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Behavioral Thermoregulation and Thermal Mismatches Influence Disease Dynamics in Amphibians

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Behavioral Thermoregulation and Thermal Mismatches Influence Disease Dynamics in Amphibians

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Amphibians are currently the most threatened vertebra taxa on the planet. Hundreds of species are thought to have gone extinct while thousands more have been listed as threatened or endangered over the past few decades. Habitat loss, invasive species, climate change, and disease are all thought to have partially contributed to these declines. Two pathogens in particular, infectious viruses in the genus *Ranavirus* (simply referred to as ranavirus) and the fungus *Batrachochytrium dendrobatidis* (Bd), have been associated with global mass mortality events of amphibians. Virulent pathogens such as these tend to impose strong selective pressures on their hosts driving the evolution of host behaviors that reduce disease.

One example of behavioral disease resistance in ectothermic hosts is behavioral fever, an acute increase in temperature preference in response to pathogen exposure. However, few studies have experimentally examined the effects of host behavior on ranaviral infections. Additionally, both field and laboratory studies testing for behavioral fever in response to Bd exposure have found conflicting results. In the Bd system, these conflicting results could, in part, be caused by some pathogen defenses of host not increasing with temperature. For example, the thermal mismatch hypothesis predicts that host species adapted to cooler temperatures might perform more poorly than the pathogen at warm temperatures, and vice versa, creating a scenario where warm- and cool-adapted hosts most often experience outbreaks at cool and warm temperatures, respectively. Here, I determine if amphibians respond to disease with behavioral fever in the

ranaviral and Bd systems and if there are species-level differences in Bd susceptibility predicted by the thermal mismatch hypothesis.

To accomplish these goals, I first needed to detect variation in thermal preferences among individuals and species. However, measuring thermoregulation can often be difficult for many reasons, including cost and confounders like moisture. I designed effective, affordable, and validated methods for measuring the thermal preferences of animals. The thermal gradient apparatus spanned temperatures from 9.29 to 33.94 °C with consistent high humidity across the entirety of the gradient. Additionally, I used simple methods for non-invasively measuring animal and substrate temperatures while avoiding feeding confounders. To validate these methods, I demonstrated that I could detect individual-level consistency and among individual variation in the preferred body temperatures of *Anaxyrus terrestris* and *Osteopilus septentrionalis*. I use this design to conduct the experimental work completed in chapters two and three of this dissertation.

Behavior fever is known to influence disease dynamics in some systems, but little work has been done in the ranavirus-amphibian system. Experimental studies, however, have demonstrated that warmer temperatures can mitigate ranaviral infections for some species. I placed *A. terrestris* in the previously described thermal gradients, recorded their temperature preferences before and after infection, and measured ranaviral loads. I found evidence of behavioral fever during the first 48h after exposure to ranavirus and that individual-level change in temperature preference was negatively correlated with ranavirus intensity. These results suggest that *A. terrestris* use behavioral fever effectively to resisting ranavirus.

Multiple field studies have documented that individual amphibians within a population that prefer warmer temperatures are less likely to be infected with the fungal pathogen Bd.

However, it is unclear whether this phenomenon is driven by behavioral fever or natural variation in thermal preference. To determine if these field patterns were the result of behavioral fever or natural variation, I placed five species of frogs in thermal gradients, recorded temperature preference over time, and measured Bd growth, prevalence, and the survival of infected animals. I found no consistent evidence of behavioral fever to Bd in any of the five tested frog species. Interestingly, individual-level and species-level differences in host temperature preferences affected Bd growth on the host and were predicted by the thermal mismatch hypothesis. For species that preferred warmer temperatures, the preferred temperatures of individuals were negatively correlated with Bd growth on hosts, while the opposite correlation was true for species preferring cooler temperatures. My results suggest that variation in thermal preference, but not behavioral fever, might shape the outcomes of Bd infections for individuals and populations, potentially resulting in selection for individual hosts and host species whose temperature preferences minimize Bd growth and enhance host survival during epidemics.

My evidence for the thermal mismatch in the Bd-amphibian system highlighted the importance of understanding how experimental temperatures might intentionally and unintentionally alter disease outcomes. To better understand how thermal mismatches and other factors impact amphibian host mortality in experiments, I conducted a meta-analysis of 58 laboratory studies on the pathogenic fungus Bd. I found that host mortality was driven by thermal mismatches across experimental studies. Hosts native to cooler environments experienced greater Bd-induced mortality at relatively warm experimental temperatures and hosts native to warmer environments experienced greater mortality at cooler experimental temperatures. I also found evidence that host exposure to novel Bd isolates increased the likelihood of Bd-induced mortality during the first two weeks after exposure. Unsurprisingly, I

found that Bd dose positively predicted Bd-induced host mortality and this effect varied across host life stages. My results suggest that thermal mismatches broadly impact Bd-induced mortality in amphibians and that researchers should carefully consider the experimental temperature relative to host thermal tolerance, the novelty of the chosen isolate, and the dose of pathogen when designing host-pathogen experiments.

Quantifying thermal tolerance and regulatory behavior in ectotherms can inform fundamental aspects of organismal physiology, behavior, and ecology that are influential in mediating the effects of both biotic and abiotic stressors. The results of this dissertation highlight the importance of working to disentangle the complicated effects of environmental factors, such as temperature, on disease dynamics, especially as emerging infectious diseases continue to cause declines in biodiversity. Additionally, my results suggest that a nuanced understanding of amphibian thermal biology, which carefully considers the potential for context dependent effects of temperature on disease dynamics, will be crucial for predicting and mitigating disease-mediated amphibian declines.

CHAPTER ONE:
AN EFFICIENT AND INEXPENSIVE METHOD FOR MEASURING LONG-TERM THERMOREGULATORY BEHAVIOR

Note to Reader

See Appendix A for full text. This chapter has been previously published in *Journal of Thermal Biology* (2016) doi: 10.1016/j.jtherbio.2016.07.016 and has been reprinted with permission from *Journal of Thermal Biology* © 2016 Elsevier Ltd, which allows authors to include their full articles in a thesis or dissertation for non-commercial purposes.

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CHAPTER TWO:
BEHAVIORAL FEVER REDUCES RANAVIRUS INFECTION IN TOADS

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Abstract

The capacity of wildlife populations to mount rapid responses to novel pathogens is crucial for reducing the impacts of disease outbreaks. Behavior, for example, is known to influence disease dynamics. However, few studies have experimentally examined the effects of host behavior on ranaviral infections, which affect at least 175 species of ectothermic vertebrates. Experimental studies have demonstrated that warmer temperatures can mitigate ranaviral infections for some species but it is unclear that any species uses behavioral fever to resist ranaviral infection. Here, we placed metamorphic (temporal block 1) or adult (block 2) Anaxyrus terrestris in thermal gradients, tested their temperature preferences before and after infection, and measured ranaviral loads of infected individuals. We found significant individual-level variation in temperature preference and evidence for behavioral fever in both metamorphic and adult A. terrestris during the first 48h after exposure. Additionally, we found that individual-level change in temperature preference was negatively correlated with ranavirus intensity and a better predictor of intensity than average temperature preference or maximum temperature reached by an individual. In other words, an increase in baseline temperature preference is more important that simply reaching a threshold temperature. These results suggest that behavioral fever appears to be effective at resisting ranaviral infections and might be a mechanism for increasing immune efficiency, not simply a way to slow viral growth directly with heat.

Introduction

Pathogens can impose strong selective pressures on their hosts, driving the evolution of host behaviors that reduce disease risk (Moore 2002, Han, Bradley et al. 2008). Many hosts cope with pathogens via plastic responses, a rapid change in a trait within the life of the host (Agrawal 2001). For example, ectothermic hosts often respond to pathogen exposure by exhibiting a behavioral fever, which is an acute increase in temperature preference (T_{pref}) (Kluger, Kozak et al. 1998). The slightly warmer environmental temperatures chosen by the host after pathogen exposure are presumably more favorable for the host than the pathogen (Reynolds, Casterlin et al. 1977, Burns, Ramos et al. 1996, Ouedraogo, Goettel et al. 2004). Understanding how rapid plastic responses, such as behavioral fever, can mitigate disease outbreaks is crucial for predicting how ectothermic host populations might be affected by emerging diseases in a changing climate.

Behavioral fever has most commonly been documented in response to bacterial and viral pathogens. An example of a deadly viral taxon is the genus *Ranavirus*, which has caused mass host mortality events contributing to host population declines in last few decades (Chinchar 2002, Brunner and Yarber 2018). Ranaviruses are known to cause frequent infection and mortality in ectothermic vertebrates globally, such as in fishes, reptiles, and amphibians (Chinchar 2002, Duffus, Waltzek et al. 2015). Some of these viruses are even capable of transmission across taxa, or interclass transmission (Brunner, Schock et al. 2004, Bandín and Dopazo 2011). Hosts likely encounter ranaviruses frequently because of their broad distributions and lack of host specificity (Miller, Gray et al. 2011), and thus ranaviruses might impose strong selective pressures on hosts.

In fact, mounting evidence suggests that hosts are adapting both immunological and behavioral strategies to combat ranaviral infections (Parris, Davis et al. 2004, Teacher, Garner et

al. 2009). For example, there is evidence that larval amphibians might prefer warmer temperatures while infected with ranaviruses, but this study did not measure temperature preference before exposure and therefore could not differentiate between preexisting differences in T_{pref} and differences caused by exposure to ranavirus (Parris, Davis et al. 2004). Additionally, this study did not measure ranaviral load or prevalence, so there is no evidence that the apparent difference in T_{pref} is effective at resisting infection. However, other studies have shown that experimentally warming ranavirus-infected hosts can reduce host mortality and viral loads (Rojas, Richards et al. 2005, Echaubard, Leduc et al. 2014) despite successful ranaviral growth in culture at these higher temperatures (Chinchar 2002). In these studies, the warming was induced by the experimenters, not the hosts, and exceeded the magnitude and duration of most behavioral fever responses. Hence, it is unclear if hosts respond to ranaviral infections with behavioral fever and whether this fever is effective at reducing ranaviral abundance on these hosts.

Here, we test whether Southern toads, *Anaxyrus terrestris*, adjust their preferred temperature after infection with ranavirus and whether any change in temperature preference reduces ranaviral growth in these hosts. To accomplish these goals, we exposed *A. terrestris* to a potent ranavirus isolate, frog virus 3 (FV3), in thermal gradients ranging in temperature from 12-33 °C (Sauer, Sperry et al. 2016) to assess individual T_{pref} before and after exposure to this virus. We also measured FV3 intensity on individual hosts to assess whether variation in T_{pref} affected FV3 growth.

Methods

Animal husbandry

Adult and metamorph *A. terrestris* were collected from Hillsborough County, Florida. *Anaxyrus terrestris* individuals were maintained in individual containers (23.5 x 16.8 x 10 cm or 11.7 dia. X 13.5 cm, respectively) on top of folded paper towels soaked with artificial spring water (Cohen, Neimark et al. 1980). These frogs were held in a laboratory maintained between 24-25°C with a 12h photoperiod for at least two weeks before the start of the experiment. The toads were fed mineral-dusted crickets *ad libitum* until the start of the experiment and their containers and paper towels were changed weekly.

Experimental design

Experiments were conducted in Tampa, Florida with two temporal blocks, the first being conducted with metamorphic and the second with adult *A. terrestris*. In each experiment, we first measured baseline non-infected temperature preference (baseline T_{pref}) in thermal gradient apparatuses (see next paragraph for the frequency of these measurements). These apparatuses were previously shown to provide variation in temperature that is independent of humidity and which does not confound amphibian and prey temperature preferences (range of 12 to 33°C; see Fig. S2 and supplemental methods, and Sauer et al. (2016) for thermal gradient construction and validation details). After measuring baseline T_{pref}, individuals were split into two groups of similar mean body masses and baseline T_{pref} (N = 53), a sham-exposed control group (metamorph: n = 12; adult n = 14) and 2) a ranavirus-exposed treatment group (metamorph: n = 13; adult n = 14).

Throughout the experiment, temperature measurements were taken each day, every four hours, between 10:00h and 22:00h (two during the lighted part of the photoperiod and two during the dark) at the center of each animal's dorsum (Rowley and Alford 2007) and the substrate adjacent to the animal using an infrared thermometer (Extech® High Temperature IR

Thermometer; accuracy: +/- 2% < 932°F). The only exception is that these measurements were not taken during feeding periods, during which all individuals were fed 10 live crickets in containment bags to prevent crickets from moving freely within the thermal gradient (see Sauer, Sperry et al. (2016) and Figure S4 for more details). Temperature measurements were taken for four days before ranavirus or sham exposure. The mean T_{pref} of those four days for each individual frog is referred to hereafter as the baseline T_{pref} ($T_{pref_{baseline}}$ in equations). Temperature measurements were taken again for two weeks (metamorph block) or four days (adult block) after ranavirus or sham exposure. We shortened our time between exposure and sampling for the adult block because we were unable to detect FV3 in the metamorphic toads which were destructively sampled two weeks after exposure. Experimental design was based on a prior experiment testing for behavioral fever in amphibians, see Sauer, Fuller et al. (2018) for more details.

Ranavirus exposures and quantification

FV3 was isolated in June of 2014, cultured at Purdue University, and shipped to Tampa, FL in January of 2015. Virus stocks used for this experiment were frozen at -80 C in Minimum Essential Medium (MEM) with a titer of $3.6x10^5$. Before exposure, virus stock was thawed and homogenized and then each individual was dosed orally with 77 µL of $2.8x10^5$ pfu (plaque forming units) or the same volume of a sham inoculum of MEM. The individuals from the metamorph block were euthanized two weeks after FV3 exposure and dissected to remove spleen, kidney, and liver for quantification of FV3 loads. The individuals in the adult block were sampled prior to exposure, to ensure they were not already infected, then sampled again four days after exposure by inserting and twirling a sterile swab in their mouth for thirty seconds and then freezing these swabs at -80°C. FV3 DNA was extracted from each metamorph and adult sample

using the Qiagen DNeasy Blood and Tissue protocol (Qiagen, Inc., Valencia, CA). To determine FV3 abundance, we used qPCR methods based on Forson and Storfer (2006), with a 250-bp fragment of the major capsid protein (MCP) gene used as a standard (gBlocks® plasmid-based standards; Integrated DNA Technologies, Skokie, IL).

Data analysis

All statistics were conducted with R 3.4.0 (Team 2017). To test for repeatability in baseline T_{pref} within individuals and variation in baseline T_{pref} among individuals, we conducted a one-way repeated measures ANOVA (*stats* package, *aov* function). This analysis tested whether baseline T_{pref} of individuals varied significantly across days (main effect of day) and whether they varied among individuals (among-individual variance). Using the ANOVA table from this analysis, we calculated repeatability or the variance explained by individual-level behavior: ($r = \frac{MS_W}{MS_W + \left(\frac{MS_W - MS_A}{n}\right)}$; where MS_W is the within-group variance component and MS_A is the among-groups variance component; (Lessells and Boag 1987).

To test for behavioral fever, we conducted multiple two-factor (treatment and time) repeated measures linear mixed effects models with individual and shelf treated as random variables (*lme4* package, *lmer* function). For each model we paired baseline T_{pref} with each post-exposure day T_{pref} (time; one model for each post exposure day) and looked for an interaction between treatment (FV3-exposed or sham-exposed) and time on z-score of ΔT_{pref}. Change in T_{pref} (ΔT_{pref}) is calculated as:

$$\Delta T_{pref} = T_{pref_{i,j}} - \overline{T_{pref_{baseline,i,J}}}$$

where $T_{pref_{i,j}}$ is the temperature preference for individual i at time point j and $\overline{T_{pref_{baseline,i,J}}}$ is the mean temperature preference of all baseline time points for individual i. We chose to use z-scores, number of standard deviations away from the mean of the ΔT_{pref} of all individuals in a day, as our dependent variable instead of simply using ΔT_{pref} to correct for the effect of change in room temperature over time, which did alter the temperature of the thermal gradients, and to compare T_{pref} between treatments within rather than across days. All individuals within a block were held in the same room at the same time, making the use z-scores an appropriate correction. A significant interaction between treatment and time would mean that the two treatments behaved differently after FV3 exposure.

To test whether individual-level T_{pref} affects ranavirus intensity (log-transformed ranavirus load of infected frogs divided by mass of the individual), we conducted multiple linear regressions (*stats* package, *glm* function; normal error distribution) with FV3 intensity as the response and either ΔT_{pref} ($\Delta T_{pref} = T_{pref_{i,j}} - \overline{T_{pref_{baseline,i,J}}}$), mean T_{pref} (mean of the four body temperature measurements per day), and maximum T_{pref} (maximum of the four body temperature measurements per day) for each of the first four days after exposure as predictors (one model per independent variable, per day; 12 total models) as well as pooled across all four days (one model per independent variable; 3 total models).

Results

There was no mortality during either block of this experiment. Before FV3 exposure, we were able to detect consistency in the baseline T_{pref} of individuals (repeatability: $r > 0.98$; Fig. S1) and variation in baseline temperature preferences among individuals (all blocks and treatments combined; main effect of individual on baseline T_{pref}: $F_{52,1537} = 7.60$, $P < 2.0\text{x}10^{-16}$). *A. terrestris*

preferred a mean temperature of 22.99℃ ± 0.30 SE. For both temporal blocks, we found evidence of behavioral fever after FV3 exposure (effect of the interaction between treatment and time on the z-score of ΔT_{pref}; metamorphs: day 1 $\chi^2 = 22.0$, $P < 0.001$ and day 5 $\chi^2 = 5.51$, $P < 0.02$; adults: $\chi^2 = 5.70$, $P < 0.02$; Fig 1 & Table S1). One day after exposure, metamorphs exposed to FV3 increased their preferred temperature by 3.52℃ ± 0.54 SE relative to controls (Fig. 1A). Two days after exposure, adults exposed to FV3 increased their preferred temperature by 1.43℃ ± 0.56 SE relative to controls (Fig. 1B). We did not test whether these magnitudes were different, because the blocks were not tested under the same conditions.

For the temporal block on metamorphs, we were unable to detect FV3 in sampled toads two weeks after exposure. For the temporal block on adults, we intentionally sampled after 4 days rather than 14 days in the hopes that we would detect FV3 before it was cleared. In this block, we did indeed detect FV3 in 92.9% of the toads. We found that ΔT_{pref} ($t_{11} = -4.89$, $P < 0.001$; Fig. 2B), T_{pref} ($t_{11} = -3.11$, $P = 0.01$; Fig. S2), and max T_{pref} ($t_{11} = -3.1$, $P = 0.004$; Fig S3) two days after exposure were all associated negatively with FV3 intensity 4 days after exposure. Additionally, ΔT_{pref} on the first, third, and fourth days after FV3 exposure was also a significant negative predictor of FV3 intensity on day four ($t_{11} = -3.19$, $P = 0.009$; $t_{11} = -2.42$, $P = 0.03$; $t_{11} = -3.78$, $P = 0.003$, respectively; Fig. 2). Finally, we found a positive effect of mean ΔT_{pref} ($t_{11} = -4.77$, $P < 0.001$) and T_{pref} ($t_{11} = -2.42$, $P = 0.03$) on FV3 intensity and but no effect of overall max T_{pref} ($t_{11} = -1.23$, $P = 0.25$) on FV3 intensity.

Discussion

We set out to determine whether *A. terrestris* responded to FV3 exposure with behavioral fever and whether fever facilitated FV3 resistance. By measuring the thermal preference of

individuals in thermal gradients both before and after exposure, we found that *A. terrestris* individuals do respond to ranavirus exposure with behavioral fever (Fig. 1). We also demonstrated that individual-level change in T_{pref} during the first 48h after exposure was the greatest predictor of FV3 load on frogs (Fig. 2). Individuals that increased their T_{pref} the most had the lowest FV3 loads. These results suggest that behavioral fever appears to be effective at resisting FV3 infections.

We demonstrated that variation in baseline T_{pref} among individuals before FV3 exposure was greater than the variation in baseline T_{pref} within individuals (Fig. S1). In other words, individuals showed consistency in their preferred temperature through time and individual toads exhibited different preferred temperatures. However, once exposed to FV3, we found that individuals moved from their baseline T_{pref} to warmer locations. For the metamorphic *A. terrestris*, this behavioral fever response peaked one day after exposure while adult behavior fever peaked two days after exposure (Fig. 1). These differences might be due to differences in body size: Both blocks were exposed to the same dose, making the dose/g higher in the metamorph than adult block. Given that there is a negative relationship between body size and metabolic rates and processes, immune and fever responses to FV3 might have been triggered more quickly in the smaller-bodied metamorphs than adults (Garner, Rowcliffe et al. 2011, Rohr, Civitello et al. 2018).

For adult *A. terrestris*, there was a significant negative correlation between ΔT_{pref}, mean T_{pref}, and maximum T_{pref} during the second day after exposure and FV3 load at four day after exposure (Fig. 2). We also found that ΔT_{pref} was significantly negatively correlated with FV3 loads across all four days we measured behavior after infection. Thus, an increase in temperature, regardless of an individual's baseline T_{pref}, helped to reduce FV3 infection. We did not find the

same overall effect of mean T_{pref} or maximum T_{pref} on FV3 load; in fact, maximum T_{pref} ended up being the worst predictor of FV3 load of the three measures (ΔT_{pref}, mean T_{pref}, and maximum T_{pref}). This result supports the hypothesis that the main purpose of fever is to increase the immune system's efficiency by raising body temperature to promote both innate and adaptive immunity (Evans, Repasky et al. 2015, Rakus, Ronsmans et al. 2017, Boltana, Aguilar et al. 2018), not to maximize absolute preferred temperature within the range that a host can tolerate. Though fever or increased temperature can slow or stop pathogen growth directly for some host-pathogen systems (Anderson, Blanford et al. 2013, Sauer, Fuller et al. 2018), any increase in body temperature from baseline T_{pref} should be beneficial to the host, assuming the increase is within the bounds of the thermal performance breadth of the host (Evans, Repasky et al. 2015, Cohen, Venesky et al. 2017, Sauer, Fuller et al. 2018). some species that are adapted to cooler climates cannot tolerate temperature increases when infected with ranavirus (Bayley, Hill et al. 2013, Brand, Hill et al. 2016).

