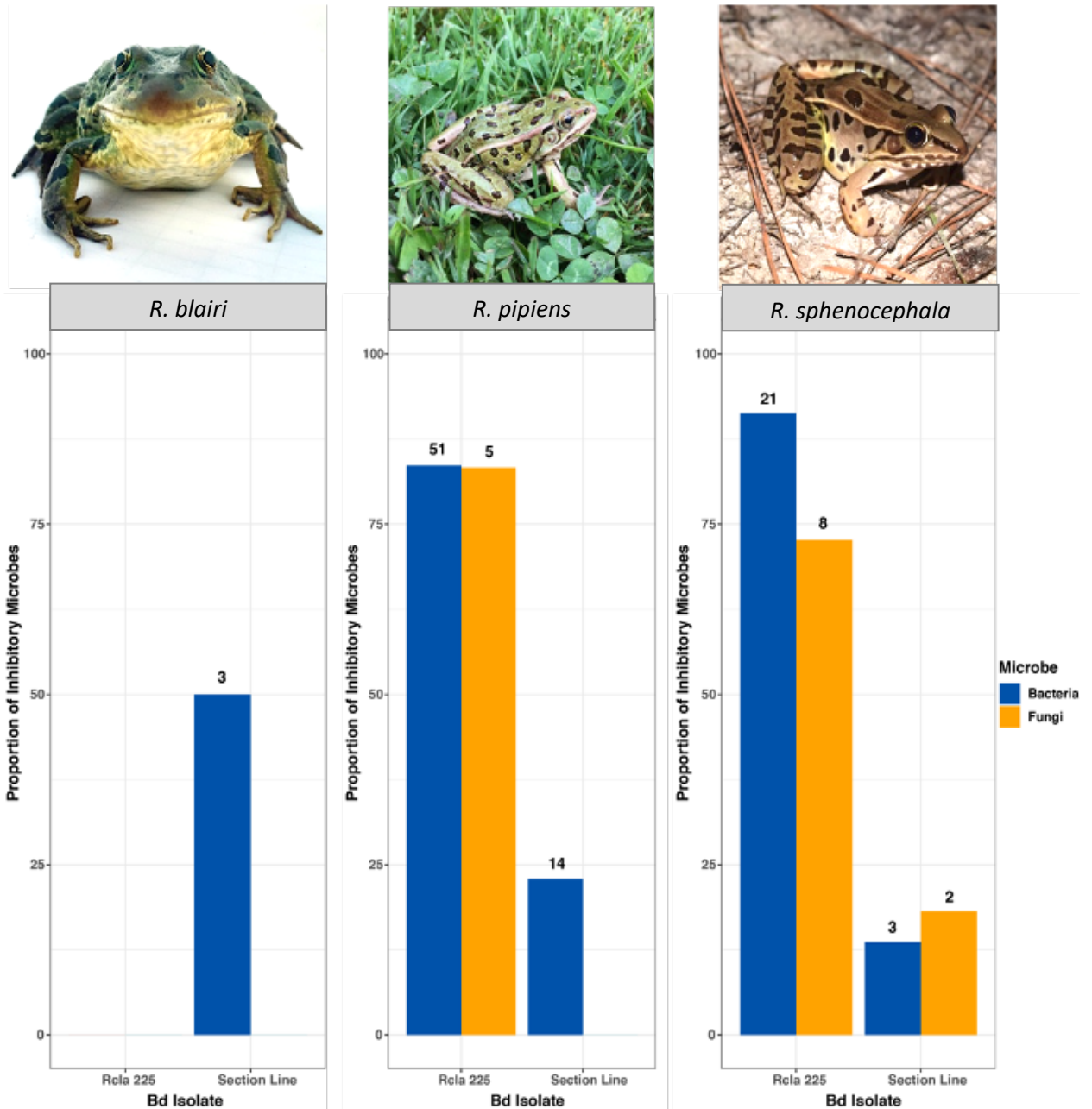


Figure 11. Relative *Bd* growth inhibition caused by secondary metabolites from fungal and bacterial isolates from leopard frogs. Numbers above the bars indicate the number of isolated bacteria and fungi from each of the three species that were inhibitory against the two *Bd* isolates.

As discussed in Le Sage *et al.* (2021), *Bd* dynamics in southern leopard frogs from TN followed seasonal fluctuations with peaks in cooler months coinciding with when host mucosal defenses were most potent against *Bd*. Although microbiome changes followed more linear trends over time, the proportion of bacteria that can function to inhibit *Bd* growth was greatest when risk of *Bd* infection was highest. Seasonality of *Bd* is thus partly regulated by annual immune rhythms including skin peptides that can alter the microbiome, and the rhythms are dominated by the effects of temperature that induce preparatory immunity in host frogs before the onset of conditions ideal for *Bd* infection. Future studies are needed to focus on the effects of

extremely warm winters or unusual weather events on DoD lands before spring migrations to breeding that could offset the evolved synchrony between amphibian immune and microbiome defense and periods of greatest infection risk.

Antimicrobial peptides (AMPs) in skin secretions

Our description of the AMPs recovered from field collected skin secretions will be presented by species and population, beginning with the Chiricahua and plains leopard frogs in New Mexico. In 2017, 43 plains leopard frogs (*R. blairi*) were sampled for antimicrobial peptides (AMPs) from the NM site number 7 on three dates (April 18, June 28, and August 31). In April, all six frogs sampled were adults, while in June, all but one of 18 frogs were juveniles and in August, all but two of 19 frogs were juveniles. The adults captured in April had greater recovered peptide concentrations (average of $18,659 \pm 6,694$ $\mu\text{g/ml}$ mucus) than the juveniles sampled in June (average of $2,448 \pm 398$ $\mu\text{g/ml}$ mucus). The two adults captured in August had the highest concentrations (average: $30,352 \pm 5,388$ $\mu\text{g/ml}$ mucus), and the juveniles sampled in August had higher concentrations (average: $6,178 \pm 2,306$ $\mu\text{g/ml}$ mucus) than those in June. Recovered peptide concentrations were not related to body condition, measured as scaled mass index (LM: $p = 0.193$). Of these 43 animals, only six were *Bd* positive. Specifically, one adult in April, one juvenile in June, and four juveniles in August. The infected adult sampled in April displayed similar peptide concentrations to the 5 uninfected animals (19,801 compared with $18,430 \pm 8,194$ $\mu\text{g/ml}$ mucus, respectively), while the infected juvenile in June appeared to have a lower concentration than the 16 uninfected juveniles (1,118 compared with $2,649 \pm 421$ $\mu\text{g/ml}$ mucus). In August, the four infected juveniles had similar peptide concentrations as the 13 uninfected juveniles (t-test: $p = 0.857$).

In 2018 and 2019, we recovered peptides from 171 Chiricahua leopard frogs (*R. chiricahuensis*) from NM sites number 5 and 8. Samples span five visits from May through October in 2018, and seven visits from February to July in 2019. Twenty-four *Bd* positive animals were sampled in 2018 and none in 2019. Of these 24 infected animals, the majority were juveniles sampled on August 3rd (17 of 21 infected juveniles). Because *Bd* infection in this dataset is confounded with site, we did not include site as a random variable in general additive models (GAM) of peptide quantities. We hypothesize that infection contributes more to variation among individuals than site (or population) in peptide quantities. In 2019, peptide quantities did not appear to differ among life stages, suggesting the lower concentrations exhibited by the infected juveniles in 2018 may be related to their seasonal infections. The top models including either daily average temperature or day of year were indistinguishable by model selection. Both models also included *Bd* infection and year. The models suggest that peptide concentrations peaked mid-year (GAM; day of year: $p < 0.001$), increased strongly when daily average temperature was above $\sim 12^\circ\text{C}$ (GAM; temperature: $p < 0.001$), and was lower in infected frogs (GAM; Infection: $p < 0.001$; Figure 12). Altogether, life stage, *Bd* infection, temperature, and day of the year are confounded, but appear to strongly predict peptide concentrations in Chiricahua leopard frogs.

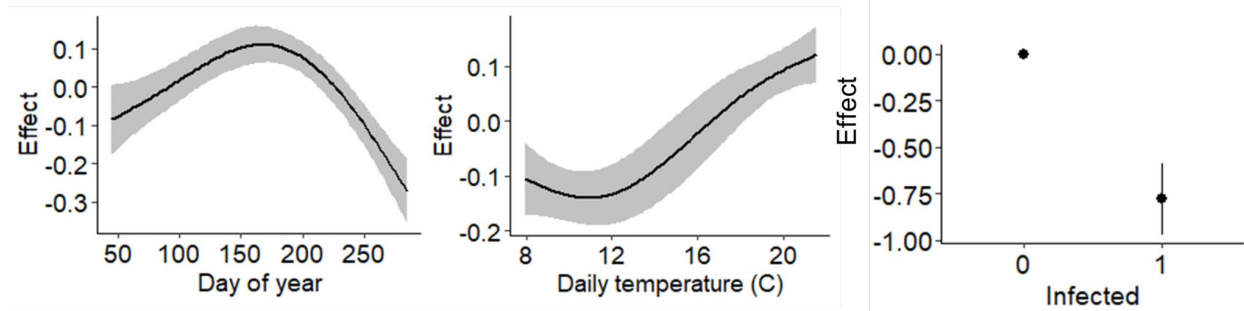


Figure 12. Recovered peptide concentrations ($\mu\text{g/ml}$ mucus) from Chiricahua leopard frogs surveyed in New Mexico in 2018 and 2019 were predicted by the two highly correlated factors, day of year (a) and temperature (b; effect shown on y-axis; GAMs fit separately). *Bd* infection had a negative effect on peptide concentrations (c). Gray shading indicates the 95% confidence interval.

When analyzing peptide spectra using matrix assisted laser desorption ionization time-of-flight (MALDI-TOF), we did not detect the known AMPs based on previous work in other populations in leopard frogs from NM (Conlon *et al.* 2011), however, we found four AMPs with similar masses that are potential isomers (Brevinin-1CHa/b: 2544 m/z, Ranatuerin-2CHb: 2890 m/z, Ranatuerin-2CHa: 2975 m/z, and Esculentin-2CHa: 3843 m/z). Of these four putative AMPs, we found that *Bd* infected frogs were less likely to express Ranatuerin-2CHa (GLM: $p = 0.002$) and had lower relative intensities of both Brevinin-1CHa/b (GLM: $p < 0.001$) and Esculentin-2CHa (GLM: $p = 0.002$). The pattern of MALDI spectra, compared as either presence/absence of peaks or relative intensity of peaks varied by day of year, year, infection status, and frog mass (PERMANOVA of Jaccard and Bray-Curtis distance matrices, respectively: $p < 0.01$).

Using serial dilutions of recovered hydrophobic peptide samples from 500 $\mu\text{g/ml}$ to 16 $\mu\text{g/ml}$, we were able to compare the minimum inhibitory concentration (MIC: concentration at which at least 95% growth compared to the positive control was inhibited) and the predicted 50% inhibitory concentration (IC₅₀: concentration estimated by fitting two parameter log-logistic curves of percent inhibition) for leopard frogs from NM. We assayed 58 and 18 Chiricahua and plains leopard frog samples, respectively, with an inter-assay CV of 3.3%, an intra-assay CV of 16.2%, and we observed no inhibition from field negative controls. We found that the majority of both species inhibited at 250 $\mu\text{g/ml}$, with six Chiricahua samples showing little to no inhibition at 500 $\mu\text{g/ml}$. The predicted 50% inhibitory concentration was different among infected and uninfected Chiricahua leopard frogs but did not vary by year or mass (ANOVA: $p = 0.003$, Year: $p = 0.271$, Mass: $p = 0.343$), suggesting secretions from infected animals may contain lower concentrations of AMPs or less effective peptides at inhibiting *Bd* growth. This capacity could be related to infected frogs expressing lower relative intensities of two of four AMPs (GLM: Esculentin-2CHa: $p < 0.001$; Brevinin-1CHa/b: $p = 0.0411$) and higher relative intensities of Ranatuerin-2CHa (GLM: $p < 0.001$). However, the IC₅₀ of secretions was positively related to only the relative intensity of Ranatuerin-2CHb (LM: $p = 0.024$). Similarly, infected frogs had lower MIC equivalents per ml of mucus than uninfected frogs (ANOVA: Infected: $p = 0.008$, Day: $p = 0.606$, Year: $p = 0.264$). With only six infected plains leopard frogs, the inhibitory capacity of secretions was only marginally related to infection (MIC; ANOVA: $p = 0.086$). Although none of the AMPs differed in relative intensity by *Bd* infection, the MIC of plains leopard frog secretions were positively correlated with the relative intensity of Brevinin-1BLa (LM: $p = 0.020$) and negatively correlated with the relative intensity

of Brevinin-1BLc (GLM: $p = 0.007$). This suggests that infection can have varying effects (i.e., suppress or enhance) on the relative intensities of AMPs, which depends on the relative intensities of other peaks in the spectra. The lowest amount of MIC equivalents (total peptide quantity divided by MIC) in mucus was 2.4, suggesting that leopard frogs surveyed here would rarely have inadequate defenses stored to inhibit *Bd* growth.

From northern leopard frogs (*R. pipiens*) in Vermont, we collected and quantified peptide secretions from 124 individuals across three years. The majority were captured at two sites (VT site numbers 3 and 6), except seven frogs from site number 7 and three frogs from site number 5. In 2017, samples were collected over six surveys, with two in spring, one in late summer, and three in fall, for a total of 46 peptide samples. In 2018, 40 samples were collected over nine surveys, but the majority were from one date in July ($N = 21$), with four surveys in spring, two in summer, and three in the fall. In 2019, 38 samples were collected over six surveys, and again the majority were collected in the summer ($N = 31$) in four surveys, and only one survey in which frogs were sampled in either spring or fall. Across all years, frogs sampled for AMPs were more likely to be infected in spring (20 of 26 total), and less likely in summer and fall (5 of 63 total and 9 of 35 total, respectively).

Recovered peptide concentrations (per ml mucus) displayed a strong seasonal pattern consistent among years in which frogs sampled in the summer had much lower concentrations than spring and fall. Note that summer samples consisted mainly of juveniles, however, in the fall juveniles and adults had similar recovered peptide concentrations (LME: $p = 0.728$). Therefore, seasonal patterns are confounded with frog body size/life stage. Peptide concentrations were predicted by the two-week lag temperature (GAMM with site as a random variable; 2-wk temperature: $p < 0.001$), in that below 10°C had a positive effect, then above 10°C , peptide concentrations were negatively associated with two-week average temperature. Infection status did not predict recovered peptide concentrations (GAMM; Infected: $p = 0.554$), however, frogs in 2018 had greater concentrations than the other years (GAMM; 2018 vs 2017: $p < 0.001$). Note temperature (derived from NicheMapR) is highly correlated with day of year, which was a significant predictor in the second-best fit model. Therefore, we analyzed the effect of temperature within season, and found that the effect of daily average temperature on peptide concentrations varied by season (average temperature $\times$ season interaction: $p = 0.031$; Figure 13). Specifically, in fall temperatures below 12°C had a positive effect, while temperatures above 12°C had a negative effect on concentrations; while in other seasons, average temperature had a slight parabolic effect. Though experimental manipulation is needed to tease these factors apart, temperature in the fall may provide the cue to ramp up peptide storage for cooler months when temperature can significantly impair peptide synthesis (Robak *et al.* 2019; Le Sage *et al.* 2021).

We are still analyzing AMP presence via MALDI for this location and hence, do not have any patterns yet to present. However, we have assayed 64 northern leopard frogs from Vermont for the capacity to inhibit *Bd* growth in vitro using a standardized serial dilution from 500 to 16 $\mu\text{g/ml}$. We assayed 26 samples from 2017, 21 from 2018, and 17 from 2019. Seven samples were not inhibitory enough at 250 or 500 $\mu\text{g/ml}$ to provide a MIC, and three of these samples did not inhibit at the 500 $\mu\text{g/ml}$ concentration. The remaining 56 samples were typically inhibitory at

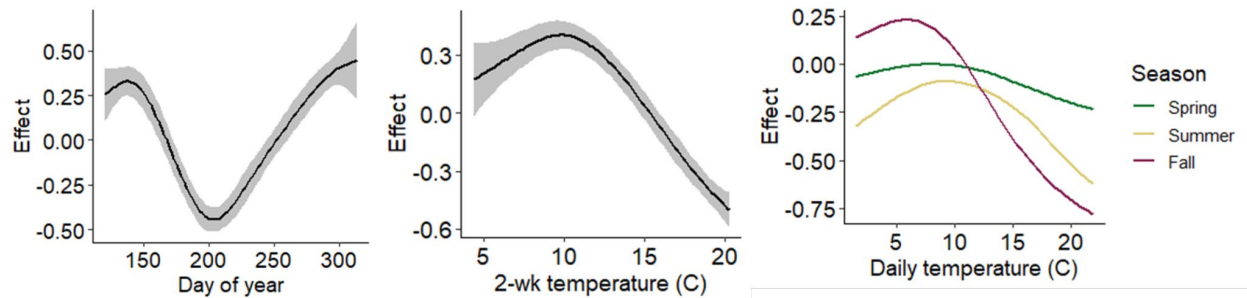


Figure 13. Recovered peptide concentrations ($\mu\text{g/ml}$ mucus) from northern leopard frogs (*R. pipiens*) surveyed in Vermont across three years were predicted by the two highly correlated factors, day of year (a) and 2-week average temperature (b; effect on y-axis; GAMMs fit separately). The effect of daily average temperature also varied by season (c). Gray shading indicates the 95% confidence interval.

