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ARTICLE
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# Localized carry-over effects of pond drying on survival, growth, and pathogen defenses in amphibians

**Emily H. Le Sage**[1] | **Michel E. B. Ohmer**[2] | **Brandon C. LaBumbard**[3] | **Karie A. Altman**[2] | **Laura K. Reinert**[1] | **Jeffery G. Bednark**[2] | **Molly C. Bletz**[3] | **Brady Inman**[1,3] | **Alexa Lindauer**[4] | **Nina B. McDonnell**[3] | **Sadie K. Parker**[2] | **Samantha M. Skerlec**[2] | **Trina Wantman**[2] | **Louise A. Rollins-Smith**[1,5,6] | **Douglas C. Woodhams**[3] | **Jamie Voyles**[4] | **Corinne L. Richards-Zawacki**[2]

[1]Department of Pathology, Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

[2]Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

[3]Department of Biology, University of Massachusetts, Boston, Massachusetts, USA

[4]Department of Biology, University of Nevada, Reno, Nevada, USA

[5]Department of Biological Sciences, Vanderbilt University, Nashville, Tennessee, USA

[6]Department of Pediatrics, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

**Correspondence**
Emily H. Le Sage
Email: e.h.lesage@gmail.com

**Present address**
Emily H. Le Sage, Department of Biology, Temple University, Philadelphia, Pennsylvania, USA

Michel E. B. Ohmer, Department of Biology, University of Mississippi, Oxford, Mississippi, USA

Karie A. Altman, Department of Biology, St. Bonaventure University, Saint Bonaventure, New York, USA.

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## Abstract

Climate change is increasing variability in precipitation patterns in many parts of the globe. Unpredictable changes in water availability can be particularly challenging for organisms that rely on precipitation-fed water sources for completing their life cycle, such as many amphibian species. Although developmental plasticity can mitigate the impacts of changing environments for some species, this strategy can come at a cost to other fitness-linked traits, such as immune function. We investigated localized variation in the capacity to respond to pond drying and evaluated whether developmental responses induced carry-over effects in disease susceptibility in three leopard frog species (*Rana* [*Lithobates*] *pipiens* and *Rana sphenocephala*; two populations each, and one population of *Rana chiricahuensis*). Using mesocosms located near the site of collection (<15 km away) in five regions spanning a latitudinal gradient, we raised tadpoles under simulated fast drying, slow drying, or constant water levels. After metamorphosis, we characterized several aspects of the skin microbiome, immune function, and response to exposure to the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*). Note that for *R. chiricahuensis,* the only carry-over effect measured was response to *Bd* exposure, for which we observed no effects of pond drying. We found that developmental plasticity in

Emily H. Le Sage and Michel E. B. Ohmer contributed equally to the work reported here.

response to drying was rare, except in the southernmost population of *R. sphenocephala*. In this location, tadpoles responded by accelerating development, and frogs with shorter larval periods developed more severe infections following *Bd* exposure post-metamorphosis, suggesting a trade-off between surviving pond drying and pathogen defense investment. In the three other locations, a lack of accelerated metamorphosis in drying treatments was accompanied by increased mortality, decreased anti-*Bd* function of the microbiome, and/or greater *Bd* infection after exposure. Overall, results suggest that faster drying conditions will likely have negative impacts on amphibians with long larval periods, both directly and indirectly via carry-over effects. Because effects of drying exposure were not uniform within a species, our findings suggest that local responses may not be generalizable to other regions of the range. These multifaceted effects of climate change on pathogen defenses are increasingly relevant as emerging infectious diseases threaten global biodiversity.

**KEYWORDS**
carry-over effects, chytridiomycosis, climate change, infectious disease, intraspecific variation, microbiome

## INTRODUCTION

In 50-year predictions for climate change impacts, even the best-case scenarios project rapid changes for freshwater ecosystems (Brooks, 2009; Cook et al., 2014). Precipitation-fed freshwater habitats, in particular, are highly vulnerable to climate variation (Winter et al., 2016) and provide essential habitat for many animals with complex life cycles (Wilbur, 1980). As such, boom-and-bust cycles in reproductive effort and juvenile recruitment in ephemeral wetlands are known to coincide with shifts in hydroperiod (i.e., the length of time a wetland retains water; Pechmann et al., 1989; Semlitsch, 2002; Stoks et al., 2014). These demographic consequences are likely driven by the sensitivity of the larval and metamorphic life stages to the environmental stress of living in variable hydroperiods. Because metamorphosis is a time of rapid, energetically demanding physiological changes, organisms often display adaptations in the timing of transitions for specific abiotic conditions and when minimum energetic reserves are met (Wilbur & Collins, 1973). Climate change, however, is likely affecting the timing and stability of specific conditions that metamorphosis depends on, highlighting the need to understand whether populations possess adaptations for coping with more variable temperature and precipitation regimes (Lowe et al., 2021).

An important adaptation for surviving in highly variable environments is phenotypic plasticity, a process by which behavioral, life history, and physiological responses induce alternative phenotypes (Stearns, 1989). Plasticity in developmental and growth rates is a hallmark of amphibian biology (Newman, 1992) that may provide a critical buffer from the effects of shortening of hydroperiod in some species (Kohli et al., 2019; Urban et al., 2014). However, only a small fraction of amphibian species has been studied under shortened hydroperiod conditions, and intraspecific variation in developmental plasticity is largely unknown (reviewed in Tejedo et al., 2010; Edge et al., 2016). Although plasticity can increase the chances of surviving in variable environments (e.g., thermal preference; Catenazzi & Kupferberg, 2017), in resource-limited cases this strategy can yield trade-offs with other performance traits (e.g., smaller body size; Wilbur, 1980). Developmental plasticity can also induce trade-offs with traits expressed later in life as carry-over effects (i.e., history affects the subsequent performance of an individual; Pechenik et al., 1998; Lindström, 1999). Indeed, exposure to pond drying typically results in faster development at a cost of a smaller body size at metamorphosis, leading to lower juvenile survival and adult fecundity in several species (reviewed in Richter-Boix et al., 2011).

Owing to an understanding that the neuroendocrine stress axis (hypothalamus–pituitary–adrenal axis) orchestrates both the accelerated metamorphosis phenotype (Denver, 1997) and the drastic immune system changes that occur with metamorphosis (Rollins-Smith, 1998),

researchers have hypothesized that carry-over effects include reduced immune investment and tolerance to additional stressors (Crespi & Warne, 2013; Gervasi & Foufopoulos, 2008; Kohli et al., 2019). In addition to the direct immunosuppressive effects of the endocrine regulators of accelerated metamorphosis (i.e., glucocorticoids), the resource-limited conditions experienced during a shortened hydroperiod may also lead to trade-offs between immune function and other performance traits (Sheldon & Verhulst, 1996). Carry-over effects may act through (and also include) changes in host-associated microbiota, which are also highly sensitive to the environmental changes associated with pond drying (e.g., warmer/fluctuating temperatures; Longo et al., 2015; Woodhams et al., 2014) and play an important role in many developmental processes including immune system priming (reviewed in Hooper et al., 2012; Lee & Brey, 2013). Regardless of whether specific host factors such as glucocorticoid levels (Uren Webster et al., 2020) or ecological community interactions (Greenspan et al., 2020) drive a change in host-associated microbiota, frogs developing under pond drying conditions may lack critical bacterial groups that provide protection from pathogens (Holden et al., 2015; Rebollar et al., 2020; Woodhams et al., 2014). These compositional changes could persist after metamorphosis (Davis et al., 2017), or alter immune development (Knutie et al., 2017), to cause carry-over effects on pathogen defenses.

In this multiscale study, we assessed responses of two populations of northern and southern leopard frog (*Rana pipiens* and *Rana sphenocephala*, respectively) and one population of Chiricahua leopard frog (*Rana chiricahuensis*) in their capacity to respond to localized pond drying conditions (i.e., five populations were tested in mesocosms <15 km from the site of origin) and evaluated carry-over effects in a comprehensive assessment of relevant pathogen defenses. We quantified pathogen defenses previously shown to affect susceptibility to *Batrachochytrium dendrobatidis* (*Bd*), a fungal pathogen associated with the disease chytridiomycosis and global population declines (Daszak et al., 1999). Following the conceptual model of Kohli et al. (2019), we hypothesized that exposure to pond drying would induce accelerated metamorphosis and carry-over effects on pathogen defenses in some populations. We predicted that populations would vary in developmental traits and larval survival in response to localized drying conditions. Further, we hypothesized that those displaying developmental plasticity in response to drying would incur a cost post-metamorphosis, such as lower survival, slower growth, reduced immune function, and/or increased *Bd* susceptibility.

# METHODS

## Project overview

Regional climate trends and predictions for our study region suggest these species will likely continue to experience reductions and fluctuations in hydroperiod even in semipermanent wetlands they inhabit (Walls et al., 2013). Specifically, predictions of climate change impacts for the study regions within the northern leopard frog's range (Vermont and Pennsylvania, USA; Figure 1) suggest a general increase in drought frequency driven by increased evapotranspiration and reduced precipitation, although these predictions are highly variable spatially (Hayhoe et al., 2007). While the climate in the study regions within the southern leopard frog's range (Tennessee and Louisiana, USA; Figure 1) now has fewer consecutive wet days and less summer precipitation, records also indicate an increase in the frequency of intense precipitation events compared with that 50 years ago (Powell & Keim, 2015). For the Chiricahua leopard frog study site (New Mexico, USA), climate trends suggest warmer and drier conditions, with drought periods increasing in frequency and duration (Cook et al., 2015).

To quantify localized responses to pond drying, we set up three replicates of each water level treatment at each location (constant level, slow drying, or fast drying; Figure 1) in the year 2018. We reared animals in mesocosm arrays until metamorphosis following the experimental design of previous work (e.g., Wilbur, 1987; see review in Edge et al., 2016), then transferred froglets to indoor facilities to quantify developmental and carry-over effects (see below). Standardized cattle tank mesocosms (i.e., same size and shade covers) near the site of egg collection (6–14.5 km apart depending on location, where we had a flat location and a water source; see Appendix S1: Table S1), as opposed to one centralized location (i.e., common garden), allowed us to expose animals to drying regimes in a seminatural, localized setting while excluding the effects of exposing animals to a foreign environment (e.g., different climate, leaf litter, and phytoplankton). Thus, differences among locations include both environmental and population effects.