While we do not have any intensity data for metamorphic *A. terrestris*, the FV3 load results from the adults provide clear evidence that behavioral fever increased *A. terrestris* resistance to FV3. Behavioral fever might be one way that amphibians resist ranaviral infections, but it is unclear how widespread that response is given the paucity of studies examining behavioral fever as a mechanism for resistance to ranaviruses (Parris, Davis et al. 2004), especially if the fairly heat-tolerant *A. terrestris* exhibits thermal regulatory behavior that is not representative of more cold-adapted species (Sauer, Fuller et al. 2018). Nevertheless, assuming that behavioral fever is primarily used as a method of improving immune efficiency and not simply a method of heat-killing the pathogen, this strategy could be employed more broadly by amphibian hosts than a strategy of simply reaching a threshold temperature to heat-kill FV3. That

is, hosts with critical thermal maxima lower than that of the pathogen might still benefit from behavioral fever as method to improve immunological resistance. However, there might also be costs of behavioral fever that might be balanced against its benefits (Sheldon and Verhulst 1996, Lochmiller and Deerenberg 2000). For example, moving to a warmer location could alter predation risk if it requires hosts to increase activity or leave refugia (Parris, Davis et al. 2004, Han, Bradley et al. 2008).

In summary, both metamorphic and adult *A. terrestris* responded to FV3 exposure with behavioral fever. Additionally, we found that adult *A. terrestris* were successful at reducing their viral loads by increasing their body temperature after exposure. Hence, behavioral fever successfully increased resistance to FV3 in *A. terrestris* and thus appears adaptive. Our results support the idea that behavioral fever is primarily used as a method of improving immunological resistance rather than simply damaging pathogens with heat. More experimental work is needed to determine how widespread ranaviral resistance via behavioral fever is in amphibian and other vertebrate taxa (fish and reptiles) affected by ranaviruses. Understanding how hosts respond to ranaviruses and how changes to environmental temperature affect these host-parasite interactions is crucial given that ranaviral outbreaks and die-offs have been increasing in recent years (Grayfer, Edholm et al. 2015).

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Figures

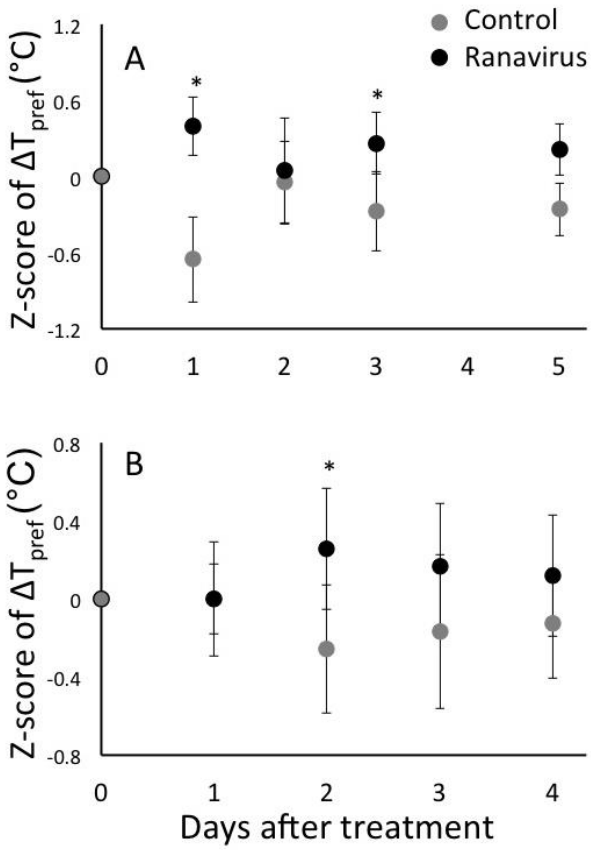

Figure 2.1. Behavioral fever in response to FV3 exposure. The z-scores for change in individual-level temperature preference ($\Delta T_{pref} = T_{pref_{i,j}} - \overline{T_{pref_{baseline,i,J}}}$) through time for **a)** metamorphic and **b)** adult *Anaxyrus terrestris* toads after they were exposed or sham exposed (control) to ranavirus. We standardized the ΔT_{pref} using z-scores to compensate for any effect of changing room temperature on the experiment. Asterisks denote time points where the exposed and sham-exposed groups differ significantly ($P < 0.05$).

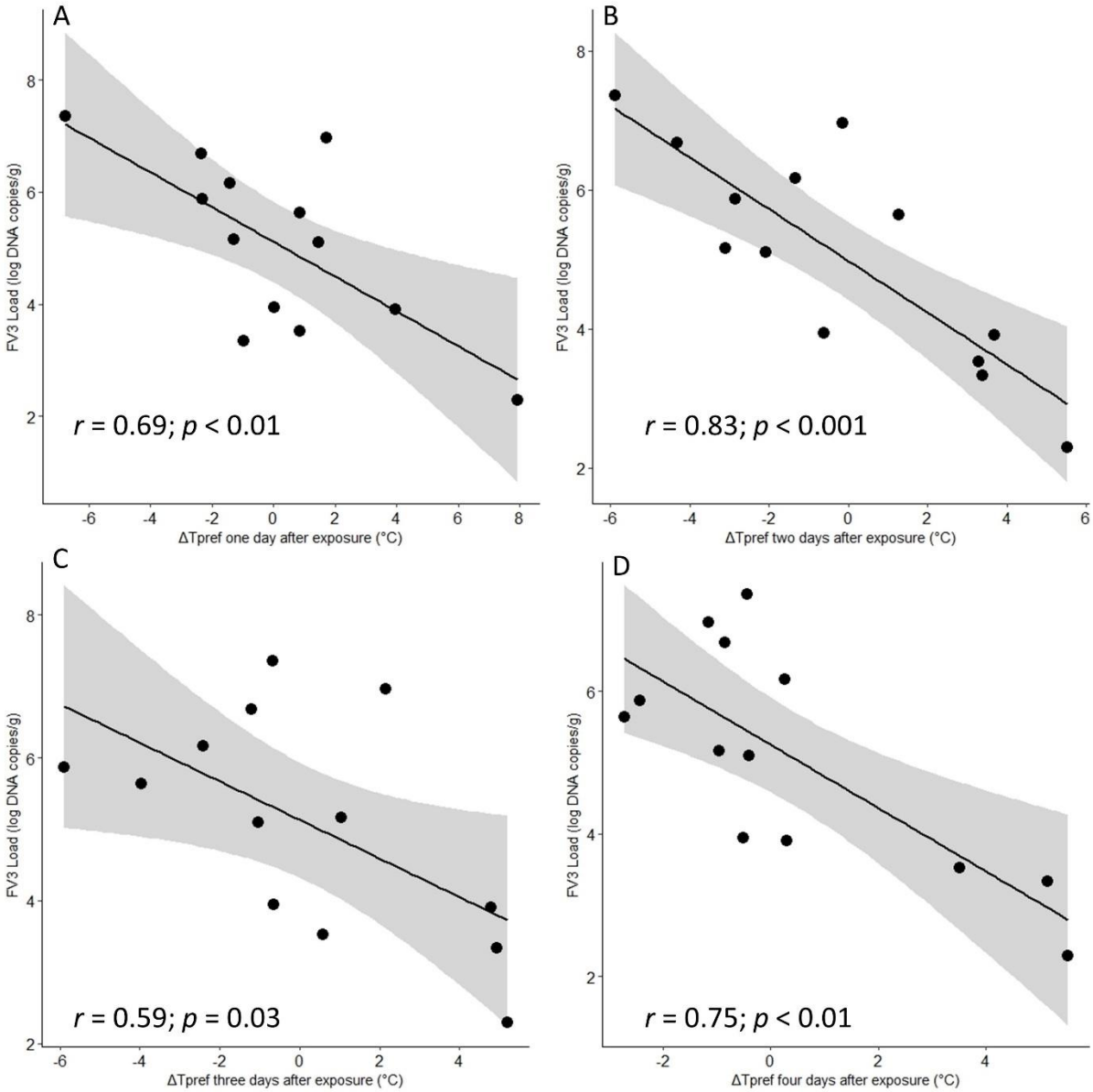

Figure 2.2. Effect of behavioral fever on FV3 loads. Relationship between change in individual-level temperature preference ($\Delta T_{pref} = T_{pref_{i,j}} - \overline{T_{pref_{baseline,i,j}}}$) **A)** one, **B)** two, **C)** three, and **D)** four days after ranaviral exposure and ranaviral loads on adult *Anaxyrus terrestris*. Frogs that exhibited the greatest increase in T_{pref} had the lowest ranaviral abundance (t_{11} = -4.89, $P < 0.001$). The shaded gray area represents the 95% confidence band.

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CHAPTER THREE:
VARIATION IN INDIVIDUAL TEMPERATURE PREFERENCES, NOT BEHAVIOURAL FEVER, AFFECTS SUSCEPTIBILITY TO CHYTRIDIOMYCOSIS IN AMPHIBIANS

Note to Reader

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CHAPTER FOUR:
INFLUENCE OF EXPERIMENTAL TEMPERATURE, DOSE, ISOLATE, AND HOST LIFE STAGE ON AMPHIBIAN SUSCEPTIBILITY TO *BATRACHOCHYTRIUM DENDROBATIDIS*

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Abstract

As emerging infectious diseases continue to cause declines in biodiversity, researchers are working to disentangle the complicated effects of environmental factors on disease dynamics, often under controlled laboratory conditions. Thus, it is important understand how experimental conditions can intentionally and unintentionally alter disease outcomes. Here, we conducted a meta-analysis of 58 laboratory studies on the pathogenic fungus Batrachochytrium dendrobatidis (Bd) to better understand which factors impact amphibian host mortality in experiments. We found that host mortality was driven by thermal mismatches, as hosts native to cooler environments experienced greater Bd-induced mortality at relatively warm experimental temperatures and hosts native to warmer environments experienced greater mortality at cooler experimental temperatures. We also found evidence that amphibian host exposure to novel Bd isolates increased the likelihood of Bd-induced mortality during the first two weeks after exposure, but that increased risk was not significant after the first two weeks. Finally, Bd dose positively predicted Bd-induced host mortality and this effect varied across host life stages, with larval amphibians experiencing lower risk of Bd-induced mortality than adults or metamorphs. Our results suggest that when designing experiments, researchers should carefully consider the experimental temperature relative to host thermal tolerance, the novelty of the Bd isolate, and the dose of Bd.

Introduction

Increases in emerging infectious diseases have caused global declines in biodiversity (Lips, Brem et al. 2006, Goulson, Nicholls et al. 2015) and have likely been exacerbated by environmental factors such as climate change and novel pathogen introductions (Rohr, Dobson et al. 2011, Langwig, Frick et al. 2012, Li, Cohen et al. 2013). To mitigate the effects of emerging infectious diseases, researchers must develop a nuanced understanding of how anthropogenic activities might influence disease dynamics (Anderson, Cunningham et al. 2004, Rohr, Dobson et al. 2011, Venesky, Raffel et al. 2014). Laboratory experiments under controlled conditions are a common approach for understanding how various environmental factors influence disease outcomes. Thus, it is important that researchers understand how using test conditions that the host is uncommonly exposed to might affect disease and alter experimental outcomes. This is especially true for host-parasite systems where experimental work is commonly used to inform conservation policy.

The pathogenic fungus *Batrachochytrium dendrobatidis* (Bd) has been associated with hundreds of global amphibian declines over the past 40 years and thus has been the focus of hundreds of studies and surveys in recent decades (Skerratt, Berger et al. 2007, Rohr and Raffel 2010). Bd infects keratinized mouthparts of larval amphibians and keratinized skin of post-metamorphic amphibians, degrading skin cells and causing the deadly disease chytridiomycosis (Berger, Speare et al. 1998, Pessier, Nichols et al. 1999, Fellers, Green et al. 2001). There is a great deal of variation in the effects of the pathogen across wild host populations, with some species experiencing declines, extirpation, or even extinction and others experiencing few to no negative impacts (Venesky, Raffel et al. 2014). This heterogeneity in mortality has led to

conflicting findings and much debate about mechanisms that might be driving these apparent differences in mortality risk among host populations (Fisher, Garner et al. 2009).

The effects of how some environmental factors affect Bd infection are relatively well understood from laboratory experiments. For example, studies have examined how individual-level behavior (Sauer, Fuller et al. 2018), body size (Carey, Bruzgul et al. 2006), diet (Venesky, Wilcoxen et al. 2012), exposure level (Carey, Bruzgul et al. 2006), and life stage impact host susceptibility to Bd. Life stage has long been recognized as a driver of Bd infection outcomes because larvae are less likely to die of Bd infection than metamorphic and adult amphibians due to a lack of keratinized skin cells outside of their mouthparts (Berger, Speare et al. 1998, Marantelli, Berger et al. 2004) but see McMahon et al. (2013). During metamorphosis, Bd moves from the mouthparts to the keratinized cells in the newly formed hind limbs, increasing the likelihood of mortality (Banks, McCracken et al. 2002, Marantelli, Berger et al. 2004, McMahon and Rohr 2015).

Conversely, the effects of some environmental factors, like Bd isolate and environmental temperature, are more contentious (Fisher, Garner et al. 2009, Venesky, Raffel et al. 2014). For example, one hypothesis suggests that host populations have adapted to local Bd isolates and experience an increase in mortality risk when novel isolates are introduced (Carey, Cohen et al. 1999, Jenkinson, Betancourt Román et al. 2016). A recent extensive genomic analysis of Bd isolates traced the origin of Bd to the Korean peninsula and dated the emergence of Bd from Korea to the early 20th century (O'Hanlon, Rieux et al. 2018). Thus, any local adaptation to Bd outside of Northeast Asia must have occurred very rapidly, which is not unrealistic as Bd can be highly virulent and impose strong selective pressures on hosts (Savage and Zamudio 2016). In fact, recent evidence suggests that some Central American amphibian populations once thought to

be extirpated because of Bd have begun to recover because of adaptive shifts in host defenses, not a reduction in Bd virulence (Voyles, Woodhams et al. 2018).

Environmental temperature is another controversial source of heterogeneity in mortality risk across populations (Fisher, Garner et al. 2009, Venesky, Raffel et al. 2014). Several studies have separately concluded that environmental temperatures should be both positively (Pounds, Bustamante et al. 2006, Bosch, Carrascal et al. 2007) and negatively (Retallick, McCallum et al. 2004, Kriger and Hero 2007) correlated with Bd prevalence and mortality risk. Recently, a more context-dependent hypothesis, the thermal mismatch hypothesis, emerged. This hypothesis proposes that host species adapted to warmer climates should be more likely to get disease at cool than warm temperatures, whereas the opposite patterns should occur for cool-adapted host species (Cohen, Venesky et al. 2017). This pattern is hypothesized because smaller-bodied pathogens generally have wider thermal breadths than their larger-bodied hosts (Rohr, Civitello et al. 2018), allowing the pathogen to outperform its hosts under thermal mismatch conditions (Cohen, Venesky et al. 2017). This hypothesis is supported by multiple laboratory and field experiments that show that warm- and cool-adapted hosts have higher Bd growth and higher field prevalences at cool and warm temperatures, respectively (Cohen, Venesky et al. 2017, Sauer, Fuller et al. 2018, Cohen, Civitello et al. In press, Cohen, McMahon et al. In revisions). However, the experimental evidence for the thermal mismatch hypothesis is confined to only three amphibian host species.

Here, we use meta-analysis to compare across experimental studies conducted in the amphibian-Bd system to assess the generality by which environmental factors affect chytridiomycosis-induced mortality in laboratory studies. Our goals were to determine if 1) Bd-induced mortality varies over time across life stages (larvae, metamorph, and adult), 2) there is an

effect of dose on host mortality, 3) the novelty of the Bd isolate affects the risk of host mortality, and 4) there is support for the thermal mismatch hypothesis across species. To accomplish these goals, we searched the published literature for Bd laboratory studies and modeled effects of life stage, dose, the novelty of Bd isolates, long-term temperature at a host's collection location, and laboratory temperature on Bd-induced mortality. We predict that 1) larvae will have lower mortality than metamorphic or adult amphibians, 2) dose will be a positive predictor of mortality, 3) there will be a positive correlation between isolate novelty and host mortality, and 4) there will be a negative interaction between long-term temperature in a host's geographic location and experimental temperature, supporting the thermal mismatch hypothesis.

Methods

Data collection

Our goal was to synthesize all experimental studies that compared amphibian hosts experimentally infected with Bd in the laboratory to unexposed controls. We located studies in Web of Science by searching for the term "*Batrachochytrium dendrobatidis"* in October 2016, producing 1,403 results. Search results first screened using the *abstract_screener* function of the *metagear* package ((Lajeunesse 2016) R 3.4.0). We included laboratory studies where there was at least one Bd- exposed treatment paired with an unexposed control group; we did not consider treatments that exposed hosts to any pesticides, additional parasites (e.g. co-infection), or other compounds. Of the treatments that met the aforementioned conditions, we also only included treatments held at a constant laboratory temperature. We only included studies in which hosts were either wild-collected or lab-reared from wild-collected parents and treatment and control mortality, sample sizes, and host and Bd isolate collection location were either available in the

manuscript or provided to us by the author when requested (final count: 58 studies; Appendix S1).

We manually extracted data from text and tables, and extracted data from figures using Plot Digitizer version 2.6.6 (plotdigitizer.sourceforge.net). In addition, we recorded other information about the biology and methodology of each study, including (if available): host species, geographic coordinates where hosts or host parents were collected, developmental stage (larvae, metamorph, or adult), percent mortality in treatment and control animals, sample sizes, Bd isolate, and geographic location of the collection site of the isolate, Bd dose (total zoospores), and duration of the experiment in days. Species nomenclature was standardized according to the IUCN (2018). Finally, for each row in our dataset, we extracted mean temperature data across fifty years (BIO1 1950-2000) from WorldClim (www.worldclim.org, *raster* package, *extract* function in R 3.4.0 (Hijmans 2014)) that corresponded to the location where each host population was collected. This long-term mean temperature represents the temperature to which the host is adapted. We also calculated the Euclidean distances between collection locations of a given host and the Bd isolates to which it was exposed to estimate the degree of novelty of an isolate to a host population (*sp* package, *spDists* function R 3.4.0 (Pebesma and Bivand 2005)).

Effect sizes

In meta-analyses, effect sizes must be calculated to provide a standardized measure of an effect across studies (Borenstein, Hedges et al. 2011). Because mortality data are binary, we calculated log odds ratios to assess the odds of mortality in the Bd-exposed animals relative to the control animals (Cox 2018), using the following equation:

$$logOR = ln\left(\frac{(D_t + p)/(A_t + p)}{(D_c + p)/(A_c + p)}\right)$$

where D_t is the number of treatment (i.e. Bd-exposed) animals that died, A_t is the number of treatment animals that survived, D_c is the number of control (i.e. sham-exposed) animals that died, A_c is the number of control animals that survive, and p (p = 1/2) is a Yate's continuity correction to avoid error in our effect sizes resulting from dividing by zero (Yates 1934) (Borenstein, Hedges et al. 2011, Cox 2018). Yate's continuity correction (p) was only added to effect sizes where an error from dividing by zero would have occurred, all other effect sizes were calculated using the same log odds ratio formula but with p omitted. When conducting analyses, odds ratios must be natural log-transformed to ensure that studies with equal but opposite effects have odds ratios that differ from zero by the same magnitude but in opposite directions (Borenstein, Hedges et al. 2011). Variance for each effect size was calculated as:

$$Var_{logOR} = \frac{1}{D_t+0.5} + \frac{1}{A_t+0.5} + \frac{1}{D_c+0.5} + \frac{1}{A_c+0.5}.$$

A log odds ratio significantly greater than 0 represents greater mortality in the treatment group than in the control, whereas a log odds ratio with error bars that overlap with 0 represents no effect of Bd exposure on host survival. For most of the studies, mortality was non-linear over time. Additionally, studies varied immensely in their duration. However, we were unable to conduct a meta-analysis of survival analyses where time is treated as a continuous variable without losing a significant portion of studies that found no effect of Bd on host mortality, increasing the risk of encountering a type I error. Therefore, we dealt with these nonlinearities and inconsistencies in experimental length by calculating log odds ratios at four arbitrarily chosen time intervals: 1-14, 15-28, 29-42, and >42 days post Bd exposure. For ease of interpretability, any study from which we could not retrieve data at each the aforementioned time intervals within the duration of that study was dropped from the analysis (e.g. a 38 day study would need data from 14, 28, and 38d to be included), meaning that all studies are included in the 1-14 d interval

and studies are dropped in each subsequent interval if they did not extend beyond the first day of that interval (e.g. a study that lasted 21 days would be included in the 1-14 d and 15-28 d time intervals). All analyses were repeated at each of these time intervals.

Meta-analytical models

All analyses were conducted in R 3.4.0 (2017). We analyzed the data using a mixed-effects meta-analysis (Equation 3; *metafor* package, *rma.mv* function (2010)) replicated once per time interval (four total meta-analytic models; Table 1). Our mixed-effects meta-analysis can be described with this regression model:

$$y_1 \dots y_k \sim \beta_1 t_1 * \beta_2 t_2 + \beta_3 d + \beta_4 l + \beta_5 x + \gamma_1 e + \gamma_1 s + y_5 b + \gamma_3 P + v_i$$

Where $\boldsymbol{y_1 \dots y_k}$ denotes log odds ratios and $\boldsymbol{v_i}$ denotes log odds ratio variance. Our primary hypotheses concerned the relationship between experimental conditions and Bd infection outcome, not simply the main effect of Bd on host mortality. Therefore, our models included four moderators: $\log_{10}$ transformed Bd dose (***d***; normally distributed continuous variable), $\log_{10}$ transformed Euclidean distance in km between host and Bd isolate collection location (***x***; normally distributed continuous variable), life stage (***l***; three-level categorical variable: larvae, metamorph, adult), and thermal mismatch ($\boldsymbol{t_1}$ & $\boldsymbol{t_2}$), represented by an interaction between long-term mean temperature of the host's collection site ($\boldsymbol{t_1}$) and the laboratory temperature ($\boldsymbol{t_2}$) at which the experiment was conducted (normally distributed continuous variables).

To avoid bias and risk of type I error, we accounted for between-study random effects (***e***), non-independence among species by including binomial species as a random effect (***s***), and non-independence among Bd isolates by including isolate (*b*) as a random effect in our models (Borenstein, Hedges et al. 2011, Civitello, Cohen et al. 2015, Rohr, Civitello et al. 2015). Finally, we accounted for correlations between effects due to the shared phylogenetic history of hosts by

including a phylogenetic correlation matrix as a random effect in our models (***P***; extracted from the Pyron and Wiens (2011) tree using the *vcv* function in the *ape* package) (Paradis, Claude et al. 2004, Viechtbauer 2010, Cohen, Lajeunesse et al. 2018). Due to the complex non-independence among effect sizes within a study (e.g. some studies had multiple effect sizes), we did not use funnel plots or rank correlation tests to assess publication bias (Lau, Ioannidis et al. 2006, Civitello, Cohen et al. 2015).