125 $\mu\text{g/ml}$, with an average of 198 ± 16 $\mu\text{g/ml}$. The inter-assay CV was 22.8% and the intra-assay CV was 35.9%, and when excluding samples with over 99% inhibition (19.5% of values) the intra-assay CV was 22.6% because differences within replicates are amplified with smaller optical density values. We observed no inhibition from field negative controls.

The predicted 50% inhibitory concentration (IC₅₀) of recovered hydrophobic secretions was related to the average temperature of the month prior to sampling (GAM; 1-mo temperature: $p < 0.001$). The IC₅₀ appears lowest (stronger capacity to inhibit) when last month's average temperature was around 10°C, therefore, secretions become less effective above and below the peak around 10°C. Whether frogs were infected with *Bd* when sampled did not affect the capacity to inhibit *Bd* growth in vitro (GAM; Infection: $p = 0.75$). The lowest amount of MIC equivalents in mucus was 8.3, suggesting that northern leopard frogs surveyed here would rarely have inadequate defenses stored to inhibit *Bd* growth.

The mucus samples from *northern leopard frogs (R. pipiens) in Pennsylvania* are still being processed and results thus far are reflective of a subset of only the quantities of secreted peptides. We analyzed 114 samples from frogs captured in 2018 and 2019 across three seasons. Note that there are only six samples that were collected in the fall, and only 22 samples from 2019. The recovered peptide concentrations in this location display a similar seasonal pattern as in VT, with the lowest quantities recovered in the summer. In general, recovered peptide concentrations are higher in spring than summer, when most of the samples were from juvenile frogs. Similar to those in VT, recovered peptide concentrations were predicted by day of year (GAM; $p < 0.001$ Figure 14), but *Bd* infection was not a significant predictor (GAM: $p = 0.40$).

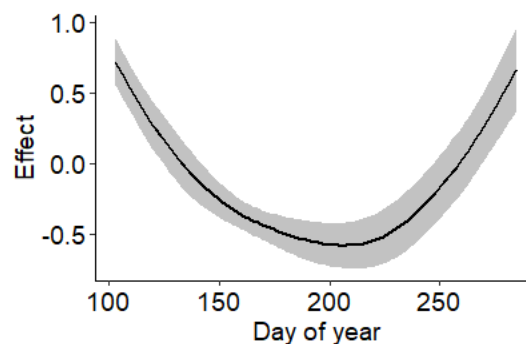


Figure 14. Recovered peptide concentrations ($\mu\text{g/ml}$ mucus) from northern leopard frogs (*R. pipiens*) surveyed in PA were predicted by day of year but not *Bd* infection (y-axis is effect of day of year predicted by GAM). Gray shading indicates the 95% confidence interval.

From southern leopard frogs (*R. sphenoccephala*) in Tennessee, we collected and quantified 449 peptide secretions across three years of sampling. These animals were sampled across 105 survey dates that ranged from mid-February to early November. More juveniles were sampled in summer than the other seasons, but the range of body sizes was similar across seasons. Recovered hydrophobic peptide quantity was related to body mass (LM: $p < 0.001$), but with a relatively low R^2 (0.146). For consistency we transformed data to concentration per ml of mucus like the other locations. Peptide concentrations were predicted by day of year and year (GAMM; $p < 0.001$, $p = 0.02$, respectively; Figure 15a), but not *Bd* infection (GAMM; $p = 0.57$), suggesting frogs had the highest concentrations in winter and fall, and the lowest in summer. The direction of temperature effects within each season was variable (GAMM; Season x 1-month temperature interaction: $p < 0.001$; Figure 15b), appearing to have a negative effect in the spring and fall, no effect in winter, and a positive effect in summer.

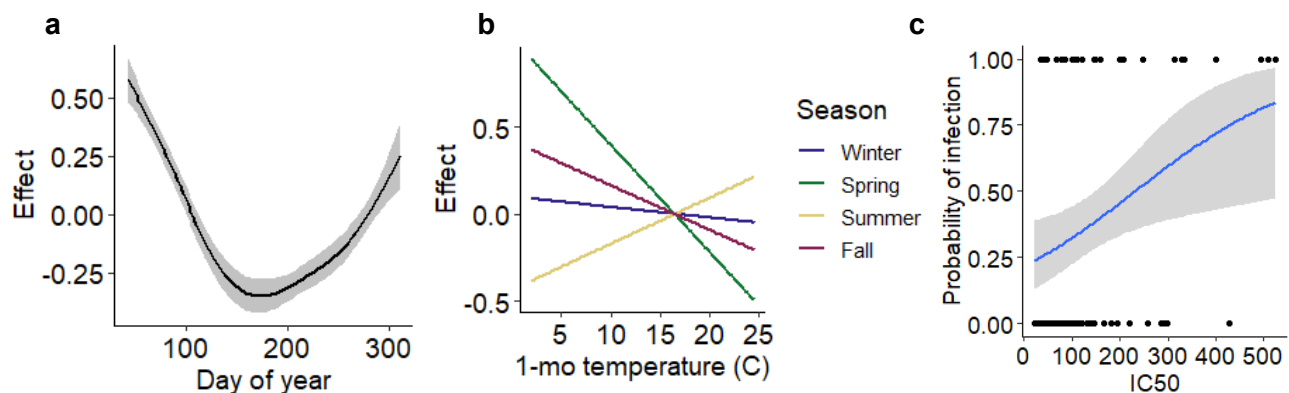


Figure 15. Recovered peptide concentrations ($\mu\text{g/ml}$ mucus) from TN southern leopard frogs (*R. sphenoccephala*) surveyed across three years were predicted by day of year (a), but not by temperature or *Bd* infection (GAMM). (b) Peptide concentrations were affected by the 1-month average temperature differently across seasons (GAMM). The effect is shown on the y-axis; GAMMs fit separately. (c) The probability of infection was predicted by the estimated 50% inhibition (GLMM), suggesting that those with less effective secretions were more likely to be infected. Gray shading indicates the 95% confidence interval.

Using MALDI-TOF mass spectrometry, we detected the presence of four previously described AMPs in southern leopard frog secretions from TN (Conlon *et al.* 1999). Of the 430 TN samples analyzed via MALDI-TOF, 81% expressed Temporin-1S, 87% expressed Brevinin-1Sa, 80% expressed Brevinin-1Sb, and 50% expressed Brevinin-1Sc. There was a seasonal pattern similar to that of total peptide concentrations in the expression of two of the four AMPs. Specifically, the probability of detecting Brevinin-1Sa and Brevinin-1Sb was predicted by day of year (GAM: Brevinin-1Sa: $p = 0.034$; Brevinin-1Sb: $p = 0.016$) and there was no effect of *Bd* infection or year (GAM: $p > 0.05$). On the other hand, the probability of detecting Temporin-1S in secretions was not related to the day of year (GAM: $p = 0.311$) but was greater in *Bd* infected frogs (GAM: $p = 0.02$) and varied by year (GAM: $p < 0.001$). The probability of detecting Brevinin-1Sc was not predicted by any of the variables in the models (GAM: day of year, temperature, *Bd* infection, year, or peptide quantity, $p > 0.05$). The probability of *Bd* infection was positively related to the relative intensity of Brevinin-1Sb and marginally to Brevinin-1Sa (GLMM; $p = 0.016$ and $p = 0.052$, respectively). Likewise, the relative intensity of two of four AMPs predicted infection probability (GLMM: Brevinin-1Sa: $p = 0.05$; Brevinin-1Sb: $p = 0.02$). This suggests infected frogs may be responding to infection with relatively more of these AMPs compared to other peptide species in their stored secretions. For 265 samples collected in 2018

and 2019, we found that the pattern of MALDI-TOF spectra, compared as either presence/absence of peaks or relative intensity of peaks varied by day of year, year, infection status, and frog mass (PERMANOVA of Jaccard and Bray-Curtis distance matrices, respectively: $p < 0.05$).

We assayed 149 southern leopard frogs from TN for the capacity to inhibit *Bd* growth *in vitro* using a standardized serial dilution from 500 to 16 $\mu\text{g/ml}$. We assayed 55 samples from 2017, 50 from 2018, and 44 from 2019. Note, 69 samples were not inhibitory enough to provide an MIC, and seven of these samples had nearly zero inhibition at 500 $\mu\text{g/ml}$. The remaining 80 samples were typically inhibitory at 250 $\mu\text{g/ml}$, with an average of $290 \pm 17 \mu\text{g/ml}$. The inter-assay CV was 13.3% and the intra-assay CV was 10.4%. We observed inhibition from some field negative controls, but there was no evidence of contamination of peptides when analyzed via MALDI-TOF.

The predicted 50% inhibitory concentration (IC₅₀) of recovered hydrophobic secretions (from a subset of GIAs performed using a standardized culture, $N = 83$) was not related to the day of year or temperature (GAMM; $p > 0.05$). *Bd*-infected frogs had a lower capacity to inhibit *Bd* growth *in vitro* (i.e., higher IC₅₀, GAMM; Infection: $p = 0.03$), and frogs in 2019 had less-effective secretions than in 2018 (GAMM; Year: $p = 0.02$). IC₅₀ was not related to total recovered peptides (LME; $p = 0.20$) but was negatively related to the relative intensity of two AMPs, Temporin-1S and Brevinin-1Sc (LME; Temporin-1S: $p = 0.046$, Brevinin-1Sc: $p = 0.041$). This suggests that secretions with greater relative intensities of these AMPs had a greater capacity to inhibit *Bd* *in vitro*. This was further supported by IC₅₀ predicting the probability of infection when including body condition, year, and 2-wk temperature or day of year (GAMM; IC₅₀: $p = 0.024$; 2-wk temperature: $p = 0.06$; day of year: $p = 0.01$). MIC equivalents did not vary by day of year, temperature or *Bd* infection, but they varied among years (GAMM; $p = 0.02$). The lowest amount of MIC equivalents in mucus was 1.4, suggesting that southern leopard frogs surveyed here would likely have very adequate defenses stored to inhibit *Bd* growth.

From *southern leopard frogs (R. sphenoccephala)* in *Louisiana*, we collected and quantified peptide secretions from 265 southern leopard frogs across three years of sampling. These animals were sampled across 75 survey dates that ranged from mid-January to early November. Note that the year 2017 does not have representatives from all seasons like the other years and consists of 13 surveys from September to October. More juveniles were sampled in summer than the other seasons, but the range of body sizes was similar across seasons. Recovered hydrophobic peptide quantity was related to body mass ($p < 0.001$) but with a relatively low R^2 (0.035). For

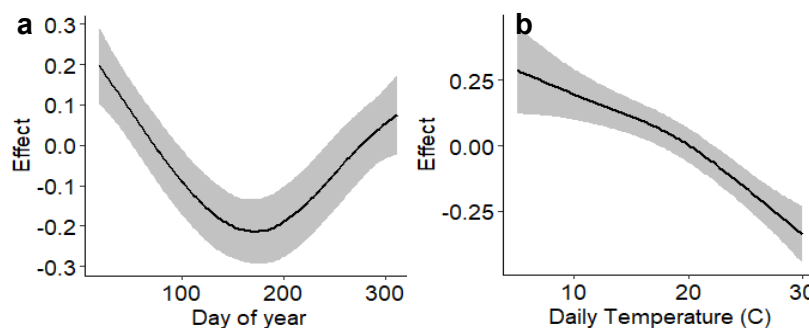


Figure 16. Recovered peptide concentrations ($\mu\text{g/ml}$ mucus) from LA southern leopard frogs (*R. sphenoccephala*) surveyed in 2018 and 2019 were predicted by day of year (a), and daily average temperature (b), but not *Bd* infection. The effect is shown on the y-axis; GAMMs fit separately. Gray shading indicates the 95% confidence interval.

consistency, we transformed data to concentration per ml of mucus like the other locations. Peptide concentrations were predicted by day of year, year (GAMM; $p < 0.001$, $p < 0.001$, respectively, Figure 16a), and the daily average temperature (GAMM; $p < 0.001$, Figure 16b), but they were not predicted by *Bd* infection status (GAMM; $p = 0.310$). Temperature and day of year were highly correlated, and these two models had similar AIC. These findings suggest frogs had the highest concentrations of peptides in winter and fall and the lowest in summer, similar to TN. There was no significant interaction between season and temperature (GAMM; season x daily temperature: $p = 0.212$), suggesting that the negative effect of temperature on recovered peptide concentrations is likely due to differences in temperature across days of the year.

MALDI-TOF mass spectrometry detected the presence of four previously described AMPs in southern leopard frog secretions from LA (Conlon *et al.* 1999). Of the 263 samples analyzed, 3% expressed Temporin-1S, 51% expressed Brevinin-1Sa, 10% expressed Brevinin-1Sb, and 13% expressed Brevinin-1Sc. These findings suggest LA southern leopard frogs are less likely to express these AMPs than those surveyed in TN. In 2018 and 2019, there were effects of day of year, *Bd* infection, or temperature on the probability of detecting each of the four AMPs in secretions (GAMM; $p > 0.05$). The probability of infection was positively related to the relative intensity of Brevinin-1Sa (GLMM: $p = 0.019$). For 214 samples collected in 2018 and 2019, we found that the pattern of MALDI-TOF spectra, compared as either presence/absence of peaks or relative intensity of peaks varied by day of year, year, and frog mass (PERMANOVA of Jaccard and Bray-Curtis distance matrices, respectively: $p < 0.05$). The dissimilarity between infected and uninfected frogs' peptide spectra was significant in relative intensity but not presence/absence of peaks (PERMANOVA: $p = 0.05$, $p = 0.10$, respectively).

We assayed 88 southern leopard frogs from LA for the capacity to inhibit *Bd* growth in vitro using a standardized serial dilution from 500 to 16 $\mu\text{g/ml}$. We assayed 10 samples from 2017 (only fall season), 36 from 2018, and 42 from 2019. Note, 37 samples were not inhibitory enough to provide an MIC, and 15 of these samples had nearly zero inhibition at 500 $\mu\text{g/ml}$. The remaining 51 samples were typically inhibitory at 500 $\mu\text{g/ml}$, with an average of 391 ± 19 $\mu\text{g/ml}$. The inter-assay CV was 13.2% and the intra-assay CV was 13.1%. We observed inhibition from some field negative controls, but there was no evidence of contamination of peptides when analyzed via MALDI-TOF. The predicted 50% inhibitory concentration (IC₅₀) of recovered hydrophobic secretions was not related to the day of year, temperature, year, or *Bd* infection (GAMM; $p > 0.05$). IC₅₀ was negatively related to total recovered peptides (LME: $p < 0.001$) but was not related to the relative intensity of any of the four AMPs (LME: $p > 0.05$). This suggests frogs with greater stored peptides have more effective secretions. MIC equivalents did not vary by day of year, temperature, or *Bd* infection, but they varied among years (GAMM; $p = 0.02$). The lowest amount of MIC equivalents in mucus was 2.8, suggesting that southern leopard frogs surveyed here would have adequate defenses stored in granular gland to inhibit *Bd* growth.