## Simulation of pond drying in mesocosm arrays

We set up nine 1135-L black polyethylene tanks (Rubbermaid, Atlanta, GA, USA) in arrays at each of five study sites in full sun: Vermont (VT), Pennsylvania (PA), Tennessee (TN), Louisiana (LA), and New Mexico (NM). We filled each tank with 600 L of water from local

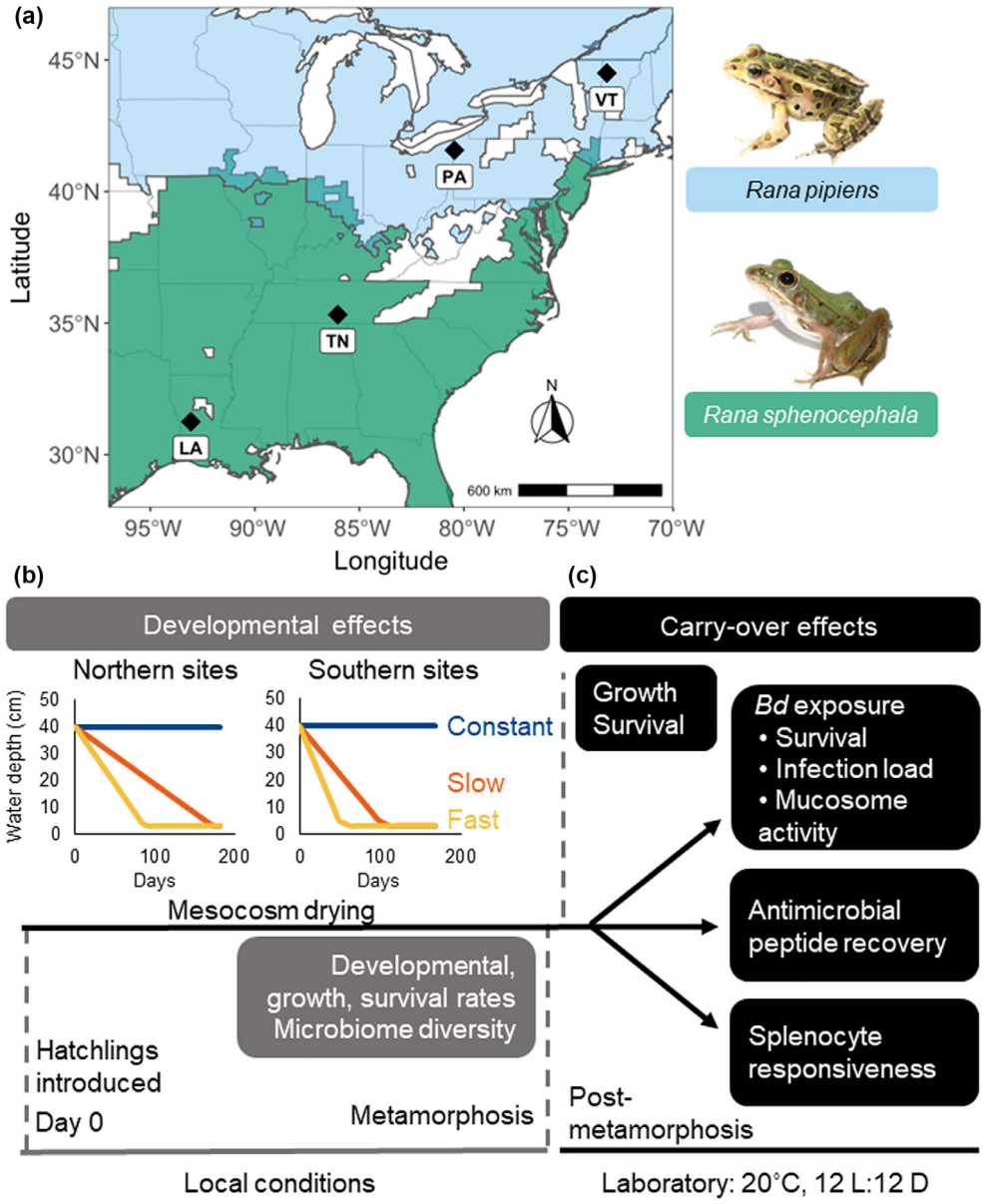


**FIGURE 1** (a) Map of mesocosm locations within each leopard frog range (northern leopard frog: Vermont = VT, Pennsylvania = PA; southern leopard frog: Tennessee = TN, Louisiana = LA; New Mexico is not pictured due to high mortality in outdoor mesocosms. An indoor experiment was conducted with a similar design; see Appendix S1). (b) Drying regimes, the mesocosm timeline, and the developmental data collected in northern and southern sites. (c) Carry-over effects that were measured post-metamorphosis. Both northern leopard frog collection sites were characterized as long hydroperiod wetlands that dry during drought years but not consistently every year, and both southern leopard frog collection sites were characterized as intermediate hydroperiod wetlands that typically dry every year. Coordinates of egg collection and mesocosm arrays, and the location of *Batrachochytrium dendrobatidis* (*Bd*) exposures are listed in Appendix S1: Table S1. Daily temperature averages in mesocosm arrays are shown in Appendix S1: Figure S1.

sources, then covered tanks with plastic 55% shade-cloth screens (1-mm mesh) to provide shade and prevent colonization by other amphibians or predators. Local water sources for each array were either well water (unchlorinated) or city water (chlorinated). If the water source was city water, we allowed the chlorine to evaporate for at least 1 week and then confirmed no chlorine remained with test strips (Tetra EasyStrips, Blacksburg, VA, USA). We then stocked tanks with 200 g dried local leaf litter, 15 g of alfalfa pellets, and 0.5 L local pond water enriched for plankton by adding sieved (80-micrometer phytoplankton net; Forestry Suppliers, Jackson, MS, USA) water from multiple transects through a local pond (<15 km away). At least 1 week after the tanks were established with food and conditioned water, we stocked tanks with tadpole hatchlings

(Gosner stage 25; Gosner, 1960) by haphazardly (i.e., selecting hatchlings by hand at random) mixing individuals from at least two clutches, to create a density of 40 tadpoles/tank. We placed two pendant temperature loggers in the water within each tank (Onset HOBO, Bourne, MA, USA): one floating 5 cm below the surface and the other held at the bottom of the tank.

We maintained the water in the constant treatment at 40 cm of depth and reduced the water in the drying treatments at one of two rates: slow or fast, which differed by location (Figure 1). We reduced water levels in the drying treatment tanks weekly beginning 7 days after animals were introduced. In Louisiana, we reduced the water level using a PVC pipe covered in mesh, set to the desired level, that allowed draining of surface water. At all other sites, we manually reduced water levels by bailing out water. We affixed meter sticks to the inside of each tank and added preconditioned water or removed water as needed to maintain the desired levels on a daily basis. Based on estimates of hydroperiod in local leopard frog ponds in each region (E. H. Le Sage & M. E. B. Ohmer pers. obs.), fast and slow tanks in the northern and Chiricahua leopard frog sites reached 3 cm at 91 and 175 days, respectively. In the southern leopard frog sites, fast and slow tanks reached 3 cm at 63 and 112 days, respectively. These drying regimes and tadpole densities were comparable with those in previous studies in northern and southern leopard frogs (Brannelly et al., 2019; Ryan & Winne, 2001). Once water levels were reduced to 3 cm in the slow and fast treatment groups, we maintained this level until 97% of animals had metamorphosed. The remaining individuals were underdeveloped tadpoles and not used in the study of carry-over effects; thus, they were euthanized at this point.

Note, few Chiricahua leopard frog tadpoles successfully completed metamorphosis by the end of the summer and the remainder subsequently overwintered in the tanks. We resumed drying treatments the following summer, but most of the tadpoles that survived overwintering still failed to undergo metamorphosis. We performed an indoor experiment with this species instead, in which water levels were reduced in indoor aquaria. In summary, we did not find an effect of reducing water levels on either developmental, survival, or *Bd* susceptibility measures in this species (see Appendix S1 for methods and results from this location).

## Evaluation of developmental responses to simulated drying

When the first tadpoles reached metamorphic climax (forelimbs emerged; Gosner stage 42), we added floating foam pieces to all tanks to ensure that froglets had access to refuge and would not drown. We collected metamorphs at or past Gosner stage 42 (i.e., stages 42–46) from the tanks daily by dipnetting and transported them in plastic containers with tank water to the local indoor animal facility. We housed frogs individually in 2.12-L plastic containers with 100–200 ml of artificial pond water (following the recipe in Wyngaard & Chinnappa, 1982) under a 12-h light cycle and at 20–21°C room temperature. We placed the containers on an incline to provide wet and dry sides and changed the water twice weekly. We fed frogs pinhead crickets dusted with a vitamin supplement (Repti Calcium; Zoo Med Laboratories, San Luis Obispo, CA, USA) two to three times weekly. Once their tails were completely absorbed, we recorded days to metamorphosis, mass (in grams), and snout–vent length (SVL in millimeters), and swabbed frogs to collect skin microbiome samples (see below).

## Microbiome analysis

We swabbed frogs with rayon swabs (MW113; Medical Wire & Equipment Co., Corsham, England) twice on each leg, and dorsal, ventral, and lateral surfaces at metamorphosis (once tails were reabsorbed) after rinsing with sterile artificial pond water. Swabs were immediately frozen at −80°C for later microbiome analysis (about 6 mo). If frogs were collected between Gosner stages 42 and 46, they were kept in the laboratory with 1 cm of water collected from their respective mesocosm until they reabsorbed tails. We extracted DNA from swabs using a DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA, USA) following the manufacturer's suggested Pretreatment for Gram-Positive Bacteria protocol. We used an initial lysozyme (20 mg lysozyme/1 ml lysis buffer) incubation step at 37°C for 1 h to lyse gram-positive bacteria. Next, we added 25 µl of proteinase K and 200 µl of buffer AL to each reaction, and then incubated at 70°C for 30 min before proceeding with the remaining kit instructions. Following DNA extractions, we conducted PCR analyses in duplicate to amplify the V4 region of the 16S rRNA gene (515F and 806R primers) following the Earth Microbiome protocol methods (Caporaso et al., 2012). Following PCR, we pooled sample amplicons and visualized them on 1.5% agarose gels. We purified and normalized samples using a Mag-Bind EquiPure Library Normalization Kit (Omega Bio-Tek, Inc., Norcross, GA, USA). We then pooled samples together and sequenced them using an Illumina MiSeq v2 300-cycle cartridge.