For each time point, we compared our final model to an intercept only model and to models with all possible combinations of the four moderators (sixteen total models per time interval) and found that the full model always performed best based on corrected Akaike information criterion. Thus, we only present the full models. Models with only one temperature variable (long-term mean temperature or experimental temperature) were not considered as we were not specifically testing for effects of either factor alone.

Results

Our literature search yielded between 206 and 78 effect sizes per time interval (1-14d: k=206; 15-28d: k=160; 29-48d: k=136; and >42d: k=78) from 58 studies and included 47 amphibian species from 11 families. Experiments used a total of 45 unique Bd isolates (Database S1). Host species were collected from North and South America, Europe, and Oceania (Fig. 1). There were no studies that met our inclusion criteria from the Middle East, Asia, or Africa (Fig. 1). Overall, Bd exposure was significantly, positively related to the odds of mortality (risk of mortality in the treatment group relative to controls; abbreviated as "logOR") during only the last two time intervals (1-14d: logOR = 0.47, z = 1.01, p = 0.31; 15-28d: logOR = 0.56, z = 0.77, p = 0.44; 29-42d: logOR = 1.36, z = 3.09, $p < 0.01$; >42d: logOR = 1.86, z = 3.98, $p < 0.01$; Table 1 & Fig. 2).

Effect of Bd exposure varied depending on host life stage (Table 1 & Fig. 2). For hosts exposed as larvae, there was no significant effect of Bd on logOR during the 1-14d or15-28d (logOR = -0.48 ±0.36 95% CI & logOR = -0.25 ±0.46 95% CI respectively; Fig. 2) time intervals, but there was an effect during the 29-42d and >42d time intervals (logOR = 0.81 ±0.51 95% CI& logOR = 2.42 ±1.26 SE, respectively; Table 1 & Fig. 2). The delayed effect of Bd on larval host may coincide with metamorphosis (see Discussion). Host exposed as adults experienced an effect of Bd during the 28-42d time interval (logOR = 0.78 ±0.57 95% CI), but there was no effect of Bd during all other time intervals (Fig.2 & Table 1). Hosts exposed as metamorphs experienced a significant positive effect of Bd on logOR during all time intervals (Fig. 2 & Table 1). Additionally, larvae had significantly lower mean logOR than the grand mean (main effect of Bd across all life stages) during the first three time intervals (1-14d: z = -3.24, p < 0.01; 15-28d: z = -3.41 p < 0.01, & >42: z = -2.10, p = 0.04) while metamorphs had significantly higher mean logOR than the grand mean during the middle two time intervals (15-28d: z = 4.50, p < 0.01 & 29-42d: z = 4.91, p < 0.01; Table 1). This suggests that the metamorph stage is, on average, the most susceptible of the three stages to Bd.

There was a significant or marginally non-significant positive relationship between log-transformed Bd dose and logOR at each time interval (1-14d: z = 2.24, p = 0.03, 15-28d: z = 1.94, p = 0.053, & 29-42d: z = 3.02, p < 0.01; >42: z = 2.70, p < 0.01; Table 1). However, we only found a significantly positive effect of log-transformed distance between host and Bd isolate collection locations on logOR during the 1-14d time interval (z = 3.03, p < 0.01); Bd novelty was not significant at any other time interval (Table 1). We found a significant negative interaction between long-term temperature and laboratory temperature (thermal mismatch effect) during the 14-28d and 29-42d time intervals (z = -2.01 p = 0.05 & 15-28d: z = -3.80, p < 0.01, respectfully;

Table 1) but no effect at the other two time intervals (1-14d and >42d). Cool-adapted hosts experienced the greatest mortality relative to controls at warm temperatures and warm-adapted hosts experienced the highest mortality relative to controls at cool temperatures (Table 1).

Discussion

Using meta-analysis, we set out to better understand the generality of experimental factors affecting mortality odds in the amphibian-Bd system. Specifically, we tested for effects of life stage, Bd dose, Bd isolate novelty, and thermal mismatches on Bd-induced mortality (measured as logOR). Our literature search highlighted a gap in laboratory studies of hosts and Bd isolates from the Middle East, Asia, and Africa (Fig. 1). As expected, our data show an overall effect of Bd on logOR as well as a positive correlation between Bd dose and logOR at every time interval (Table 1). Bd-induced mortality increased with each time interval, suggesting that longer experiments were more likely to produce a positive effect of Bd exposure on host mortality than shorter experiments, despite the fact that more resistant hosts are most likely to be overrepresented in experiments lasting >42 days as experiments with less resistant hosts might end sooner because of host mortality. We also found that the effect of Bd exposure on logOR varied across life stage (Table 1, Fig. 2). Additionally, we only saw a positive effect of Bd novelty on logOR during the first two weeks after exposure (Table 1). Finally, we found support for the thermal mismatch hypothesis 15 to 42 d after Bd exposure (Table 1).

Our literature search yielded high numbers of effect sizes for all time intervals (k>77 for all intervals) and included large numbers of amphibian species and families as well as a large number of unique Bd isolates. However, North American and European hosts and Bd isolates were overrepresented in our analyses with less than 10% of our effect sizes representing host

species collected from Central and South America or Oceania, and 0% from Asia, Africa or the Middle East. Because we were testing for the thermal mismatch hypothesis, we did not include studies using captive-bred hosts in our study because we feared that they might have adapted to laboratory temperature. This excluded many studies that used hosts native to Oceania and Central America (not surprising given the high number of threatened, endangered, and extirpated species in those regions). We were unable to find any studies that tested wild-collected or captive-bred Asian or African host species or Bd isolates that met our inclusion criteria. It is imperative that researches conduct laboratory experiments on species from these neglected areas, especially considering the strong genetic support for Bd originating from Northeastern Asia and spreading globally within the last 100 years (O'Hanlon, Rieux et al. 2018).

Unsurprisingly, we found that the main effect of Bd on logOR increased over time and was significant after 28d post-exposure. We also found a positive correlation between Bd dose and logOR at every time interval. These results are unsurprising as Bd has been implicated in hundreds of amphibian declines or extinctions (Berger, Speare et al. 1999, Fisher 2012) and is noted for its ability to cause rapid localized die-offs of hosts. However, logOR did vary across life stage. Larvae were less susceptible to Bd than metamorphs or adults. Hosts exposed as larvae experienced no significant effect of Bd until 29 days after exposure. The adverse effects that occurred after day 29 are likely because many of the larvae in these studies began to metamorphose during these later time intervals (Banks, McCracken et al. 2002). As larval amphibians infected with Bd metamorphose, Bd moves from the mouthparts to the budding hind limbs, which have keratinized skin (Marantelli, Berger et al. 2004, McMahon and Rohr 2015). Amphibians exposed as metamorphs were significantly more likely to experience mortality after Bd exposure than the larval or adult stages. Metamorphosis is energetically costly and there are

likely energetic trade-offs occurring between development and the immune system, which could make metamorphic amphibians more susceptible to Bd-induced mortality than larvae or adults (Rollins-Smith 1998, Warne, Crespi et al. 2011).

During the first two weeks after exposure, Bd isolate novelty, measured as the distance between host and Bd collection sites, positively predicted logOR. This result suggests that the anthropogenic spread of Bd isolates (pathogen pollution) may increase risk of outbreaks and declines in naïve amphibian populations (Daszak, Cunningham et al. 2003). It has been hypothesized that novel isolates might kill hosts before their immune systems recognize and defend against the pathogen or that novel pathogens might be immunosuppressive (Carey, Cohen et al. 1999). Additionally, there is evidence that amphibians can develop defenses to local Bd isolates in relatively short periods of time (McMahon, Sears et al. 2014). These hypotheses may explain why there was no effect of isolate novelty after 14 days post exposure. However, empirical evidence for these hypotheses is minimal and mortality was relatively low during the first two weeks after exposure when compared to all following time periods. It is difficult to draw confident conclusions about mechanism from this result as the meta-analysis only tested for differences in logOR and did not incorporate any measures of immunological response or function.

It is possible that novel isolates are more likely to be hypervirulent to the test host than local isolates, driving the positive effect of Bd isolate novelty on logOR during the first two weeks after exposure. To address this concern we first attempted to categorize each isolate by lineage using text from the study or the isolate phylogeny database published by O'Hanlon et al (2018). Secondly, we determine if studies using isolates belonging to the hypervirulent Global Pandemic Linage (GPL) had, on average, greater distances between the host and Bd-isolate

collection locations than studies using isolates belonging to non-hypervirulent lineages (Farrer, Weinert et al. 2011). We were able to categorize 24 of the 45 unique isolates by linage (accounting for 65.5% of the 206 effect sizes in the 1-14 d time interval); 17 of those (which account for 63.1% of effect sizes) belonged to the GPL lineage while only 4 (0.02% of effect sizes) belonged to a non-hypervirulent lineage. The studies that used Bd isolates belonging to non-hypervirulent linages, on average, had further distances between the host and Bd isolate collection sites (mean distance = 1551 km) than studies using isolates from the hypervirulent lineage, GPL (mean distance = 447 km), or isolates that we were unable to categorize (mean distance = 258 km). Thus, it is unlikely that isolates from the hypervirulent lineage, GPL, are driving the positive effect of Bd isolate novelty on logOR during the first two weeks after exposure as novel isolates are not more likely to belong to the hypervirulent lineage.

We found evidence supporting the thermal mismatch hypothesis between 14 and 42 days after Bd exposure. Cool-adapted populations had the highest Bd-induced mortality at warm temperatures and warm-adapted populations had the highest mortality at cool temperatures (Cohen, Venesky et al. 2017) (Table 1). These results, which confirm previous evidence for the important of thermal mismatches in this system, suggest that predicted increases in environmental temperatures caused by climate change might place cool-adapted species at greater risk of disease-related declines than warm-adapted species (Cohen, Venesky et al. 2017, Sauer, Fuller et al. 2018, Cohen, Civitello et al. In press, Cohen, McMahon et al. In revisions). It is unlikely that our results are driven by experiments that were purposely conducted at extreme temperatures as only five of the 58 studies included in this analysis purposely manipulated environmental temperatures. The majority of studies simply conducted their experiments at an arbitrary room temperature.

Researchers might be inadvertently over or underestimating Bd growth and host mortality by conducting their experiments at arbitrarily chosen room temperatures (Cohen, Venesky et al. 2017, Sauer, Fuller et al. 2018, Cohen, Civitello et al. In press, Cohen, McMahon et al. In revisions), rather than a temperature to which the host is adapted. Outside of the mounting evidence for the thermal mismatch hypothesis in the Bd-amphibian system, there is large body of research showing that environmental temperatures impact experimental outcomes in other amphibian-disease systems (Rojas, Richards et al. 2005, Venesky, Raffel et al. 2014, Brand, Hill et al. 2016, Cohen, Venesky et al. 2017). Researchers should consider how experimental temperatures and host thermal preferences and tolerances impact the results and conclusions drawn from laboratory experiments. Where possible, we encourage researchers to select temperatures that are ecologically relevant for their host-pathogen system.

Our results suggest that life stage, dose, isolate, and environmental temperature should be carefully considered when designing experiments as all four can alter experimental outcomes. Additionally, more research is needed on hosts and Bd isolates from Africa and Asia. Additional research is also needed to better understand how rapid adaptation to local Bd isolates and increased susceptibility to novel isolates might be shaping heterogeneity in host population susceptibility globally (McMahon, Sears et al. 2014, Jenkinson, Betancourt Román et al. 2016, Voyles, Woodhams et al. 2018). Finally, our results are consistent with the thermal mismatch hypothesis, suggesting that there are context-dependent effects of environmental temperature on amphibian mortality in this system. This result highlights the need for researchers to carefully consider the thermal tolerances and optima of their host species before choosing an experimental temperature to avoid the confounding effect of thermal mismatch.

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Figures

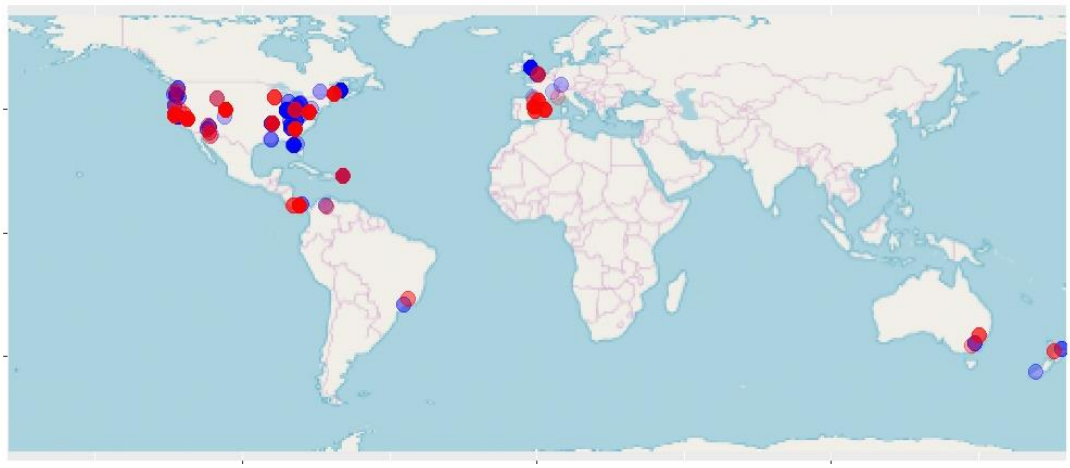

Figure 4.1. Map of host species and Bd collection locations. Map showing where hosts (blue points) and Bd isolates (red points) were collected from for studies included in the meta-analysis. All points have the same opacity; locations where points appear darker indicate spatial overlap.

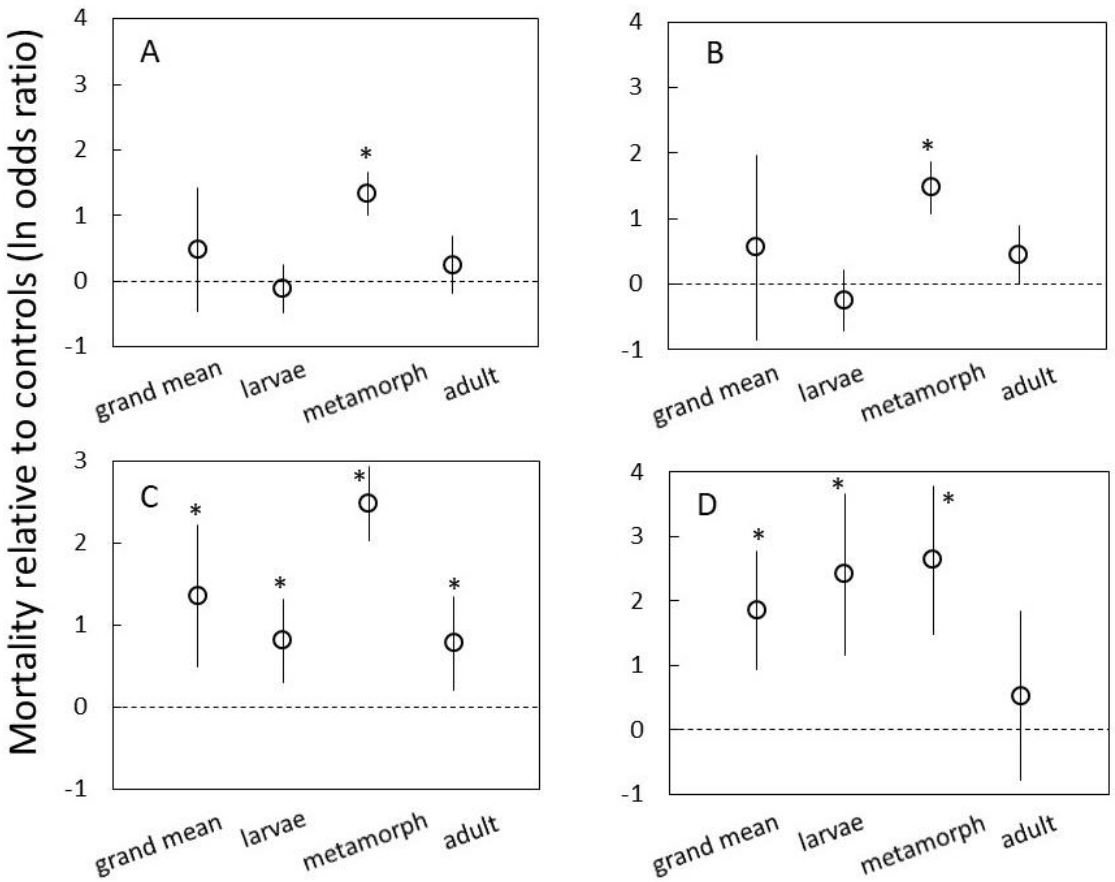

Figure 4.2. Main effect of life stage on mortality (larvae, metamorph, or adult ; presented as log odds ratio) (A) between 1 and 14 days, (B) between 15 and 28 days, (C), between 29 and 42 days, or (D) more than 42 days after exposure to Bd. The first point in each panel represents the grand mean for that time block. Asterisks indicate significant differences from zero (grey dotted line) or an effect of Bd on mortality risk. Error bars represent 95% confidence intervals.

Tables

Table 4.1. Results of the multivariate random effects meta-analytic models for each of the four time intervals analyzed post Bd exposure. The main effect of Bd on mortality is indicated by the intercept where the coefficient represents the grand mean for each time interval as an odds ratio. For continuous variables, asterisks indicate a slope that deviates significantly from zero where the sign of the coefficient indicates the direction of the effect. For categorical variables (life stage only: larvae, metamorph, and adult), asterisks indicate a significant difference from the grand mean with the sign of the coefficient indicating the direction of the difference from the grand mean.

	Coefficient	SE	z value	p value	
1-14d					
Intercept	0.484	0.480	1.009	0.313	
logDose	0.206	0.092	2.243	0.025	*
logDistance	0.438	0.145	3.026	0.003	*
Larvae	-0.601	0.185	-3.242	0.001	*
Metamorph	0.839	0.167	5.034	<0.001	
Adult	-0.238	0.221	-1.076	0.282	
LongTermTemp	-0.001	0.012	-0.104	0.917	
LabTemp	0.046	0.077	0.598	0.550	
LongTermTemp*LabTemp	0.000	0.001	-0.257	0.797	
15-28d					
Intercept	0.557	0.723	0.770	0.441	
logDose	0.219	0.113	1.935	0.053	
logDistance	-0.128	0.191	-0.668	0.504	
Larvae	-0.805	0.236	-3.408	0.001	*
Metamorph	0.916	0.204	4.497	<0.001	*

Adult	-0.111	0.233	-0.476	0.634	
LongTermTemp	0.017	0.013	1.366	0.172	
LabTemp	0.177	0.100	1.774	0.076	
LongTermTemp*LabTemp	-0.001	0.001	-2.009	0.045	*
29-42d					
Intercept	1.359	0.440	3.090	0.002	*
logDose	0.411	0.136	3.024	0.003	*
logDistance	-0.246	0.221	-1.116	0.264	
Larvae	-0.547	0.261	-2.097	0.036	*
Metamorph	1.127	0.229	4.919	<0.001	*
Adult	-0.580	0.290	-2.001	0.045	*
LongTermTemp	0.049	0.015	3.247	0.001	*
LabTemp	0.339	0.111	3.053	0.002	*
LongTermTemp*LabTemp	-0.003	0.001	-3.798	<0.001	*
>42d					
Intercept	1.862	0.468	3.982	<0.001	*
logDose	0.598	0.222	2.698	0.007	*
logDistance	-0.377	0.367	-1.027	0.304	
Larvae	0.554	0.641	0.864	0.388	
Metamorph	0.777	0.585	1.327	0.185	
Adult	-1.331	0.667	-1.995	0.046	*
LongTermTemp	-0.046	0.046	-1.000	0.317	
LabTemp	-0.288	0.229	-1.256	0.209	
LongTermTemp*LabTemp	0.001	0.002	0.622	0.534	

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APPENDICES

Appendix A: Chapter One Reprint

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An efficient and inexpensive method for measuring long-term thermoregulatory behavior

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ABSTRACT

Thermoregulatory ability and behavior influence organismal responses to their environment. By measuring thermal preferences, researchers can better understand the effects that temperature tolerances have on ecological and physiological responses to both biotic and abiotic stressors. However, because of funding limitations and confounders, measuring thermoregulation can often be difficult. Here, we provide an effective, affordable (~$50 USD per unit), easy to construct, and validated apparatus for measuring the long-term thermal preferences of animals. In tests, the apparatus spanned temperatures from 9.29 to 33.94 °C, and we provide methods to further increase this range. Additionally, we provide simple methods to non-invasively measure animal and substrate temperatures and to prevent temperature preferences of the focal organisms from being confounded with temperature preferences of its prey and its humidity preferences. To validate the apparatus, we show that it was capable of detecting individual-level consistency and among individual-level variation in the preferred body temperatures of Southern toads (*Anaxyrus terrestris*) and Cuban tree frogs (*Osteopilus septentrionalis*) over three-weeks. Nearly every aspect of our design is adaptable to meet the needs of a multitude of study systems, including various terrestrial amphibious, and aquatic organisms. The apparatus and methods described here can be used to quantify behavioral thermal preferences, which can be critical for determining temperature tolerances across species and thus the resiliency of species to current and impending climate change.

1. Introduction

Quantifying thermoregulatory behavior in ectotherms can elucidate fundamental aspects of organismal physiology, behavior, and ecology (Huey and Stevenson, 1979; Bauwens et al., 1990; Hutchison and Dupré, 1992; Hertz et al., 1993; Blouin-Demers and Weatherhead, 2001; Angilletta et al., 2010). Specifically, determining thermal preferences and tolerances is critical for understanding how individuals mediate both biotic and abiotic stressors. Indeed, thermoregulatory behavior has been observed in almost all ectothermic taxa, including reptiles (Monagas and Gatten, 1983; Burns et al., 1996), amphibians (Kluger, 1977; Hutchison and Murphy, 1985), bony fishes (Reynolds et al., 1976, 1977), and invertebrates (Bicego et al., 2007). By seeking external sources of heat or refuge in cool places, ectotherms can regulate their metabolism to facilitate feeding and digestion (Ayers and Shine, 1997), reproduction (Christiansen and Bakke, 1968), growth (Lillywhite et al., 1973; Sinervo and Adolph, 1989; Calsbeek and Sinervo, 2002a), immune function and disease resistance (Blanford and Thomas, 1999; Mondal and Rai, 2001; Rohr et al., 2013), territory selection and defense (Calsbeek and Sinervo, 2002b), mate search and mating (Calsbeek and Sinervo, 2002b), and many other physiological functions (Bennett, 1980). Hence, measuring thermoregulatory behaviors and temperature preferences is important to understanding many aspects of the fundamental biology of ectotherms.