In summary, seasonality in peptide defenses is a shared trait among three species of leopard frog, suggesting that similar to other aspects of immunity, these defenses may be entrained to seasonal temperature and day length cues. Across all locations, greater stored AMP defenses were typically observed around the peak in *Bd* prevalence in leopard frogs. Specifically, northern (*R. pipiens*) and southern (*R. sphenocéphala*) leopard frogs exhibited the peak in potential *Bd* defenses and infection risk during cooler seasons, while the Chiricahua leopard frog (*R. chiricahuensis*) uniquely stored greater amounts of peptide defenses during warmer months when infection risk was highest in this subset of surveyed animals. Peptide defenses, both stored

concentrations and inhibitory capacity, of young Chiricahua leopard frogs decreased with *Bd* infection, although those sampled contained great enough concentrations to inhibit *Bd* growth. Similarly, infected frogs in TN had lower inhibitory capacities of secretions than uninfected frogs, despite similar peptide amounts. In TN and VT, temperature effects on inhibitory capacities of leopard frog secretions depended on season, while in NM, warmer temperatures coincided with more effective secretions and no temperature effects were observed in LA. From these findings, we expect extreme warm or cool spring temperatures to decrease stored peptide effectiveness in VT, and warmer springs to decrease stored peptide amounts in TN, though more research is needed to examine the role of temperature variability on peptide defenses. Management strategies should focus on reducing the risk of infection to young Chiricahua leopard frogs, which exhibit reduced peptide defenses when infected.

Objective 2. Impacts of Current and Future Climates on Amphibian-*Batrachochytrium* Dynamics

Task 1: Climate impacts on immune development

We predicted that populations of *R. sphenocephala* from LA and *R. chirichauensis* from NM, which are adapted to climates in some of the warmest (LA, NM) and driest (NM) areas of these species' current ranges, would be better able to respond to environmental cues and speed the development of immune defenses against *Batrachochytrium* pathogens than frogs that are adapted to cooler climates (where pond drying presumably occurs more slowly and predictably).

We conducted a pond-drying experiment in five locations to provide local managers of DoD lands relevant guidance for mitigating the impacts of climate change-induced shortened hydroperiods, as described above (see *Effects of hydroperiod on development of immune defenses*). We demonstrated that populations from distant regions of these species' ranges differed in their capacity to respond developmentally to drying and in the resulting carry-over effects of drying on juvenile survival, size, and pathogen defenses. In summary, northern and southern leopard frogs did not exhibit developmental plasticity, except the southernmost location (LA) of southern leopard frogs, which exhibited accelerated development in response to drying. Northern leopard frogs in either location did not display an accelerated developmental response, however, exposure to drying resulted in developmental stress in the form of smaller mass at metamorphosis (VT) and increased larval (VT, PA) and juvenile (PA) mortality. In LA and VT, drying exposure resulted in a different microbiome composition, but not greater dispersion. We also found that in general, warmer water temperatures at metamorphosis were related to a lower predicted anti-*Bd* function of the microbiome. In terms of carry-over effects, LA frogs with a faster time to metamorphosis and TN frogs exposed to drying displayed more severe *Bd* infections when exposed after metamorphosis. These findings corroborate previous work in these species documenting negative consequences of pond drying, including reduced larval survival and smaller sizes at metamorphosis, and expand on this work to show that the effects of drying during development persist after metamorphosis and can increase the severity of *Bd* infections. The following paragraphs provide some more detailed results from this study.

Northern leopard frogs (*R. pipiens*) did not display developmental plasticity in response to reduced water levels (LMM, VT: moderate drying: $p = 0.213$, fast drying: $p = 0.085$; PA: moderate drying: $p = 0.994$, fast drying: $p = 0.547$) and instead experienced decreased survival (GLMM: VT: moderate drying: $p = 0.004$, fast drying: $p = 0.126$; PA: moderate drying: $p = 0.387$, fast drying: $p = 0.034$). These findings indicate that the tadpoles experienced developmental stress under the prescribed drying regimes. Further, VT frogs in drying conditions

were smaller at metamorphosis compared to those in constant water levels, despite similar developmental rates (treatment x days to metamorphosis: moderate drying: $p = 0.010$, fast drying: $p < 0.001$). VT frogs that survived drying also exhibited a different microbiome composition at metamorphosis (PERMANOVA Unweighted UniFrac; VT: $R^2 = 0.028$, Pseudo-F = 1.659, $p = 0.004$). Specifically, frogs from slow drying mesocosms—the treatment with the lowest survival—displayed a more diverse skin microbial community that had lower anti-*Bd* function than those reared in constant water level conditions (Beta regression: moderate drying: $p = 0.029$, fast drying: $p = 0.280$). Warmer water temperatures also predicted lower anti-*Bd* function for both VT (VT Beta regression; Water Temperature: $\beta = -0.276$, $p < 0.001$; Figure 17A) and PA frogs. In PA, the interaction with drying treatment (PA Beta regression; water temperature x fast drying: $\beta = 0.225$, $p = 0.025$) suggested no effect of temperature in fast, and a

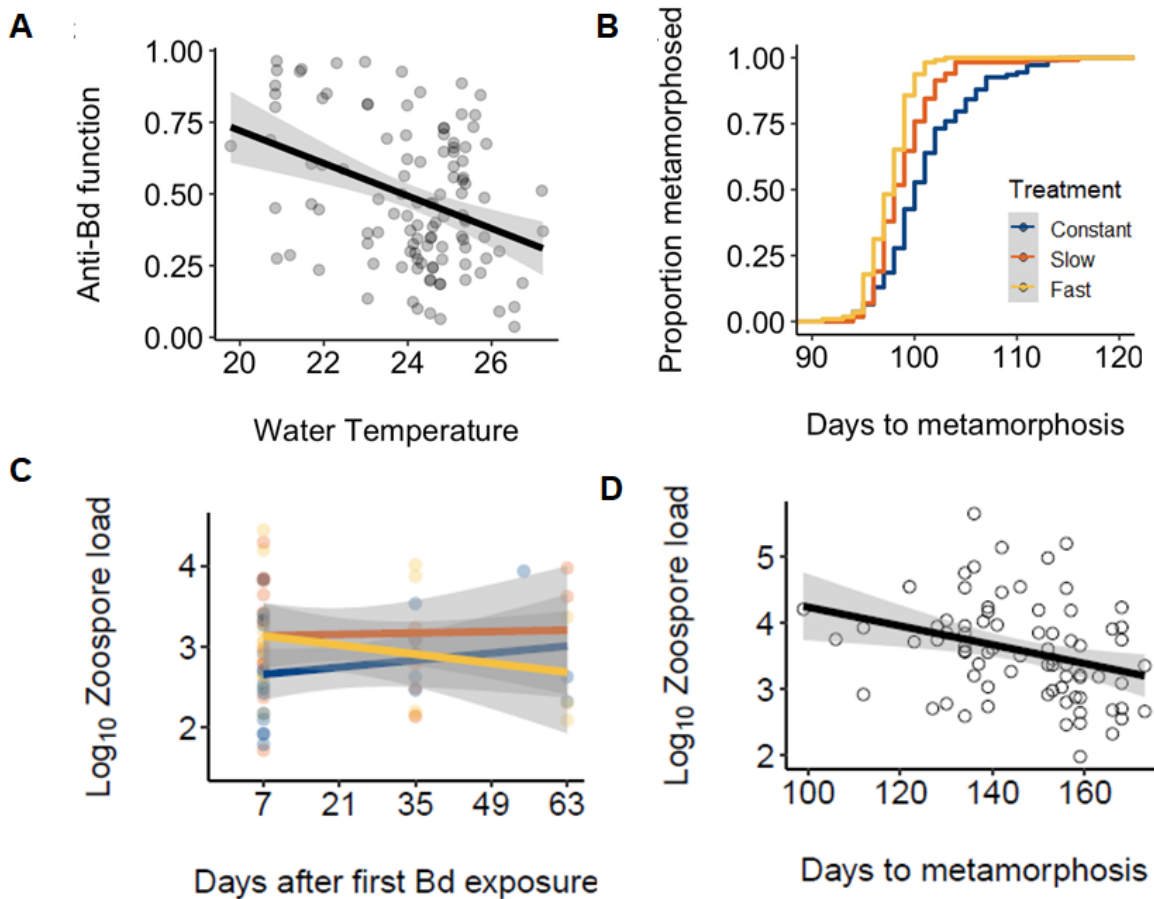


Figure 17. Developmental and carry-over effects of exposure to pond drying in northern and southern leopard frogs. A) VT frogs had lower anti-*Bd* function of the skin microbiome when water temperatures were warmer at metamorphosis. B) LA frogs accelerated metamorphosis in response to drying. C) In response to *Bd* exposure after metamorphosis, TN frogs raised under drying conditions had more severe infections than those raised under constant water levels. D) Louisiana frogs with a longer larval period, regardless of treatment, exhibited lower *Bd* loads when exposed after metamorphosis. In panels A, C, & D, points represent individuals and lines represent linear regressions and 95% confidence intervals. In B & C, points and lines are colored according to drying treatment.

negative effect of temperature in slow and constant treatments. Carry-over effects of drying included reduced survival to 60 days post-metamorphosis in PA frogs (Cox proportional hazards: PA; fast drying: $p < 0.001$; moderate drying: $p < 0.001$), suggesting drying regimes affected resources or energy stores available to survive through metamorphosis. Despite no effect of drying on developmental rate or spleen cellularity, PA frogs that developed faster had lower responsiveness of splenocytes to a T-cell mitogen, PHA (LMM: days to metamorphosis: $p = 0.036$), similar to the skin-swelling PHA response displayed by frogs previously reared in drying conditions from this location (Brannelly *et al.* 2019). Localized effects were apparent in survival following *Bd* exposure; specifically, 80% of VT *Bd*-exposed frogs died by the end of the experiment, whereas none from PA died and 96% cleared infections. Because water temperatures were similar throughout development in these locations, temperature was likely not driving population differences. Altogether, the potential for reduced juvenile recruitment and adult fitness in northern leopard frogs under more variable hydroperiods appears likely.

In Chiricahua leopard frogs (*R. chiricahuensis*), drying treatment did not affect time to or size at metamorphosis, nor did it affect survival during development or after metamorphosis. There was no difference in larval period of frogs reared under fast drying (LMM: $p = 0.35$, mean = 56.8 ± 2.4 d) or moderate drying (LMM: $p = 0.73$, mean = 61.4 ± 2.4 d) hydroperiods as compared to the larval periods of frogs developing under constant water depths (mean = 60.4 ± 2.6 d). In addition, there was no significant difference in body size among drying treatments. Chiricahua leopard frog metamorphs from control, slow drying, and fast drying tanks had mean masses of 2.07 ± 0.09 g, 2.02 ± 0.08 g, and 1.89 ± 0.06 g, respectively, and had mean body lengths of 23.3 ± 0.4 mm, 23.6 ± 0.3 mm, and 22.8 ± 0.3 mm, respectively; however, drying treatment did not affect mass at metamorphosis (ANOVA: drying term, $p = 0.61$; drying x larval period, $p = 0.10$) or SVL at metamorphosis (ANOVA: drying term, $p = 0.23$; drying x larval period, $p = 0.09$). Survival to metamorphosis was high (99% survival), and the two tadpole deaths that occurred in fast drying tanks were not dependent on drying treatment (Fisher's exact test, $p = 0.33$). The four deaths that occurred between metamorphosis and *Bd* exposure were split among drying treatment groups and were also not dependent on hydroperiod ($\chi^2 = 0.46$, $p = 0.79$). Reduced hydroperiod did not affect survival probability to *Bd* exposure or *Bd* infection intensity. There was no difference in *Bd* load among drying treatments per sample day (ANOVA: day 14 $p = 0.08$; day 41 $p = 0.74$; day 73 $p = 0.12$; day 102 $p = 0.12$; day 121 $p = 0.74$). Further, infection intensity decreased over time despite repeated exposures on days 0, 59, and 112. After infection intensity peaked two weeks after initial exposure at 105 zoospore equivalents (ZE) across drying treatments, *Bd* loads decreased to very low levels (<100 ZE) in one third of exposed frogs between days 73 and 121. Against predictions, juvenile *R. chiricahuensis* did not develop severe or lethal chytridiomycosis despite becoming infected. As such, there was no effect of drying treatment, *Bd* exposure, or their interaction on survival probability (Cox proportional hazards, LRT = 1.06, df = 5, $p = 1$). We observed $<1\%$ mortality ($n = 10$ of 120 frogs) over the course of the 136-day exposure experiment. These deaths were split evenly between *Bd*- and *Bd*+ individuals, with seven of the deaths occurring within the first 14 days after exposure (three *Bd*- individuals, four *Bd*+ individuals). Due to the low death rate, even split between *Bd* exposure group deaths, and number of deaths occurring too early for lethal chytridiomycosis development in most species, we do not attribute these deaths to *Bd* infection.

In summary, our study adds to the growing concern that climate change will exacerbate the impact of chytridiomycosis, in that a greater frequency of pond drying will likely increase the intensity of infection in recently metamorphosed frogs. The general lack of developmental

responses to drying, reduced survival in northern leopard frogs, and greater disease susceptibility in southern leopard frogs suggest that more variable hydroperiods may lead to population-level consequences. Local management responses that lengthen the hydroperiod and connectivity among habitats would have the potential to decrease the carry over effects of climate change induced by shortened hydroperiods on these species. More research is needed to test these management strategies in order to effectively balance the potentially beneficial effects of reducing persistence of *Bd* in more permanent hydroperiod wetlands with negative effects on disease susceptibility.

Local adaptation to climate

We conducted a reciprocal transplant experiment between TN and LA to examine whether southern leopard frog (*R. sphenoccephala*) populations exhibit local adaptation or plasticity in metamorphic and immune defense traits across their distribution. Water temperatures in the LA mesocosms were significantly warmer on average than the TN mesocosms (20.5°C in TN, 22.6°C in LA, March-July 2019; LME $\beta = 252.07$, SE = 21.89, $p < 0.0001$). However, mesocosms experienced greater daily temperature fluctuations in TN than in LA (LME $\beta = -128.23$, SE = 26.46, $p < 0.0001$), largely as a result of cooler overnight temperatures. At 35 days after adding tadpoles to mesocosms, there was an effect of environment, and an environment x population interaction, for both developmental stage and size (mass), providing evidence for

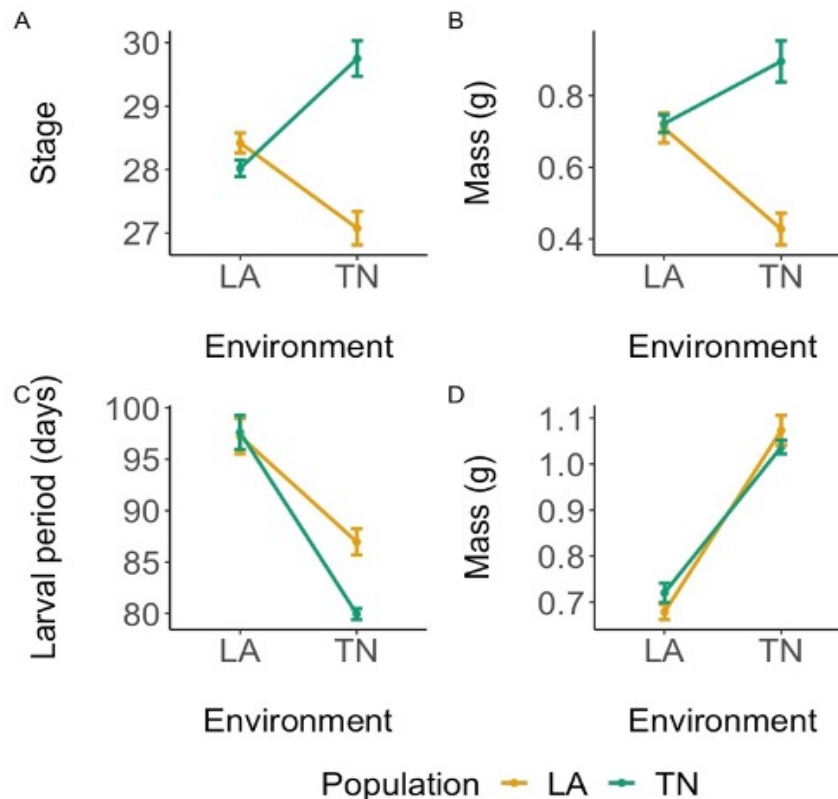


Figure 18. Two-way plots of the effects of population origin (LA or TN) and rearing environment on tadpole development and growth. Stage (A) and mass (B) of tadpoles at 35 days post experimental start show a population x environment interaction, with tadpoles growing and developing slower in a foreign environment. At metamorphosis, larval period (C) and mass (D) only showed strong effects of rearing environment, with animals demonstrating long larval periods and low mass at metamorphosis when raised in LA versus TN. Error bars show standard error of the mean.

local adaptation or maternal effects in both early development and growth rates (Figure 18; stage x environment: $\beta = -0.049$, SE = 0.022, $p = 0.040$, environment x population: $\beta = 0.11$, SE = 0.031, $p = 0.0036$; mass x environment: $\beta = -0.28$, SE = 0.10, $p = 0.014$, environment x population: $\beta = 0.012$, SE = 0.095, $p = 0.006$). Results were similar at 59 days into the experiment for development stage (LME stage x environment: $\beta = 0.12$, SE = 0.026, $p < 0.001$, environment x population: $\beta = 0.10$, SE = 0.034, $p = 0.009$), but environmental effects had overtaken any population effects on growth rate, with tadpoles reared in TN significantly larger in mass than animals reared in LA (LME mass x environment: $\beta = 1.14$, SE = 0.20, $p < 0.001$).