We processed the raw Illumina 16S rRNA amplicon data and quality-filtered using QIIME 2 v2020.2 (Bolyen et al., 2019). We classified the reads into suboperational

taxonomic units (sOTUs) using the Deblur workflow (Amir et al., 2017). Within Deblur, we trimmed reads to 150 bp and assigned bacterial taxonomy using the Greengenes 13_8 99% OTU reference classifier (McDonald et al., 2012). The sOTUs assigned as "mitochondria" and "chloroplast," as well as contaminant sOTUs with more than 20 reads in DNA extraction and PCR negative controls, were removed. For analyses, we used 4,459,748 sequences. Next, we rarified the dataset at 3400 sequences per sample to retain most samples and to normalize read counts across samples, leaving 281 samples for microbiome analysis. We generated several metrics for the smaller datasets to explore differences in alpha (sOTU richness and faith phylogenetic distance) and beta (Bray–Curtis, Jaccard, and unweighted and weighted UniFrac) diversity.

We estimated anti-*Bd* function by comparing sequences with those in the Antifungal Isolates Database (Woodhams et al., 2015). This database currently contains over 6000 bacteria tested for the capacity to inhibit *Bd* growth and isolated from globally distributed amphibian species. Like most predictive databases (e.g., BugBase and PICRUSt), there is the potential for some biases to exist; however, the database consists of a wide range of host species, seasons, climates, and *Bd* invasion histories from which isolates were collected. Using the vsearch cluster-features-closed-reference script (Rognes et al., 2016), we identified sequences from our experimental frogs with a 99% match to bacterial isolates previously shown to inhibit *Bd* growth in culture by at least 80% compared with that in controls. The proportion of each individual's total reads that matched those in this antifungal database is hereafter referred to as "anti-*Bd* function." In addition, we calculated the number of sOTUs with predicted anti-*Bd* function, termed "anti-*Bd* richness" below. Note, these estimates of microbial community function come with uncertainty, because the inhibitory function of microbial secondary metabolites can differ among *Bd* isolates (Antwis & Harrison, 2018), and 16S rRNA sequences may not faithfully indicate secondary metabolite production. However, we posit that these estimates may be useful for between-group comparisons (Langille et al., 2013), and function can be a phylogenetically conserved trait (Goelen et al., 2020). We were also interested in whether these individuals from distant regions of their ranges shared core microbiomes (see Results in Appendix S1).

## Evaluation of carry-over effects of simulated drying

We assessed carry-over effects on disease susceptibility in three separate subsets of animals in which we estimated one of the following measures: cellular immunity, antimicrobial peptides, or response to *Bd* exposure. We randomly assigned animals (within mesocosm block) into these groups to standardize the range of developmental timing and animal size represented in each. Before any manipulation, we measured each frog once for mass and SVL to calculate post-metamorphic (juvenile) growth rate, and these measurements ranged between 20 and 80 days depending on the experimental group (cellular immunity, antimicrobial, or *Bd* exposure). In order to compare survival post-metamorphosis among drying treatments, we recorded daily mortality before the experimental manipulations began.

Cellular immunity estimates consisted of five measures: T-cell responsiveness to PHA (phytohemagglutinin, a standardized mitogen), B-cell responsiveness to heat-killed *Escherichia coli*, total lymphocyte count in spleens, total lymphocyte count in thymuses, and circulating white blood cells in blood samples. To estimate stored antimicrobial peptide defenses, we induced skin secretions from a subset of juveniles around one and two months post-metamorphosis for two measurements per individual (for more details on cellular immunity and antimicrobial peptide methods and results; see Appendix S1). We exposed the third subset of frogs to either sham (control) or *Bd* inoculum to estimate survival and infection dynamics.

## *Bd* exposure

We standardized *Bd* isolate and passage history across populations and species. We exposed frogs to a low-passage (12–14 passages since isolation) *Bd* isolate called "Section Line," which falls within the Global Panzootic Lineage (Piovia-Scott et al., 2015). This *Bd* isolate was originally obtained from a juvenile Cascades frog (*Rana cascadae*) in Northern California in 2011 and is highly pathogenic to *R. cascadae* and *R. sphenocephala* (Holden et al., 2015; Piovia-Scott et al., 2015). We shipped aliquots of the cryopreserved Section Line *Bd* isolate to each laboratory (Appendix S1: Table S1), where they were revived and grown under identical conditions (Boyle et al., 2003). We cultured the Section Line *Bd* isolate in T-broth culture flasks (1% tryptone) and incubated it at 21°C. When zoospores were needed for exposure, we seeded T-broth agar plates from culture flasks (Boyle et al., 2003). We made zoospore inoculum by flooding plates with sterile artificial pond water after 4–7 days of growth and then harvested zoospores using a sterile nylon 20-μm filter to remove zoosporangia. Similarly, we used flooded sterile tryptone agar plates to obtain an inoculum that did not contain zoospores for sham-exposed (control) frogs.

Before exposure, we measured mass and SVL to standardize size when randomly assigning frogs to sham or *Bd*

exposure groups. We exposed frogs at a standardized age by staggering exposures and distributed equal proportions of cohorts that metamorphosed at fast, moderate, and slow rates within the sham and *Bd* exposure groups. The first exposure to *Bd* occurred between 24 and 92 (median: 47) days post-metamorphosis, and exposures continued every other week, for a total of four exposures per frog (days 0, 14, 28, and 42). Following Holden et al. (2015), we used an exposure dose of $1 \times 10^6$ zoospores per frog, except for the second exposure, which was $1.5 \times 10^6$ zoospores per frog. Zoospore doses were quantified using a hemocytometer. We exposed froglets for 24 h in small 59-ml (2-ounce) condiment cups filled with 10 ml of inoculum, a level that prevented frogs from climbing out of inoculum but kept them from drowning. After exposure, we monitored frogs daily for signs of chytridiomycosis and mortality (e.g., lethargy, irregular skin sloughing, abnormal posture, and inappetence; Voyles et al., 2009). We swabbed frogs from all populations for *Bd* on days 7, 35, and 63 after initial exposure, except in Vermont, where the final swab time point was day 49 instead of 63 due to high mortality. Using fine-tip rayon swabs (see above), we swabbed frogs five times across each leg, and on the dorsal, ventral, and lateral sides of the body (Hyatt et al., 2007). We also swabbed any animals that died when they were found during daily health checks. We stored all swabs at −20°C until processing. At the final swabbing time point, we soaked a subset of frogs in sterile artificial pond water, which was frozen and later tested for the mucosome capacity to kill *Bd* zoospores (see Appendix S1 for detailed methods).

## Quantifying *Bd* load in infected frogs

We extracted DNA from swabs using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD, USA) following the manufacturer's protocol for animal tissue samples with two modifications. First, we incubated swabs for 30 min., vortexed, centrifuged, and then repeated this sequence again. Second, we eluted samples twice with 100 µl of elution buffer for a final volume of 200 µl of eluted DNA. We used quantitative PCR (qPCR) to quantify *Bd* DNA, using a protocol that followed Boyle et al. (2004). To assess and combat reaction inhibition, we included TaqMan Exogenous Internal Positive Control Reagents (Applied Biosystems, Foster City, CA, USA) and bovine serum albumin (final concentration 400 ng/µl; Garland et al., 2010) in each reaction well (Kriger et al., 2006). We included positive and negative extraction control samples on each plate, as well as *Bd* standards ranging from 2.1 to $2.1 \times 10^6$ gene copies/µl (Pisces Molecular, Boulder, CO, USA). We tested samples in singlicate to keep costs low (Kriger et al., 2006). We converted *Bd* load (in DNA copies) per 5-µl reaction well to *Bd* DNA load per swab, then to zoospore quantity per swab (see Appendix S1 for detailed methods).

## Statistical analysis

We performed all statistical analyses using R (R Core Team, 2020). We fit all linear models using maximum likelihood and used likelihood-ratio tests for comparisons of nested models (package: base; function: ANOVA; R Core Team, 2020). We treated sites as independent and thus analyzed them separately, unless otherwise noted. Where appropriate, we treated mesocosm tank as a random variable unless otherwise noted (e.g., when random effects caused singularity). We used AIC values to select the best models and reported *p* values of all variables included in the best model regardless of statistical significance.

We analyzed differences in mesocosm water temperature at both the top and bottom logger positions (daily average, minimum and maximum temperatures, as well as daily thermal range) between mesocosm locations of each species using linear mixed-effects models (LMM) with the three-way interaction between treatment, day, and site as the fixed factor, days nested in site as a random effect, and an autocorrelation structure of order 1 (package: nlme, function: lme; Pinheiro et al., 2021). We used Tukey's post hoc tests to determine differences across treatments and sites (package and function: emmeans; Lenth, 2020).

We analyzed differences in time to and size (SVL and mass) at metamorphosis and larval growth rate (mass × days to metamorphosis interaction) among treatments for each population using linear mixed models (LMMs). To determine differences in larval survival, we performed generalized LMMs with binomial distributions (package: lme4, function: glmer; Bates et al., 2015). The response term was a two-vector variable of the number that survived and the number that failed to survive to the end of the mesocosm period. The fixed effect was treatment. We analyzed differences in survival to 60 days post-metamorphosis among treatments and with mass at metamorphosis as a covariate with Cox proportional hazard regression models, with mesocosm included as a cluster effect (package: survival; function: coxph; Therneau & Grambsch, 2000; Therneau, 2021). We compared post-metamorphic growth using a LMM of mass by the interaction between drying treatment and age (days after metamorphosis), and individual ID nested within mesocosm tank as a random variable.

To analyze microbiome diversity, we compared models with the following predictors: drying treatment, average treatment water temperature over the 2 days before metamorphosis (Bletz et al., 2017), days to

metamorphosis, and the interactions between treatment and either days to metamorphosis or temperature, with mesocosm tank as a random variable. Average water temperature and days to metamorphosis were confounding variables in the southern leopard frog sites (Tennessee and Louisiana), so they were not included in the same model during model comparison. For alpha diversity, we compared generalized LMMs of sOTU richness (comparing negative binomial, Gaussian, and Poisson distributions with "lmer" and "glmer" and a Bobyqa optimizer when needed) and LMMs of Faith's phylogenetic distance. We compared microbiome anti-*Bd* richness using generalized LMMs (comparing negative binomial, Gaussian, and Poisson distributions) and anti-*Bd* function using beta regression models (package glmmTMB; beta_family (link = "logit")). Because the constant treatment group was set as the reference level for this variable, we used "summary()" when reporting interactions to demonstrate the difference in slope of drying treatment groups compared with constant. For beta diversity, we performed a linear decomposition model analysis (ldm function in the LDM package; Hu & Satten, 2020) on each of the beta-diversity metrics. For statistically significant models, we used the "pairwise. adonis" function for the post hoc testing (Oksanen et al., 2019) and the "betadisper" function to see whether there was significant dispersion of community structure, or alternatively a shift in the community composition. We performed a differential abundance analysis using LDM to assess treatment effects only in sites with significant differences in beta diversity. Differential sOTUs with adjusted $p$ values less than 0.01 were selected for comparisons of relative abundance across treatments. Finally, we generated taxonomy plots in *R* to show the relative abundance of the top bacterial families.