Quantification of thermal preferences can also inform issues relevant to applied biology. For instance, many anthropogenic factors can alter the thermal environment posing threats to the performance of organisms. Global climate change is the most obvious (Deutsch et al., 2008; Seebacher and Post, 2015), but there are other examples as well. For instance, deforestation, or more generally the loss of shading caused by habitat destruction, can greatly increase the temperatures to which organisms are exposed (Gordon, 2003). Moreover, infectious diseases, many of which are introduced or exacerbated by humans, often induce behavioral fevers (preference of warmer temperatures in response to pathogen exposure) in ectotherms (Blanford and Thomas, 1999) that can be important for resisting infections and reducing the adverse

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consequences on hosts (Pörtner, 2002; Raffel et al., 2006; Lafferty, 2009; Rohr and Raffel, 2010; Rohr et al., 2011a, 2013). Hence, determining the bounds of thermoregulatory abilities among ectothermic populations will be critical for predicting the impacts of widespread anthropogenic change (Addo-Bediako et al., 2000; Deutsch et al., 2008; Seebacher and Post, 2015).

Although thermal preferences are an important determinant of many aspects of temperature-dependent physiology of ectotherms, experimentally determining thermal preferences can be challenging and expensive. A standard method for determining thermal preferences is to place focal animals in a thermal gradient chamber and monitor body temperatures over some length of time (Row and Blouin-Demers, 2006; Weatherhead et al., 2012). In the absence of other stimuli (e.g. food, conspecifics, etc.), the assumption is that the focal animal will spend the majority of time within their preferred temperature range (Hertz et al., 1993). However, there can be many confounding factors that can make the results of these trials difficult to interpret, particularly when the duration of these trials exceed the food deprivation limits of the focal organism. For example, because environmental temperature is correlated positively with evaporative water loss, temperature gradients are often confounded with moisture gradients (Malvin and Wood, 1991; Rohr and Palmer, 2013). Given that organisms must maintain moisture in addition to preferred temperatures (Bellis, 1962; Rohr and Madison, 2003; Rohr and Palmer, 2005), confounding moisture and temperature gradients makes it challenging to assess whether true temperature preferences are being quantified.

Additionally, longer-term trials that require feeding the test animal can pose additional confounders. Ectotherms often prefer warmer temperatures during digestion (Lillywhite et al., 1973; Greenwald and Kanter, 1979) making it challenging to discriminate baseline and digestion-related temperature preferences. Live prey provided as a food source might prefer temperatures outside of the preferred temperature range of the focal organism, confounding the temperature preference of the prey and test organism. Finally, invasive temperature measurement techniques, such as dermally attached loggers, brain implants, or thermometer probes, are used commonly in longer-term experiments, but they can alter behavior and thus can compromise measurements of true temperature preferences (Rowley and Alford, 2007).

Here, we provide an inexpensive, efficient, and validated method for measuring thermoregulatory behavior in the laboratory for extended periods of time while controlling for humidity, disturbance, and other confounders. Using supplies found in most hardware stores, we constructed thermal gradient apparatuses for less than $50 USD, spanning temperatures from 9.29 to 33.94 °C (for supply list see Table S1). The methodological details and results presented here demonstrate that our apparatus and methods (1) maintain consistent high humidity across the entire temperature gradient, (2) allow for long-term maintenance of animals in the temperature gradient, (3) do not confound temperature preference of the prey with the focal organism, (4) measure temperature using minimally invasive techniques, and (5) can detect consistency in the thermal preferences within individuals but differences in preferences among individuals and species, a prerequisite for quantifying temperature preferences and behavioral thermoregulation. At a time when measuring temperature tolerance across species is critical to assess the ability of organisms to respond to climate change and other stressors, our method provides an affordable, easy to implement, effective way to measure thermal responses across a wide range of species of varying sizes.

2. Materials and methods

2.1. Apparatus

We constructed 28 thermal gradient apparatuses using $274 \times 8 \times 12$ cm aluminum downspout gutters cut in half along the longest and widest sides yielding final internal dimensions of $137 \times 8 \times 6$ cm (Fig. 1a and b). Each apparatus was insulated using foam windowsill insulation with holes cut where the metal meets a heat or cooling source (Fig. 1c and d) and was capped at the ends using Styrofoam and silicon sealant (Fig. 1b). The top of each apparatus was sealed using five 27×10 cm Plexiglas® windows resting on window weather-stripping. These Plexiglas® windows were held in place by small duct tape hinges (Fig. 1b). The windows allow ambient light to pass through and give the experimenter access to each section of the apparatus with minimal disturbance to the organism. Each window was secured to prevent animals from escaping using twine and cord locks (Fig. 1b).

2.2. Maintaining temperature and humidity

The warmer ends of the apparatuses rested on a gradient of 7.62 cm 10 W heat tape (Flexwatt Industrial Sales®, Maryville, TN) controlled by a bulb-and-capillary thermostat (Selco Products Co., Orange, CA). The temperature gradient was created by adhering six 132 cm strips of heat tape to a piece of plywood (60.96×132.08 cm) at increasing distances from the end of the apparatus (Fig. 1c). The cooler end of each apparatus rests on a frozen (−80 °C) gel pack (32 oz No-Sweat, Temperatsure Inc., Reno, NV) (Fig. 1d). Ice packs sat on windowsill insulation and were replaced every 12 h. Plywood height was adjusted to the height of the ice packs to level the apparatuses. Two pieces of wood ($5.08 \times 10.16 \times 137$ cm) rested against the outside of the outermost apparatuses to prevent heat loss (Fig. 1a). Each shelf of apparatuses was covered by two large sheets of Plexiglas to further insulate the apparatuses while maintaining the desired photoperiod (Fig. 1a). Organic sphagnum moss substrate, kept saturated with artificial spring water (Cohen et al., 1980), was used to maintain constant high humidity throughout each apparatus.

2.3. Maintaining animals and taking temperature measurements

In separate trials, we housed a total of 36 Southern toads (*Anaxyrus terrestris*, mean mass: 0.61 g; ±0.01 SE), and 25 Cuban tree frogs (*Osteopilus septentrionalis*, mean mass: 7.67 g; ±0.35 SE) in the apparatuses (one animal per apparatus) for three weeks during the trials. For both species, we used an ecologically relevant temperature gradient of 12–33 °C (US Climate Data, 2016). All animals were fed 10 live crickets twice a week in containment to prevent crickets from moving freely within the apparatuses. The feeding containers were constructed of quart-size zip-top bags with plastic coated paper clips adhered to the outside for structure (Fig. S1). Feeding containers were placed in the thermal gradient apparatuses at the location each individual was found prior to feeding. After seven hours of confinement, quantity of crickets eaten was recorded for each individual to monitor feeding success throughout the duration of the experiment. No temperature measurements were taken on feeding days because of the limited movement allowed by focal animals and their prey during feeding.

Temperature measurements were taken with an Extech® High Temperature Infrared Thermometer (accuracy: ±2% of rdg < 932 °F), which uses a laser to non-invasively measure temperatures and minimize disturbance to the animal. In amphibians, this method is comparable to cloacal measurements taken via thermally sensitive radio-transmitters (Rowley and Alford, 2007). At each temperature measurement, we located the individual,

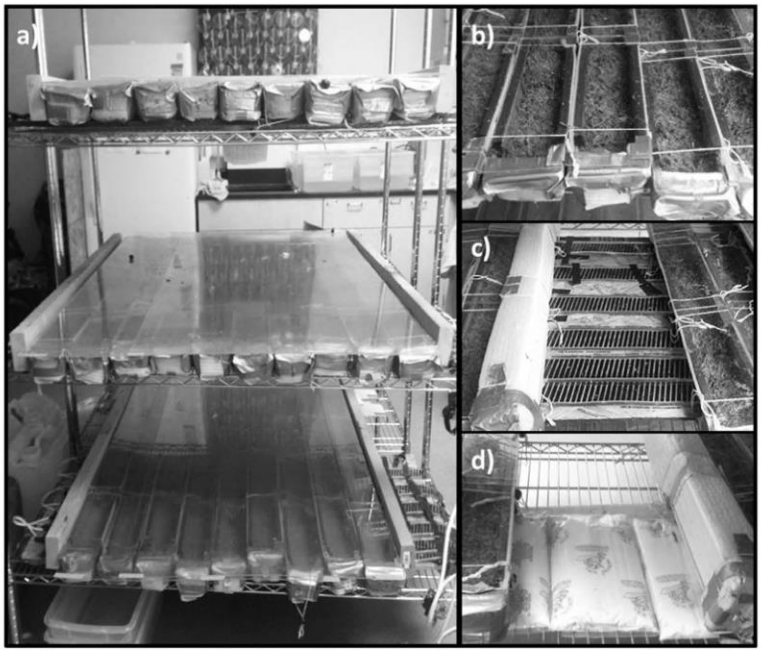

Fig. 1. Temperature gradient apparatuses. **(a)** The entire set up of thermoregulatory apparatus, showing insulating 2 × 4's and large Plexiglas covers. **(b)** View of the sphagnum moss interior and small Plexiglas window sealing each apparatus. **(c)** Heat tape gradient and bottom of apparatus showing the space where the aluminum meets the heat tape. **(d)** Ice packs and bottom of apparatus showing the space where the aluminum meets the ice packs.

opened the appropriate Plexiglas window section directly above the animal, and then measured the body temperature of the animal and the temperature of the substrate as close as possible to where the animal was found with the infrared thermometer. Given that some animals can move regularly, measuring both the temperature of the substrate and animals offers insight into whether the animal has been at a given location long enough for it body temperature to conform to environmental temperature. The animals did not respond to the infrared laser in any observable manner. Temperature measurements were taken every four hours, four times a day between 1000 h and 2200 h, five days a week, for three weeks totaling 100 body temperature measurements per individual. The four hour time intervals within a day and a 12 h gap between days was chosen to allow ample opportunity for organisms to move between measurements within and among days. Measurements were averaged within a day to meet the assumptions of normality (i.e. central limit theorem).

2.4. Validating temperature and humidity

To determine the relationship between location in the gutters and temperature, time of day and temperature, and humidity and temperature, we monitored the substrate surface temperature and humidity of seven randomly selected apparatuses over time using five equally spaced Thermochron iButtons® (Maxim Integrated Products, Inc.) and five equally spaced Xintiandi™ Hygrometers (accuracy ± 5%) while no animals were in the apparatuses.

2.5. Statistical analyses

To test for a temperature and humidity gradient across the gutters, we regressed spatial location of the iButtons against the associated temperature and humidity measurements (using the *lm* function in R). To assure that shelf location and any associated variation in access to light did not influence results, we included shelf as a predictor in all analyses. To test for individual consistency in temperature variation within individuals and variation in body temperature preferences among individuals, we conducted a one-way repeated measures ANOVA blocking by shelf (using Statistica, Statsoft, Tulsa, OK). This analysis tested whether temperature preferences of individuals varied significantly across days (main effect of day) and whether temperature preferences varied among individuals (within-individual variance, s^2). Additionally, we calculated repeatability (Lessells and Boag, 1987), the proportion of the variance explained by the individual (Falconer and Mackay, 1995). For each analysis, residuals were normally distributed and met the assumptions of the analyses. Results are presented as mean ± 1 SE.

3. Results

Our apparatuses maintained an average thermal gradient between 12.0 and 33.4 °C (± 0.36 and 0.28 °C) (Fig. 2) across 135 cm with a mean daily range of 9.29–33.94 °C (± 0.08 and 0.01 °C) (mean room temperature: 21.16 ± 0.07 °C). While change in temperature was slightly more pronounced in the warmest 27 cm of the apparatus, the temperature gradient was generally even across the remaining length (Fig. 2). The saturated moss maintained humidity between 84.1% and 90.7% (± 0.65%) throughout the apparatuses (Fig. 2), essentially functioning as a wick, drawing moisture from the cool to warm end to maintain the constant humidity. In fact, although temperature significantly declined across the five iButton locations (χ^2=408.56, df=1, $p \leq 0.001$),

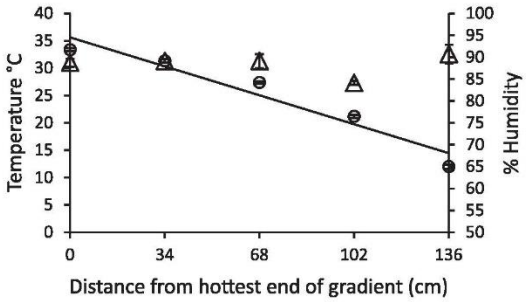

Fig. 2. Substrate surface temperature (circles and trend line) and humidity (triangles) gradients as a function of the distance from the warmest end of the apparatus. At the five equidistant locations receiving iButtons and hygrometers, average temperature ranged from 12.0 to 33.4 °C (±0.36 and 0.28 °C SE), whereas average humidity ranged from 84.1% to 90.7% (±0.65% SE). Temperature declined significantly as distance from the warmest end of the apparatus increased (χ^2=408.56, df=1, $p \leq 0.001$), but, there was no significant change in humidity across this temperature gradient (χ^2=0.16, df=1, p=0.69) nor was there a significant impact of location on humidity (χ^2−0.01, df−1, p−0.91). Points indicate means ± 1 standard error of seven replicates.

humidity did not significantly change across this temperature gradient (χ^2=0.16, df=1, p=0.69), nor was there a significant impact of location on humidity (χ^2=0.01, df=1, p=0.91; Fig. 2).

Over the course of a 12-h period, melting ice packs only moderately altered the temperature of the coldest third of the apparatuses (they experienced an average 4.12 ± 0.31 °C shift in temperature twice daily; Fig. 3a). These daily temperature fluctuations likely did not affect temperature preferences as the remaining two thirds of the apparatuses were not impacted and the thawing only altered temperatures for a very short amount of time. Both within and across days, temperature fluctuations in the apparatuses were minimal (Fig. 3a and b). Mean temperature preferences of individual *A. terrestris* ranged from 22 to 27 °C with a mean (± SE) overall preference of 23.8 °C (± 0.17 °C; Fig. 4a) and *O. septentrionalis* ranged from 19 to 27 °C with a mean (± SE) overall preference of 22.8 °C (± 0.50 °C; Fig. 4b). Importantly, using our apparatuses, we were able to detect consistency in the temperature preference of individual *A. terrestris* and *O. septentrionalis* (*A. terrestris* main effect of day: F=1.32, df=3, p=0.27; *O. septentrionalis* main effect of day: F=0.26, df=3, p=0.86) but variation in temperature preferences among *A. terrestris* (within-individual variance: s^2=17.5; repeatability: r=0.99; Fig. 4a) and *O. septentrionalis* (within-individual variance: s^2=9.5; repeatability: r=0.99; Fig. 4b). We also determined that there was no main effect (F=0.782, df=2, p=0.466) or interacting effect of shelf location on body temperature preference over time (F=0.357, df=5, p=0.838), therefore, we dropped shelf from the model.

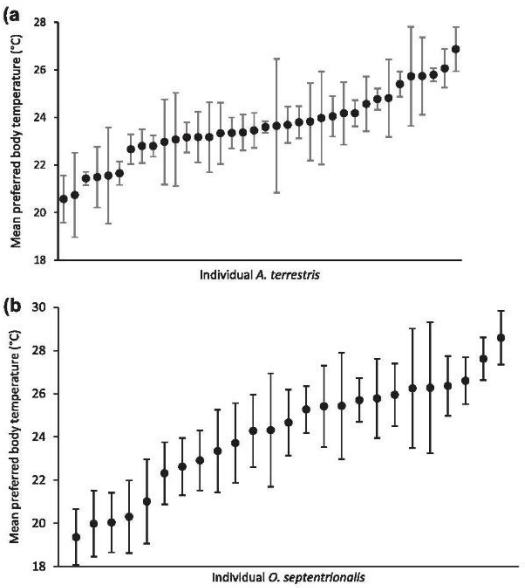

Fig. 4. Plot of individual (a) *Anaxyrus terrestris* (n=36) and (b) *Osteopilus septentrionalis* (n=25) preferred body temperature. *A. terrestris* and *O. septentrionalis* mean preferred body temperatures were 23.8 °C (± 0.17 °C) and 22.8 °C (± 0.50 °C), respectively. Individuals exhibited consistency in their preferred body temperature (main effect of day: F=1.32, df=3, p=0.27 and F=0.26, df=3, p=0.86) but there was significant variation among individuals in preferred body temperature (within-individual variance and repeatability: s^2=17.5, r=0.99 and s^2=9.5, r=0.99).

4. Discussion

Here we offer a validated, inexpensive, and efficient way to

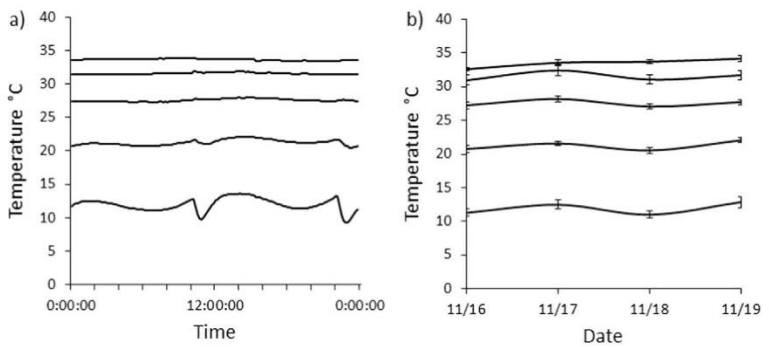

Fig. 3. Variation in substrate surface temperature within and across gutters and within and across days. **(a)** Mean substrate surface temperature for five equally spaced positions in the apparatuses (n=7) over 24 h. The variation in the curves in the coldest section of the apparatuses is a product of the ice packs melting and being replaced every 12 h, which shifted temperature 4.12 °C (± 0.31 °C SE) twice daily. **(b)** Mean (± SE) temperature for five equally spaced positions in the apparatuses (n=7) over 4 days, showing the consistency in measurements at each location. There was no significant effect of date on temperature across the gradient (F=0.03, df=3, p=0.88).

quantify the long-term thermal preferences of animals while avoiding moisture and feeding confounders. We tested *A. terrestris* and *O. septentrionalis* preferred body temperatures using a novel design for a thermal gradient apparatus. We found that the apparatus functioned well, with apparatus temperatures, temperature variation, and humidity being relatively uniform over time. As further evidence for apparatus functioning, variation in the preferred body temperatures of *A. terrestris* and *O. septentrionalis* within individuals was minimal compared to variation among individuals. Being able to quantify individual consistency and variation among individuals in preferred body temperatures is a pre-requisite for quantifying temperature preferences and behavioral thermoregulation. Additionally, variation among individuals within a population is the raw material on which selection acts, thus, measuring this variation is critical for predicting how populations might adapt to climate change (Rowley and Alford, 2013). The design detailed here could be used for a variety of thermal ecology applications.

Nearly every aspect of our design can be easily modified to meet the needs of individual researchers. Temperature range of the apparatuses can be shifted or expanded by altering the heating and cooling sources. The thermostat used in these experiments can decrease the temperature of the heat tape down to room temperature and, with alternative thermostats, Flexwatt heat tape can reach temperatures over 40 °C. Additionally, the cooler end of the gradient could be maintained at a more stable temperature using a cold water cooling system or by replacing the ice packs more frequently to maintain temperatures closer to our minimal temperature of 9.3 °C. Because our apparatuses are set up on shelves, there may be slight differences in light intensity across shelves. Any differences in light intensity can be dealt with by adding additional lighting to each shelf or by randomly distributing individuals across shelves and including shelf as a block in any subsequent statistical analyses. The most substantial drawback to this design is the labor intensive nature of the temperature measurements, given that an infrared heat gun is used to noninvasively record each measurement. More invasive forms of temperature measurements that log body temperature continuously (e.g. surgically implanted monitors) might be more appropriate for some studies and our design can also accommodate these forms of measurements.

Depending on the physiology of the focal animals, apparatus humidity can easily be modified. Although we kept humidity relatively high to accommodate the needs of amphibians, alternative substrates can easily be used to accommodate a wide variety of focal taxa (e.g. cotton, sand, soil, mulch, or paper towels), such as arthropods, lizards, snakes, and even small mammals. Because of the duration of the trials, we found it necessary to feed our test animals. The feeding containment bag design we employed is simple and easy to replicate. All of the *A. terrestris* and *O. septentrionalis* in our study fed successfully in their feeding containers, finishing 78.9% ($\pm$0.33% SE) and 75.7% ($\pm$0.02% SE) of their crickets within the seven-hour feeding period. If needed, the containers could be easily cleaned and bleached for reuse, thereby facilitating use in studies testing for behavioral fever in response to infections (Blanford and Thomas, 1999).

The expansive application potential of our thermal gradient apparatuses, coupled with the straightforward, effective, and affordable design, makes them ideal for measuring thermoregulatory behavior. Unlike most thermal gradient apparatuses used in thermal biology studies (Klein et al., 1992; Burns et al., 1996; Zdanovich, 2006; Lourdais et al., 2013; Lara-Reséndiz et al., 2015), we were able to create a broad gradient representing an ecologically relevant temperature range, control for humidity, avoid feeding confounders, avoid invasive temperature measurement techniques, and maintain animals for substantial time periods. While we did not control for spatial preferences within the apparatuses in our validation trials, testing for temperature preferences while controlling for spatial distribution within each apparatus could easily be done by alternating the direction of the temperature gradient on each shelf or by including identical apparatuses held at a constant temperature. The overall cost of each thermal preference apparatus was $48.20 USD (see Table S1 for a list and cost breakdown of all the supplies). Additionally, we were able to capture individual-level preferred body temperatures of 36 *A. terrestris* and 25 *O. septentrionalis* over the course of three weeks and have used the apparatuses for additional studies, successfully detecting host behavioral fever responses to pathogens (unpublished).

Body temperature is remarkably influential to almost every facet of physiological performance in both endothermic and ectothermic organisms (Huey and Kingsolver, 1989; Angilletta et al., 2010). The importance of understanding thermoregulation and thermal biology will only increase as organisms face new anthropogenic stressors and threats, such as climate and land use change (Rohr et al., 2011b; Rohr and Palmer, 2013). Hence, we believe our method for measuring thermoregulation will facilitate future research in the continually expanding field of thermal biology.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jtherbio.2016.07.016.

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Appendix B: Supplementary Materials: Chapter One

Supplementary Figures

Figure B1. Feeding containment apparatus. Feeding containment apparatus with one adult *Osteopilus septentrionalis* for size comparison.

Supplementary Tables

Table B1. Cost and source of materials. All the materials needed to build the thermal gradient apparatuses are included. Each apparatus costs $40.91 USD, the feeding apparatuses cost $0.24 USD each, and the IR thermometer costs $179.99. In total the entire set up costs $48.20 per apparatus.