By the time animals metamorphosed, only environmental effects remained for both development and growth rate, with significantly shorter larval periods (LME environment: $\beta = -$

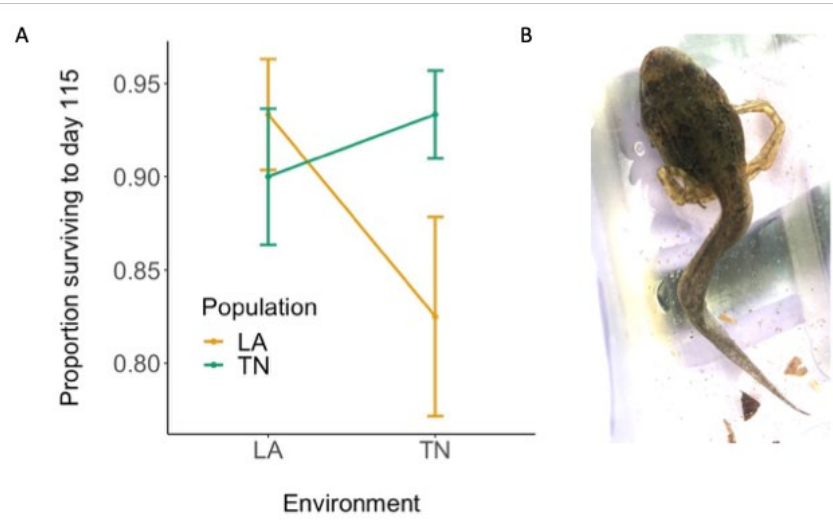


Figure 19. (A) Proportion of southern leopard frog tadpoles surviving to day 115 of the mesocosm experiments in each location. Developmental deformities such as (B) were only observed in TN tadpoles raised in LA. Error bars show the standard error of the mean.

0.044, SE = 0.016, $p = 0.017$) and larger mass (LME environment: $\beta = 0.40$, SE = 0.078, $p < 0.001$) at metamorphosis for frogs reared in TN, regardless of population origin. This indicates that these metamorphic traits are developmentally plastic in both populations. However, this plasticity came at a cost for frogs raised in foreign environments. LA animals raised in TN were less likely to survive to metamorphosis (GLM environment: $\beta = 1.089$, SE = 0.41, $p = 0.007$; environment x population: $\beta = -1.53$, SE = 0.61, $p = 0.012$; Figure 19A), and TN animals raised in LA were more likely to metamorphose with severe deformities (missing and non-functional limbs; X^2 : $p = 0.005$; no deformities seen in other treatments; Figure 19B).

A month after metamorphosis, frogs reared in the foreign environment had greater splenocyte counts than when reared in their local environment (LME: environment x population: $p = 0.037$), while thymocyte counts were not different among populations or environments (LME: population: $p = 0.093$, environment: $p = 0.076$). The T-cell proliferation response (to a standardized mitogen, phytohemagglutinin) was also greater in the LA compared to TN population (LME: $p = 0.009$).

For animals placed in growth chambers post-metamorphosis, we found effects of developmental environment and population on growth rate, with animals raised in LA having faster growth rates than those raised in TN, resulting in LA-raised animals ‘catching up’ in size

to TN-raised animals by 80 days post-metamorphosis, regardless of chamber temperature (LM environment: $\beta = -0.077$, SE = 0.0082, $p < 0.001$; population: $\beta = 0.020$, SE = 0.008, $p = 0.014$). While the effect of environment was strongest on growth rate, within each rearing environment, animals from the TN population had faster growth rates than animals from the LA population (LME environment x days in chamber: $\beta = -0.037$, SE = 0.004, $p < 0.001$; population x days in chamber: $\beta = 0.013$, SE = 0.0040, $p = 0.014$). Conversely, we found stronger effects of population origin on measures of immune function, with animals from the LA population having greater plasma bacterial killing ability (population: $\beta = -16.73$, SE = 6.02, $p = 0.014$), and those from the TN population recovering greater amounts of peptides post-depletion (population: $\beta = 153.41$, SE = 73.07, $p = 0.039$). AMP expression also substantially varied by population, specifically the LA population expressed only one of the four known AMPs, while TN frogs expressed all four. Further, TN frogs were more likely to express Brevinin-1Sc when raised in the mismatch environment (environment x chamber interaction: $p = 0.027$). Overall, appears that growth rate is developmentally plastic in southern leopard frogs, allowing animals to compensate for stressful developmental conditions and small sizes at metamorphosis. However, most

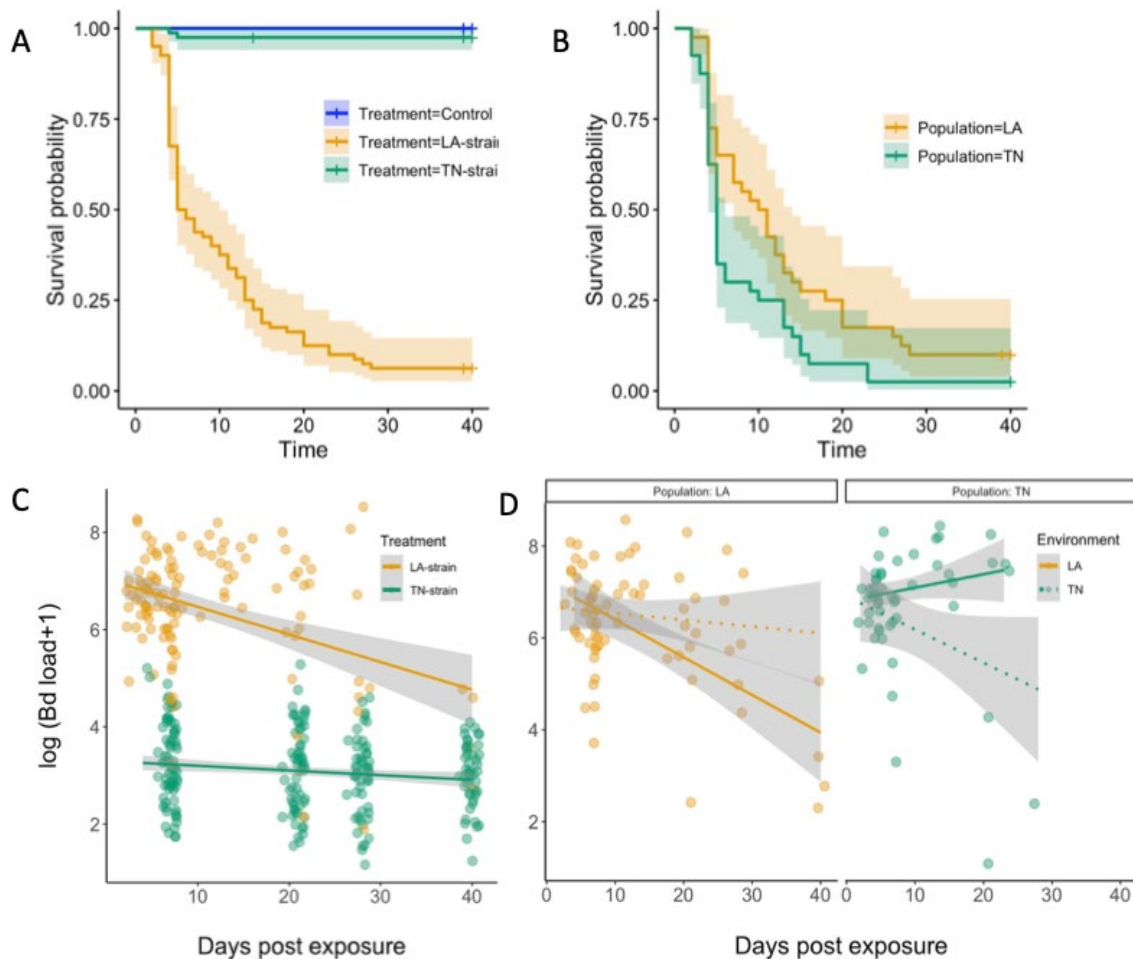


Figure 20. Survival post-*Bd* exposure was most strongly influenced by *Bd* isolate, with the LA isolate resulting in almost 100% mortality (A). Tadpoles that originated from TN, regardless of rearing environment, experienced faster mortality when exposed to the LA isolate (B). *Bd* load was most strongly driven by isolate as well, with higher loads on animals exposed to the LA isolate (C). Of animals exposed to the LA isolate, TN animals raised in LA had higher loads (D). Shaded areas indicate 95% confidence intervals.

measures of immune function are less plastic and variable depending on population of origin, regardless of developmental and post-metamorphic environment, though some were more robust when experiencing a mismatch between pre- and post-metamorphic environment.

For frogs exposed to *Bd* at room temperature post-metamorphosis, probability of survival was driven by *Bd* isolate, rather than population origin or developmental environment, with 97.5% surviving exposure to the TN isolate, and only 6.3% surviving exposure to the LA isolate (Figure 20). All frogs exposed to the LA isolate developed infections (Cox proportional hazards: strain: $\beta = -4.71$, SE = 0.730, $p < 0.001$; population: $\beta = 0.59$, SE = 0.18, $p = 0.001$), and at much higher infection loads (LME: strain: $\beta = -3.22$, SE = 0.12, $p < 0.001$), demonstrating that *Bd* isolates from different regions can vary greatly in pathogenicity. TN froglets died at a faster rate (Cox proportional hazards: population $\beta = 0.57$, SE = 0.19, $p = 0.002$), and had higher infection loads (LME: population: $\beta = 0.84$, SE = 0.34, $p = 0.028$), than LA froglets when exposed to the LA isolate. This demonstrates that there may be some degree of local adaptation to regional *Bd* strains, though we used only two strains in our study, and further research is necessary to determine how generalizable our results are. From a management perspective, this work indicates that the anthropogenic transfer and spread of *Bd* among amphibian populations (e.g., on equipment, or via the movement of animals) should be avoided, as non-local isolates may be more pathogenic than local isolates.

Effects of future climate on pathogen defenses

Researchers disagree as to rising temperatures due to global climate change will ease or worsen the impact of *Bd* on amphibian populations. Some have hypothesized that climate change will worsen *Bd*'s effects, based on the idea that increasing variability of temperatures and precipitation may change the host-pathogen dynamics (Yeh *et al.* 2009). Others have predicted the opposite, that climate change will lessen the impact of *Bd* on its hosts given that *Bd* is a cold-adapted fungus and warmer temperatures are associated with faster amphibian immune responses (Rohr and Raffel 2010). To test the hypothesis that elevated environmental temperatures during the larval period negatively affect the ability of newly-metamorphosed froglets (the life stage most susceptible to chytridiomycosis; Rollins-Smith 1998) to defend themselves against *Bd*, we experimentally raised larvae from two populations of each of two species (northern leopard frog, *R. pipiens*, from VT and PA and southern leopard frog, *R. sphenoccephala*, from TN and LA) under either their current or simulated future local pond temperatures. We then tested whether exposure to warmer temperatures during development, as predicted under global climate change, impacts later-life fitness and the development of the amphibian immune system. We predicted that frogs that developed under future (warmer) climate conditions would have lower survival, a smaller size at metamorphosis, reduced immune function, and greater susceptibility to *Bd* relative to those developing under current (cooler) climate conditions.

In each population and temperature treatment, over half of all tadpoles did not survive to metamorphosis and none of the *R. sphenoccephala* tadpoles from TN survived to metamorphosis. We therefore could not collect post-metamorphic data from the TN population. There were differences among populations and species in terms of which temperature treatment experienced greater survival and only for *R. pipiens* from Louisiana was survival to metamorphosis reduced in the future temperature treatment, as we had predicted (Figure 21). The larval periods for animals from the three populations that survived to metamorphosis were shorter in the future temperature treatment than in the current temperature treatment (Cox proportional hazards: $p <$

0.001). The shortest larval periods we observed were similar across species and populations (range: 127 – 153 d) for animals from the future temperature treatments whereas the longest larval periods (range 241 – 321 d) for animals from the current temperature treatments differed more dramatically, with some *R. pipiens* individuals from VT taking more than 300 d to metamorphose. Regardless of population of origin, *R. pipiens* that developed in the future temperature treatment had lower mass at metamorphosis than animals that developed in the current temperature treatment (LME: VT $p < 0.001$; PA $p < 0.001$). However, for *R. sphenoccephala* from LA there was not a significant difference in mass between metamorphs from the current and future temperature treatments (LME: $p = 0.531$). Animals from the future temperature treatment were also smaller in size (snout-vent length, or SVL) at metamorphosis than animals from the current temperature treatment (ANOVAs: VT *R. pipiens*: $p < 0.001$; PA *R. pipiens*: $p < 0.001$; LA *R. sphenoccephala*: $p = 0.034$). The smaller size and mass of frogs reared under future climate conditions suggests that there will be fitness costs to developing as larvae under future temperatures.

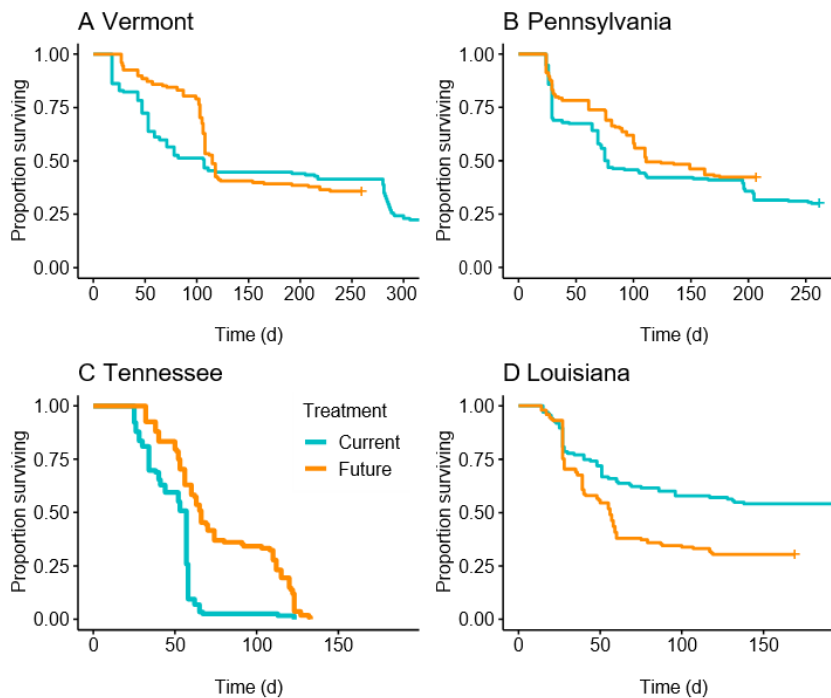


Figure 21. Larval survival curves for A. VT *R. pipiens*, B. PA *R. pipiens*, C. TN *R. sphenoccephala*, and D. LA *R. sphenoccephala*. Day 0 is the day when the tadpoles were placed inside temperature treatments (at Gosner stage 25).

After metamorphosis, froglets that developed as larvae under simulated future temperatures tended to secrete lower quantities of skin peptides and fewer of the AMPs known to be effective against *Bd*. The *R. pipiens* from VT (LMs: one-month post-metamorphosis: $p = 0.038$; two-months post-metamorphosis: $p = 0.008$) and the *R. sphenoccephala* from LA (LMs: one-month post-metamorphosis: $p = 0.030$, two-months post-metamorphosis: $p = 0.017$) that developed in the future temperature treatments secreted fewer total peptides than the frogs that developed in the current temperature treatments (Figure 22) whereas for *R. pipiens* from PA there was not a difference in secreted peptides among temperature treatments (LM: $p \geq 0.442$). The pattern for AMP secretion was similar, with more AMPs detected from *R. pipiens* from VT

(GLM: one-month post-metamorphosis: $p = 0.024$, two-months post-metamorphosis: $p < 0.001$) when they had been reared under the current temperature treatment than under the future temperature treatment but no difference among treatments for *R. pipiens* from PA (GLMs: $p \geq 0.086$). The *R. sphenoccephala* from LA did not secrete enough AMPs at either time point to enable a statistical analysis. Thus, while the effect of temperature was not consistent for all populations, our study suggests that developing in a warmer than normal temperature can inhibit the development of mucosal peptides, an important component in the innate defense against *Bd*.