We analyzed two aspects of *Bd* infection in each population in exposure trials: prevalence (i.e., the proportion of animals infected) and the intensity of infection (i.e., *Bd* load) for exposed groups. Although a zero-inflated negative binomial model would analyze these aspects of infection simultaneously, these models often failed to converge when fit to our data. Therefore, we analyzed the prevalence and the intensity of infection separately. We used a generalized LMM with a binomial distribution (package: lme4, function: glmer; Bates et al., 2015) to assess the effects of days post-exposure and drying treatment on the proportion of individuals that became infected in each population. We analyzed the effects of days post-exposure and drying treatment on *Bd* load ($\log_{10}$[zoospores + 1]) of infected individuals from each location (individuals with *Bd* loads of 0 were excluded) using a LMM (package: lme4, function: lmer; Bates et al., 2015). We were also interested in whether anti-*Bd* function and richness in the microbiome at metamorphosis were related to infection intensity or the probability of infection at the first swab (7 days). Thus, we conducted LMMs of *Bd* load ($\log_{10}$[zoospores + 1]) of only infected individuals and generalized LMM with a binomial distribution for the probability of infection, with each aspect of the microbiome analyzed in separate models.

All models of *Bd* infection included a random effect of individuals nested within mesocosm. Each model also included either days to metamorphosis or days between metamorphosis and first *Bd* exposure (days to exposure) as a covariate, with one exception. The model describing effects of drying treatment and days after exposure on the proportion of infected individuals in the Tennessee location failed to converge when either time to metamorphosis or time to exposure was included. Therefore, we excluded these covariates from this analysis. We used Tukey's pairwise comparisons (package: multcomp, function: glht; Hothorn et al., 2008) when drying treatment significantly influenced a response variable.

# RESULTS

## The effect of drying on water temperature

Water temperatures in all fast-drying and most slow-drying tanks reached higher daily maximums (LMM; Tennessee/Louisiana: Fast × Time: $\beta = 0.047$, SE = 0.0027, $p < 0.001$, Slow × Time: $\beta = 0.054$, SE = 0.0031, $p < 0.001$; and Vermont/Pennsylvania: Fast × Time: $\beta = 0.043$, SE = 0.0058, $p < 0.001$) and lower daily minimums than did constant water level tanks (LMM; Tennessee/Louisiana: Fast × Time: $\beta = -0.025$, SE = 0.0012, $p < 0.001$, Slow × Time: $\beta = -0.032$, SE = 0.0014, $p < 0.001$; Vermont/Pennsylvania: Fast × Time: $\beta = -0.041$, SE = 0.0045, $p < 0.001$, Slow × Time: $\beta = -0.013$, SE = 0.0061, $p = 0.038$). Overall, fast-drying tanks had larger daily temperature ranges than constant tanks, reaching higher and lower temperatures daily, as seen in previous studies using this design (e.g., Leips et al., 2000). Specifically, from north to south, fast-drying tanks had greater daily temperature ranges by 2.0–7.8°C than constant tanks, with minimum and maximum differences of 1.5–2.8°C and 0.5–5.0°C, respectively, resulting in 0.4–0.7°C cooler daily average temperature.

Across northern sites, mean daily water temperatures (LMM: $\beta = 0.30$, $p = 0.51$) and daily variability (LMM: $\beta = 0.63$, $p = 0.22$) were similar. However, the constant treatment demonstrated lower daily temperature variation in Pennsylvania than in Vermont (Tukey's pairwise comparison: $\beta = -0.79 \pm 0.255$, $t = -3.1$, $p = 0.025$; Appendix S1: Figure S1).

In the southern sites, Louisiana animals in all treatments experienced lower temperatures on average

than Tennessee animals, due to the experiment starting 40 days earlier in Louisiana (see Appendix S1: Table S1 for experimental timelines; LMM: $\beta = -3.25$, SE = 0.70, $p < 0.001$; Louisiana mean = 19.0°C, Tennessee mean = 22.6°C). Daily temperature variability was higher in the fast- and slow-drying treatments than in the constant water level treatment in both sites (LMM; Fast × Time: $\beta = 0.073$, SE = 0.0035, $p < 0.001$, Slow × Time: $\beta = 0.091$, SE = 0.0045, $p < 0.001$). However, when the first animals metamorphosed in Louisiana and Tennessee, the average mesocosm water temperatures were similar (LA: 21.04, TN: 22.2°C; Appendix S1: Figure S1).

## Developmental responses of northern leopard frogs to pond drying

Vermont and Pennsylvania northern leopard frogs did not accelerate development in response to drying (LMM; Vermont—slow: $p = 0.213$, fast: $p = 0.085$; Pennsylvania—slow: $p = 0.994$, fast: $p = 0.547$; Figure 2a,d). However, drying resulted in smaller sizes at metamorphosis and slower larval growth rates in Vermont (LMM; Slow × Days to metamorphosis: $\beta = -0.940 \pm 0.705$, $p = 0.010$, fast: $\beta = -1.684 \pm 0.678$, $p < 0.001$; Figure 2b), and frogs that had longer larval periods metamorphosed at larger sizes in Pennsylvania ($\beta = 0.131 \pm 0.085$, $p = 0.008$; Figure 2e). Also, exposure to slow drying in Vermont and fast drying in Pennsylvania resulted in lower larval survival relative to the other treatments (GLMM; Vermont—slow: $\beta = -1.231 \pm 0.826$, $p = 0.004$, fast: $p = 0.126$; Pennsylvania—slow: $\beta = p = 0.387$, fast: $\beta = -1.639 \pm 1.510$, $p = 0.034$). Specifically, 30% and 42% of tadpoles did not survive the Vermont fast- and slow-drying treatments, respectively, compared with 18% mortality in the constant treatment. In Pennsylvania, 12% did not survive the fast-drying treatment compared with about 3% and 5% larval mortality in the constant and slow-drying treatments, respectively.

## Northern leopard frog microbiome responses to pond drying

In Vermont, but not Pennsylvania, alpha diversity in the microbiome at metamorphosis varied by treatment

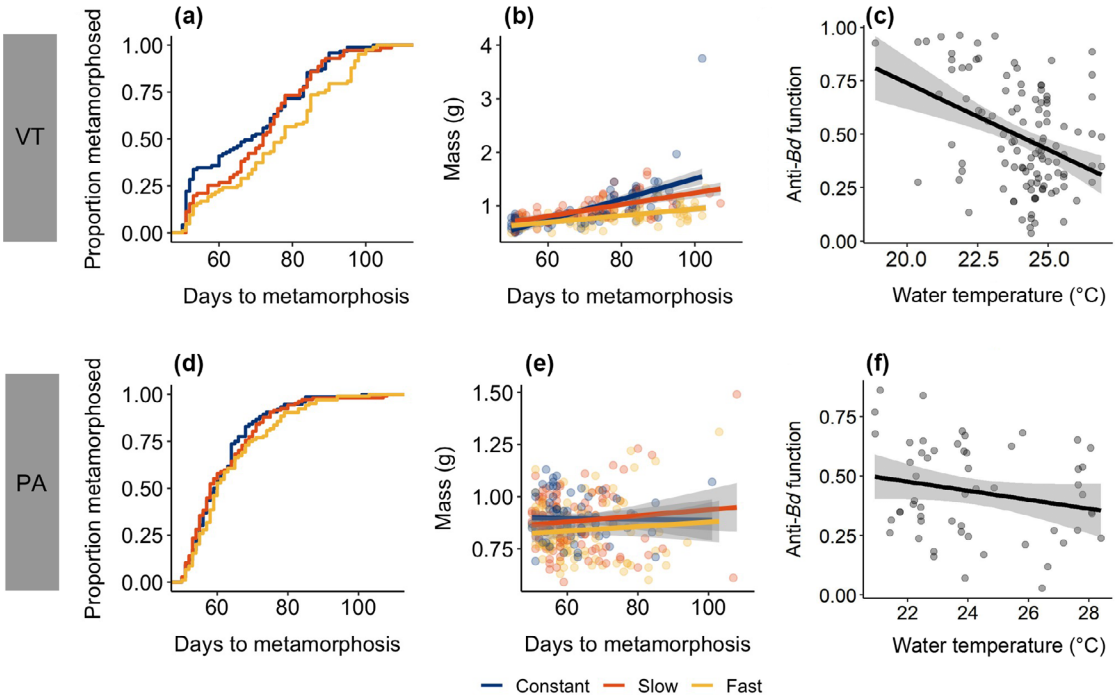


**FIGURE 2** Northern leopard frogs in Vermont (VT; panels a–c) and Pennsylvania (PA; panels d–f) did not display developmental plasticity when exposed to pond drying compared with those exposed to constant water levels. (a, d) Cumulative proportion of frogs reaching metamorphosis by days after introducing tadpoles to mesocosms. (b, e) Larval growth rates shown as individual mass (in grams) at metamorphosis by time to metamorphosis, with lines and shading representing linear regression models and 95% CIs. Vermont frogs exposed to fast drying grew slower than those to constant water levels (b). Sample sizes for developmental and growth rates in Vermont: constant = 116, slow = 84, and fast = 97; and in Pennsylvania: constant = 77, slow = 111, and fast =105. (c, f) Microbiome anti-*Batrachochytrium dendrobatidis* (*Bd*) function was negatively related to water temperature in Vermont but not Pennsylvania. Sample sizes for microbiome in Vermont: constant = 42, slow = 34, and fast = 37; and in Pennsylvania: constant = 20, slow = 18, and fast = 17. Note that axes vary among panels.

and was negatively related to days to metamorphosis (LMM of sOTU richness in Vermont—days to metamorphosis: $\beta = -2.162 \pm 0.547$, $p < 0.001$, slow: $p = 0.475$, fast: $\beta = 48.576 \pm 16.108$, $p = 0.032$; in Pennsylvania—slow: $p = 0.502$, fast: $p = 0.208$; and LMM of phylogenetic diversity in Vermont—days to metamorphosis: $\beta = -0.254 \pm 0.055$, $p < 0.001$, slow: $p = 0.375$, fast: $\beta = 4.936 \pm 1.790$, $p = 0.039$; in Pennsylvania—slow: $p = 0.464$, fast: $p = 0.379$). Specifically, Vermont frogs exposed to fast drying or those with shorter development times had higher sOTU richness and phylogenetic diversity than those raised in constant or had slower development. In Pennsylvania, alpha diversity was similar across drying treatments. Anti-*Bd* function in Vermont did not vary across treatment but decreased with temperature and increased with days to metamorphosis (LMM for the proportion of anti-*Bd*—days to metamorphosis: $\beta = 0.008 \pm 0.002$, $p < 0.001$, temperature: $\beta = -0.044 \pm 0.014$, $p = 0.002$; Figure 2c). In Pennsylvania, anti-*Bd* function did not vary by drying treatment (LMM; slow: $p = 0.499$, fast: $p = 0.836$; Figure 2f). Anti-*Bd* richness was predicted by the interaction between drying treatment and days to metamorphosis in Vermont (LMM; Fast × Log(days to metamorphosis): $\beta = 0.396 \pm 0.171$, $p = 0.022$, Slow × Log(days to metamorphosis): $p = 0.217$), where anti-*Bd* richness increased by days to metamorphosis in fast-drying, and was similar across days in slow-drying and constant treatments. Anti-*Bd* richness did not differ across drying treatment in Pennsylvania (LMM; slow: $p = 0.232$, fast: $p = 0.760$).