	Quantity	Cost/unit	Total	Source
Construction of the apparatuses				
Downspout gutter (3.048 cm)	8	13.37	106.96	Lowes
Window sill insulation (8.9 cm x 22.86 m)	5	5.95	29.75	Lowes
Rubber weatherseal (0.95 cm x 5.18 m)	14	9.87	138.18	Lowes
Plexiglass (1.54 x 1.83 m)	4	65.97	263.88	Lowes
Styrofoam sheet (1300 cm^2)	1	6.93	6.93	Amazon Prime
100% silicon caulk (4-pack)	1	17.89	17.89	Lowes
Cord locks (100-pack)	3	9.25	27.75	Etsy (scrubhatlady)
Twine (60.96 m)	1	2.08	2.08	Amazon Prime
Sphagnum moss (2.56 cm^3)	10	15.88	158.80	Lowes
10-watt FlexWatt heat tape (7.62 cm x 22.86 m)	1	169.85	169.85	ABDragons
Bulb-and-capillary thermostat	3	28.85	86.55	Selco Products
Lead-free electronics solder	1	4.12	4.12	Amazon Prime
Soldering iron	1	9.00	9.00	Amazon Prime
Liquid electrical tape (4 oz)	1	5.99	5.99	Amazon Prime
Electrical tape	2	0.72	1.44	Lowes
Duct tape (41.15 m roll)	1	4.98	4.98	Lowes
Non-polarized wire (76.2 roll)	1	34.17	34.17	Lowes
2-prong plugs	3	1.10	3.30	Lowes
Terminal wire connectors (12 pack)	1	2.98	2.98	Lowes
Pine sheathing (plywood)	2	7.95	15.90	Lowes
32 oz no-sweat ice packs (case of 18)	6	9.18	55.08	Temperatsure
Total cost for thermal gradient apparatuses:		1145.58		
Cost per apparatus:			40.91	
Feeding apparatuses				
Quart sized zip lock bags (100 pack)	1	8.48	8.48	Amazon Prime
Plastic coated paper clips (1500 pack)	1	5.65	5.65	Amazon Prime
Packing tape (4-pack)	1	9.81	9.81	Amazon Prime
Total cost for feeding apparatuses:		23.94		
Cost per apparatus:			0.24	
Thermometer				

IR thermometer	1	179.99	179.99	Forestry Suppliers
Total one-time cost for permanent set up:			1349.51	
Total cost per apparatus:			**48.20**	

Appendix C: Supplementary Materials: Chapter Two

Supplementary Figures

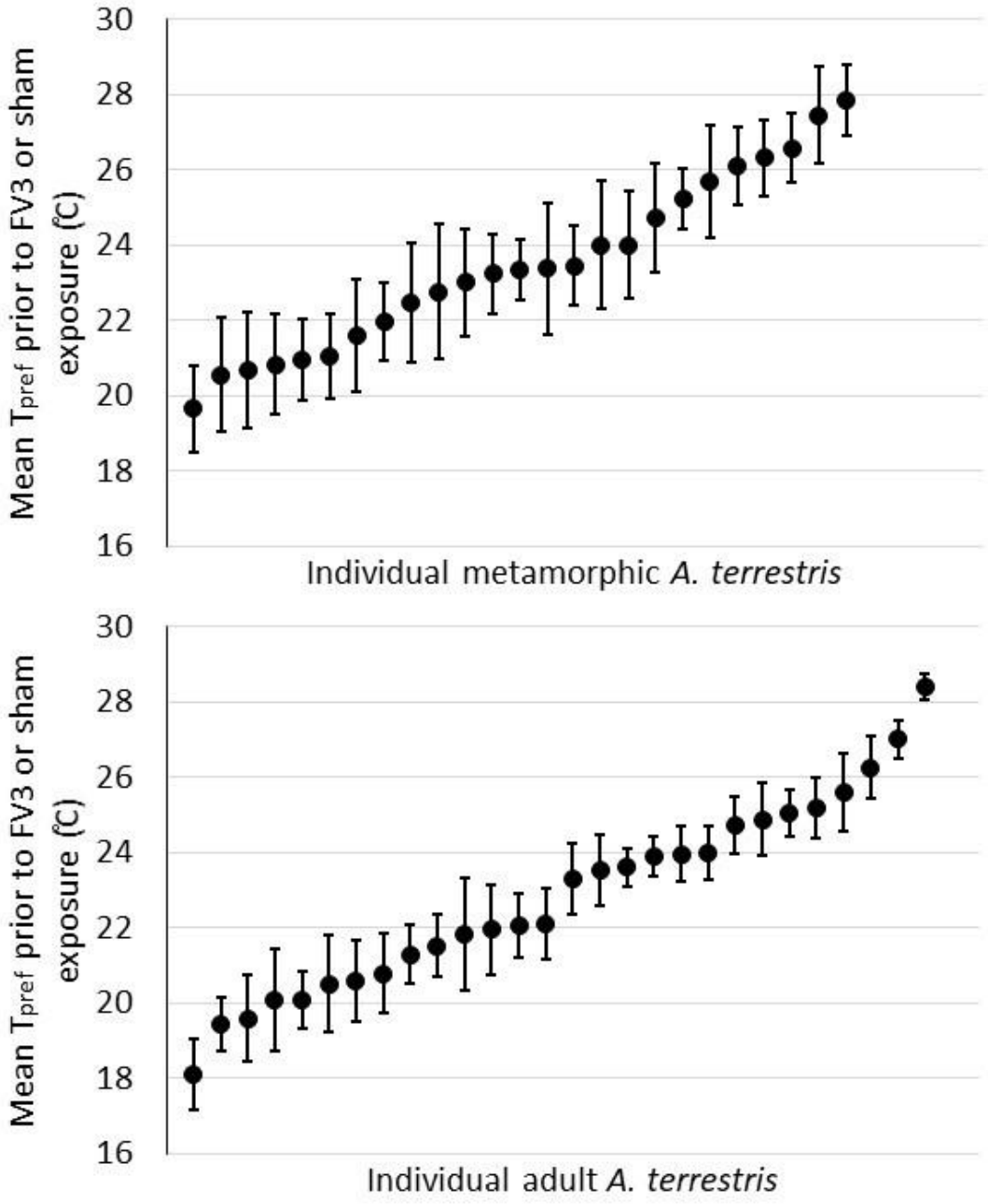

Figure C1. Individual level temperature preferences. Plot of individual A) metamorph (n=25) and B) adult (n=28) *A. terrestris* prior to FV3 or sham exposure. Error bars represent ±1 SE. We were able to detect consistency in the baseline Tpref of individuals (repeatability: r > 0.98)

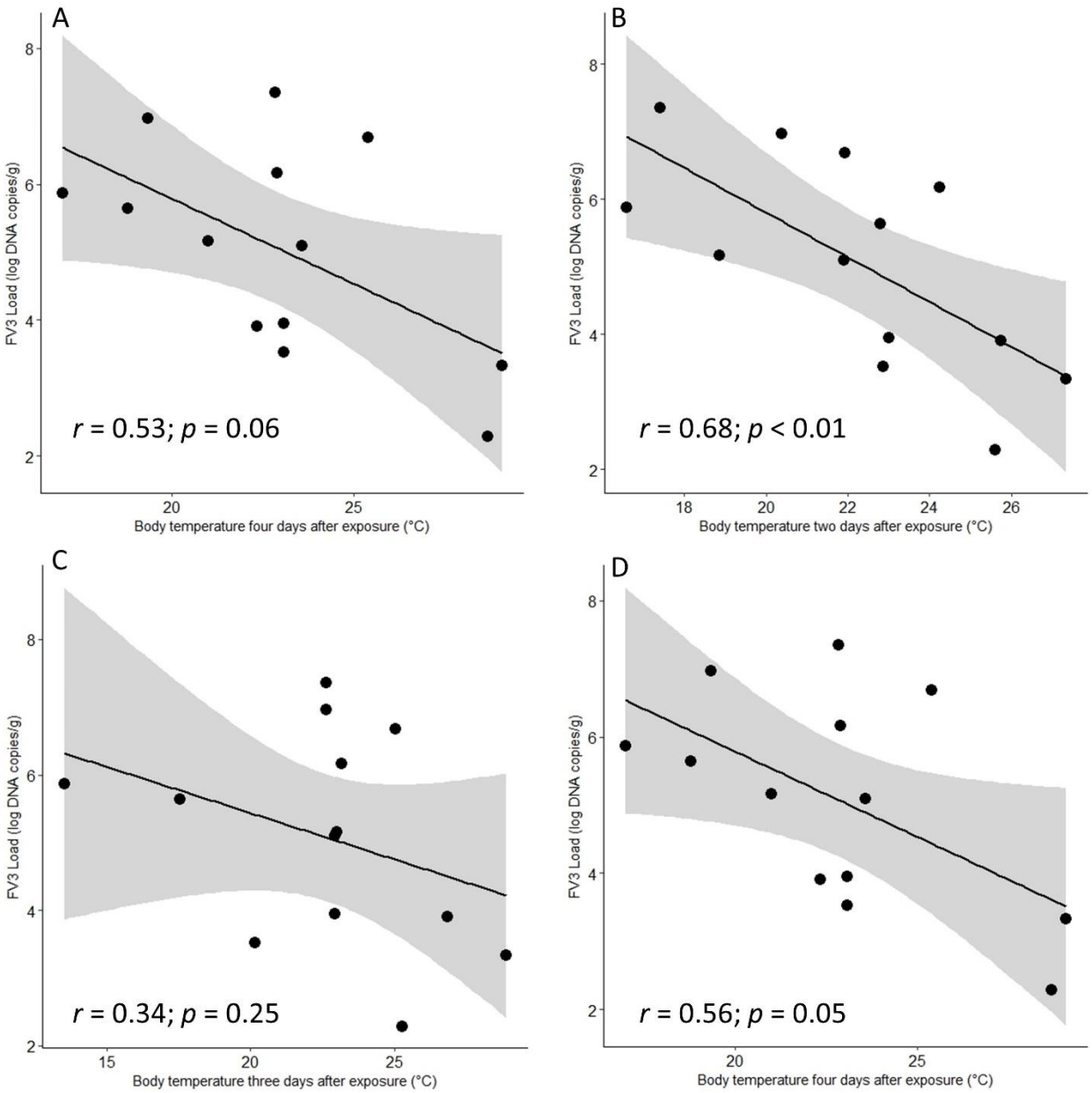

Figure C2. Effect of mean body temperature on FV3 load A) one, B) two, C) three, and D) four days after exposure to FV3 in adult *Anaxyrus terrestris* four days after exposure.

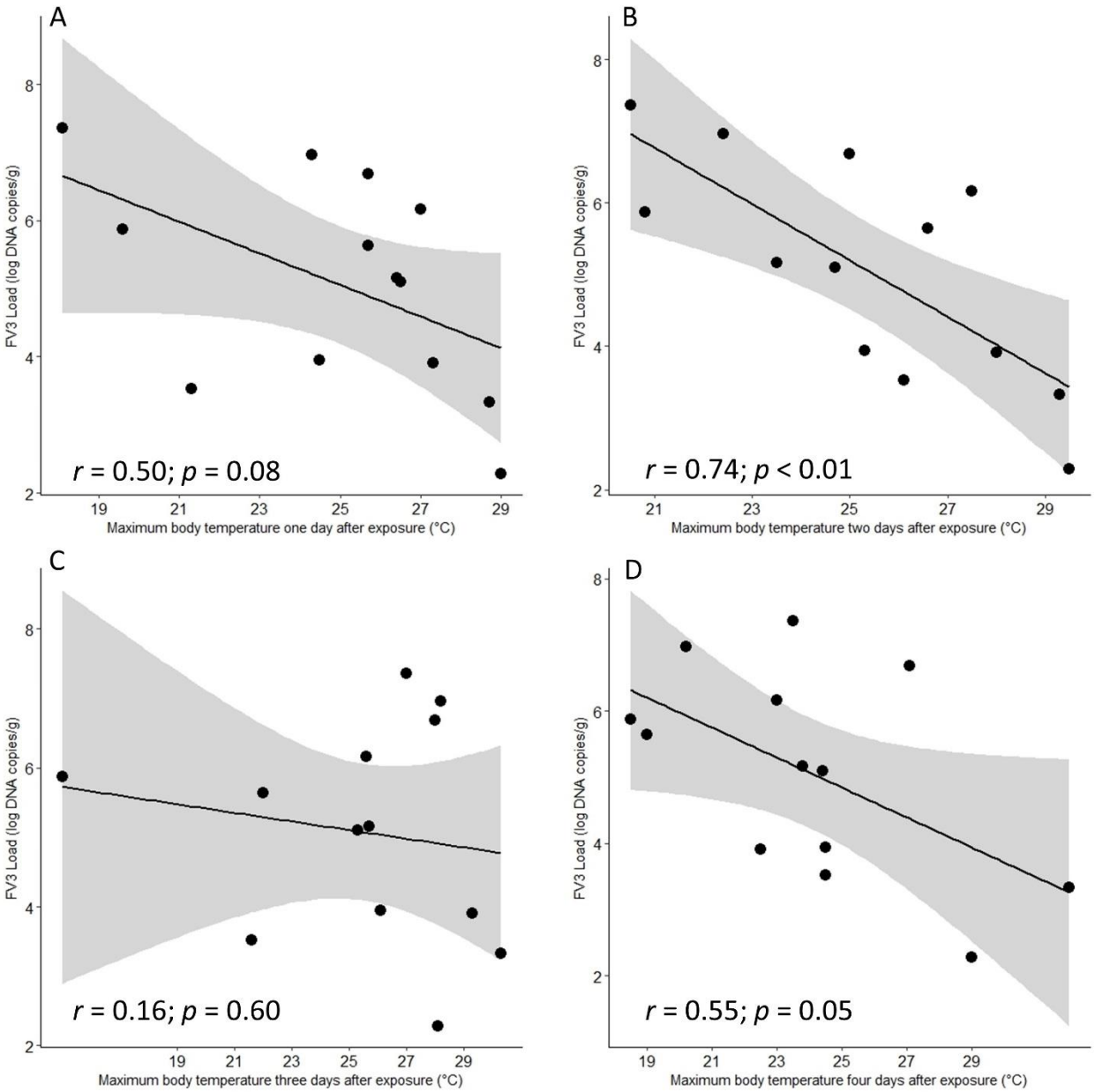

Figure C3. Effect of maximum body temperature on FV3 load A) one, B) two, C) three, and D) four days after exposure to FV3 in adult *Anaxyrus terrestris* four days after exposure.

Figure C4.

Figure C4. Feeding containment apparatus with one adult *Osteopilus septentrionalis* for size comparison. Constructed of quart-size zip-top bags with plastic coated paper clips adhered to the outside for structure. Figure and caption adapted from Sauer et al. (2016).

Supplementary Tables

Table C1. Test statistics for the effect of the interaction between two treatment (Bd exposed or sham exposed) and time (before exposure and day after exposure) on change in temperature preference ($\Delta T_{pref} = T_{pref_{i,j}} - \overline{T_{pref_{baseline,i,j}}}$) on days 1-5 after treatment exposure. The Tpref data were standardized using z-score to compensate for any effect of changing room temperature on the experiment. Significance is denoted by *

Block	Day	χ^2 value	*p* value	
Metamorph	1	22.01	0.001	*
	2	0.00	0.994	
	3	0.16	0.692	
	5	5.51	0.019	*
Adult	1	5.70	0.017	*
	2	2.29	0.130	
	3	3.49	0.062	
	4	1.24	0.266	

Table C2. Regression results for the effect of change in temperature preference ($\Delta T_{pref} = T_{pref_{i,j}} - \overline{T_{pref_{baseline,i,j}}}$), mean body temperature, and maximum body temperature on day two after FV3 exposure on FV3 loads in adult *Anaxyrus terrestris* on day four after exposure. Significance is denoted by *

	Day	*df*		*F* value	*p* value	
ΔTemp	1	11		-3.19	0.0086	*
	2	11		23.90	0.0005	*
	3	11		5.86	0.0340	*
	4	11		14.31	0.0030	*
Body Temp	1	11		4.37	0.0605	
	2	11		9.68	0.0099	*
	3	11		1.47	0.2510	
	4	11		5.03	0.0465	*
Max Temp	1	11		3.65	0.0824	
	2	11		13.02	0.0041	*
	3	11		0.29	0.5982	
	4	11		4.75	0.0519	

Appendix D: Chapter Three Reprint

PROCEEDINGS B

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Research

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Variation in individual temperature preferences, not behavioural fever, affects susceptibility to chytridiomycosis in amphibians

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The ability of wildlife populations to mount rapid responses to novel pathogens will be critical for mitigating the impacts of disease outbreaks in a changing climate. Field studies have documented that amphibians preferring warmer temperatures are less likely to be infected with the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*). However, it is unclear whether this phenomenon is driven by behavioural fever or natural variation in thermal preference. Here, we placed frogs in thermal gradients, tested for temperature preferences and measured *Bd* growth, prevalence, and the survival of infected animals. Although there was significant individual- and species-level variation in temperature preferences, we found no consistent evidence of behavioural fever across five frog species. Interestingly, for species that preferred warmer temperatures, the preferred temperatures of individuals were negatively correlated with *Bd* growth on hosts, while the opposite correlation was true for species preferring cooler temperatures. Our results suggest that variation in thermal preference, but not behavioural fever, might shape the outcomes of *Bd* infections for individuals and populations, potentially resulting in selection for individual hosts and host species whose temperature preferences minimize *Bd* growth and enhance host survival during epidemics.

1. Introduction

Increases in emerging infectious diseases over the last few decades have caused global declines in biodiversity [1,2]. Anthropogenic global climate change is predicted to influence human and wildlife disease dynamics worldwide, possibly exacerbating these disease-driven declines [3,4]. One reason that climate change might affect disease dynamics is because the infectivity and virulence of pathogens, as well as host resistance and tolerance of infection can vary with climatic conditions [5]. This is especially true for ectothermic hosts, which have only a limited ability to regulate body temperature independent of environmental temperatures and can struggle to combat stressors, such as disease, when exposed to sub-optimal temperatures [6–8]. Additionally, individual ectothermic hosts can vary in their preferred temperatures, which can affect their susceptibility to infections [9]. Hence, epidemics could select for host individuals and species that inherently prefer temperatures that facilitate tolerance and/or resistance to pathogens, a process that would occur across generations [9,10].

Hosts can also cope with pathogens using plasticity, which is a change in host physiology (e.g. acquired immunity), morphology, or behaviour during the life of the host, and thus occurs within rather than across generations. For instance, upon infection, ectothermic hosts could modify their temperature preferences

(via behavioural thermoregulation), selecting environmental temperatures that are unfavourable for the parasite, ideal for host defences, or both. Ideally, this plasticity in response to infection should be differentiated from preferred temperatures in the absence of infections. Understanding the extent to which host populations can mount rapid plastic responses to pathogens might be critical for predicting the impacts of continued widespread disease outbreaks in a changing climate.

Many ectothermic hosts exhibit a type of plasticity called behavioural fever, which is when a host increases its temperature preference (T_{pref}) in response to pathogen exposure [11–13]. Behavioural fever has most commonly been documented in response to bacterial and viral pathogens, which tend to grow well at high temperatures [14]. In these cases, behavioural fever tends to increase host immune responses, which is believed to provide a net benefit to the host despite the increased pathogen growth at the higher temperature [14]. If behavioural fever is effective against thermophilic pathogens, it might be even more effective against psychrophilic (cold-loving) pathogens because the higher temperatures might both stimulate host immunity and be directly detrimental to pathogen growth.

An example of a relatively cold-tolerant pathogen is the fungus *Batrachochytrium dendrobatidis* (*Bd*). *Bd* causes the disease chytridiomycosis, is associated with global amphibian declines [7,15], grows best in culture under cool conditions between 18°C and 22°C, and can be cleared from some hosts when held above 25°C for extended periods of time [16–19]. In fact, field studies have documented little to no *Bd* in populations associated with hot springs and relatively warm low-elevations, even when surrounding or adjacent high-elevation populations have high prevalence [20–22].

Not surprisingly, several studies suggest that *Bd* dynamics are influenced by temperature [16,19,23,24], but whether amphibians respond to *Bd* with behavioural fever in the field and laboratory remains controversial. Multiple field studies correlating amphibian body temperature and *Bd* infection have shown that individual amphibians with higher body temperatures are less likely to be infected with *Bd* relative to individuals with lower body temperatures within the same population [9,21,25]. One hypothesis for this pattern is that some but not all individuals preferred microhabitats with temperatures that were unfavourable for *Bd*, regardless of whether they were infected [9]. By contrast, other researchers have hypothesized that these field patterns were the result of amphibians intentionally moving to warmer microhabitats to resist infection (i.e. behavioural fever) [25]. Two laboratory experiments tested for *Bd*-induced behavioural fever and reported mixed results. The first experiment found no evidence of *Bd*-induced behavioural fever in toad tadpoles [26]. The second study claimed to have provided evidence for *Bd*-induced behavioural fever in adult amphibians, but it had low statistical power and consequently could not conclusively support or rule out a behavioural fever response [27].

These conflicting laboratory and field results might be partly a product of the effectiveness of pathogen defences of some host species not increasing with temperatures. For example, the thermal mismatch hypothesis predicts that host species adapted to warmer temperatures might perform more poorly than the pathogen at cool temperatures, and vice versa, creating a scenario where warm- and cool-adapted hosts most often experience outbreaks at cool and warm temperatures, respectively [24,28]. There is support for this hypothesis in the amphibian-*Bd* system [24].

Here, we attempt to address the controversy regarding whether anuran amphibians tend to adjust their preferred temperature when infected with *Bd*. Our goals were to determine if: (i) there was individual-level variation in T_{pref} within the tested species, (ii) there were correlations between T_{pref} and *Bd* growth within and among the tested species of frogs, (iii) there was any support for the thermal mismatch hypothesis, and (iv) any tested amphibian species changed their T_{pref} in response to *Bd* exposure. To accomplish these goals, we exposed five species of adult frogs (Cuban tree frogs, *Osteopilus septentrionalis*, southern toads, *Anaxyrus terrestris*, Panamanian golden frogs, *Atelopus zeteki*, northern cricket frogs, *Acris crepitans*, and American toads, *Anaxyrus americanus*) to *Bd* in thermal gradients ranging in temperature from 9°C to 34°C [29] to assess individual T_{pref} before and after *Bd* exposure. We also measured *Bd* growth on individuals over time to assess whether any variation in T_{pref} affected *Bd* growth.