When we compared aspects of the adaptive immune system among frogs from our current and future temperature treatments, as predicted, we sometimes found greater concentrations of immune cells in frogs from the current temperature treatment. For example, frogs from VT that had developed in the current temperature treatment had more circulating white blood cells one month after metamorphosis (LM: $p = 0.005$) but not at two months after

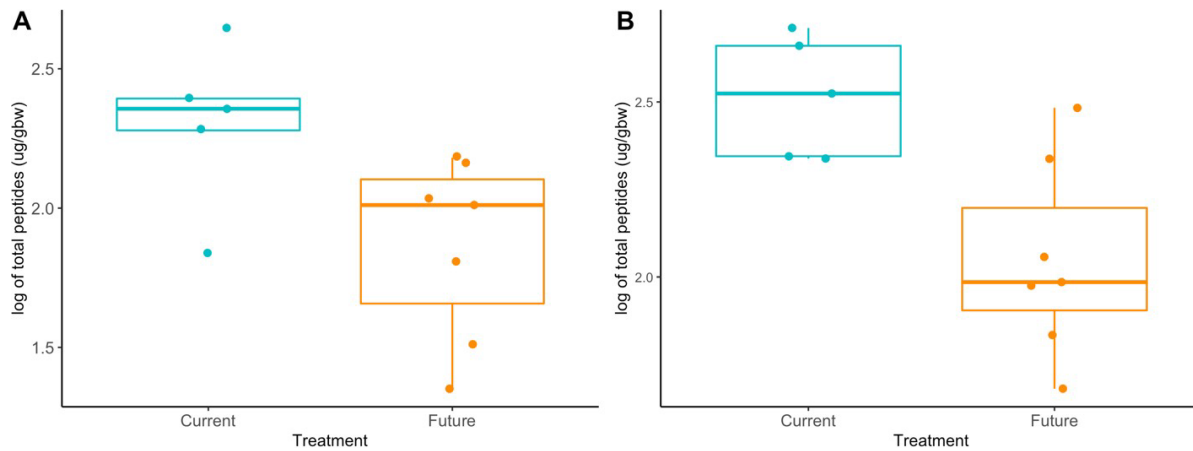


Figure 22. Box plots showing total peptides secreted (in μg per g body weight, or $\mu\text{g/gbw}$) for *R. pipiens* from VT at (A) one-month post-metamorphosis and (B) two months post-metamorphosis. For each box plot the center line indicates the median and the upper and lower extents of the box represent the 75th and 25th quartiles, respectively. The whiskers extend no further than 1.5 times the inter-quartile range.

metamorphosis (LM: $p = 0.220$). However, for frogs from PA and TN there were no differences in white blood cell counts between frogs that had developed in current and future temperature treatments (LMs: $p \geq 0.067$). However, when we counted thymocytes (white blood cells within the thymus), and splenocytes (white blood cells within the spleen) we sometimes found greater numbers in frogs reared in the future temperature treatment. For example, frogs two months post-metamorphosis from PA (LM: $p = 0.004$) and LA (LM: $p = 0.002$) had more thymocytes if they had developed in the future temperature treatment and frogs from PA at two months post-metamorphosis had more splenocytes if they had developed in the future temperature treatment (LM: $p = 0.002$), but more often we saw no difference in thymocyte or splenocyte counts among frogs from the different temperature treatments (LMs: $p \geq 0.059$). Across populations and species, we also found no effect of temperature treatment on T-lymphocyte proliferation (LMs: $p \geq 0.091$). However, contrary to predictions, at two months post-metamorphosis, we sometimes found greater B-lymphocyte proliferation in frogs that had developed in the future temperature treatment [e.g., in VT frogs (LM: $p = 0.033$), but not in PA or LA frogs (LMs: $p \geq 0.398$)]. In summary, the response of the adaptive immune system to the stress of development under future climate scenarios appears to be complex and will require further research in order to predict with

any certainty.

When we exposed frogs from the two temperature treatments to *Bd* we predicted we would find greater infection probability, pathogen load, and mortality among frogs reared under the more stressful future temperature conditions. However, we rarely saw differences in infection (yes/no) or pathogen load among frogs from the two temperature treatments. Only in PA *R. pipiens* did we see a difference in infection probability (GLMM: $p = 0.041$) and pathogen load (GLMM: $p < 0.001$), and contrary to our predictions both of these disease variables were greater in frogs that had developed under current climate conditions. Despite greater infection probability, however, the PA frogs reared under current temperatures did not suffer greater mortality due to *Bd* than frogs reared under future climate conditions (Cox proportional hazards: $p = 0.239$). The frogs from VT that were reared under future temperatures, however, had greater

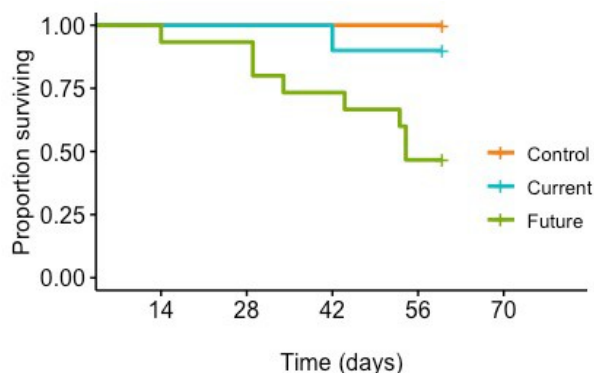


Figure 23. Survival curves for *R. pipiens* from VT that were exposed to *Bd* (labelled by temperature treatment) and for all frogs (regardless of temperature treatment) in the control (sham exposure) group from week one after metamorphosis (Gosner stage 46) until day sixty after the first *Bd* exposure.

mortality after exposure to *Bd* than their current temperature counterparts (Cox proportional hazards: $p = 0.023$, Figure 23) despite having similar *Bd* loads (GLMM: $p = 0.294$). These mismatches between mortality and pathogen load suggest that developing under stressful climate conditions may affect the ability of amphibian hosts to tolerate *Bd* infection (i.e., pathogen tolerance).

In summary, our experiment showed that larval development under elevated temperatures, like those predicted with global climate change, can have negative impacts the fitness of juvenile amphibians (the most susceptible life stage to *Bd*) and can also impact the development of immune defenses that are key to fighting *Bd* and other emerging pathogens. While we saw different effects of elevated temperatures on these parameters across populations and species, in many cases, the detected differences point to the possibility of compromised immune defenses and greater *Bd*-related mortality in animals forced to develop in warmer climates. This suggests that management actions that promote the health and development of newly metamorphosed animals, the life stage where many of the immune defenses important to life on land are being developed, will go a long way toward mitigating the interacting impacts of global climate change and disease on amphibian populations.

Effects of climate on virulence traits

We collected five *Bd* isolates from our DoD regions and investigated their responses to temperature in current and future thermal conditions (i.e., simulated to mimic temperatures we anticipate with climate change). In the first part of this project we used growth assays to generate

a thermal performance curve for each of isolates from the five DoD regions. In the second part of the project, we used experimental evolutions and serially passaged the isolates in current and future thermal conditions and in thermal conditions.

To understand the differences among the five isolates in their thermal responses, we generated thermal performance curves that spanned the known *Bd* thermal range (4-27°C). We observed important similarities and differences in many of the parameters that we measured to understand *Bd* virulence traits. Namely, all isolates exhibited maximum viability at 21°C. However, there were differences among isolates in their mean viability at the T_{opt} of 21°C (ANOVA: $p < 0.001$, Figure 24). The isolate from LA exhibited the greatest mean viability in the T_{opt} , 21°C, as well as at every other temperature treatment except 27°C. The isolate from VT exhibited the lowest viability except in the low temperature treatments of 4°C and 12°C.

Bd viability in high and low temperature treatments was lower than at the T_{opt} for all isolates. However, we found that there were differences among the *Bd* isolates in their viability in both low and high temperature conditions (Figure 24). For the lowest temperature treatment,

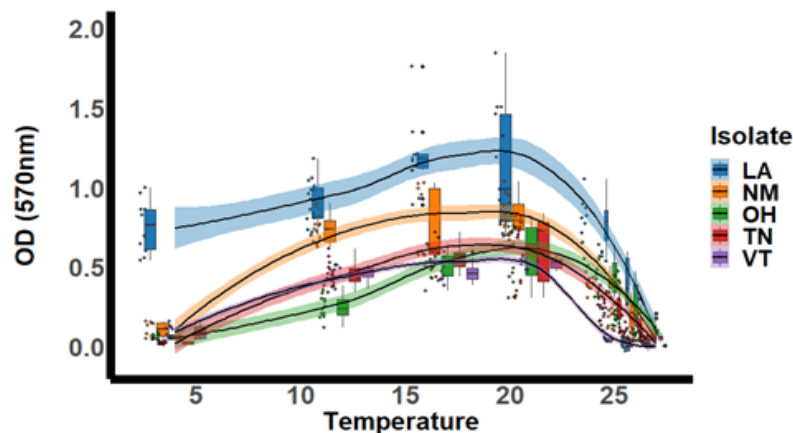


Figure 24. Thermal performance curves for isolates of *Bd* collected across a latitudinal gradient. Data show mean viability ($\pm$ SE) by isolate. Shaded areas indicate the standard error of the mean. For boxplots, the center line indicates the median and the box contains the 25th to 75th quartiles. Whiskers extend no further than 1.5 times the inter-quartile range.

all isolates exhibited minimal growth at 4°C but there were differences among the isolates in viability at that temperature (ANOVA: $p < 0.001$). The isolates also differed in their responses to high temperature treatments. The isolate from PA region (referred to as OH in figures) exhibited significantly greater viability at the highest temperature treatment of 27°C (ANOVA: $p < 0.001$; Game's Howell, $p < 0.01$), whereas the isolate from VT had low viability at 26°C and was not viable at 27°C.

We also measured zoospore densities for all of the *Bd* isolates in all temperatures because the capacity to generate high zoospore densities is thought to be a critical factor for disease development. We found patterns in our measures of zoospore densities that differed from those in our viability assays (Figure 25). Specifically, two of the isolates produced their maximum zoospore densities at the low temperatures of 4°C and 12°C. Notably, for the NM isolate, zoospore densities were highest at 4°C and were dramatically lower at all other temperatures. Accordingly, the NM isolate exhibited the highest fecundity (zoospores per viability measure) at 4°C. All isolates exhibited a similar pattern, with higher fecundity in lower temperatures, but it was most pronounced in the NM isolate at 4°C. In addition, we found that the time to maximum zoospore densities differed among isolates at 4°C (ANOVA: $p < 0.001$) and 21°C (ANOVA: $p <$

0.001). We also found that, although the cultures were viable and growth measurements increased at the higher temperatures of 25°C, 26°C, and 27°C, no zoospores were produced.

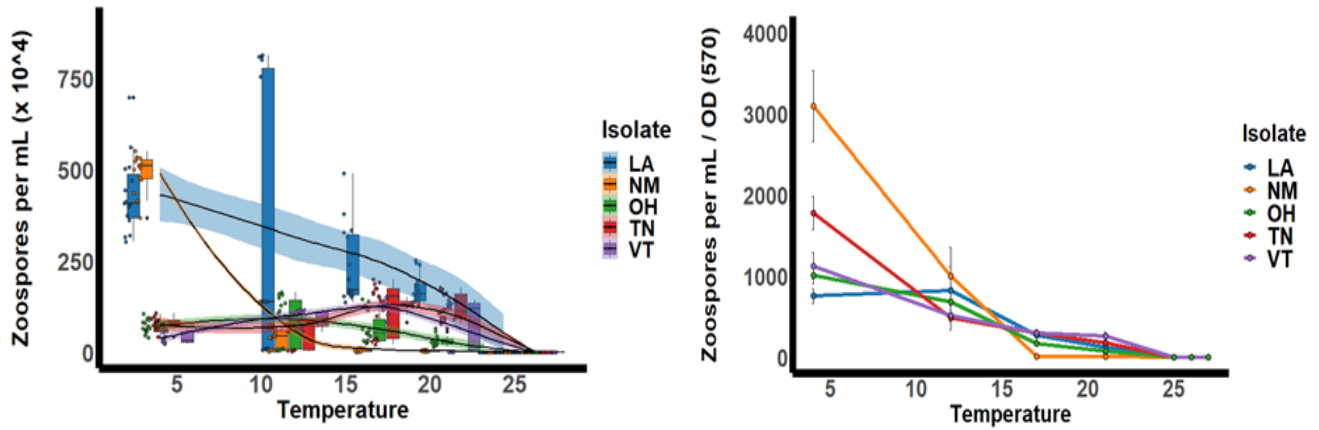


Figure 25. Thermal performance curves for zoospore densities for five isolates of *Bd* collected from five DoD sites. Data show mean zoospore densities and fecundity (calculated as optical density / zoospore densities) (+/- SE) by isolate over the thermal range for *Bd* (4-27°C). Shaded areas around curves and error bars indicate the standard error of the mean. For boxplots, the center line indicates the median and the box contains the 25th to 75th quartiles. Whiskers extend no further than 1.5 times the inter-quartile range.

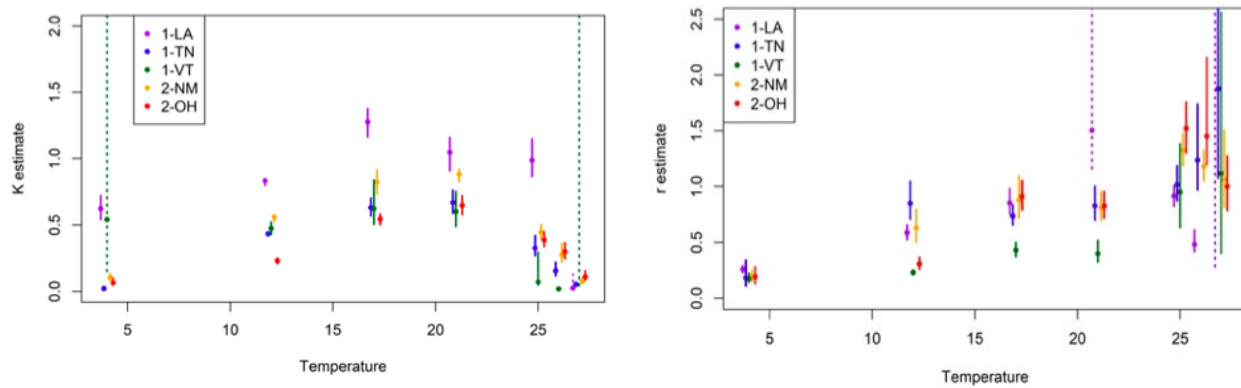


Figure 26. Logistic growth curve parameter estimates for each isolate for growth rate r and carrying capacity (K) estimates. Solid circles indicate the best fit parameter values and vertical bars show the 95% confidence intervals. The dashed vertical bars indicate situations in which the confidence interval for the parameter of interest had a poorly resolved upper end point due to parameter identifiability issues. Not shown, for clarity: VT-26°C r estimate is 8.58, CI = (4.61, 14.12). Error bars indicate 95% confidence intervals.

We also calculated growth rates (r) and carrying capacities (K) across temperatures for each isolate. The overall trend for all isolates is r estimates that increase and then plateau for temperatures up to 21°C (Figure 26). However, for higher temperatures (25-27°C), the r estimates are larger and more variable both across isolates and in terms of having larger confidence intervals. The initial growth rates at the higher temperatures, however, do not yield much long-term growth. The corresponding K estimates also increase and plateau at approximately 21°C, but then markedly decline at the higher temperatures. The combined effect

at these high temperatures is a short-lived exponential growth phase that quickly reaches a relatively low upper bound.

To create current and future temperature conditions for serial propagation of *Bd* isolates, we used programmable environmental chambers to simulate thermal conditions. We based the simulated temperature treatment on two data sets that calculated the daily average body temperatures of wild amphibians (Figure 27). The simulated treatments model current and future daily average body temperatures of wild amphibians in the area that one or more of the isolates were collected from. We selected these temperature conditions by using field-collected data for a month when *Bd* prevalence is high and when host densities are high due to normal congregations for breeding. We also simulated future conditions for amphibian habitats in the field (to ~1 km spatial resolution; http://www.worldclim.org/cmip5_30s).