Beta diversity differed by treatment and days to metamorphosis in northern leopard frogs in Vermont (LDM of unweighted UniFrac—treatment: F.tran = 0.059, $p < 0.001$, days to metamorphosis: F.tran = 0.051, $p < 0.001$, Treatment × Days to metamorphosis: F.tran = $p = 0.156$; Appendix S1: Figure S2), and by days to metamorphosis in Pennsylvania (treatment: $p = 0.167$, days to metamorphosis: F.tran = 0.033, $p = 0.049$, Treatment × Days to metamorphosis: $p = 0.768$; Appendix S1: Figure S2). Specifically, the community composition of Vermont microbiomes differed between the fast and constant, and between the slow and constant treatments (pairwise Adonis; constant–fast: pseudo-$F = 2.013$, $p = 0.005$; constant–slow: pseudo-$F = 1.749$, $p = 0.014$). However, Vermont frogs did not differ in dispersion between treatments (betadisper: pseudo-$F = 1.200$, $p = 0.305$). The LDM differential abundance analysis revealed that 18 sOTUs in Vermont and 1 sOTU in Pennsylvania varied in richness between drying treatments (LDM; Bray–Curtis sOTU false discovery rate (FDR)-adjusted $p$ values ≤0.01; Figure 3a,c, and for individual plots; see Appendix S1: Figure S3). Of the 18 sOTUs that differed in richness among Vermont treatments, 12 matched to isolates in the cultured database, and 11 were categorized as anti-*Bd*. The 1 sOTU that differed in Pennsylvania treatments was also categorized as anti-*Bd*.

## Developmental responses of southern leopard frogs to pond drying

Southern leopard frogs from Louisiana and Tennessee differed in their responses to drying. Tennessee frogs displayed no difference among treatments in development time (LMM; slow: $p = 0.274$, fast: $\beta = -0.014 \pm 0.0135$, $p = 0.089$; Figure 4a) nor mass at metamorphosis (LMM; slow: $p = 0.463$, fast: $p = 0.131$; Figure 4b), and did not experience reduced larval survival (LMM; slow: $p = 0.185$, fast: $p = 0.942$). By contrast, Louisiana frogs accelerated development in response to drying, with those in slow and fast treatments metamorphosing 8 and 20 days faster on average, respectively, than in the constant treatment (LMM; slow: $\beta = -0.023 \pm 0.014$, $p = 0.016$, fast: $\beta = -0.060 \pm 0.013$, $p < 0.001$; Figure 4d). Frogs in the Louisiana drying treatments had slower larval growth rates than in the constant treatment (LMM; Fast × Days to metamorphosis: $\beta = -1.407 \pm 0.572$, $p < 0.001$, Slow × Days to metamorphosis: $\beta = -1.480 \pm 0.586$, $p < 0.001$; Figure 4e), resulting in smaller masses at metamorphosis (LMM; fast: $\beta = 2.872 \pm 1.230$, $p = 0.004$, slow: $\beta = 3.005 \pm 1.268$, $p = 0.004$). Specifically, frogs in the constant treatment were on average (SD) 0.685 g (0.122) at metamorphosis, while those in the fast- and slow-drying treatments were 31%–32% smaller (0.475 g [0.093] and 0.463 g [0.069], respectively). The fast-drying treatment also reduced larval survival in Louisiana (GLMM; fast: $\beta = -1.104 \pm 0.751$, $p = 0.004$, slow: $-0.743 \pm 0.778$, $p = 0.062$). Specifically, 23% and 18% of tadpoles did not survive exposure to fast and slow drying, respectively, while only 9% did not survive in the constant water treatment in Louisiana. In Tennessee, larval mortality was 5%–6% across treatments.

## Southern leopard frog microbiome responses to pond drying

Bacterial alpha diversity in the microbiome at metamorphosis increased with days to metamorphosis in Louisiana but did not differ in Tennessee (LMM of sOTU richness in Louisiana: days to metamorphosis: $\beta = 0.508 \pm 0.250$, $p = 0.046$; LMM of sOTU richness in Tennessee: slow: $p = 0.481$, fast: $p = 0.641$; LMM of phylogenetic diversity in Louisiana: days to metamorphosis: $\beta = 0.075 \pm 0.028$, $p = 0.009$; and LMM of phylogenetic

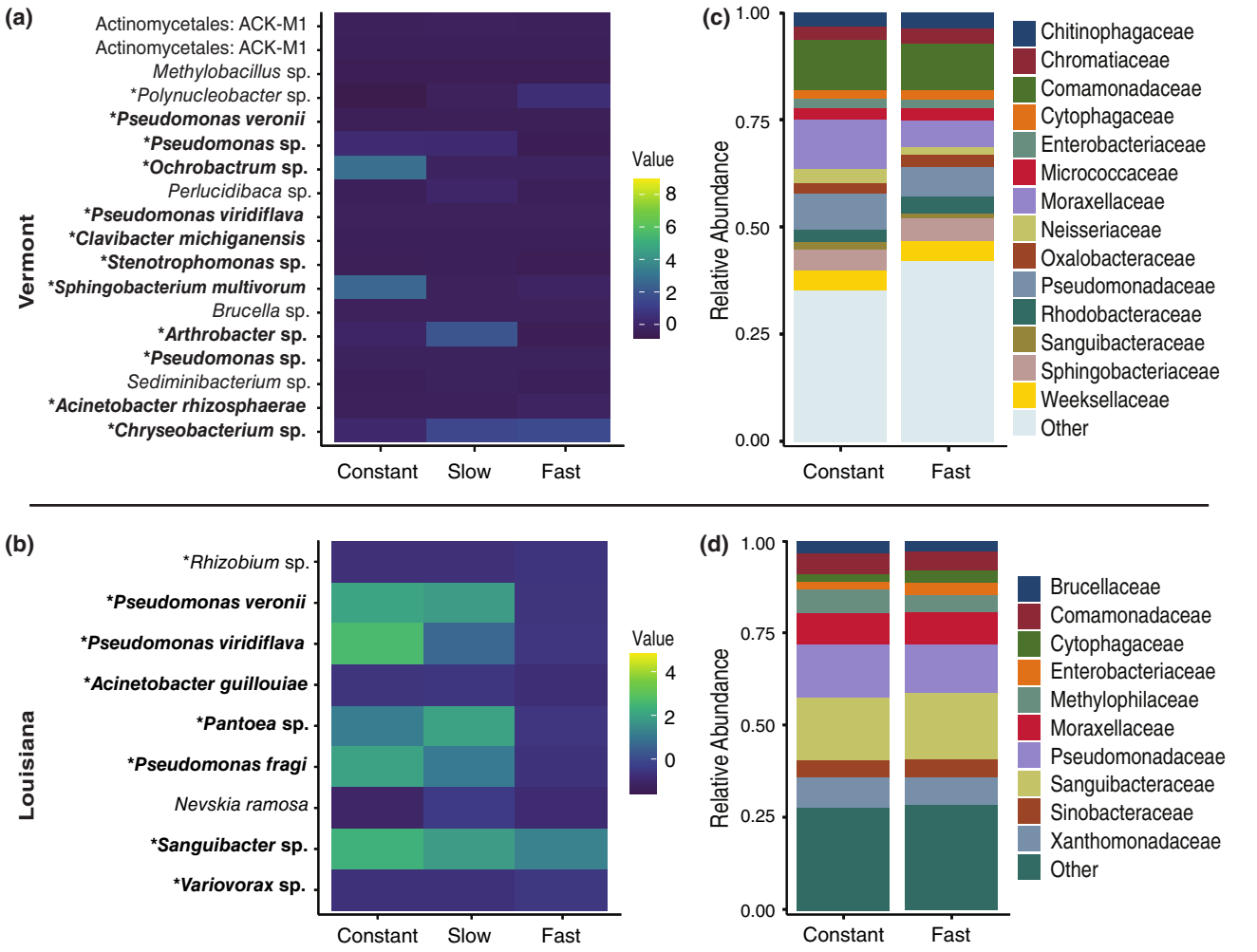


**FIGURE 3** Linear decomposition model (LDM) for differentially abundant sOTUs of northern leopard frogs in Vermont (a) and southern leopard frogs in Louisiana (b) across drying treatments. The most significant 18 Vermont taxa and 9 Louisiana taxa at the species level (if possible) represent the largest positive and negative changes in relative abundance across the drying treatments (LDM with FDR selection criteria of 0.01). sOTUs with an * indicate that they matched to the culture database and those in bold matched to anti-*Batrachochytrium dendrobatidis* (*Bd*) isolates. Each sOTU is scaled across samples, and the mean magnitude differences are shown in the legend as the value. See Appendix S1: Table S2 for FDR-adjusted *p* values for each sOTU. Taxonomic summary plot showing the relative abundance of the top 15 bacterial families for Vermont (c) and top 11 families for Louisiana (d) constant and fast-drying treatments. The "Other" category combines all other families together that were not determined to be members of the top relative abundance (sOTUs with less than 250 in Vermont and 290 in Louisiana summed reads across samples). Sample sizes for microbiome in Vermont: constant = 42, slow = 39, and fast = 38; and in Louisiana: constant = 25, slow = 21, and fast = 21.

diversity in Tennessee: slow: $p = 0.717$, fast: $p = 0.669$). In Tennessee, neither anti-*Bd* richness nor function varied by treatment (LMM of anti-*Bd* sOTUs: slow: $p = 0.336$, fast: $p = 0.933$; LMM for the proportion of anti-*Bd*: slow: $p = 0.423$, fast: $p = 0.214$; Figure 4c). Anti-*Bd* function in Louisiana was negatively related to days to metamorphosis and temperature (LMM [fit separately]; days to metamorphosis: $\beta = -0.004 \pm 0.001$, $p < 0.001$; temperature—$\beta = -0.024 \pm 0.010$, $p = 0.02$; Figure 4f), and anti-*Bd* richness did not vary by treatment (LMM; slow: $p = 0.174$, fast: $p = 0.179$).