2. Methods

(a) Thermoregulation experiments

Experiments were conducted at the three locations: *O. septentrionalis* and *An. terrestris* experiments took place in Tampa, FL, *An. americanus* and *Ac. crepitans* experiments took place in Champaign, IL, and *At. zeteki* experiments took place in New Orleans, LA. See the electronic supplementary material, methods for details regarding animal collection and maintenance as well as protocols regarding *Bd* exposures and measuring *Bd* growth on hosts. In each experiment, we first measured individual baseline non-infected T_{pref} in thermal gradient apparatuses. All species except for *At. zeteki* (thermal gradient range: 19°C to 38°C; see the electronic supplementary material, methods for more details and description) were in thermal gradient apparatuses that were previously shown to provide variation in temperature that is independent of moisture/humidity and which does not confound amphibian and prey temperature preferences (12°C to 33°C see the electronic supplementary material, figure S4 and methods; and Sauer *et al.* [29] for thermogradient construction and validation details). After measuring non-infected T_{pref}, individuals were split into three treatment groups with similar mean body masses and non-infected T_{pref}: (i) a sham-exposed control group that was allowed to thermoregulate, (ii) a *Bd*-exposed group that was allowed to thermoregulate, and (iii) a *Bd*-exposed non-regulating group where each individual was held at their individual preferred body temperature (*O. septentrionalis*), at the population-level temperature preference (*Ac. crepitans*, *An. americanus*, *An. terrestris*), or at acclimation temperature (*At. zeteki*) by transferring them to temperature-controlled Styrofoam incubators (electronic supplementary material, figure S6) or environmental chambers (see the electronic supplementary material, methods).

Throughout the experiment, temperature measurements were taken each day, every four hours, four times a day, between 08.00 h and 22.00 h using an infrared thermometer [30] (Micro-Epsilon ThermoMeter LS (accuracy: ±0.75%) for *At. zeteki* and an Extech® High Temperature IR Thermometer (accuracy: ±2% < 932°F) for all other species) from the centre of each animal's dorsum [30] and from the substrate adjacent to the animal, except for during feeding periods (see the electronic supplementary material, methods for details on feeding). Temperature measurements were taken for at least four days before *Bd* or sham exposure and for at least two weeks after these exposures. Experiments were conducted using multiple temporal blocks to ensure adequate sample sizes (see the electronic supplementary material, table S2 for sample sizes for each temporal block in each experiment).

Osteopilus septentrionalis has previously been shown to acquire immunological resistance to *Bd* after a previous exposure and

clearance [17], so we tested whether this species could acquire the ability to exhibit a behavioural fever response to *Bd*. We exposed half of the *O. septentrionalis* to *Bd* and half to a sham inoculate, held all individuals at 23°C for 10 days, and then shifted all frogs to 30°C for 14 days for heat clearance [16]. After confirming that all individuals were uninfected, we proceeded with the T_{pref} trials previously described but with six treatments, *Bd*-naive versus *Bd*-experienced animals crossed with the three treatment groups previously described (mean $n = 6$, $N = 37$).

We were concerned that, by placing frogs into the thermal gradients immediately after *Bd* inoculations, they could quickly select a high temperature to clear the infection before it successfully established. Consequently, we conducted a separate experiment on *An. terrestris*, where individuals received *Bd* or sham exposures. We then held them at 17°C for 7 days to ensure that there was *Bd* establishment followed by considerable pathogen population growth, and then placed them into the thermogradients to test for behavioural fever as described above.

(b) Data analysis

All statistics were conducted with R 3.4.0 [31]. To test for repeatability within individuals in T_{pref} and variation in T_{pref} among individuals before infection, we conducted a one-way repeated measures ANOVA (*stats* package, *aov* function). This analysis tested whether temperature preferences of individuals varied significantly across days (main effect of day) and whether temperature preferences varied among individuals (within-individual variance, s^2). Additionally, we calculated repeatability (see the electronic supplementary material, methods for formula), the proportion of the variance explained by the individual [32].

We used a weight of evidence approach to test for behavioural fever across species (three-factor: treatment, time and species) and within species (two-factor: treatment and time) we conducted multiple repeated measures ANOVAs with individual treated as a random variable (*stats* package, *aov* function, assuming normal error distribution). For each model, we paired all pre-exposure days with each post-exposure day (time; one model for each post-exposure day) and looked for an interaction between treatment and time on ΔT_{pref} (the difference between mean pre-exposure T_{pref} of each animal and its T_{pref} at each time point). We then assessed significance using the Benjamini–Hochberg (B-H) procedure [33].

We also tested for an effect of infection intensity (log-transformed *Bd* load divided by mass of the individual) on ΔT_{pref} (difference between mean pre-exposed T_{pref} and T_{pref} during the 24 h after being swabbed) on *At. zeteki* and *An. terrestris* by conducting a linear mixed-effects model with individual as a random effect (*nlme* package, *lme* function). Individual-level *Bd* growth rates for *An. terrestris* were determined by first calculating infection intensity by dividing *Bd* loads (DNA copies) by individual mass, then log transforming infection intensity, then extracting the slope parameter from a generalized linear model of each individual's infection intensity over time (*stats* package, glm function; time in days). *Bd* growth rates for *At. zeteki* were determined by first calculating log infection intensity using the aforementioned methods then extracting the growth parameter from a logistic growth model of each individual's infection intensity over time (*bbmle* package, *mle2* function; time in weeks; see the electronic supplementary material, methods for model). Growth models for each species were chosen based on a visual examination of the shape of *Bd* load data over time. To test the influence of individual-level T_{pref} on *Bd* growth, we conducted a linear regression with the previously calculated *Bd* growth rates as the response and an individual's mean T_{pref} for the 7 days following *Bd* exposure as the predictor (*stats* package, glm function). To test for differences in *Bd* intensity (main effect of treatment) and growth (interaction between treatment and time) between regulating and non-regulating exposed treatments over time, we conducted a two-factor (treatment and time) ANOVA with individual included as a random effect (*nlme* and *stats* packages, lme function). We also ensured there was no effect of body mass on T_{pref} by conducting a one-way repeated measures ANOVA for these two species.

Additionally, we tested for reductions in *Bd* prevalence over time. To do this, we calculated prevalence for all species using animals from the *Bd*-exposed treatment and then ran a one-way ANOVA for each species separately to determine if there was a significant change in prevalence from week 1 to week 2. We also ran a two-factor (species and treatment) ANOVA for each of the two weeks followed by Tukey's *post hoc* multiple comparison tests to assess differences in prevalence between species and treatments (regulating or non-regulating) (stats package, Tukey HSD function). Tukey's *post hoc* multiple comparisons tests were also used to assess differences when a treatment had more than two levels (*multcomp* package, glht function). Finally, to test for differences in survival among treatments, we conducted a Cox-proportional hazards model (*survival* package, coxph function).

3. Results

(a) Temperature preferences across individuals and species

Before *Bd* exposure, we were able to detect consistency in the T_{pref} of individuals (repeatability: $r > 0.90$ for all species; electronic supplementary material, table S1) and variation in temperature preferences among individuals (electronic supplementary material, table S1) and across species ($F_{4,158} = 6.82$, $p < 1.0 \times 10^{-4}$). *Atelopus zeteki* (mean T_{pref}: 20.8°C ± 0.65 s.e.) and *An. americanus* (21.3°C ± 0.43) preferred significantly cooler temperatures than *Ac. crepitans* (23.4°C ± 0.61) and *An. terrestris* (23.5°C ± 0.65). *Osteopilus septentrionalis* (22.5°C ± 0.70) preferred moderate temperatures and was not significantly different from any other species (figure 1). To ask whether these T_{pref} might be an artefact of differences in acclimation temperature, we tested for a correlation between acclimation temperature and species-level T_{pref} and found no trend ($t_4 = 0.60$, $p = 0.59$), but the power of this analysis is admittedly low.

(b) Behavioural fever

When we adjusted our alpha for multiple comparison tests, we found no evidence of behavioural fever after exposure to *Bd* for the omnibus test across species (interaction between treatment and time, $p <$ B-H critical value; figure 2*a*; electronic supplementary material, figure S1 and table S2). If we looked at individual species, we found no evidence of behavioural fever or shifts in T_{pref} for *An. americanus*, *An. terrestris*, or *At. zeteki* (interaction between treatment and time $p >$ adjusted threshold; figure 2*a*; electronic supplementary material, figures S1 and S2 and table S2). There were some days with significant interactions between treatment and time for *O. septentrionalis* (days 3 and 10 for the treatment group were significantly warmer; electronic supplementary material, figure S2 and table S2) and *Ac. crepitans*. For *Ac. crepitans*, the control frogs preferred significantly warmer temperatures than the *Bd*-exposed frogs, (days 6–11, 13, 17; electronic supplementary material, figure S1 and table S2), which is inconsistent with behavioural fever. Additionally, infection intensity had no effect on T_{pref} in the species where quantitative PCR was conducted (main effect of intensity on T_{pref} for *An. terrestris*: $\beta = 0.06$, $p = 0.38$ and *At. zeteki*: $\beta = 0.03$, $p = 0.44$). Despite evidence that *O. septentrionalis* can acquire immunological resistance to *Bd* after previous clearance of infections [18], previous exposure to *Bd* did not alter the T_{pref} of *O. septentrionalis* when infected

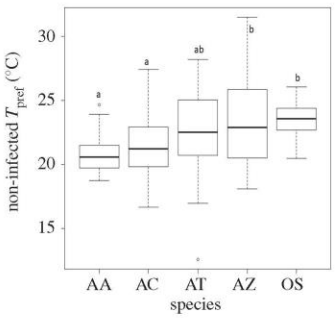

Figure 1. Temperature preferences (T_{pref}) for *Atelopus zeteki* (AZ), *Anaxyrus americanus* (AA), *Osteopilus septentrionalis* (OS), *Acris crepitans* (AC), and *Anaxyrus terrestris* (AT) prior to *Batrachochytrium dendrobatidis* exposure. Species marked with the same letter do not have significantly different T_{pref} based on a Tukey's HSD multiple comparison test ($p > 0.05$). Centre lines represent medians, boxes are first and third quartiles, and whiskers are highest and lowest points.

with *Bd* a second time (figure 2*b*; electronic supplementary material, figure S2 and table S3).

(c) *Batrachochytrium dendrobatidis* abundance and disease susceptibility

For thermoregulating *An. terrestris* and *At. zeteki*, we found that individual T_{pref} during the first week after *Bd* exposure had a significant effect on *Bd* growth rate in the thermal gradients over the course of the three week experiment. *Atelopus zeteki*, which preferred the coolest temperatures, showed a positive relationship between individual T_{pref} and *Bd* growth rate ($F_{1,11} = 4.73$, $p = 0.05$; figure 3*a*), indicating that *Bd* grew better on this species at warmer temperatures. *Anaxyrus terrestris*, which preferred the warmest temperatures, showed a negative relationship between individual T_{pref} and *Bd* growth ($F_{1,11} = 8.86$, $p = 0.01$; figure 3*b*). We also tested for an effect of mass on T_{pref} for these two species and found no effect (*At. zeteki*: $F_{1,26} = 1.02$, $p = 0.32$; *At. terrestris*: $F_{1,29} = 0.05$, $p = 0.82$). We were unable to calculate *Bd* growth rates for *O. septentrionalis* owing to low *Bd* prevalence.

There were no detectable differences in *Bd* loads or *Bd* growth rates between regulating and non-regulating *Bd*-exposed groups (*An. terrestris* main effect of treatment: $\beta = 0.78$, d.f. = 35, $p = 0.36$; interaction between treatment and time: $\beta = -0.22$, d.f. = 63, $p = 0.58$ and *At. zeteki* main effect of treatment: $\beta = 0.68$, d.f. = 24, $p = 0.42$; interaction between treatment and time: $\beta = 0.01$, d.f. = 24, $p = 0.94$; see the electronic supplementary material, figure S3). However, there were differences in prevalence across species and within species across weeks (figure 4). Two week prevalences ranged from 100% for *At. zeteki* to 0% for *O. septentrionalis*. For *At. zeteki*, prevalence remained a constant 100% between week 1 and 2 of the experiment, whereas for *Ac. crepitans* prevalence dropped from 89% to 27% over this time period (figure 4). *Atelopus zeteki* was the only species with substantial *Bd*-induced mortality and there was no significant difference in the survival curves between regulating and non-regulating treatment groups (100% and 100% mortality and 25.1 and 20.3 mean days alive, respectively; $\beta = 0.45$, $p = 0.08$; electronic supplementary material, figure S4). The maximum mortality for any of the other species was 15% in the non-regulating *An. americanus* (electronic supplementary material, figure S4).

4. Discussion

We set out to determine if the tested species of amphibians showed any individual- or species-level variation in T_{pref}, if variation in T_{pref} among individuals or species was correlated with *Bd* growth on frogs, whether relationships between T_{pref} and *Bd* growth were consistent with the thermal mismatch hypothesis, and if any of the tested species responded to *Bd* infections by increasing their T_{pref}. We were able to detect differences in T_{pref} among individuals within a species, as well as differences in T_{pref} across species. Our methods for testing T_{pref} were identical for all species but *At. zeteki* and we found no evidence that acclimation temperature impacted species-level T_{pref}. Moreover, given that *Ac. crepitans* was acclimated to the lowest temperature and had one of the highest preferred temperatures and *At. zeteki* was acclimated to one of the higher temperatures and had the lowest preferred temperature, any undetected effect of acclimation temperature was probably small relative to any inherent species-level differences in temperature preference. We demonstrated that individual-level T_{pref} was correlated with *Bd* growth on frogs and that differences in species-level T_{pref} predicted the direction of this correlation. Though there were some effects of treatment on T_{pref} in two of the five species, we were unable to detect a significant behavioural fever response to *Bd* exposure across species. Our experimental findings suggest that previously reported field patterns correlating body temperature with *Bd* infection [9,25,34] were probably owing to standing variation in T_{pref}, where frogs that preferred warmer temperatures were less likely to be infected because of reduced *Bd* exposure and/or reduced *Bd* growth. Our study, with experiments performed across three laboratories and five species, is probably the most comprehensive test for behavioural thermoregulatory responses to *Bd* exposure in amphibian hosts.

Importantly, for each species, we demonstrated that variation among individuals in T_{pref} was greater than the variation in T_{pref} within an individual through time. That is, there was variation among individuals in their T_{pref}. Individuals often found a suitable thermal microhabitat and continuously chose that preferred temperature, even after being moved to the centre of the gradient each night. This variation among individuals represents the raw material upon which natural selection can act. Assuming that T_{pref} is heritable [35] via genetic or maternal effects [36], it stands to reason that over time a selective sweep could eliminate some of this variation, resulting in a change in average T_{pref} and a decrease in *Bd* prevalence [19]. Other disturbances that select for T_{pref} or reduce thermal microhabitat availability, such as climate change, deforestation, or disease, might also lead to population-level shifts in thermal microhabitat selection [37,38].

Additionally, we confirmed previous findings by detecting differences in T_{pref} among species that probably reflect their adaptations to environmental temperatures [24]. For example, *At. zeteki* was our coolest-preferring species and, not surprisingly, it is native to cool, mid-elevation sites in Central America where daily air temperatures remain in the mid to low-twenties (°C) year round [25]. By contrast, *An. terrestris* was our warmest preferring species, and it is native to warm, low elevation sites in the southeastern United States where mean temperatures in the summer reach into the high-twenties with average daily highs in the low-thirties (°C) [24]. While this study used slightly different methods to measure T_{pref} across these two species, we previously published that *At. zeteki* might prefer even cooler

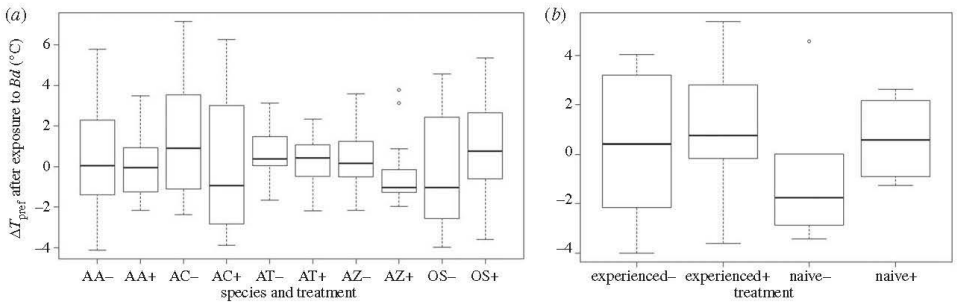

Figure 2. Change in temperature preferences (ΔT_{pref}) after exposure to *Bd* across all time points for *Atelopus zeteki* (AZ), *Anaxyrus americanus* (AA), *Osteopilus septentrionalis* (OS), *Acris crepitans* (AC), and *Anaxyrus terrestris* (AT) after frogs were (+) or were not (−) exposed to *Batrachochytrium dendrobatidis*: when all frogs were naive to *Bd* (*a*) or when half the OS were naive and half were previously exposed and cleared of *Bd* (*b*). Centre lines represent medians, boxes are upper and lower quartiles, and whiskers are highest and lowest points.

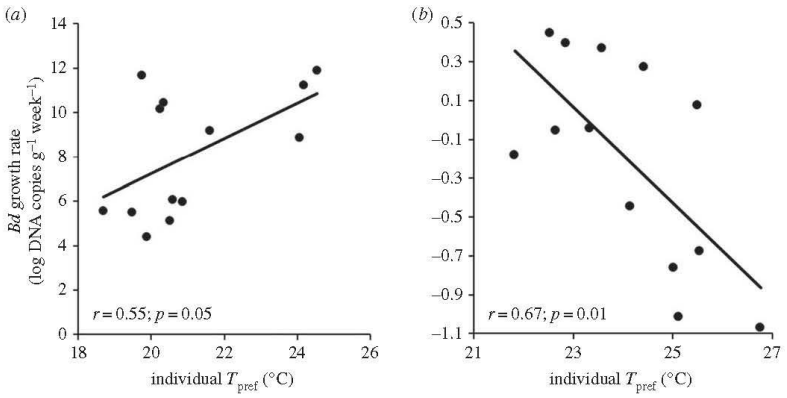

Figure 3. Relationship between individual-level temperature preference (T_{pref}) and *Batrachochytrium dendrobatidis* (*Bd*) growth on frogs for (*a*) *Atelopus zeteki* and (*b*) *Anaxyrus terrestris*. *Atelopus zeteki*, which preferred the coolest temperatures (figure 1), showed a positive relationship between *Bd* growth rate on individual hosts and host T_{pref} ($F_{1,11} = 4.73$, $p = 0.05$), indicating that *Bd* grew better on this species at warmer temperatures. By contrast, *Anaxyrus terrestris*, which preferred the warmest temperatures (figure 1), showed a negative relationship between *Bd* growth on individual hosts and host T_{pref} ($F_{1,11} = 8.86$, $p = 0.01$), indicating that *Bd* grew better on this species at cooler temperatures.

temperatures (T_{pref} 17.85 ± 0.14°C) [24] when tested using methods identical to those used for *An. terrestris* in this study. In this previous experiment, much lower minimum temperatures were available for *At. zeteki* to select (average low of 12°C compared to 19°C) than in the current experiment, which is probably why it had a lower temperature preference.

Although we experimentally tested for behavioural fever in both of the species that have been previously thought to respond to *Bd* exposure with fever (*At. zeteki and An. americanus*) [25,27], there was no evidence that those species or, for that matter, any of the five species exhibited a behavioural fever response to *Bd*. While our experimental results suggest that *At. zeteki* individuals which prefer warmer temperatures experience more rapid *Bd* growth, previous field studies showed that warmer *At. zeteki* were less likely to be infected with *Bd* than cooler preferring individuals in the population [25]. This inconsistency could be explained by differences in exposure given that *Bd* is considered saprophytic. In the absence of a host, *Bd* may persist better at low temperatures. If so, then *At. zeteki* which prefer warmer temperatures might have lower exposure to *Bd*. However, once exposed, *Bd* might grow faster on *At. zeteki* at higher than at lower temperatures.

We found that one species, *Ac. crepitans*, appeared to decrease preferred temperature after infection. The change in preferred temperature, however, did not appear to be beneficial to the host or pathogen as there was no difference in prevalence or survival between frogs in the regulating and non-regulating treatments. After prior exposure and heat clearance, individuals of *O. septentrionalis*, a species known to acquire immunological resistance to *Bd* [17], did not alter their thermoregulatory behaviour significantly. When we lumped the four treatments into exposed and sham-exposed, we did find that the *Bd*-exposed animals were warmer than the sham-exposed animals on day 3 and again on day 10. However, the day 3 differences were largely owing to the naive sham-exposed group sharply decreasing in temperature; there was no difference between the experienced sham-exposed and two *Bd*-exposed groups. Like the drop in

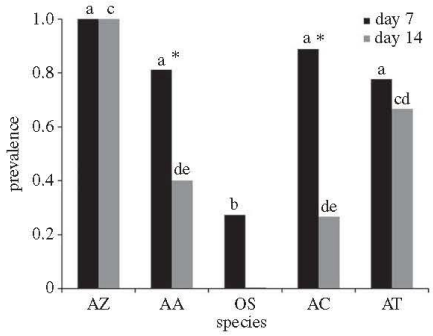

Figure 4. Prevalence of *Batrachochytrium dendrobatidis* (*Bd*) infection one and two weeks after pathogen exposure in *Atelopus zeteki* (AZ), *Anaxyrus americanus* (AA), *Osteopilus septentrionalis* (OS), *Acris crepitans* (AC), and *Anaxyrus terrestris* (AT) when they were free to roam in a temperature gradient. Within week means with different letters are significantly different from one another based on Tukey's HSD test ($p < 0.05$). Asterisks denote species that showed significant drops in prevalence from week 1 to week 2 based on an ANOVA (AA: $F_{1,22} = 6.33$, $p = 0.02$; AC: $F_{1,22} = 20.84$, $p < 0.0001$).

temperature preference observed for *Ac. crepitans*, this change in preferred temperature did not appear to be beneficial to the host or pathogen as there was no difference in prevalence or survival between frogs in the regulating and non-regulating treatments. Hence, both of these changes are possibly spurious and do not appear to be biologically significant. We also found that allowing *Bd* to grow on hosts for a week before introducing them to the thermal gradients had no effect on the likelihood of exhibiting behavioural fever.

Our results suggest that previous field associations between host temperatures and *Bd* abundance were probably a result of the pre-existing variation in T_{pref}, rather than a change in thermoregulatory behaviour in response to infection. That is, frogs which already preferred warmer temperatures were less likely to be infected because their warmer temperatures caused them to either experience reduced *Bd* growth or avoid *Bd* exposure altogether. These results do not suggest that amphibians are incapable of behavioural fever, only that the species of anurans we tested did not respond to *Bd* with a behavioural fever response. In contrast to fungi, viral and bacterial pathogens have been shown to induce behavioural fevers in amphibians [39,40] as well as other ectothermic vertebrate and invertebrate hosts [11,12]. Additionally, our study controlled for moisture to avoid confounding T_{pref} with moisture preference. Thus, we cannot draw any conclusions about amphibians attempting to resist *Bd* infection by 'drying-out', a strategy that could be as effective at as behavioural fever [41].