Following 45 passages of *Bd* in these two thermal conditions, we compared the maximum viability and zoospore densities for two lineages (one propagated in current conditions, “Current”, and one propagated in future conditions, “Future”) in three thermal treatments: stable 21°C (Figure 28), fluctuating at current temperatures, 5-25°C (Figure 28), and fluctuating in future temperatures, 8-28°C (Figure 28). We found that, in several cases, the maximum viabilities of the Future lineages were higher for several isolates across multiple temperature

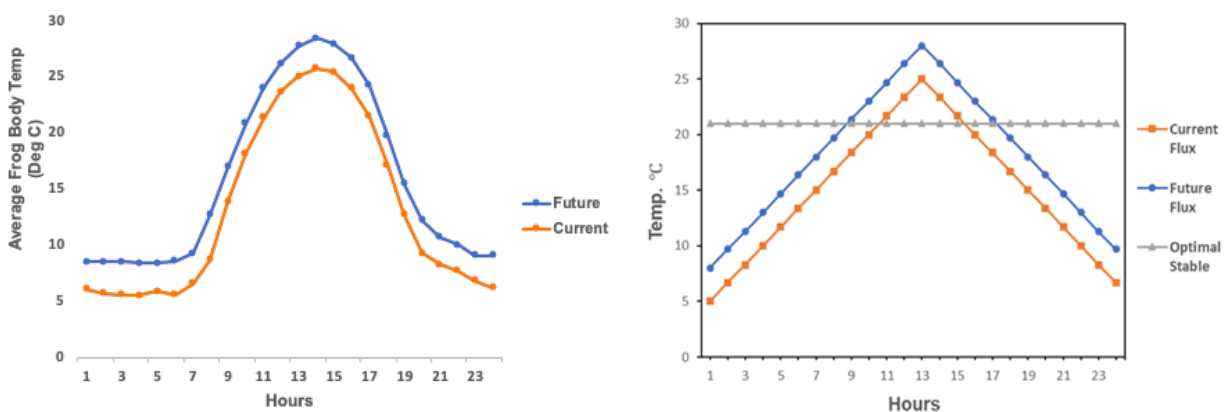


Figure 27. The future and current thermal profiles that we used for programmable environmental chambers for the serial passaging of *Bd* isolates. Temperature profiles are based on data for a month in the spring for the PA site, and this temperature profile can be modeled based on data for other months across all other sites. The current temperature profile fluctuates between 5-25°C in a 24-hour period. The future temperature profile fluctuates between 8-28°C in a 24-hour period.

treatments. For example, the Future lineage for the VT isolate had higher maximum viability in all temperature treatments (21°C: $p < 0.001$; 5-25°C: $p < 0.001$; and 8-28°C: $p < 0.001$). We observed a similar pattern with the Future lineage of TN isolate, except that results were not significant in 21°C and the 8-28°C fluctuating temperature treatment (21°C: $p = 0.31$; 5-25°C: $p < 0.001$; and 8-28°C: $p = 0.14$). In addition, there was one notable inconsistency for viability of the two lineages for the isolate from NM. Specifically, the Current lineage had higher viability in both fluctuating temperature treatments (5-25°C: $p < 0.001$; and 8-28°C: $p < 0.01$) compared to the future lineage.

We observed a similar pattern with the Future lineage of LA isolate, except that results were not significant in the 8-28°C fluctuating temperature treatment (21°C: $p < 0.01$; 5-25°C: $p = 0.047$; and 8-28°C: $p = 0.18$). We found again that there was an inconsistency for the maximum

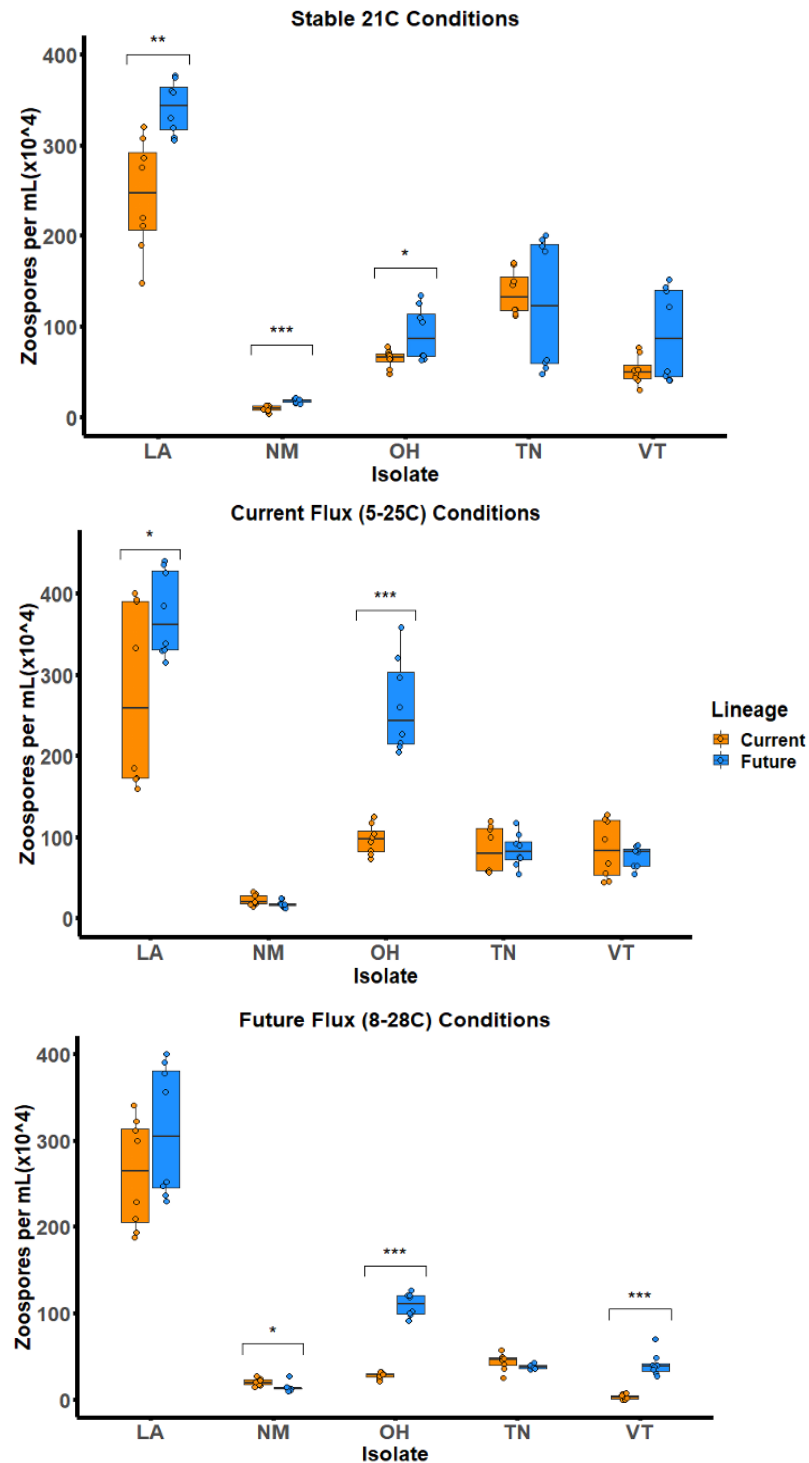


Figure 28. Boxplots show the population viability of the two evolved lineages for each isolate in the three thermal treatments: stable optimal conditions (21°C), the current fluctuating conditions (5-25°C), and the future fluctuating conditions (8-28°C). Each boxplot consists of data from a 2-day peak period during the thermal experiment. Significance labels are shown with * marks to indicate degrees of significance between lineages as follows: * <0.05, ** <0.01, *** <0.001. For boxplots, the center line indicates the median and the box contains the 25th to 75th quartiles. Whiskers extend no further than 1.5 times the inter-quartile range.

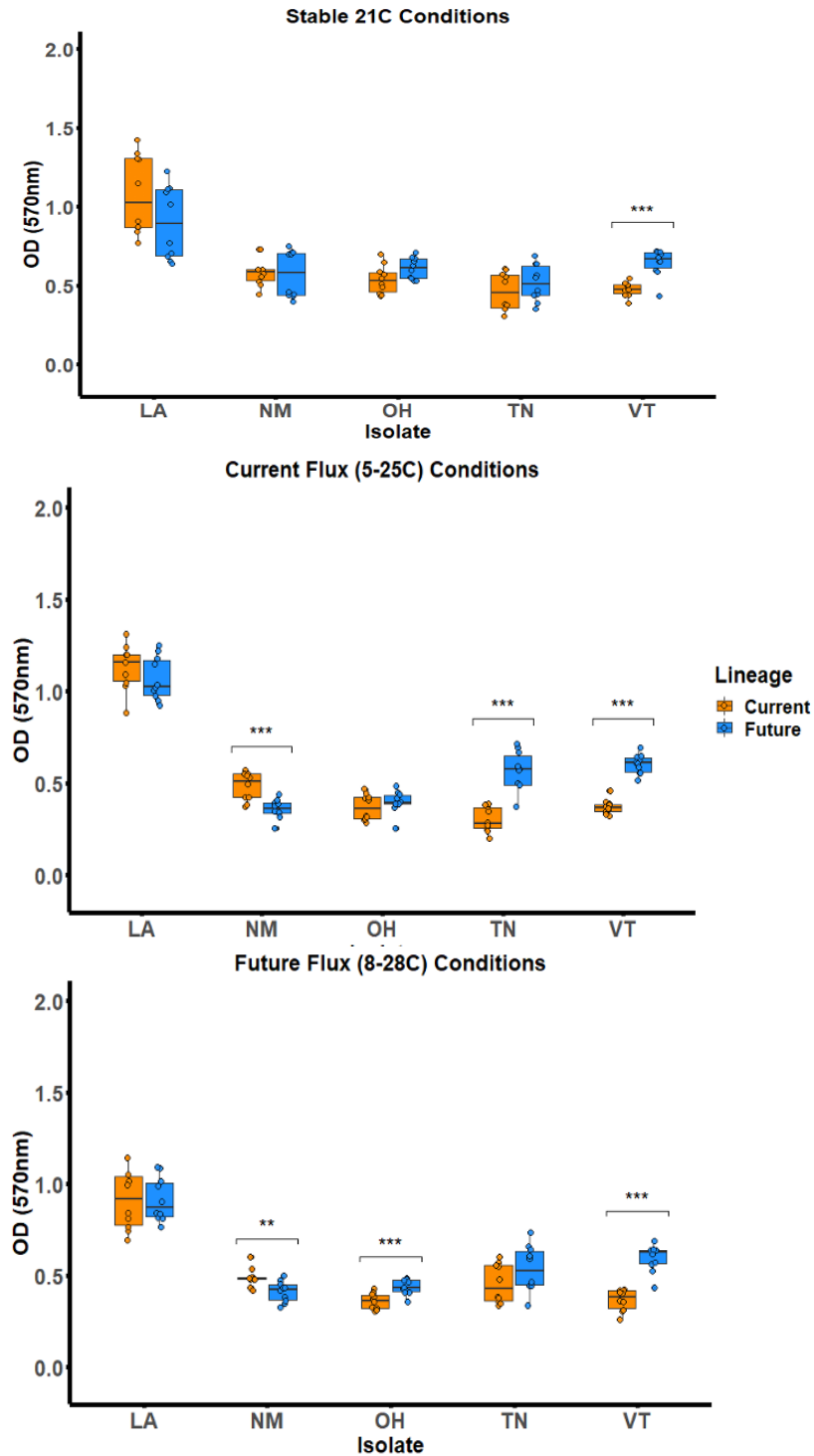


Figure 29. Boxplots show the zoospore densities of the two evolved lineages for each isolate in the three thermal treatments: stable optimal conditions (21°C), the current fluctuating conditions (5-25°C), and the future fluctuating conditions (8-28°C). Each boxplot consists of data from a 2-day peak period during the thermal experiment. Significance labels are shown with * marks to indicate degrees of significance between lineages as follows: * <0.05, ** <0.01, *** <0.001. For boxplots, the center line indicates the median and the box contains the 25th to 75th quartiles. Whiskers extend no further than 1.5 times the inter-quartile range.

zoospore densities for the NM isolate; the Current lineage had higher maximum zoospore densities in the 8-28°C fluctuating temperature treatment ($p = 0.03$) but the Future lineage had higher densities in the stable 21°C treatment ($p = 0.08$).

Effects of community structure on development of immune defenses

We predicted that co-occurrence of *R. pipiens* with infection-tolerant *Bd*-reservoir hosts (e.g., green frogs, *R. clamitans*) would increase disease risk and that *Bd*-resistant hosts (e.g., gray treefrogs, *Hyla versicolor*) would act as zoospore sinks and decrease disease risk (Searle *et al.* 2011, Gahl *et al.* 2012). In addition, we predicted that changes in community composition would affect infection susceptibility and tolerance through trait-mediated indirect effects, including host mucosal defenses, skin microbiota, and developmental variables (mass, larval duration, survival probability). To examine the impact of host community composition on infection prevalence and host development, two tadpole mesocosm experiments were conducted in the summers of 2019 and 2020 at our VT field, where *R. pipiens* were raised in combination with different assemblages of naturally co-occurring *H. versicolor* and *R. clamitans* tadpoles (as described in methods, above). In 2020, no mesocosm tadpoles became infected with *Bd* following introduction of newt-exposed *R. pipiens*, despite 90% infection prevalence (qPCR assay) in *N. viridescens* used for exposure. Accordingly, results were analyzed based on the four community treatments which received newt-exposed tadpoles, and the *Bd* control treatment (1) was excluded. We found that community composition did not affect post-metamorphic survival (2019), larval survival probability, or larval duration. However, *R. pipiens* reared with the putative pathogen sink species, *H. versicolor*, exhibited greater body mass when compared to individuals reared in single-species or with *R. clamitans* at the same total density, in the absence of *Bd* (2020). This suggests that positive effects of *H. versicolor* on *R. pipiens* mass are not retained in the presence of *Bd*, despite any sink effects of the former species.

In 2019, mean mass at metamorphosis was 2.014g (sd = 1.18g) and mean larval period was 61.98 days (sd = 15.9 days). In 2020, mean mass at metamorphosis was 1.264g (sd = 0.4g) and mean larval period was 38.08 days (sd = 9.07). Overall tadpole survival was 78% in 2019 and 91% in 2020. Community treatment did not have a significant effect on metamorph mass in 2019 (Wald: $p = 0.25$), but in 2020 the leopard frogs in the treatment with gray treefrogs (LIPI+HYVE treatment) had a significantly greater mass at metamorphosis (GLMM: $p < 0.01$; Wald: $p < 0.01$) than the single-species (LIPI) treatment (Tukey: $p = 0.07$), the treatment with green frogs (LIPI+LICL, Tukey, $p = 0.01$), and three-species treatment (Tukey: $p = 0.10$) (Figure 30). Treatment still had an effect on metamorph mass in the 2020 experiment after the removal of a LIPI+HYVE outlier (Wald: $p < 0.01$). Community treatment did not have a significant effect on larval duration (Wald: 2019 $p = 0.32$, 2020 $p = 0.09$), but metamorphosis in the 2020 experiment was confined to a smaller time window than 2019, peaking on Day 32, when 35% of *R. pipiens* had completed metamorphosis (Figure 31). Body mass was positively correlated with larval duration in both years (Fisher's exact test: 2019 $p \leq 0.01$, 2020 $p < 0.01$). Community treatment did not affect tadpole survival in 2019 (Wald: $p = 0.21$) or 2020 (Wald: $p = 0.21$) (Figure 32), nor survival probability in the 8 weeks following metamorphosis in 2019 (Cox proportional hazards: $p = 0.27$; Figure 33). Furthermore, 2019 tadpole survival in single- and mixed-species *Bd* treatments did not differ from the *Bd*-free control treatment (Dunnnett test: $p > 0.19$), and there were no significant differences in larval period, (GLMM: $p = 0.30$), metamorph mass (GLMM: $p = 0.81$), or survival (GLMM: $p = 0.58$), between *Bd*-exposed and control single-species communities.

