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# PHYSIOLOGICAL, ECOLOGICAL, AND MICROBIAL FACTORS SHAPING THERMAL TOLERANCE AND PERFORMANCE IN ECTOTHERMIC VERTEBRATES

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PHYSIOLOGICAL, ECOLOGICAL, AND MICROBIAL FACTORS SHAPING THERMAL  
TOLERANCE AND PERFORMANCE IN ECTOTHERMIC VERTEBRATES

by

Jason W. Dallas

B.S., Rider University, 2014  
M.S., Shippensburg University, 2017

A Dissertation  
Submitted in Partial Fulfillment of the Requirements for the  
Doctor of Philosophy Degree

School of Biological Sciences  
in the Graduate School  
Southern Illinois University Carbondale  
August 2023

DISSERTATION APPROVAL  
PHYSIOLOGICAL, ECOLOGICAL, AND MICROBIAL FACTORS SHAPING THERMAL  
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A Dissertation Submitted in Partial  
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in the field of Zoology

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AN ABSTRACT OF THE DISSERTATION OF

Jason Dallas, for the Doctor of Philosophy degree in Zoology, presented on June 14, 2023, at Southern Illinois University Carbondale.

TITLE: PHYSIOLOGICAL, ECOLOGICAL, AND MICROBIAL FACTORS SHAPING THERMAL TOLERANCE AND PERFORMANCE IN ECTOTHERMIC VERTEBRATES

MAJOR PROFESSOR: Dr. Robin Warne

Temperature represents a major driving force in biology as it influences essential functions across multiple levels of biological organization. The role of temperature is especially important for ectothermic animals, whose biotic processes are dependent on both body and environmental temperature. Assessing the relationship between temperature and organismal performance represents an important research direction as temperatures continue to warm under anthropogenic climate change.

Chapters two and three are focused on a recently colonized population of the invasive Mediterranean House Geckos at the northern edge of their invasion front. These chapters examine the ecological and physiological factors that enable these lizards to persist in a cooler and more temperate environment than their native range. The thermal breadth of a reptile greatly influences its ability to tolerate a thermally variable environment, particularly when environmental options are limited for behavioral thermoregulation. These chapters explore the thermal performance of this species, and the results show that the eurythermality of these geckos promotes their rapid colonization of novel environments despite experiencing prolonged periods of cool temperatures.

Chapters four, five, and six, by contrast, shift focus to larval amphibians to explore the constraints and factors underlying plasticity in acclimation to temperature extremes. As habitats continue to warm with climate change, ectotherms with limited capacity to thermoregulate, such

as larval amphibians in shallow ponds, will be under a heightened threat of heat stress and mortality. Resultantly, identifying different factors that can increase organismal heat tolerance would reduce the risk of overheating and promote survival. Chapters four, five, and six explore this topic by measuring the critical thermal maximum ( $CT_{max}$ ) of larval wood frogs. Chapter four focuses on the tradeoff between basal  $CT_{max}$  and plasticity of  $CT_{max}$  and its consequences for how a larval anuran responds to an acute heat shock. Chapter five examines the role a viral pathogen, ranavirus, has on larval  $CT_{max}$ . Surprisingly, a lethal dose of ranavirus did not reduce  $CT_{max}$  which goes against the common pattern of pathogenic infections lowering host heat tolerance. Lastly, chapter six explores the relationship between the gut microbiota and host  $CT_{max}$  with a particular focus on cross-species microbiota transplants. In line with our prediction, transplanting the gut microbiota of a heat-tolerant donor species promoted greater  $CT_{max}$  in the heat-sensitive recipient species.

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## CHAPTER 1

### INTRODUCTION

Environmental temperature is a key abiotic driver in the biology of all organisms with particular importance in ectotherms, which account for > 95% of all animal life. The reliance on behavioral thermoregulation, rather than endogenous metabolic heat production results in practically all aspects of ectothermic physiology being strongly temperature-dependent (Huey 1982, Angilletta Jr et al. 2002b, Angilletta Jr 2009). One taxonomic group that has received particular focus in terms of their thermal physiology is the herpetofauna – the amphibians and reptiles – which exhibit great diversity in their ability to modulate their body temperature in response to fluctuating environmental conditions (Bogert 1949, Bodensteiner et al. 2020, Munoz 2022). Understanding the thermal biology of these organisms is particularly important in the face of anthropogenic climate change, because as environmental temperatures continue to warm, herpetofauna risk reaching their thermal limits with consequences for performance and persistence (Somero 2010, Duarte et al. 2012, Huey et al. 2012, Cohen et al. 2019, Cox et al. 2022). Because herpetofauna have essential roles as both predators and prey, temperature-induced declines in their performance and population will likely have negative consequences for their ecological communities.