We demonstrated that differences in species-level T_{pref} could predict the direction of the correlation between T_{pref} and *Bd* growth. The coolest preferring species (*At. zeteki*) had high *Bd* growth rates at relatively warm body temperatures, whereas the warmest preferring species (*An. terrestris*), had high *Bd* growth rates at relatively cool body temperatures. This result is consistent with the thermal mismatch hypothesis, which suggests that cool- and warm-adapted hosts might be more susceptible to disease outbreaks at abnormally warm and cool temperatures, respectively. This is hypothesized to occur because pathogens generally have wider thermal tolerances than their hosts [42], allowing them to outperform hosts under thermal mismatch conditions [24]. In addition to documenting temperature-dependent species-level variation in *Bd* susceptibility, our data also show that variation in T_{pref} among individuals can drive individual-level variation in disease susceptibility within a species. While field evidence showing variation in susceptibility and prevalence of *Bd* can be driven by variation in environmental temperature across individuals [9,25] and populations [21,43], there are very few studies that experimentally test how individual T_{pref} can drive differences in disease susceptibility within a population for this or any host–pathogen system.

In summary, none of the five host species tested exhibited a clear behavioural fever response to *Bd* infection but there were differences in individual-level T_{pref} that affected *Bd* growth. Additionally, we found species-level differences in the direction of the effect of individual-level T_{pref} on *Bd* growth that were consistent with the thermal mismatch hypothesis [24]. These results suggest that variation in T_{pref} within a population might be vital to buffer a species or populations against extirpation when a temperature-sensitive pathogen sweeps through an environment. Variation in T_{pref} might be more easily maintained in an ectothermic population when there are a wide variety of thermal microhabitats available. Thus, degradation of the thermal environment and microhabitat availability might reduce the ability of a species or population to buffer against temperature sensitive pathogens.

Ethics. *Atelopus zeteki* were obtained and used with permission from the Maryland Zoo, *An. terrestris* and *O. septentrionalis* were collected under permit with the Florida Fish and Wildlife Conservation Commission, and *An. americanus* and *Ac. crepitans* were collected under permit with the Illinois Department of Natural Resources. Experimental methods were approved by the Tulane, University of South Florida, and University of Illinois International Animal Care and Use Committees (protocols 0430R, 14112, and W IS00000548, respectfully).

Data accessibility. Data available from the Dryad Digital Repository at http://dx.doi.org/10.5061/dryad.643r37b [44].

Authors' contributions. E.L.S., C.L.R.-Z., J.H.S. and J.R.R. conceived ideas and designed experiments, E.L.S. and J.R.R. oversaw experiments in Tampa, FL, C.L.R.-Z. and J.S. oversaw experiments in New Orleans, LA, J.H.S. and R.C.F. oversaw experiments in Champaign, IL, E.L.S. and J.R.R. conducted statistical analyses, and E.L.S. and J.R.R. wrote the paper with comments and edits from R.C.F., C.L.R.-Z. and J.H.S. All authors agreed to submission of the manuscript and accept the responsibility for the accuracy and integrity of the manuscript.

Competing interests. We declare we have no competing interests.

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Appendix E: Supplemental Information: Chapter Three

Supplementary Methods

Animal collection and maintenance

Adult *O. septentrionalis* and post-metamorphic *An. terrestris* were collected from Hillsborough County, Florida, *An. americanus* and *Ac. crepitans* were collected from Champaign County, IL, and *At. zeteki* were obtained from the Maryland Zoo (Baltimore, MD). All individuals were swabbed and tested for *Bd* using PCR or qPCR to verify that they were free of *Bd* before the start of the experiment. *Anaxyrus americanus* and *Ac. crepitans* were maintained in 38-liter aquaria (51.4L x 26.7W x 32.1H cm; separated by sex and species) stocked with small rock and pools containing water in a laboratory maintained between 18-21 °C. *Osteopilus septentrionalis* and *An. terrestris* were maintained in individual containers (11.7 dia. x 13.5 cm) on top of folded paper towels soaked with artificial spring water (Cohen, Neimark et al. 1980) in a laboratory maintained between 23-25 °C. *Atelopus zeteki* were maintained in individual containers (30 x 19.5 x 20.5 cm) containing 2 cm of filtered tap water with an inverted plastic bowl for climbing in a laboratory maintained at 20-22 °C. All laboratories maintained a 12h photoperiod prior to and during experiments. All animals were maintained in the aforementioned laboratory conditions for at least 14 days prior to the start of the experiment. The frogs were fed mineral-dusted crickets *ad libitum* until the start of the experiment, and their containers and paper towels were changed once or twice weekly.

Thermoregulation experiments

For experiments involving *An. americanus, Ac. crepitans*, *An. terrestris,* and *O. septentrionalis*, temperature in the thermal gradients was maintained via Flexwatt heat tape (Flexwatt Industrial Sales®, Maryville, TN) controlled using a bulb-and-capillary thermostat

(Selco Products Co., Orange, CA) and frozen (-80° C) gel packs (32oz No-Sweat, Temperatsure Inc., Reno, NV) that were replaced every 12h. For *At. zeteki*, temperature gradients were maintained using Repti heat cables (Zoo Med Laboratories Inc. San Luis Obispo, CA) controlled using a thermostat (Exo Terra, Rolf C. Hagen Inc., Montreal, CA) and an aquarium chiller (AquaEuroUSA, Gardena, CA). Non-regulating *At. zeteki* were kept in Conviron Adaptis environmental chambers at 20 °C in plastic containers (30 x 19.5 x 20.5 cm) with 2 cm of water and inverted plastic bowls for climbing/hides.

During the two days when thermoregulation was not monitored, all individuals were fed 10 live crickets in containment bags to prevent crickets from moving freely within the thermogradient (Figure S2; see Sauer et al 2016 (2016) for more details).

Bd exposures and growth on hosts

Individuals of all species (except *At. zeteki*) received 1 mL of deionized water pipetted on to their dorsal surface that was rinsed from either 1% tryptone agar plates that were (isolate SRS 812) or were not (sham) growing *Bd*. Each *Bd*-exposed frog received $1.0x10^5$ zoospores. For *At. zeteki*, individuals were bathed for 24 h in 240ml of a solution with (isolate JEL 411 from Panama) or without (sham) $5.0x10^4$ *Bd* zoospores.

All individuals were swabbed immediately before, one week after, and two weeks after *Bd* or sham exposure. During each swabbing event, a sterile swab was passed over the ventral surface from snout to vent and each leg from hip to toe five times before being frozen at -80°C. *Bd* DNA from swabs of all species but *At. zeteki* was extracted using Prepman Ultra. Swabs from *At*. zeteki were extracted using Qiagen's DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA) following the protocol for animal tissue and using a final elution volume of 200µL. To determine *Bd* abundance on *An. terrestris*, *At. zeteki*, and *O. septentrionali*s, we used qPCR

methods based on Boyle *et al* (2004) with plasmid-based standards (Pisces Molecular Boulder, CO). To determine *Bd* prevalence on *An. americanus* and *Ac. crepitans*, *Bd* presence was determined using traditional PCR (Annis, Dastoor et al. 2004). All individuals were massed and euthanized with buffered MS-222 and stored at -80°C at the end of the experiment.

Data analyses

Code used in R to determine individual-level logistic growth-rates for *At. zeteki.* The code performs the following functions in order of line: 1) defines individuals as unique, 2) creates a blank matrix, 3) sets up the loop code to create a row in the matrix for each individual, 4) defines the starting load to be functionally zero, 5) subsets the data points within individuals, 6) runs a logistic growth model for each individual, 7) starts a list of the parameters which will be added the matrix, 8) defines how many times the model will be run and how much the parameters can vary, 9) creates a vector of parameters, 10) binds those parameters to the matrix.

```
AZ = unique(AZgrowth2$ID)
AZmatrix=matrix(nrow=0, ncol=5)
For(i in 1:length(AZ))
 try({N0=.001
 dataset = subset(AZgrowth2, ID ==AZ[i])
 fit = mle2(Log.load~dnorm(mean=(N0*K)/(N0+(K-N0)*exp(-r*week)), sd=sigma),
 start=list(K=1, r=1, sigma=1),
 control=list(maxit=50000, parscale=c(K=1, r=1, sigma=1)), data =dataset)
parameters=c(as.character(dataset$ID[1]), as.numeric(coef(fit))
AZmatrix=rbind(AZmatrix, parameters)})
```

Supplementary Figures

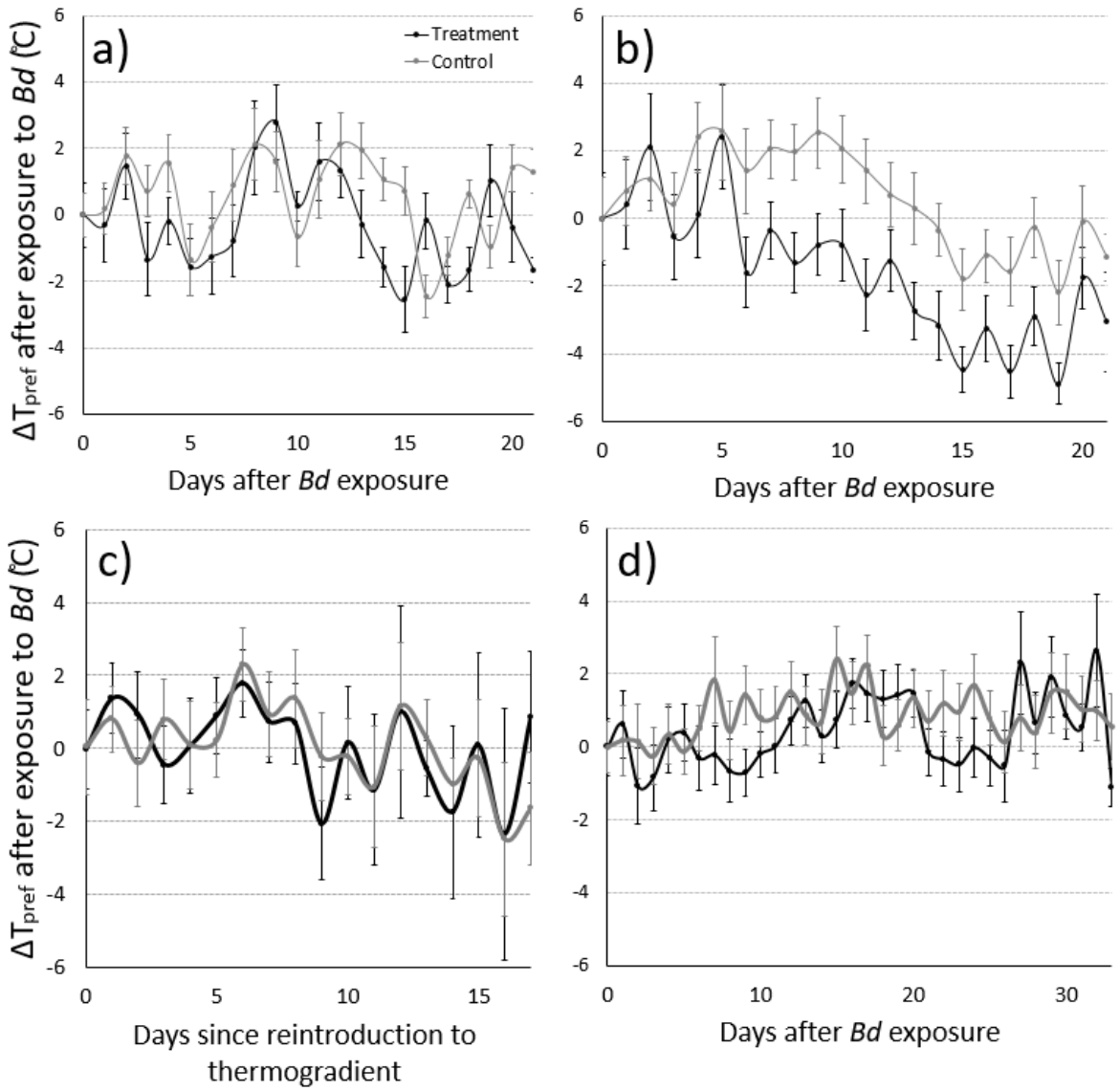

Figure E1. Change in T_{pref} after exposure to *Bd* (black lines) or sham-exposure (grey lines) plotted over time for **a)** *An. americanus*, **b)** *Ac. crepitans*, **c)** *An. terrestris*, and **d)** *At. zeteki*. Error bars indicate ±1 SE.

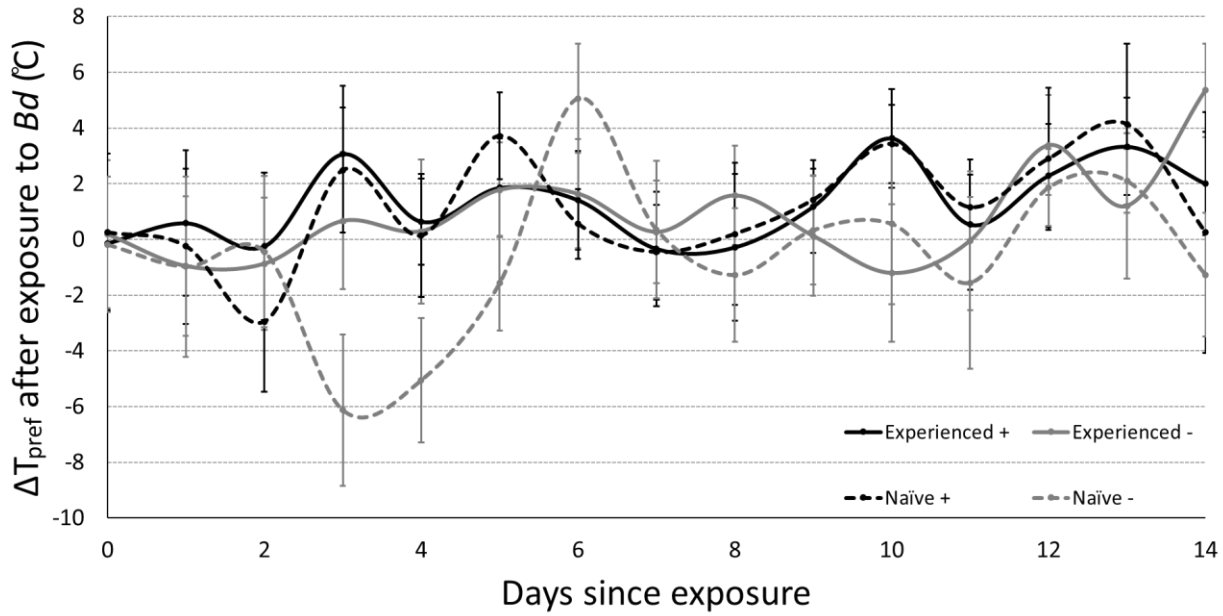

Figure E2. Change in T_{pref} after exposure to *Bd* (+) or sham-exposed (-) plotted over time for *O. septentrionalis* when half the individuals were naïve and half were previously exposed and cleared of *Bd* (experienced). Error bars indicate ± 1 SE.

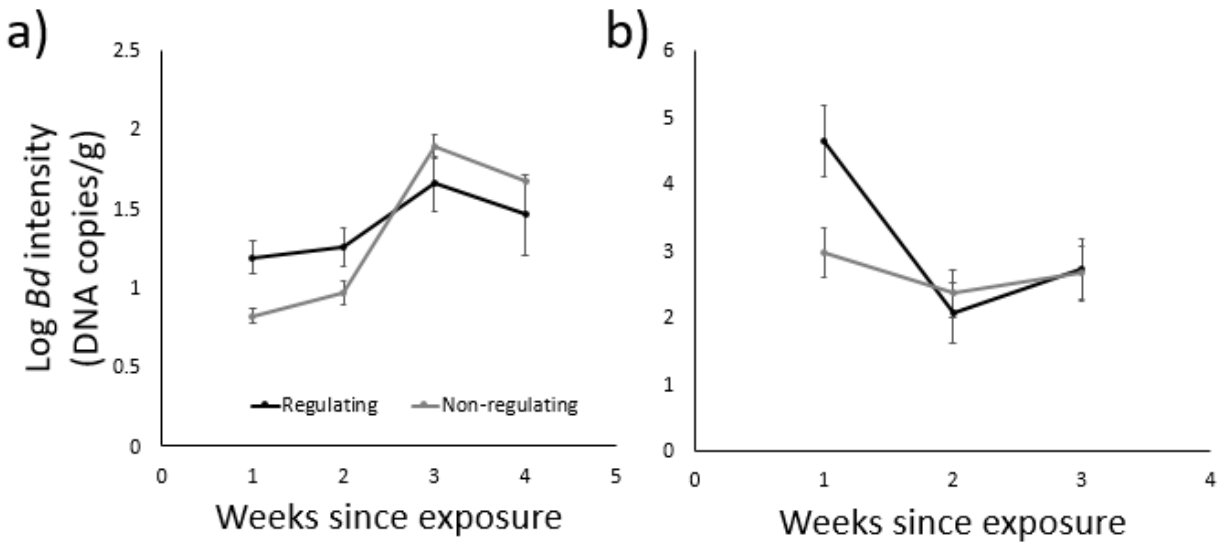

Figure E3. Log *Bd* infection intensity (log DNA copies/g) for regulating exposed and non-regulating exposed treatments plotted over time for **a)** *At. zeteki* and **b)** *An. terrestris*. Error bars indicate ± 1 SE.

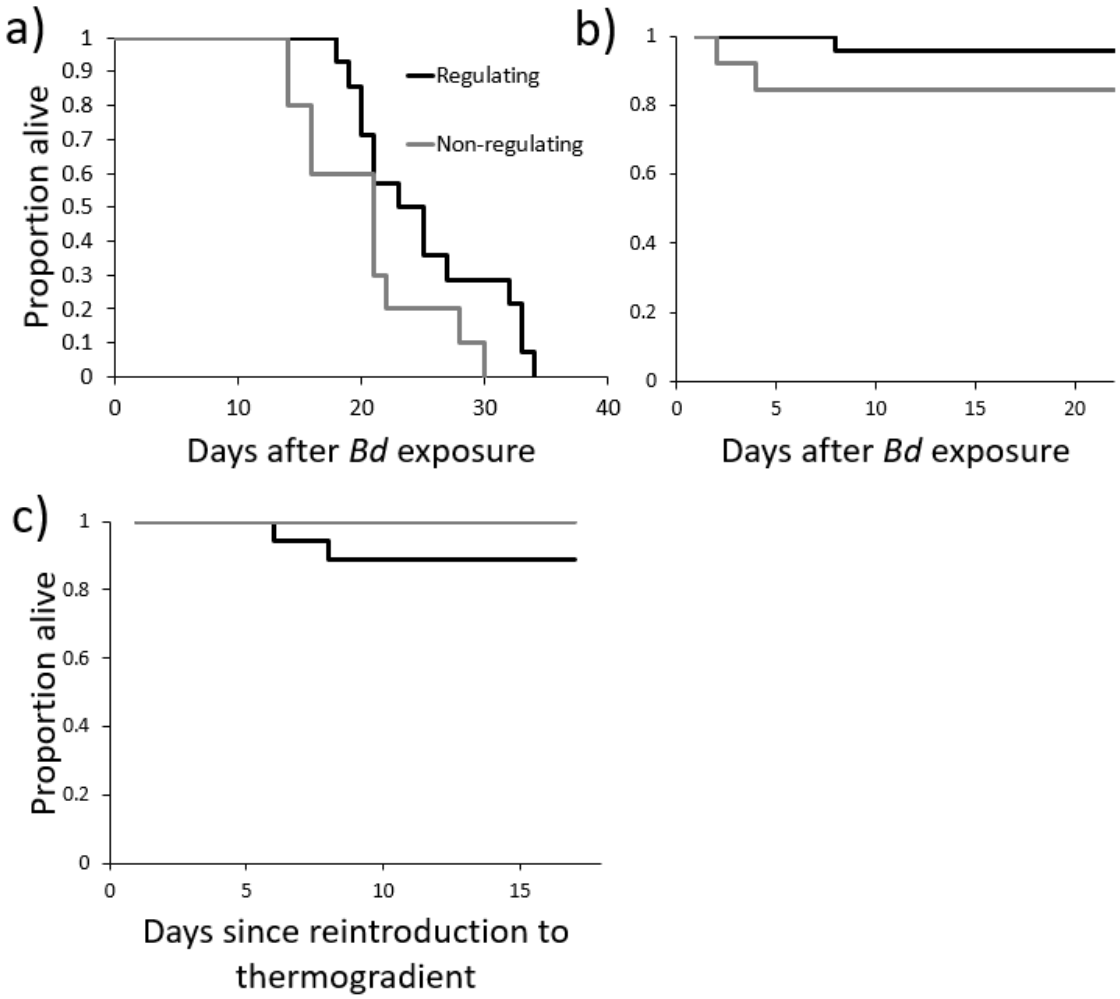

Figure E4. Survival curves for regulating exposed (black lines) and non-regulating exposed (grey lines) treatments plotted over time for **a)** *At. zeteki* **b)** *Ac. crepitans*, and **c)** *An. terrestris*

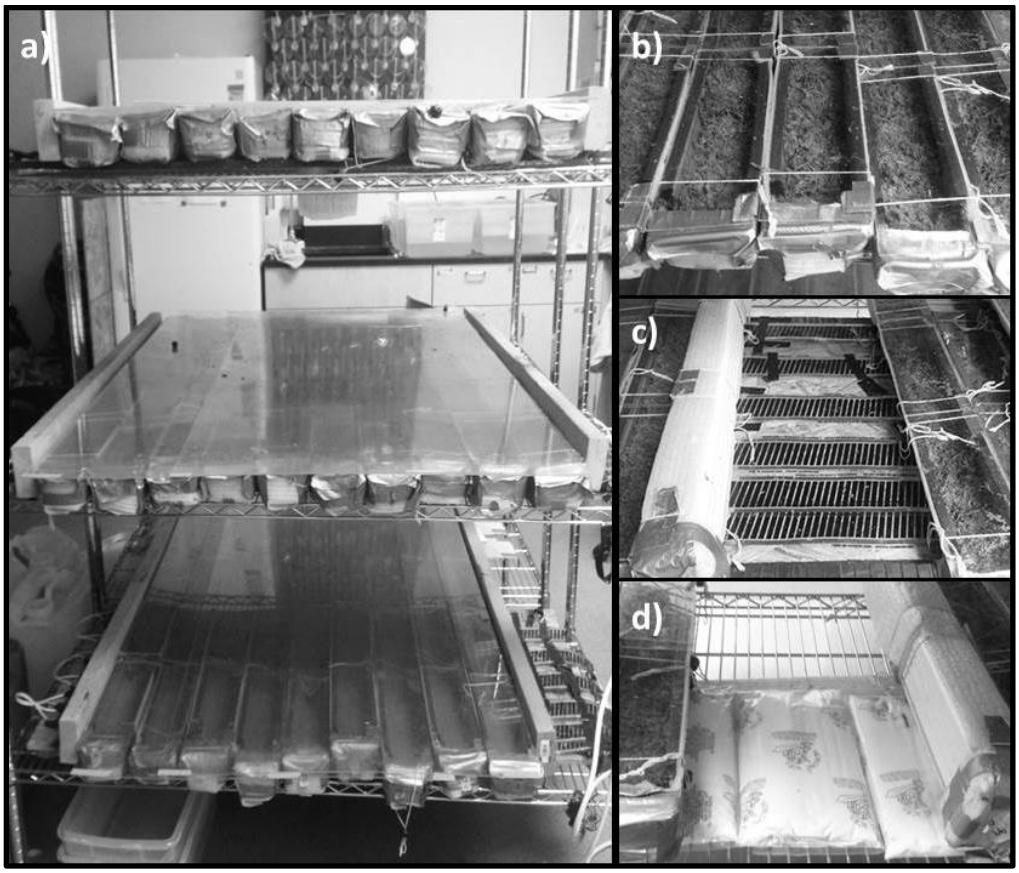

Figure E5. Temperature gradient apparatuses used for *An. americanus*, *Ac. crepitans*, *An. terrestris*, and *O. septentrionalis*. a) The entire set up of thermoregulatory apparatus, showing insulating 2x4's and large Plexiglas covers. **b)** View of the sphagnum moss interior and small Plexiglas window sealing each apparatus. **c)** Heat tape gradient and bottom of apparatus showing the space where the aluminum meets the heat tape. **d)** Ice packs and bottom of apparatus showing the space where the aluminum meets the ice packs.