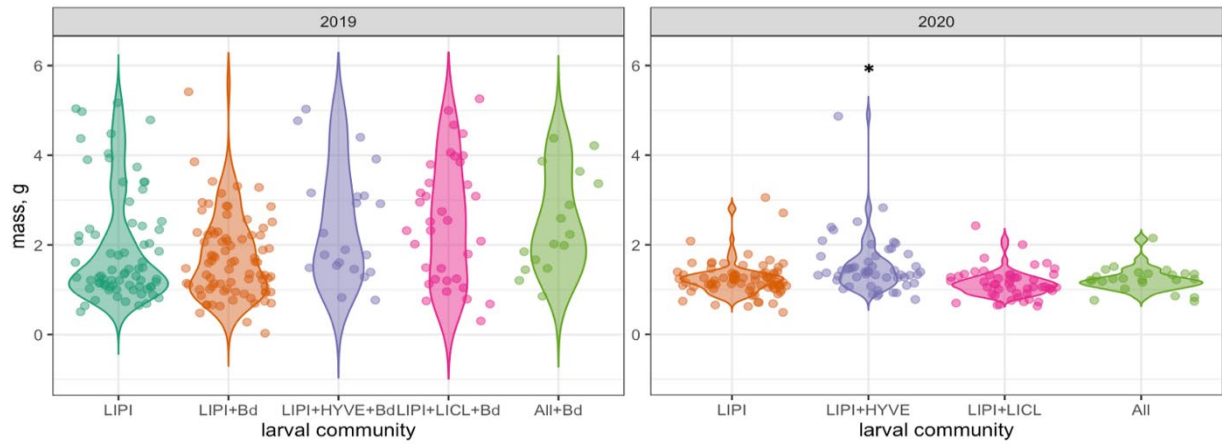


Figure 30. Body mass of *R. pipiens* at metamorphosis for community treatments in 2019 and 2020 represented as violin plots.

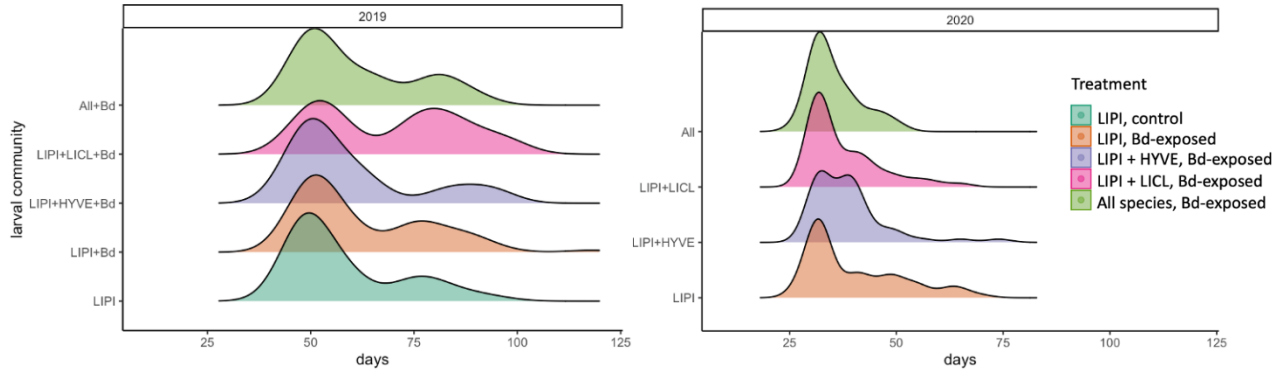


Figure 31. Time to metamorphosis for *R. pipiens* in community treatments in 2019 and 2020. LIPI = *R. pipiens*, HYVE = *H. versicolor*, LICL = *R. clamitans*.

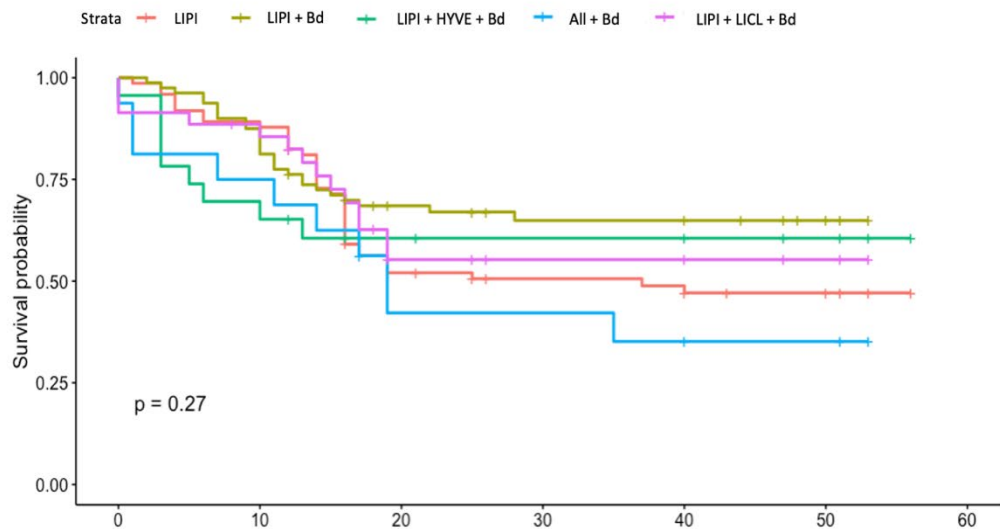


Figure 32. Survivorship of *R. pipiens*, as a proportion of total animals surviving to metamorphosis, for community treatments in 2019 and 2020. LIPI = *R. pipiens*, HYVE = *H. versicolor*, LICL = *R. clamitans*.

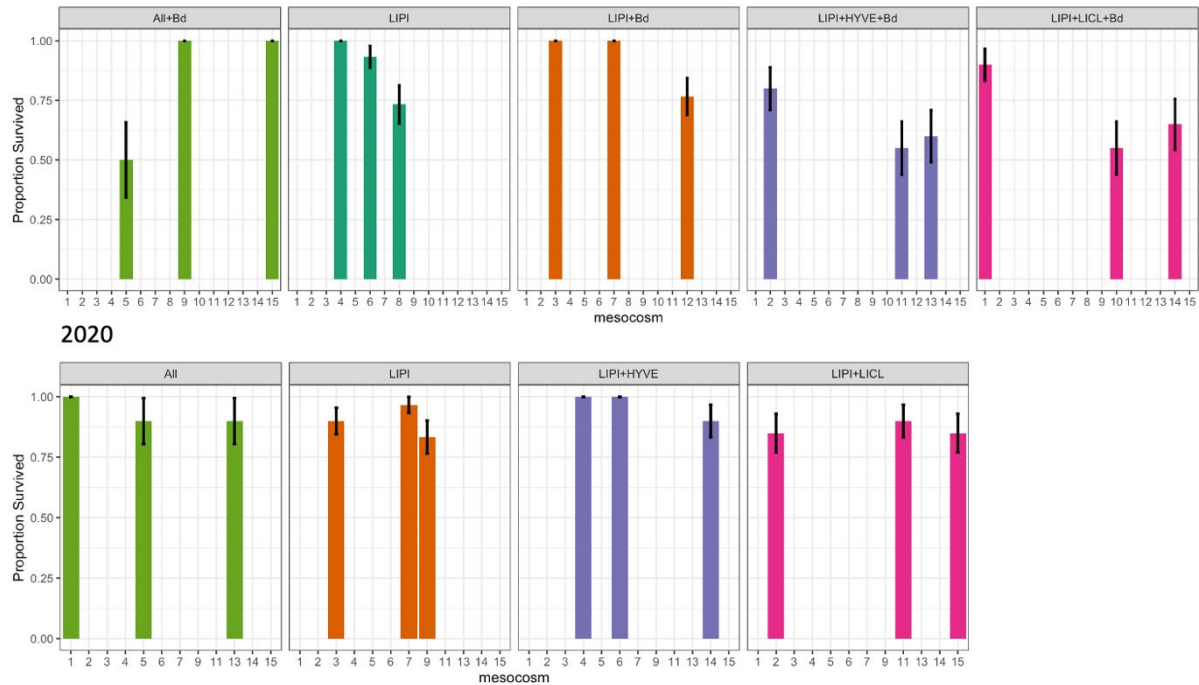


Figure 33. Post-metamorphic survivorship for *R. pipiens* from 2019 community treatments from 0 – 57 days after tail resorption. LIPI = *R. pipiens*, HYVE = *H. versicolor*, LICL = *R. clamitans*. Error bars indicate the standard error of the mean.

In 2019, *Bd* was not detected in exposed *R. pipiens* at 2-weeks post-exposure (n=82) or 4-weeks post-exposure to *Bd* (n=58). Of *Bd* exposed *R. pipiens* sampled 6-weeks post-exposure (n=16), 25% tested positive for *Bd* (mean *Bd* load= 244.71 DNA copies, SD = 112.68 DNA copies). At 8-weeks post-exposure, none of the remaining *R. pipiens* tadpoles were *Bd*-positive (n=11). Of all *Bd*-exposed metamorphs (n= 157), 5.10% were positive (mean *Bd* load = 307.33 DNA copies, SD = 156.20 DNA copies). There were not sufficient *Bd* positives to test for treatment differences in infection prevalence or load. In 2020, 19 of the 20 *N. viridescens* *Bd*-source animals were qPCR positive (mean *Bd* load= 598,757.42 DNA copies, SD = 527,426.39 DNA copies). Despite these loads, no newt-exposed tadpoles became infected with *Bd* and no larvae or metamorphs from 2020 mesocosms tested positive.

In summary, we found limited support for *R. pipiens* tadpoles becoming infected or maintaining infection with *Bd* under VT summer mesocosm conditions. This is consistent with our other infection trials in this species. We expect that management of tadpole community composition is unlikely to affect *R. pipiens* tadpole infection prevalence under summer field conditions, and *R. pipiens* larvae are unlikely to serve as a seasonal reservoir of *Bd*. However, future work could explore the potential of host community management for mitigating disease outbreaks in other species, life stages, or seasons with greater infection potential. Similar mesocosm experiments could be implemented with target species that are more susceptible to *Bd* infection in order to better understand the role of the host community in transmission dynamics. Despite negative *Bd* infection results in 2020, we did observe differences in *R. pipiens* mass at metamorphosis between community treatments. Such effects may have arisen from niche differences, competition, or facilitation between tadpole species or their symbiotic microbiota. Future

investigations of how tadpole community structure affects skin microbiomes and their anti-Bd function may inform our understanding of disease dynamics and immune development.

Objective 3. Modeling Disease Risk Under Alternative Climate and Management Scenarios

We developed a novel modeling approach, “reduced IPMs”, which are low dimension approximations of full host-parasite Integral Projection Models (IPMs) that can facilitate the fitting of IPMs to population-level disease data of the sort that we have collected in our field surveys (Wilber *et al.* IN REVISION). In our reduced IPM, *Bd* load is modeled on a log scale. We demonstrated that the reduced IPMs did an excellent job at capturing the dynamics of the full IPM, especially when the pathogen load distribution predicted by the full IPM follows a normal distribution (on a log scale). Therefore, our reduced IPMs are appropriate models for *Bd* dynamics, because the data from our field surveys (along with numerous other empirical studies) find that the *Bd* loads for infected individuals are usually well approximated by a log-normal distribution. The reduced IPMs are tractable models that allow for analytical calculations of population-level parameters, such as R_0 , the basic reproductive number of a pathogen, which determines when a pathogen can invade a host population (and which can inform pathogen control efforts). The reduced IPM illustrates that R_0 for a parasite is a product of the lifespan and “reproduction” of the parasite in each stage of its life-cycle, where the production of the environmental parasite stage depends on mean pathogen load. We further found that when host mortality depends on pathogen load, the reduced IPM predicts that a virulence-transmission trade-off can emerge, where R_0 is maximized at intermediate within-host pathogen growth rates. However, the presence and strength of the virulence-transmission trade-off is determined by the nature of load dependent host survival. Overall, we found that reduced IPMs provide a useful framework to expand our theoretical and data-driven understanding of how within-host processes affect population-level disease dynamics.

We then customized our reduced IPM to model the dynamics of *Bd* in the two leopard frog species, *R. pipiens* and *R. sphenoccephala*, found at our VT, PA, TN, and LA field sites (Figure 34). We found that demographic processes associated with changes in transmission risk, such as leaving and returning to aquatic habitats during breeding and hibernation, largely accounted for changes in *Bd* prevalence over the course of a season. In other words, once we accounted for how temperature affected *Bd* load dynamics, we generally did not need to invoke any additional temperature dependence in transmission rates to describe the seasonal changes in *Bd* prevalence. For three of the locations, TN, PA, and VT, models without any effects of temperature on host susceptibility predicted that, consistent with the observed data, *Bd* prevalence declined to low values when post-metamorphic leopard frogs spent less time in the water following breeding, but then rapidly returned to high levels at the onset of the breeding season. Populations from LA were an exception. For this site, our model predicted that a significant negative effect of temperature on transmission rate improved model predictions, even after accounting for temperature dependence in *Bd* load dynamics. Across all locations, our best fit models predicted a negative effect of temperature on *Bd* growth rate, such that log *Bd* load on post-metamorphic leopard frogs was predicted to be lowest during the warmest months (June, July, August) and highest during the coldest months (December, January, February; Figure 35).

Our best fit models predicted that, for all locations, instantaneous R_0 (the basic reproduction number for a pathogen) was highest in the coolest months when leopard frogs were present in the aquatic habitat. While this was not the time when post-metamorphic density was highest (which occurred during warmer months following larval recruitment), this was the time when mean log *Bd* load and prevalence were greatest. The higher mean *Bd* load in cooler months

compared to warmer months (by orders of magnitude in all locations) easily offset lower host density and led to high instantaneous values of R_0 (the basic reproduction number for a pathogen at a given moment in time). In other words, leopard frog populations were most susceptible to *Bd* invasion in the cooler months.

Our analysis of seasonal R_0 predicted that leopard frogs could maintain *Bd* in the population deterministically at the highest host densities we explored and when *Bd*-induced host mortality was zero. However, when *Bd*-induced mortality was minimal, but present, it was unlikely that southern leopard frogs in TN or PA could maintain *Bd* in the population even at high host densities; median seasonal R_0 values were less than one and deterministic simulations of models using median parameter estimates and no external zoospore pool led to eventual *Bd* extinction. In contrast, our models predicted that leopard frogs in LA could still deterministically maintain *Bd* in the population when *Bd*-induced mortality was present; seasonal R_0 values were greater than one and deterministic simulations of models using median parameter estimates and no external zoospore pool led to *Bd* persistence. Importantly, however, stochastic simulations with realistic population sizes, where frog and *Bd* birth, death, and infection processes occurred with some probability each time step as opposed to the deterministic analyses described above, suggest it is highly unlikely that leopard frogs in any location could maintain *Bd* on their own for greater than 10 years. Given that *Bd* is unlikely to persist on leopard frogs alone at our field sites, these results highlight the importance of incorporating the rest of the amphibian host community into our models when considering intervention strategies for controlling *Bd*. This next stage of our modeling work is currently underway.