Microbiome beta diversity differed by treatment and days to metamorphosis in frogs from Louisiana, but not Tennessee (LDM of unweighted UniFrac in Louisiana: treatment: F.tran = 0.076, $p < 0.001$, days to metamorphosis: F.tran = 0.062, $p < 0.001$, Treatment × Days to metamorphosis: $p = 0.794$; in Tennessee: treatment: $p = 0.837$, days to metamorphosis: $p = 0.463$, Treatment × Days to metamorphosis: $p = 0.551$; Appendix S1: Figure S2). Louisiana frogs did not differ in dispersion between treatments (betadisper: $p = 0.883$) but displayed a difference in bacterial community composition between constant and fast-drying treatments (pairwise.adonis: pseudo-$F = 2.171$, $p = 0.006$). The LDM differential abundance analysis revealed that nine sOTUs in Louisiana varied in richness

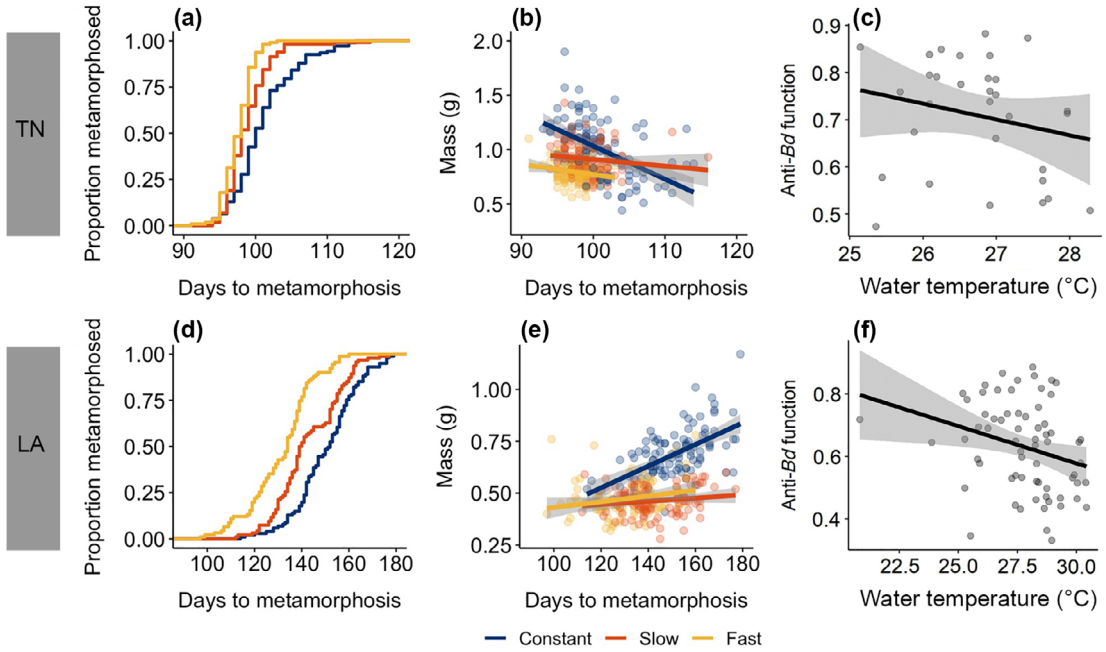


**FIGURE 4** Southern leopard frogs in Tennessee (TN; panels a–c) and Louisiana (LA; panels d–f) varied in their developmental response to pond drying compared with those to constant water levels. Drying did not impact time or survival to metamorphosis (a) and size at metamorphosis (b) in Tennessee frogs. However, Louisiana frogs in drying treatments experienced lower survival and developed faster (d), and metamorphosed at a smaller size (e) than those in the constant treatment. (a, d) Cumulative proportion of frogs reaching metamorphosis by days after introducing tadpoles to mesocosms. (b, e) Larval growth rates shown as individual mass (in grams) at metamorphosis by time to metamorphosis, with lines and shading representing linear regression models and 95% CIs. Sample sizes for developmental and growth rates in Tennessee: constant = 113, slow = 118, and fast = 117; and in Louisiana: constant = 101, slow = 95, and fast = 92. (c, f) Microbiome anti-*Batrachochytrium dendrobatidis* (*Bd*) function was negatively related to water temperature in Louisiana but not Tennessee frogs. Sample sizes for microbiome in Tennessee: constant = 9, slow = 7, and fast = 15; and in Louisiana: constant = 25, slow = 21, and fast =21. Note that axes vary among panels.

between drying treatments (LDM of Bray–Curtis sOTU FDR-adjusted $p$ values ≤0.01; Figure 3b,d; for individual plots; see Appendix S1: Figure S3). Of the nine sOTUs that differed, eight were matched to isolates in the cultured database and seven were categorized as anti-*Bd*. For core microbiome comparisons among all locations; see Appendix S1.

## Carry-over effects of drying on northern leopard frogs

Drying did not affect post-metamorphic growth rates in Vermont (LMM: $p = 0.094$; Figure 5a), whereas in Pennsylvania, juveniles from the fast-drying treatment grew slightly faster than those from the constant treatment (LMM; Fast × Time: $\beta = 0.002 \pm 0.001$, $p = 0.020$, Slow × Time: $p = 0.568$; Figure 5d). In Vermont, mass at metamorphosis, but not drying treatment, was negatively related to juvenile survival (COXPH; mass: $\beta = -4.852 \pm 0.630$, $p < 0.001$, fast: $p = 0.090$, slow: $p = 0.868$; Figure 5b). Drying resulted in reduced juvenile survival in Pennsylvania, regardless of mass (COXPH; mass: $p = 0.266$; fast: $\beta = 1.249 \pm 0.641$, $p < 0.001$, slow: $\beta = 1.339 \pm 0.630$, $p < 0.001$; Figure 5e). Specifically, Pennsylvania frogs from the fast- and slow-drying treatments were 1.3× more likely to die within 60 days post-metamorphosis than frogs from the constant treatment. When exposed to *Bd*, drying exposure did not affect infection dynamics (probability of infection in Vermont: fast: $p = 0.754$, slow: $p = 0.583$, days since first exposure: $p = 0.831$, age at first exposure [days]: $p = 0.26$; in Pennsylvania: fast: $p = 0.530$, slow: $p = 0.610$, days since first exposure: $\beta = -0.066 \pm 0.031$, $p < 0.001$, age at first exposure [days]: $p = 0.554$; and $\log_{10}$ *Bd* load in Vermont: fast: $p = 0.637$, slow: $p = 0.136$, days since first exposure: $\beta = -0.036 \pm 0.016$, $p < 0.001$, age at first exposure [days]: $p = 0.373$; in Pennsylvania: fast: $p = 0.566$, slow: $p = 0.537$, days since first exposure: $p = 0.327$, days to metamorphosis: $p = 0.724$). However, survival post-exposure and *Bd* clearance varied substantially between Vermont and Pennsylvania frogs. Specifically, 80% of Vermont *Bd*-exposed frogs died by the end of the experiment, whereas none from Pennsylvania died and 96% cleared infections (Figure 5c,f). None of the measured aspects of the microbiome nor mucosome viability predicted initial *Bd* load or the probability of infection (see Results in Appendix S1). Drying also had no effect on

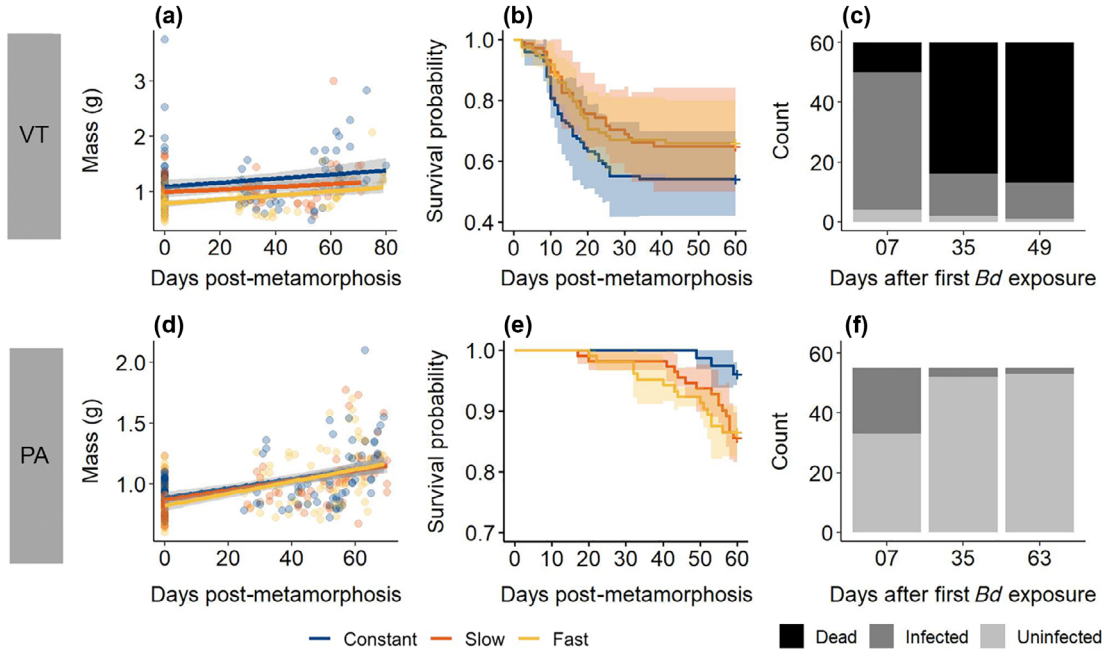


**FIGURE 5** Carry-over effects of pond drying in northern leopard frogs from Vermont (VT; panels a–c) and Pennsylvania (PA; panels d–f) included lower survival in Pennsylvania (e), where frogs from the fast-drying treatment exhibited catch-up growth post-metamorphosis (d). (a, d) Post-metamorphic growth in mass (in grams) of frogs from each drying treatment (day 0 = day of metamorphosis). Sample sizes for juvenile growth in Vermont: constant = 50, slow = 43, and fast = 45; and Pennsylvania: constant = 58, slow = 61, and fast = 64. (b, e) Cumulative proportion of frogs in each drying treatment that survived to 60 days post-metamorphosis shown as survival curves and 95% CIs. (c, f) Proportion of *Batrachochytrium dendrobatidis* (*Bd*)-exposed frogs that survived and were uninfected (light gray), survived and were infected (medium gray), and were dead (black) at each time point after the first of four *Bd* exposures. Sample sizes for *Bd* exposure in Vermont: constant = 23, slow = 18, and fast = 20, and Pennsylvania: constant = 21, slow = 18, and fast = 19. Note that axes vary among panels.

the immune measures analyzed in a subset of animals at one and two months post-metamorphosis (see Results in Appendix S1), although PHA response was greater in frogs with longer developmental periods than in Pennsylvania frogs (LMM; days to metamorphosis: $\beta = 0.034 \pm 0.014$, $p = 0.036$, days post-metamorphosis: $p = 0.453$; Appendix S1: Figure S4).