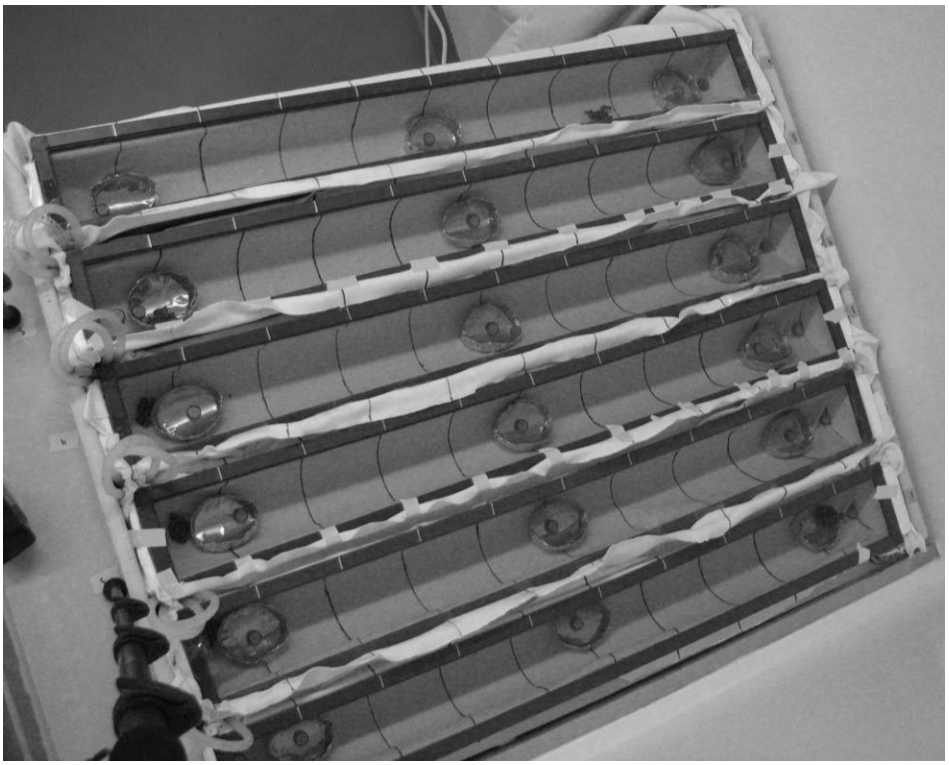

Figure E6. Temperature gradient apparatuses used for *At. zeteki* with animals in the gradients. Each is covered with a mesh lids and lined with wet fabric.

Figure E7. Individual temperature controlled incubators internal dimensions 37x21x13 cm; Marko Foam Products®, Salt Lake City, UT) (Raffel, Romansic et al. 2013) controlled using a bulb-and-capillary thermostat (Selco Products Co., Orange, CA) and maintained at their mean preferred temperature via Flexwatt heat tape (Flexwatt Industrial Sales®, Maryville, TN). **a)** Incubators on shelves; **b)** top view of incubator showing heat tape and bulp-and-capillary thermostat; **c)** top view with towel added to buffer animals from the heat tape; **d)** top view of complete incubator with lid, containing two animal containers. Figure and caption adapted from Raffel et al (2013).

Figure E8. Feeding containment apparatus with one adult *Osteopilus septentrionalis* for size comparison. Constructed of quart-size zip-top bags with plastic coated paper clips adhered to the outside for structure. See Sauer et al 2016 (2016) for more details.

Supplementary Tables

Table E1. Repeatability results. Prior to exposure, all species showed variation among individuals (among-individual variance) and consistency within individuals (repeatability). Repeatability, the proportion of the variance explained by the individual, is calculated by: $r = s^2/(s^2+s^2A)$. Where, s^2 is the within-group variance component, means squared within, and s^2A is the among-groups variance component, difference between means squared across and s^2 divided by n. Values for s^2 and s^2A are taken from the results of an ANOVA looking at the main effect of time on behaviour (T_{pref}) that incorporates individual as a random effect.

	df within (*n*)	Main effect of day (*p* value)	among-individual variance (s^2A)	repeatability (*r*)
All species	153	0.73	0.14	0.99
An. americanus	37	0.11	0.83	0.95
Ac. crepitans	37	0.25	0.26	0.99
An. terrestris	30	0.04	1.25	0.90
At. zeteki	27	0.65	0.35	0.97
O. septentrionalis	22	0.50	1.62	0.97

Table E2. Effect of the interaction between two binomial predictors: treatment group (regulating exposed and regulating sham-exposed) and time (before exposure and day x after exposure) on T_{pref}. Benjamini-Hochberg adjusted critical value given in the last column. Significance is denoted by *

	Day	*F* value	*p* value	critical value
All species	1	0.67	0.415	1.48E-03
	2	0.07	0.786	2.81E-03
	3	0.03	0.854	3.05E-03
	4	0.03	0.860	3.07E-03
	5	1.61	0.206	7.36E-04
	6	6.30	0.013	4.70E-05
	7	6.19	0.014	4.96E-05
	8	3.78	0.054	1.92E-04
	9	5.88	0.017	5.89E-05
	10	0.00	0.979	3.49E-03
	11	0.22	0.638	2.28E-03
	12	2.95	0.088	3.15E-04
	13	0.80	0.372	1.33E-03
	14	2.92	0.090	3.20E-04
An. americanus	1	0.03	0.872	2.08E-03
	2	0.00	0.984	2.34E-03
	3	2.59	0.110	2.62E-04
	4	0.54	0.465	1.11E-03
	5	0.01	0.943	2.24E-03
	6	0.65	0.422	1.00E-03
	7	3.06	0.082	1.96E-04
	8	0.11	0.744	1.77E-03
	9	0.50	0.482	1.15E-03
	10	0.22	0.644	1.53E-03
	11	0.12	0.729	1.74E-03
	12	0.85	0.358	8.52E-04
	13	2.39	0.124	2.95E-04
	14	3.15	0.078	1.85E-04
	15	4.54	0.035	8.24E-05
	16	4.59	0.034	8.02E-05
	17	0.13	0.723	1.72E-03
	18	2.54	0.113	2.69E-04
	19	2.75	0.100	2.37E-04
	20	0.85	0.359	8.55E-04
	21	1.59	0.210	4.99E-04
Ac. crepitans	1	0.62	0.431	1.03E-03

	2	1.44	0.232	5.53E-04	
	3	1.22	0.271	6.45E-04	
	4	2.76	0.099	2.35E-04	
	5	0.51	0.478	1.14E-03	
	6	11.43	0.001	2.18E-06	*
	7	6.61	0.011	2.64E-05	*
	8	8.94	0.003	7.71E-06	*
	9	15.46	0.001	2.38E-06	*
	10	10.99	0.001	2.71E-06	*
	11	10.82	0.001	2.95E-06	*
	12	4.00	0.047	1.13E-04	
	13	9.77	0.002	5.05E-06	*
	14	5.42	0.021	5.05E-05	
	15	4.39	0.038	9.00E-05	
	16	2.73	0.101	2.40E-04	
	17	5.80	0.017	4.10E-05	*
	18	3.61	0.059	1.41E-04	
	19	4.92	0.028	6.64E-05	
	20	1.62	0.205	4.87E-04	
	21	1.99	0.160	3.81E-04	
An. terrestris	1	0.49	0.485	1.51E-03	
	2	2.23	0.138	4.31E-04	
	3	1.73	0.190	5.94E-04	
	4	0.01	0.933	2.92E-03	
	5	0.49	0.485	1.51E-03	
	6	2.23	0.138	4.31E-04	
	7	1.73	0.190	5.94E-04	
	8	0.01	0.933	2.92E-03	
	9	0.49	0.485	1.51E-03	
	10	2.23	0.138	4.31E-04	
	11	1.73	0.190	5.94E-04	
	12	0.01	0.933	2.92E-03	
	13	0.49	0.485	1.51E-03	
	14	2.23	0.138	4.31E-04	
	15	1.73	0.190	5.94E-04	
	16	0.01	0.933	2.92E-03	
	17	0.49	0.485	1.51E-03	
At. zeteki	1	0.43	0.514	7.79E-04	
	2	0.83	0.365	5.53E-04	
	3	0.13	0.716	1.08E-03	
	4	0.00	0.995	1.51E-03	
	5	0.49	0.485	7.35E-04	
	6	0.71	0.400	6.06E-04	

	7	4.39	0.038	5.73E-05	
	8	1.27	0.262	3.97E-04	
	9	5.16	0.024	3.70E-05	
	10	1.01	0.316	4.79E-04	
	11	0.66	0.418	6.33E-04	
	12	0.65	0.421	6.38E-04	
	13	0.26	0.612	9.27E-04	
	14	0.15	0.704	1.07E-03	
	15	3.16	0.077	1.17E-04	
	16	0.12	0.730	1.11E-03	
	17	0.69	0.407	6.17E-04	
	18	0.98	0.325	4.92E-04	
	19	0.59	0.445	6.74E-04	
	20	0.08	0.783	1.19E-03	
	21	1.27	0.262	3.97E-04	
	22	1.59	0.210	3.18E-04	
	23	1.28	0.260	3.94E-04	
	24	1.85	0.176	2.67E-04	
	25	0.34	0.559	8.47E-04	
	26	0.00	0.964	1.46E-03	
	27	2.57	0.111	1.68E-04	
	28	0.51	0.476	7.21E-04	
	29	0.71	0.402	6.10E-04	
	30	0.00	0.949	1.44E-03	
	31	0.02	0.891	1.35E-03	
	32	2.20	0.140	2.12E-04	
	33	0.00	0.954	1.45E-03	
O. septentrionalis	1	1.13	0.290	1.04E-03	
	2	0.12	0.733	2.62E-03	
	3	12.60	0.001	1.82E-06	*
	4	4.53	0.035	1.24E-04	
	5	3.76	0.054	1.94E-04	
	6	3.08	0.081	2.90E-04	
	7	0.27	0.606	2.16E-03	
	8	0.07	0.791	2.83E-03	
	9	0.89	0.348	1.24E-03	
	10	10.87	0.001	4.32E-06	*
	11	1.56	0.213	7.61E-04	
	12	0.00	0.996	3.56E-03	
	13	3.07	0.082	2.93E-04	
	14	0.37	0.542	1.94E-03	

Table E3. Sample sizes and block break downs across species and blocks.

	N	Regulating treatment (*n*)	Regulating control (*n*)
An. americanus			
Block 1	19	10	9
Block 2	19	6	13
Total	38	16	22
Ac. crepitans			
Block 1	4	2	2
Block 2	14	6	8
Block 3	20	10	10
Total	34	16	18
An. terrestris			
Block 1	14	7	7
Block 2	17	11	6
Total	31	18	13
At. zeteki			
Block 1	12	6	6
Block 2	12	6	6
Block 3	4	2	2
Total	16	8	8
O. septentrionalis			
Experienced	13	7	6
Naïve	10	4	6
Total	23	11	12

Supplementary References

Annis, S. L., F. P. Dastoor, H. Ziel, P. Daszak and J. E. Longcore (2004). "A DNA-based assay identifies Batrachochytrium dendrobatidis in amphibians." Journal of Wildlife Diseases **40**(3): 420-428.

Boyle, D., D. Boyle, V. Olsen, J. Morgan and A. Hyatt (2004). "Rapid quantitative detection of chytridiomycosis (Batrachochytrium dendrobatidis) in amphibian samples using real-time Taqman PCR assay." Diseases of aquatic organisms **60**: 141-148.

Cohen, L. M., H. Neimark and L. Eveland (1980). "Schistosoma mansoni: response of cercariae to a thermal gradient." The Journal of parasitology **66**(2): 362-364.

Raffel, T. R., J. M. Romansic, N. T. Halstead, T. A. McMahon, M. D. Venesky and J. R. Rohr (2013). "Disease and thermal acclimation in a more variable and unpredictable climate." Nature Climate Change **3**(2): 146-151.

Sauer, E. L., J. H. Sperry and J. R. Rohr (2016). "An efficient and inexpensive method for measuring long-term thermoregulatory behavior." Journal of Thermal Biology **60**: 231-236.

Appendix F: Supplemental Material: Chapter Four

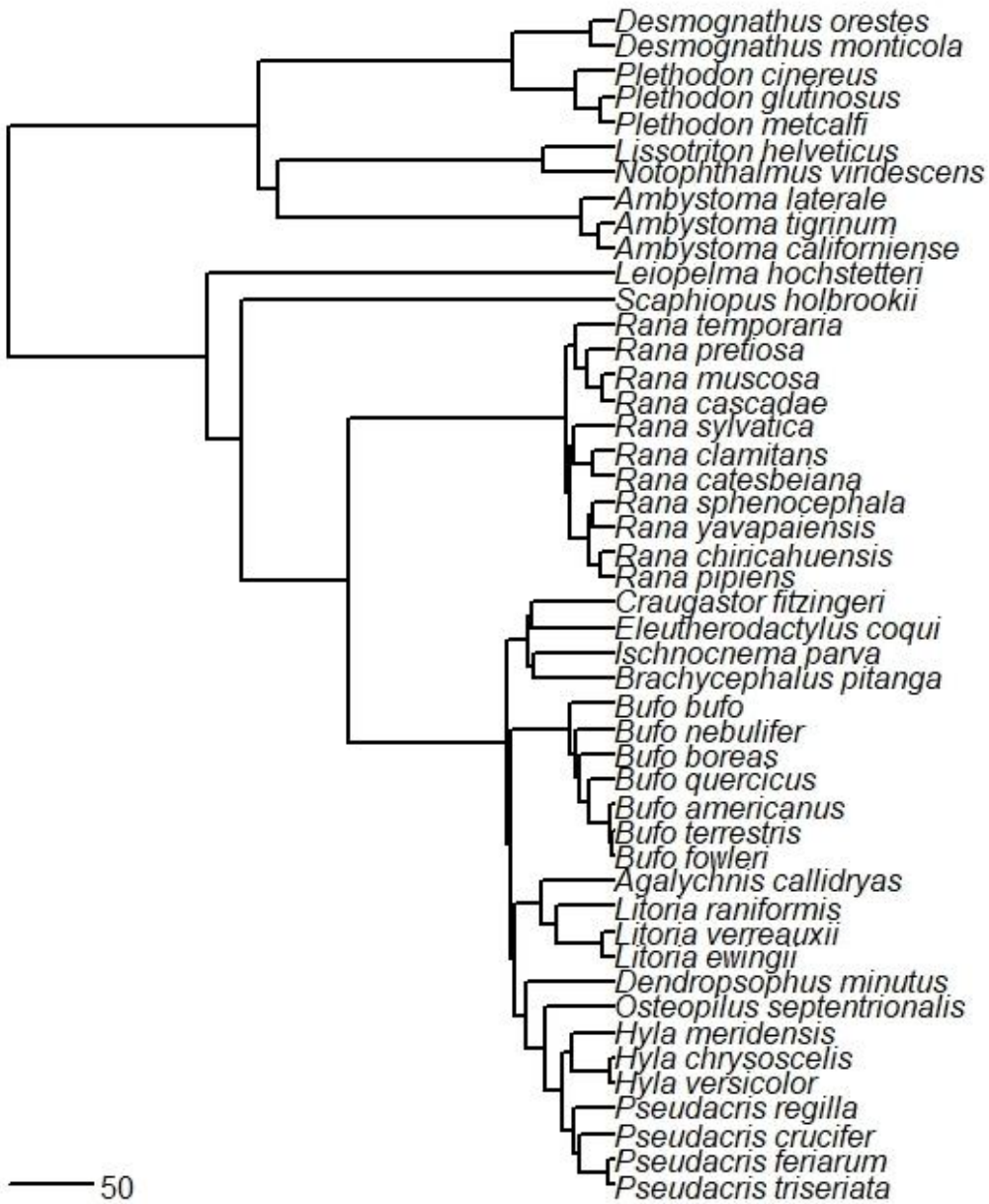

Figure F1. Phylogenetic tree of species included in the meta-analysis. The 58 studies included in this meta-analysis included 47 amphibian species from 11 families. We extracted our tree from the Pyron and Wiens (2011) tree and used it to construct a phylogenetic correlation matrix which was included as a random effect in our models.

Appendix F: Institutional Animal Care and Use Committee Approval Letter

USF UNIVERSITY OF SOUTH FLORIDA

RESEARCH INTEGRITY AND COMPLIANCE
INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

MEMORANDUM

TO:	Jason Rohr,
FROM:	Farah Moulvi, MSPH, IACUC Coordinator Institutional Animal Care & Use Committee Research Integrity & Compliance
DATE:	6/30/2014
PROJECT TITLE:	Interactions between Batrachochytrium dendrobatidis infection and behavioral thermoregulation in amphibians
FUNDING SOURCE:	USF department, institute, center, etc.
IACUC PROTOCOL #:	W IS00000548
PROTOCOL STATUS:	**APPROVED**

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC **APPROVED** your request to use the following animals in your **protocol for a one-year period beginning 6/27/2014:**

Frog/Anaxyrus terrestris (Egg masses)	14
Frog/Anaxyrus quercicus (Egg masses)	14
Frog/Hyla cinerea (Egg masses)	14
Frog/Osteopilus septentrionalis (Egg masses)	14
Frog/Acris gryllus (Egg masses)	14
Frog/Gastrophryne carolinensis (Egg masses)	14
Frog/Hyla squirella (Egg massses)	14
Frog/Hyla femoralis (Egg masses)	14
Frog/Acris crepitans (Egg masses)	14
Frog/Scaphiopus holbrookii (Egg masses)	14
Frog/Anaxyrus americanus (Egg masses)	14
Salamander/Plethodon cinereus (Egg masses)	14

Please take note of the following:

• IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system.

After three years, all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.

• **All Comparative Medicine pre-performance safety and logistic meetings must occur prior to implementation of this protocol.** Please contact the program coordinator at compmed@research.usf.edu to schedule a pre-performance meeting.

• **All modifications to the IACUC-Approved Protocol must be approved by the IACUC prior to initiating the modification.** Modifications can be submitted to the IACUC for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and justified in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application.

• **All costs invoiced to a grant account must be allocable to the purpose of the grant.** Costs allocable to one protocol may not be shifted to another in order to meet deficiencies caused by overruns, or for other reasons convenience. Rotation of charges among protocols by month without establishing that the rotation schedule credibly reflects the relative benefit to each protocol is unacceptable.

• **The PI must assist the IACUC with tracking wild animal field research activities.** The PI must report episodes of wild animal use, the approximate range of taxa, and the approximate numbers of animals encountered or used at intervals appropriate to the study but at least once a year.

RESEARCH & INNOVATION • RESEARCH INTEGRITY AND COMPLIANCE
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
PHS No. A4100-01, AAALAC No.58-15, USDA No. 58-15
University of South Florida • 12901 Bruce B. Downs Blvd., MDC35 • Tampa, FL 33612-4799
(813) 974-7106 • FAX (813) 974-7091

Appendix G: Institutional Biosafety Committee (IBC) Approval

Jason Rohr, Ph.D.
Biology
SCA 110

10/12/2016

IBC Study number: **1334 Infectious Agent**

IBC Study Title: ***Amphibian, Trematodes and Pesticides; Completion of Trematode Life Cycles; Interactions between Batrachochytrium dendrobatidis infection and behavioral thermoregulation in amphibians***

Dear Dr. Rohr:

Your above entitled registration application was reviewed by the Institutional Biosafety Commitee (IBC) at its **09/20/2016** meeting. The IBC acknowledges you have fulfilled the IBC review requirements. The IBC hereby grants the approval for this project. In addition, please take note of the following:

- Approval is for a one-year period beginning: **10/12/2016**
- The Principal Investigator shall not modify any research project approved by the IBC until that proposed modification has been registered with and approved by the IBC.
- Report any significant problems, violations of the CDC/NIH Guidelines, or any significant research-related accidents and illnesses to Research Integrity and Compliance at 974-0954.
- All operations must be conducted at Biosafety Containment Level Practices as described in your application in accordance with the NIH/CDC publication Biosafety in Microbiological and Biomedical Laboratories, 5th Edition.
- When using research animals with an IBC registered biological agent, initiation of the animal component of the study is contingent upon the completion of and approval by the Institutional Animal Care and Use Committee (IACUC) process.
- IBC registrations are approved for a one-year period at the end of which, an annual renewal/amendment application must be submitted for years two (2) and three (3) of the protocol. A new registration application must be reviewed and approved by the Full Committee every three (3) years.

If you have any questions regarding the status of this project, please contact Research Integrity and Compliance at 974-0954.

Sincerely,

BA: dh
cc:DSR

Andrew Cannons, Ph.D.
Chairperson
Institutional Biosafety Committee

USF RESEARCH & INNOVATION• RESEARCH INTEGRITY & COMPLIANCE •
INSTITUTIONAL BIOSAFETY COMMITTEE
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