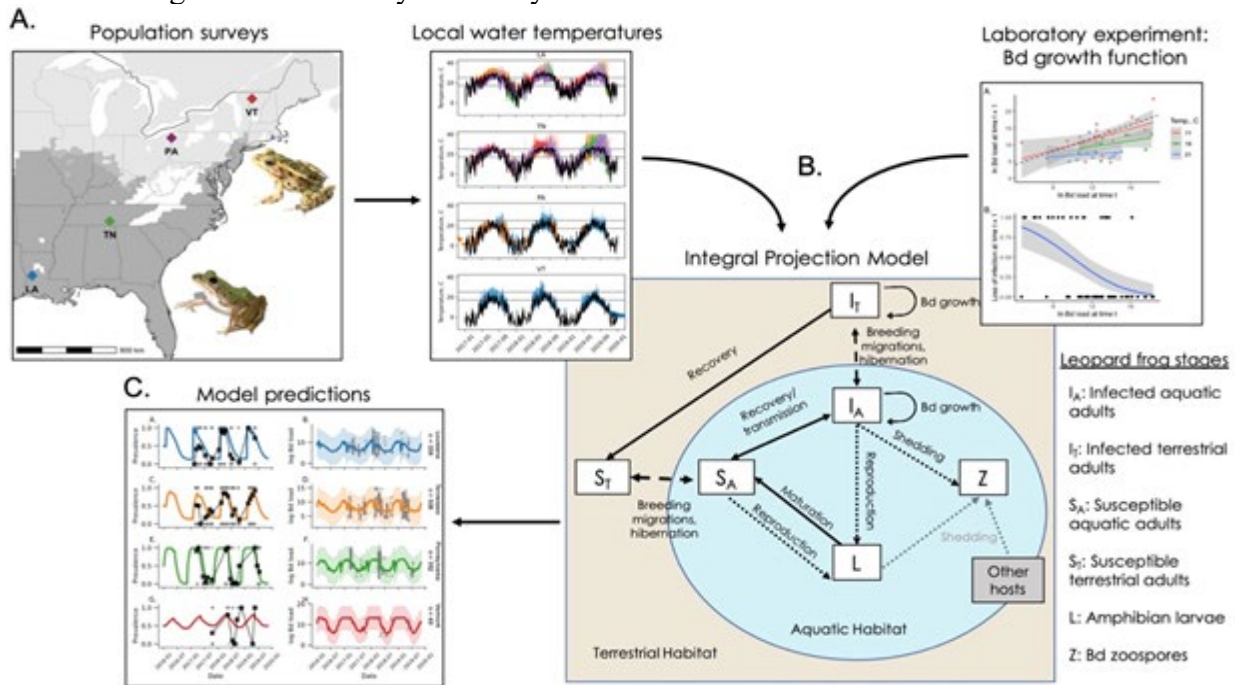


Figure 34. We combined population surveys from four geographic regions (A), local temperature data, laboratory infection experiments, and a novel mathematical model (B) to understand temperature-dependent infection dynamics of southern (*R. sphenoccephala*) and northern leopard frogs (*R. pipiens*) infected with the fungal pathogen *Bd* (C). Using our model, we quantified the contributions of seasonal demographic processes (e.g., breeding migrations) and seasonal infection processes (e.g., temperature-dependent pathogen growth on hosts) to seasonal fluctuations in *Bd* prevalence and infection intensity. We also used our models to quantify the potential of leopard frogs to act as maintenance species for *Bd*.

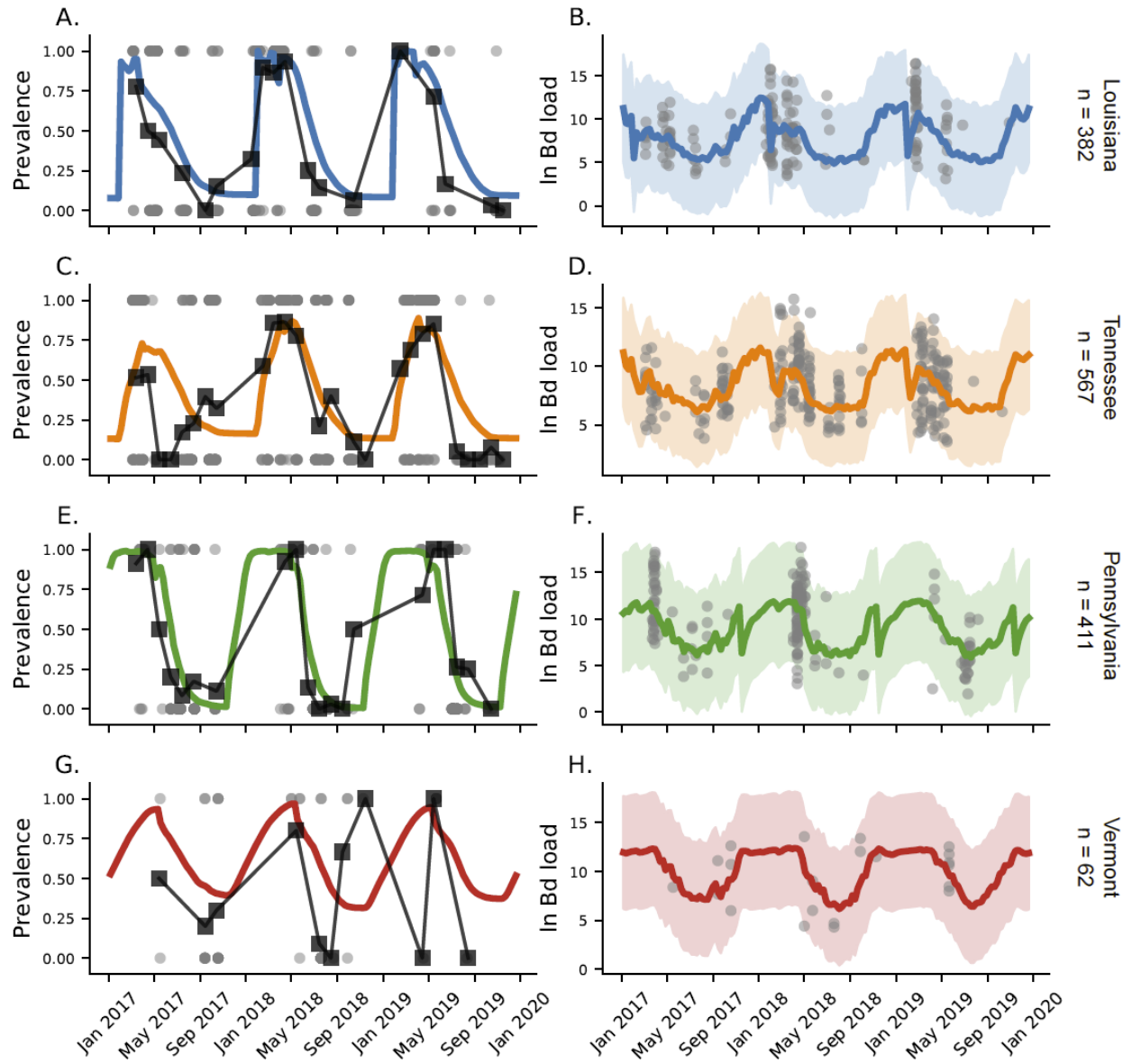


Figure 35. Best fit model predictions and observed *Bd* prevalence and load data from four locations. Each row gives the observed and predicted infection trajectories from a given location. The number of post-metamorphic leopard frogs sampled for *Bd* at each location is given under each location name. The colored lines give the predictions from the model with the lowest deviance information criteria (DIC) for each location under an assumption of low host density (0.0003 per m³). The shaded region gives the 95% prediction interval of log *Bd* load from the best model. For the *Bd* prevalence plots, gray points indicate whether a sampled host was positive (1) or negative (0) for *Bd* when sampled. Black points and lines give the mean monthly *Bd* prevalence on leopard frogs, pooled across all sites sampled within a location. For log *Bd* load plots, gray points give the observed *Bd* load for sampled leopard frogs that were *Bd* positive.

Conclusions and Implications for Future Research/Implementation

From 2017-2019, we sampled over 14,000 amphibians, including members of 50 species (30 frogs/toads and 20 salamanders) across five distinct climates/habitats for *Bd* and *Bsal*. At the same time, we collected individual- and site-level environmental data, enabling us to test for correlations between climate and the ecology of this host-pathogen interaction. We did not detect *Bsal*, the more recently discovered of the two pathogens, on any of the > 14,000 amphibians we sampled for this project. Thus, our results offer support for the idea that this pathogen has not yet invaded these locations of the United States. This is good news, as the arrival of *Bsal* to naive amphibian communities in the U. S. could have dire consequences, especially for salamander species, the taxon that has been most adversely affected by this pathogen in Europe (Martel *et al.* 2013, 2014). Management actions aimed at keeping this pathogen from reaching DoD lands will be important. Coordination with working groups of the North American *Bsal* Task Force (<https://www.salamanderfungus.org/>), a group of scientists and conservation practitioners who have been working together on management, policy and research implications of *Bsal* infection of amphibians, may be the most effective way for the DoD to develop and implement plans to prevent the spread of this pathogen onto its lands.

While we did not detect *Bsal*, we did detect the other amphibian *Batrachochytrium*, *Bd*, at all field sites and in the majority of species for which we had an adequate sample size to permit its detection. The *Bd* we detected was not only ubiquitous, but also genetically diverse. Ours was the first broad-scale comparison of contemporary *Bd* genetic variation within the U.S. Because we worked from skin swab samples we were able to sequence *Bd* at fine spatial scales, and replicate this sequencing across diverse localities within the U.S. The large amount of *Bd* genetic diversity we were able to document in this study, and especially the mix of *Bd* lineages seen both within and among wetlands, was surprising. Our results suggest that *Bd* has likely been coexisting with amphibian hosts in the U.S. for some time and that strains are easily moved from one place to another, likely by human activity or non-amphibian species (e.g., birds; Garmyn *et al.* 2012) as amphibians do not generally disperse widely. Further study is needed to understand the history of *Bd*'s introduction and spread within the U.S. and to better understand the origin of the 'mixed' lineages we detected at many field sites. Our experimental evolution study with *Bd* lineages isolated from our study regions showed that *Bd* can adapt to new climates through shifts in its thermal physiology. This suggests that we cannot count on global warming to limit the range of this historically temperature-sensitive and cool-adapted pathogen. Future studies comparing the virulence of particular lineages, in combination with the genomic tools we have developed and employed here, will be valuable to the DoD in monitoring and mitigating the movement and spread of particularly lethal *Bd* variants.

Our field surveys documented clear seasonal patterns in both *Bd* infection and the mucosal defenses that amphibian hosts employ to fight against this pathogen. The prevalence of *Bd* infection in the amphibian community showed a distinct seasonal pattern, peaking in the early spring and hitting its lowest point in the summer, at all but our VT and NM field sites. However, we did see strong seasonality in infection intensity in NM, which was largely driven by the leopard frogs in the amphibian community. The lack of seasonality in *Bd* infections in VT could stem from the short active season for amphibians at this site. Generally, degree of seasonality in *Bd* infection decreased with increasing latitude (and a shorter active season). Our focal species group for Objective 2, the leopard frog species complex, showed a similar pattern of seasonal

variation in prevalence and infection intensity, with *Bd* prevalence reaching near 100% in the spring at some sites.

Given the thermal dependence of *Bd* growth (Piotrowski *et al.* 2004; Voyles *et al.* 2017), it is not surprising that *Bd* prevalence at our study sites was associated with several environmental variables. Temperature, and to a lesser extent other environmental variables like pH and canopy cover, seem to be key determinants of seasonal infection dynamics. Amphibian body temperature at the time of capture non-linearly predicted *Bd* prevalence at the population level, with lower prevalence at low and high temperatures, following the thermal performance curve of *Bd* growth (Piotrowski *et al.* 2004). Furthermore, one month prior water temperature, which has been shown to predict *Bd* prevalence in the field previously (Bell *et al.* 2020), was also a strong predictor of *Bd* prevalence at our sites. Finally, we saw an interactive effect between water temperature and both pH and canopy cover, demonstrating that there are additional environmental variables that may influence *Bd* infection in nature. Previous work has demonstrated that closed canopies can increase disease prevalence (Roznik *et al.* 2015). We found this to be interactive with water temperature, in that low canopy cover results in greater thermal extremes, and therefore lower *Bd* prevalence at those extremes. This information can be used to make species and site-level predictions about *Bd* infection risk. For example, management actions that provide access to warmer terrestrial microhabitats, which allows for faster clearance of *Bd* infections, could be an option for helping amphibians through cooler months of the year, which often coincide with peaks in *Bd* prevalence and infection intensity in amphibian communities.

We documented for the first time that the mucosal defenses of amphibian hosts in the U. S. exhibit their own seasonal patterns, and in many cases these defenses peak just before or during the seasonal peak in *Bd* infections providing a sort of ‘preparatory immunity’. The proportion of bacteria in the mucosal microbiome that can function to inhibit *Bd* growth, and the secretion of mucosal peptides effective against *Bd*, were greatest either just before or coincident with the time of year when risk of *Bd* infection was highest. Seasonality of *Bd* thus appears to be partly regulated by annual immune rhythms including skin peptides that can alter the microbiome, and the rhythms are dominated by the effects of temperature that induce preparatory immunity in host frogs before the onset of conditions ideal for *Bd* infection. Future studies are needed to clarify the effects of extreme climate anomalies, which could offset the evolved synchrony between amphibian immune and microbiome defense and periods of greatest infection risk. In places like NM, where endangered Chiricahua leopard frogs (*R. chiricahuensis*) with reduced peptide defenses are particularly susceptible to developing heavy *Bd* infections, management strategies should focus on reducing the risk of infection to the most vulnerable life stages (e.g., newly metamorphosed froglets) and at the time of year when infections are most prevalent (often the coolest months of the amphibian active period).

Our experiments demonstrated that larval development under stressful climate conditions, including pond drying, elevated temperatures, and non-local climates, often has a cost in terms of reduced fitness up to and shortly after metamorphosis. However, we also documented negative impacts of climate stressors experienced during the larval stage on mucosal defenses and *Bd* susceptibility many months after metamorphosis. These results add to the growing concern that climate change will exacerbate the impact of chytridiomycosis as more rapid pond drying and elevated water temperatures expected under global climate change can increase the intensity of *Bd* infection in recently metamorphosed frogs. These results suggest that management efforts focused on limiting the impact of climate stressors on amphibian larvae may be effective in

bolstering the ability of juvenile amphibians, the most susceptible life stage, to fight *Bd* infections. Our reciprocal transplant experiment, in which newly metamorphosed froglets were exposed to both local and non-local *Bd* strains, also suggested the potential for the adaptation of hosts to their local *Bd* strains. From a management perspective, this indicates that the anthropogenic transfer and spread of *Bd* among amphibian populations (e.g., on equipment, or via the movement of animals) should be avoided, as non-local isolates may be more pathogenic than local isolates to amphibian populations.

The outcome of *Bd* infection on an individual amphibian is determined by a race between the rate of progression of the fungal infection on the individual host, and the ability of the host to mount effective defenses against the infection. Both processes can be strongly affected by environmental conditions, host life stage, and by specific attributes of both the host species and *Bd* strain. To investigate the potential impacts of alternative climate and management scenarios on chytridiomycosis, we used two approaches to develop and parameterize data-driven, mechanistic models of the impacts of climate on disease dynamics at the individual and community levels. In the first *Experiment-based models* approach, we are using the detailed experiments in Objectives 1 and 2 to determine the appropriate forms of each of the functions in the models, and to determine how temperature affects the model parameters. In the second *Field survey-based models* approach, we are fitting flexible Integral Projection Models to the patterns of prevalence and *Bd* load through time from the field survey data from each of the five regions. We developed a novel reduced dimension host-parasite Integral Projection Model (“reduced IPM”) approach that facilitated the fitting of IPMs to population-level disease data. We fit reduced IPMs to the field survey data from two leopard frog species found at our field sites, and found that demographic processes associated with changes in transmission risk, such as leaving and returning to aquatic habitats during breeding and hibernation, largely accounted for changes in *Bd* prevalence over the course of a season.

The next steps of our modeling work that are currently underway are to (a) finalize parameters for our *Experiment-based models*, now that the data are available from all of our laboratory experiments and measurements, (b) incorporate the rest of the amphibian host community into our *Field survey-based models*, and (c) use both of our modeling approaches to explore the efficacy of various management strategies to reduce the severity of chytridiomycosis outbreaks and/or increase the probability of persistence of the amphibian populations.

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Appendices

A. List of Scientific/Technical Publications

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- Bletz, M. C., B. C. LaBumbard, E. H. Le Sage, and D. C. Woodhams. Extraction-free detection of amphibian pathogens from water baths. *In review*.
- Brannelly, L. A., D. P. Wetzel, M. E. B. Ohmer, L. Zimmerman, V. Saenz and C. L. Richards-Zawacki. 2020.. Evaluating environmental DNA as a tool for detecting an amphibian pathogen using an optimized extraction method. *Oecologia* 194: 267-281.
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- Sheets, C. N., D. R. Schmidt, P. J. Hurtado, A. Q. Byrne, E. B. Rosenblum, C. L. Richards-Zawacki, and J. Voyles. 2021. Thermal performance curves of multiple isolates of *Batrachochytrium dendrobatidis*, a lethal pathogen of amphibians. *In press in Frontiers in Veterinary Science*

- Wilber, M., M. E. B. Ohmer, K. Altman, L. Brannelly, V. Saenz, E. Le Sage, B. LaBumbard, N. McDonnell, A. Muniz Torres, C. Nordheim, F. Pfab, C. L. Richards-Zawacki, L. Rollins-Smith, J. Voyles, D. Wetzel, D. Woodhams, and C. Briggs. Once a reservoir always a reservoir? Seasonality affects the pathogen maintenance potential of amphibian hosts. *In review*.
- Wilber, M. Q., Pfab, F., Ohmer, M. E. B., and Briggs, C. J. Integrating infection intensity into within- and between-host pathogen dynamics: implications for invasion and virulence evolution. *In revision at The American Naturalist*.