## Carry-over effects of drying on southern leopard frogs

Drying affected post-metamorphic (juvenile) growth rates in both populations of southern leopard frogs (LMM; log[mass] in Tennessee: Fast × Time: $\beta = 0.002 \pm 0.001$, $p < 0.001$, Slow × Time: $p = 0.859$; in Louisiana: Fast × Time: $\beta = -0.002 \pm 0.001$, $p = 0.007$, Slow × Time: $\beta = -0.003 \pm 0.001$, $p = 0.005$; Figure 6a,d). Specifically, Louisiana juveniles from both slow- and fast-drying treatments had slower growth rates than those from constant treatments (i.e., lower regression slope), and Tennessee frogs in the fast-drying treatment had reduced growth rates in comparison with those in constant treatments. Not enough mortality occurred 60 days after metamorphosis in Tennessee (models failed), and Louisiana frogs exposed to drying survived similar to those exposed to the constant treatment (Cox PH; slow: $p = 0.176$, fast: $p = 0.486$). Both Louisiana and Tennessee frogs were able to clear *Bd* infections in spite of repeated exposures and did not exhibit significant mortality or signs of chytridiomycosis. The prevalence of infection was similar across drying treatments (Tennessee: slow: $p = 0.984$, fast: $p = 0.904$, days since first exposure: $\beta = -0.070 \pm 0.027$, $p < 0.001$; Louisiana: slow: $p = 0.788$, fast: 0.441, days since first exposure: $\beta = -0.057 \pm 0.023$, $p < 0.001$, days to metamorphosis: $p = 0.231$; Appendix S1: Figure S6). However, Tennessee frogs exposed to drying had greater *Bd* loads than those reared in constant water levels (LMM; slow: $\beta = 0.688 \pm 0.544$, $p = 0.014$, fast: $\beta = 0.668 \pm 0.559$, $p = 0.020$, days since first exposure: $p = 0.729$, age at first exposure (days): $p = 0.979$; Figure 6b). Specifically, frogs reared in the constant treatment had lower *Bd* loads than those reared in either fast- or slow-drying treatments (Tukey's test, $z = 2.48$, $p = 0.035$, and $z = 2.34$, $p = 0.05$, respectively), but fast- and slow-drying treatments did not differ ($p = 0.997$). While drying effects were not found in Louisiana frogs, there was a negative correlation between *Bd* load and days to metamorphosis (LMM; days to

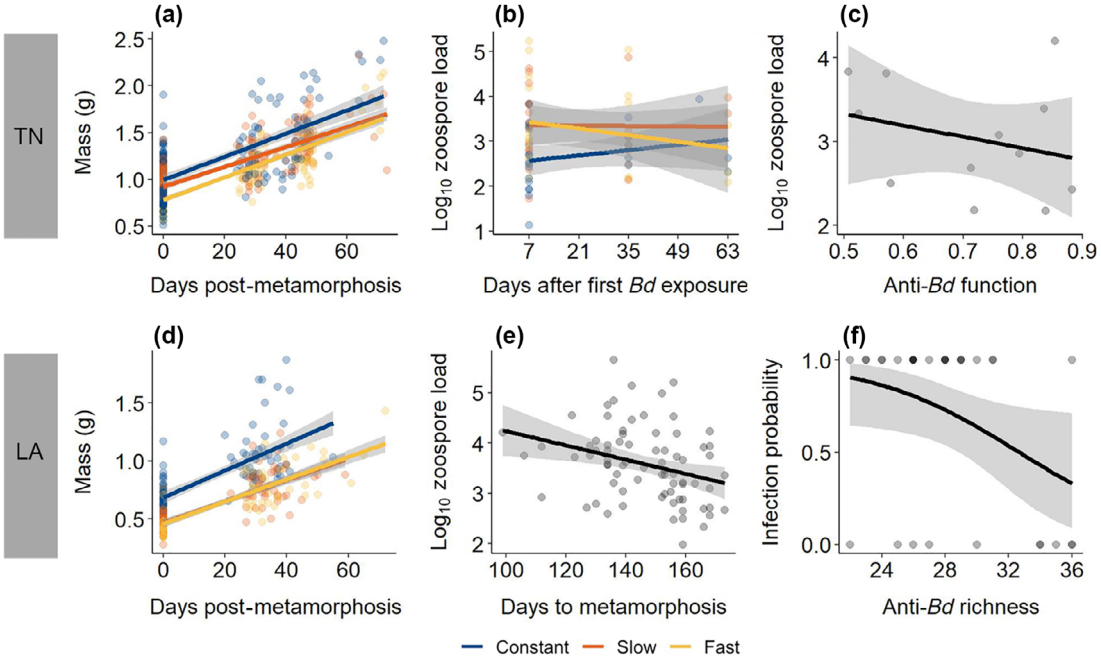


**FIGURE 6** Carry-over effects of pond drying in southern leopard frogs from Tennessee (TN; panels a–c) and Louisiana (LA; panels d–f) included slower post-metamorphic growth in both populations (a, d) and higher *Batrachochytrium dendrobatidis* (*Bd*) loads in Tennessee (b). (a, d) Post-metamorphic growth in mass (in grams) of frogs from each drying treatment (day 0 = day of metamorphosis). Sample sizes for juvenile growth in Tennessee: constant = 83, slow = 91, and fast = 88; and Louisiana: constant = 51, slow = 47, and fast = 45. (b) *Bd* load (zoospore equivalents) of infected Tennessee frogs by days after the first of four exposures in each treatment. Sample sizes for *Bd* loads of infected frogs in Tennessee: constant = 16, slow = 16, and fast = 17. (c) *Bd* load at 7 days post-exposure by anti-*Bd* function at metamorphosis in infected Tennessee frogs ($n = 12$). (e) *Bd* load by days to metamorphosis in infected Louisiana frogs ($n = 48$). (f) Infection probability at 7 days after first exposure by anti-*Bd* richness at metamorphosis of Louisiana frogs ($n = 33$). Lines represent linear (a–e) and logistic (f) smooths with 95% CIs. Note that axes vary among panels.

metamorphosis: $\beta = -0.013 \pm 0.012$, $p = 0.047$, slow: $p = 0.405$, fast: $p = 0.984$, days since first exposure: $p = 0.332$; Figure 6e). Drying did not affect post-metamorphic survival (Cox-PH failed due to low mortality in Tennessee; in Louisiana:Slow: $p = 0.176$, fast: $p = 0.486$) nor any of the specific immune responses measured in a subset of animals at one and two months post-metamorphosis (see Results in Appendix S1). Even Louisiana frogs, which accelerated their developmental rates in response to drying, had similar amounts of recovered peptides, splenocyte and thymocyte counts, and T-cell responsiveness in those raised in fast-drying compared with those in constant water level treatments.

In several cases, aspects of the microbiome at metamorphosis were related to infection dynamics post-metamorphosis. Specifically, Tennessee frogs with greater anti-*Bd* function had lower infection intensities 7 days after the first exposure (only *Bd*-positive frogs; LMM for the proportion of anti-*Bd*: $\beta = -2.916 \pm 0.859$, $p = 0.039$; Figure 6c) and Louisiana frogs with greater anti-*Bd* richness were less likely to be infected at 7 days after the first exposure (GLMM; anti-*Bd* sOTUs: $\beta = -0.212 \pm 0.204$, $p = 0.042$; Figure 6f).

## DISCUSSION

We hypothesized that exposure to pond drying would induce accelerated metamorphosis and carry-over effects and that populations would vary in response to localized drying conditions. We demonstrate support for these hypotheses in that populations from across these species' ranges differ in their capacity to respond developmentally to shortened hydroperiods and in the resulting carry-over effects on juvenile survival, growth, and pathogen defenses. In general, northern, southern, and Chiricahua leopard frogs did not demonstrate developmental plasticity, except for the southernmost location (Louisiana) of southern leopard frogs, which exhibited accelerated development in response to drying. Despite a lack of accelerated metamorphosis, exposure to drying resulted in developmental stress in the form of smaller mass at metamorphosis (Vermont), and increased larval mortality (Vermont and Pennsylvania) and juvenile mortality (Pennsylvania). In Louisiana and Vermont, drying exposure resulted in skin microbiome composition that differed from those raised in constant levels. We also found that in Vermont and Louisiana, higher water temperatures at

metamorphosis were related to a lower predicted anti-*Bd* function of the microbiome. In terms of carry-over effects, Louisiana and Tennessee frogs raised in drying regimes displayed more severe infections when exposed to *Bd* after metamorphosis. Meanwhile, Chiricahua leopard frogs did not display carry-over effects in response to *Bd* exposure (no other traits were tested; see Appendix S1). Note that differences among northern and southern leopard frogs could also be a result of the varying drying regimes, which were slower for the northern leopard frogs. These findings corroborate previous work in these species documenting negative consequences of pond drying, including reduced larval survival and smaller sizes at metamorphosis (Ryan & Winne, 2001; Wilbur, 1987), and expand on this work to show that the effects of drying persist even months after metamorphosis. Site-specific pond drying responses also demonstrate that the consequences of pond drying cannot be generalized across the range of one species.