The general pattern of temperature-dependent physiological performance across most herpetofauna is the thermal performance curve (TPC). (Huey and Stevenson 1979, Schulte et al. 2011). The typical shape of an ectothermic TPC follows an “inverted-U” shape with the endpoints representing the thermal tolerance range of an organism; these are the critical thermal (CT) temperatures at which physiological performance reaches zero and mortality is assumed to occur at temperatures outside of this range. Of particular focus considering climate change is CT



maximum ( $CT_{max}$ ) which is associated with latitude (Sunday et al. 2019) and biological traits including hydration status (Plummer et al. 2003, Guevara-Molina et al. 2020), diet quality (Bujan and Kaspari 2017, Zhao et al. 2022), and exposure to physiological stressors (Rodgers et al. 2021, Chuang et al. 2022). There is a great deal of evidence indicating that  $CT_{max}$  is plastic (Bodensteiner et al. 2020) and that plasticity increases in  $CT_{max}$  are projected to be beneficial under warming scenarios as a buffer against heat mortality (Hoffmann et al. 2013, Gunderson et al. 2017, Markle and Kozak 2018, Rohr et al. 2018, Morley et al. 2019). This indicates the significance of exploring various abiotic and biotic mechanisms that can plastically shift the  $CT_{max}$  of herpetofauna upwards.

Beyond thermal tolerance range, the TPC is denoted by a gradual, linear increase in performance as temperatures increase until reaching its maximum at a given temperature deemed the thermal optimum ( $T_{opt}$ ). Beyond the  $T_{opt}$ , performance rapidly declines until reaching the  $CT_{max}$ . The shape of the TPC displays great variation among species from the two extremes of stenothermal and eurythermal which reflects either a narrow or broad shape, respectively. The differences in TPC shape are often dependent on the variation in environmental temperature the organism experiences with greater thermal variability (i.e., temperate climates) promoting a broader shape (Dowd et al. 2015). Inherently, eurythermality maximizes performance across a greater range of temperatures relative to stenothermal organisms. While the TPC shape often differ among species, there is evidence that it varies among different physiological traits as well (Kellermann et al. 2019, Ørsted et al. 2022). As TPCs attempt to act as a proxy for measuring the temperature-dependence of organismal fitness, determining the most relevant performance metric can influence any conclusions made. For instance, Ørsted et al. (2022) showed that TPCs

of somatic growth are more stenothermal than other traits including metabolic rate, indicating the need to examine the TPCs of multiple fitness-related traits.

To address multiple aspects of herpetofaunal thermal physiology, I used two study organisms, the Mediterranean House Gecko (*Hemidactylus turcicus*) and larval wood frogs (*Lithobates sylvaticus*). The former is an invasive species that is expanding its invasive range throughout the United States into cooler environments, while the latter is a wide-ranging anuran.

In chapter 2, I explored if eurythermality is a trait beneficial to invasive herpetofauna as they colonize novel environments with unique climates. To address this, I examined the thermal performance of a recently colonized population of Mediterranean House Geckos at the leading edge of its invasive range. I measured how maximum sprint speed and a broad immune measure – the inflammatory immune swelling response to phytohemagglutinin (PHA) injection - changed across a thermal gradient. While sprint speed is a commonly used metric in thermal performance analyses as a proxy for individual fitness, the thermal performance of the PHA response represents an *in vivo* immune assay that has received less attention relative to *in vitro* assays. The geckos displayed eurythermality in both traits, indicating that their broad performance breadth likely promotes their northward expansion in the United States. This result provides further support for the importance of eurythermality as a key determinant in the success of invasive herpetofauna.

Building upon the laboratory results, chapter 3 describes the field thermal biology of Mediterranean House Geckos across an active season in southern Illinois. I measured various aspects of gecko thermal biology and ecology to determine how they persisted in a suboptimal climate relative to warmer climates in their invasive habitat core. Despite being active at temperatures below their preferred temperature range that restricted their performance for

multiple months, the geckos in Illinois were able to exhibit traits, such as triple-clutching and growth rates, that resembled those seen in much warmer environments. Resultantly, the prolonged period of cool temperatures does not place a significant cost on Mediterranean House Geckos, signifying their ability to successfully colonize novel climates.

Shifting focus towards a different study system, chapter 4 details the relationship between basal thermal tolerance and plasticity, also known as the tolerance-plasticity tradeoff hypothesis. This hypothesis was proposed by van Heerwaarden and Kellermann (2020) and suggests that ectotherms that display higher basal thermal tolerance (i.e.,  $CT_{max}$ ) have reduced plasticity to adjust their  $CT_{max}$ . This negative relationship has been suggested to place a greater burden under projected global warming as plasticity is predicted to buffer threatened populations from climate change (Morley et al. 2019). Utilizing larval wood frogs, I sought to identify if they followed the tolerance-plasticity tradeoff hypothesis by measuring the effect of heat hardening, defined as increased tolerance of high temperatures through short-term exposure to elevated environmental temperatures. This was tested in individuals acclimated to one of two temperatures (low vs high) for different acclimation periods (short vs long). In line with the tolerance-plasticity tradeoff hypothesis, heat hardening was most pronounced in larvae that had the lowest basal  $CT_{max}$ . This result indicates that acclimation to warmer temperatures promotes warmer basal  $CT_{max}$  in larval anurans but limits their ability to tolerate further acute heat stress.