## Developmental and carry-over effects of pond drying in northern leopard frogs

Northern leopard frogs did not display developmental plasticity in response to reduced water levels and instead experienced decreased survival. Vermont frogs in drying conditions were smaller at metamorphosis than those in constant water levels, despite similar developmental rates. Further, Vermont frogs from slow-drying mesocosms—the treatment with the lowest survival—displayed a more diverse skin microbial community and had lower anti-*Bd* function than those reared in constant water level conditions. Carry-over effects of drying included reduced survival to 60 days post-metamorphosis in Pennsylvania frogs, suggesting drying regimes affected resources or energy stores available to survive through metamorphosis. Despite no effect of drying on developmental rate or spleen cellularity, Pennsylvania frogs that developed faster had lower responsiveness of splenocytes to a T-cell mitogen, PHA, similar to the skin-swelling PHA response displayed in frogs previously reared in drying conditions at this location (Brannelly et al., 2019). We found localized effects in survival in northern leopard frogs following *Bd* exposure, although further research is needed to determine whether location or population effects explain why Vermont frogs suffered substantial mortality, while Pennsylvania frogs quickly cleared infections. Because water temperatures were similar throughout development in these locations, temperature likely did not drive observed population differences. Altogether, it appears likely that shortened hydroperiods as a result of climate change could reduce juvenile recruitment in northern leopard frogs.

## Developmental and carry-over effects of pond drying in southern leopard frogs

While previous work in southern leopard frogs found developmental plasticity in response to drying (Parris, 2000; Ryan & Winne, 2001), we demonstrate that the capacity for this response varies by location. Frogs in Tennessee did not display developmental plasticity but rather a narrow range of larval periods across treatments. By contrast, frogs in Louisiana responded to drying by increasing their developmental rate, but they experienced higher larval mortality and metamorphosed at a smaller size than those raised under constant water levels. Potentially, these smaller frogs would have elevated predation risk and reduced fecundity and survival to adulthood (Semlitsch et al., 1988; Berven, 1990, but see Earl & Semlitsch, 2013). Even 2 months after metamorphosis with ad libitum food and faster growth rates, frogs from both locations exposed to drying did not catch up to the size of those raised under constant water levels.

We hypothesized that microbiome composition could play a role in *Bd* susceptibility, based on estimates of anti-*Bd* function (Woodhams et al., 2014; Woodhams et al., 2015). We demonstrate support for this hypothesis in that Tennessee frogs with greater anti-*Bd* function at metamorphosis had lower infection intensities when exposed to *Bd* as juveniles. Similarly, Louisiana frogs with greater anti-*Bd* richness at metamorphosis were less likely to be infected when exposed to *Bd* as juveniles. Several other studies have also found that the bacterial community composition on amphibian skin is related to host survival when exposed to *Bd* (e.g., Becker et al., 2011; Robak & Richards-Zawacki, 2018; Woodhams et al., 2007). However, more research is needed to understand whether these aspects of the skin microbiome obtained at metamorphosis are maintained over the juvenile life stage. Overall, both Tennessee frogs exposed to drying and Louisiana frogs with faster developmental rates experienced greater susceptibility to *Bd*, adding to the growing evidence for the potential synergistic effects of climate change and disease on population health (Adams et al., 2017; Moura-Campos et al., 2021).

## Effects of pond drying on the skin microbiome

Developmental and environmental factors often underlie changes in amphibian microbiomes, which play an integral role in physiological processes that influence adult fitness, including pathogen defenses (Hooper et al., 2012; Knutie et al., 2017). However, the direction of the correlation between developmental time and alpha diversity varied across locations. In Vermont, bacterial alpha richness

was higher in those exposed to fast drying and those with shorter development times, whereas Louisiana frogs displayed greater richness with longer development times. Individuals with a longer larval period could have been exposed to more variable conditions (such as fluctuating water temperatures), potentially leading to a greater turnover in the microbiome, and/or increased the probability of colonization by new bacteria from the environment, thus increasing richness and diversity. In addition, we found anti-*Bd* function was lower with higher temperatures at metamorphosis in Vermont and Louisiana, suggesting that warming larval conditions could carry over to affect *Bd* susceptibility later in life. These temperatures are within the range at which *Bd* growth occurs (Longcore et al., 1999) and the range at which infection was previously observed (Le Sage et al., 2021; Voordouw et al., 2010), suggesting possible negative consequences of warmer water on resistance against *Bd*. This finding parallels that of the previous work that has identified temperature as an important driver of the composition and anti-*Bd* function of amphibian skin microbiomes (Kueneman et al., 2019).

In addition to richness, the bacterial composition was also affected by exposure to pond drying in Vermont and Louisiana, and Pennsylvania to a degree. These compositional changes, observed as differential abundance among treatment groups, were unique in representative taxa and the direction of relative change among locations. In Vermont, some of the differentially abundant microbes that varied in relative abundance across drying treatments were *Acinetobacter rhizosphaerae* (increasing from constant to fast), *Chryseobacterium* sp. (increasing from constant to fast), *Ochrobactrum* sp. (decreasing from constant to fast), *Polynucleobacter* sp. (increasing from constant to fast), several *Pseudomonas* species, and *Sphingobacterium multivorum* (decreasing from constant to fast). In Pennsylvania, a *Sanguibacter* species was differentially abundant across drying treatments, with relative abundance increasing from the constant to the fast treatments. In Louisiana, several species of *Pseudomonas* (*Pseudomonas fragi*, *Pseudomonas veronii*, and *Pseudomonas viridiflava*) and a *Sanguibacter* species all decreased in relative abundance from the constant to the fast treatments. The same *Sanguibacter* species differed in both Pennsylvania and Louisiana. The same sOTUs of *P. veronii* and *P. viridiflava* differed in both Vermont and Louisiana. Species of *Chryseobacterium*, *Ochrobactrum*, *Pseudomonas*, and *Sphingobacterium* have been shown to have broadscale inhibitory effects against several microbial pathogens, including *Bd* (Assis et al., 2020; Becker et al., 2015; Jiménez et al., 2019; Muletz-Wolz et al., 2019), suggesting shortened hydroperiods could impact a host's ability to prevent infection or decrease infection severity. Assis et al. (2020) similarly found higher relative abundance of *Ochrobactrum* and *Sphingobacterium* in *Proceratophrys boiei* captured in cooler continuous forest habitats compared with that in warmer fragmented forest habitats. However, even though some microbes decreased in relative abundance in response to pond drying, others increased, such as *A. rhizosphaerae* and several *Chryseobacterium* and *Pseudomonas* species, which have also been shown to have anti-*Batrachochytrium* properties that increase during the warmer periods of the year but may be ineffective at cooler temperatures (Bletz et al., 2017; Daskin et al., 2014; Longo & Zamudio, 2017; Muletz-Wolz et al., 2017; Woodhams et al., 2015). Overall, these compositional changes could signify a loss of symbionts with fast drying, which could have lasting effects on microbiome function. Although frogs exposed to pond drying did not exhibit signs of dysbiosis (e.g., greater dispersion; Zaneveld et al., 2017), our results suggest that in some locations, longer developmental time, and cooler and constant water levels alter microbiome composition and promote greater anti-*Bd* function at metamorphosis.

## CONCLUSIONS

Although northern and southern leopard frogs typically breed in semipermanent wetlands, climate change has already increased the likelihood of both droughts and floods within their ranges (Hayhoe et al., 2007; Powell & Keim, 2015) leading to more variable hydroperiods (Brooks, 2009). 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 negative population-level effects (Urban et al., 2014). We predict that these effects could become more pronounced as climate changes lead to more variable precipitation and could interact with local land management practices, as variation in transpiration rates of tree species can impact wetland hydroperiod (McNulty et al., 2019). Because carry-over effects were localized and not consistent within species, our findings suggest that more than one population/location assessment is needed to better inform management decisions. In general, lengthening the hydroperiod of important habitat—but not so far as to create permanent wetlands—would improve recruitment across all locations and reduce the potential negative consequences of higher susceptibility in southern leopard frogs. Future work investigating the role of local adaptation, maternal effects, and plasticity is needed to quantify the variation in adaptive capacity to prior and future changes in the length of hydroperiods. These empirical estimates of climate-induced mortality

and disease susceptibility can also be informative for mathematical modeling of population and transmission dynamics to better predict the impact of both climate change and chytridiomycosis.

The potential for synergistic effects of climate change and chytridiomycosis is a major focus of amphibian conservation research (reviewed in Rollins-Smith, 2017; e.g., Moura-Campos et al., 2021). So far, most studies have focused on climate change-related increases in temperature mean and variability effects on chytridiomycosis (Cohen et al., 2019; Raffel et al., 2013). Here, we add that a greater frequency of pond drying could cause higher *Bd* susceptibility in recently metamorphosed frogs. Given that pond drying likely coincides with additional stressors such as warmer temperatures, greater evaporative water loss, lower food availability, increased competition, and reduced water quality (Greenberg & Palen, 2021; Walls et al., 2013), further work investigating the severity of drought impacts on amphibian populations is warranted.

## AUTHOR CONTRIBUTIONS

Corinne L. Richards-Zawacki, Louise A. Rollins-Smith, Douglas C. Woodhams, Jamie Voyles, Michel E. B. Ohmer, and Emily H. Le Sage designed the experiment and the conceptual framework. Michel E. B. Ohmer, Emily H. Le Sage, Brandon C. LaBumbard, and Karie A. Altman conducted experiments and collected the data with substantial help from Laura K. Reinert, Jeffery G. Bednark, Brady Inman, Alexa Lindauer, Nina B. McDonnell, Sadie K. Parker, Samantha M. Skerlec, and Trina Wantman. Michel E. B. Ohmer, Emily H. Le Sage, Brandon C. LaBumbard, and Karie A. Altman wrote the manuscript and analyzed the data. All authors wrote the manuscript and interpreted the findings.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

Data (Le Sage, 2022) are available from Dryad: https://doi.org/10.5061/dryad.2547d7wsp.

## ETHICS STATEMENT

This research was conducted according to the Institutional Animal Care and Use Committee protocols from Vanderbilt University Medical Center (Protocol number M1600250-00), University of Pittsburgh (Protocol number IML-18052950), University of Massachusetts (Protocol number 2014003), and University of Nevada Reno (Protocol number 00698). Permission to collect eggs was granted by the Pennsylvania Fish and Boat Commission (Permit 2018-01-0360), the Tennessee Wildlife Resources Agency (Permit 3912), the Arnold Air Force Base, the Louisiana Department of Wildlife and Fisheries (Scientific and Collecting Permit LHNP-18-005), the Vermont Fish and Wildlife Department (Permit SR-2016-17), and the New Mexico Department of Game and Fish (Permit 3615) and the USFWS Native Threatened Species Recovery Permit for Chiricahua Leopard Frog Research (TE17901C-0).

## ORCID

*Emily H. Le Sage* https://orcid.org/0000-0002-2815-6651
*Corinne L. Richards-Zawacki* https://orcid.org/0000-0002-4212-041X

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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