Emerging infectious diseases pose a great threat to global biodiversity and have been the causative agent of population declines and species extinctions (Pounds et al. 2006, Hoyt et al. 2021). As temperatures are projected to warm under climate change, the potential negative interaction between pathogenic infections and host heat tolerance represents an important research topic (Hector et al. 2023). Pathogens often trigger an inflammatory response that can

negatively affect heat tolerance through increased production of reactive oxygen species (Sears et al. 2011), which can disrupt mitochondrial activity and membrane homeostasis (Slimen et al. 2014) and lead to lower thermotolerance. Therefore, it is often found that pathogenic infections lead to a decline in heat tolerance (Hector et al. 2021), however there are few limited host-pathogen systems in which this relationship has been studied. To address this gap in our knowledge, In chapter 5, I explored how ranavirus, a prominent pathogen of ectothermic vertebrates (Gray and Chinchar 2015), can alter the heat tolerance of larval wood frogs. Despite experimental infections with an LD<sub>100</sub> concentration of ranavirus, there was no noticeable decline in CT<sub>max</sub> suggesting that infected larvae may choose to maintain their heat tolerance to promote selection of warmer temperatures enabling behavioral fever.

While multiple variables can influence host heat tolerance, one feature that has been largely overlooked until recently is the gut microbiota. The microbiome is a highly complex and diverse assemblage of bacteria, fungi, viruses, and parasites that live within and on their host and contribute to various critical aspects of host biology (McFall-Ngai et al. 2013, Warne and Dallas 2022) and species conservation (Dallas and Warne 2022). There is evidence that transplanting the gut microbiota of individuals acclimated to warm temperatures to conspecifics at lower temperatures results in greater heat tolerance in the recipients (Moghadam et al. 2018, Doering et al. 2021, Baldassarre et al. 2022). However, the degree to which cross-species gut microbiota transplants can impart beneficial effects towards greater heat tolerance has not been examined. In chapter 6, I studied how the gut microbiota, specifically the gut bacteria, of larval wood frogs influences their CT<sub>max</sub> following early life antibiotic exposure. Specifically, I wanted to determine if transplanting the gut microbiota of larval green frogs, a species with high CT<sub>max</sub>, to wood frogs, a species with a low CT<sub>max</sub>, would improve the CT<sub>max</sub> of the latter. I showed that

larval wood frogs that received the green frog inoculation had the highest  $CT_{max}$  compared to other antibiotic-treated wood frogs, in support of cross-species microbiome transplants being a potential mechanism to improve thermotolerance.

## CHAPTER 2

### EURYTHERMIC SPRINT AND IMMUNE THERMAL PERFORMANCE AND ECOLOGY OF AN EXOTIC LIZARD AT ITS NORTHERN INVASION FRONT

#### Introduction

Thermal performance of immunity has been relatively understudied in ectotherms, especially in the context of invasiveness, or in relation to other fitness-related traits and thermoregulatory patterns in the field. For herpetofauna, environmental temperature is a critical component in habitat selection and functional performance (Blouin-Demers and Weatherhead 2001, Row and Blouin-Demers 2006, Doody and Moore 2010, Dorcas et al. 2010). As a result, the northern expansion of invasive tropical herpetofauna is expected to be restricted by temperate climates. Despite this, subtropical species are colonizing temperate regions that exhibit cooler temperatures outside of their native ranges. Range expansion and persistence in novel environments include behavioral use of climatically advantageous microhabitats (Dobkin et al. 1989, Meshaka Jr 1996), and distinctive physiological traits that allow for high phenotypic plasticity (Meshaka Jr et al. 2005, Kolbe et al. 2014). Urban areas, commonly associated with reduced herpetofaunal biodiversity (French et al. 2018), provide suitable habitat for exotic reptiles (Battles and Kolbe 2019) and the urban heat island effect can buffer against seasonally variable climates (Tiatragul et al. 2017). Quantifying the thermal ecology of invasive herpetofauna can elucidate how some species are able to expand their ranges into temperate areas through maximizing performance over a wide thermal breadth and/or behavioral adaptations to maintain high body temperatures ( $T_b$ ). It is likely that these mechanisms, along with urban heat islands, can facilitate the colonization of invasive species into higher latitudes.

Invasive species are often generalists that have a wide tolerance of environmental conditions and/or plasticity in their responses to changing conditions. Indeed, the native geographic range of successful invaders is often larger than non-invasive species (Pyšek et al. 2009, Latella et al. 2010, Gallagher et al. 2011, Peoples et al. 2017), which reflects their evolutionary history of adaptation to greater variability in climatic conditions (Allen et al. 2013, González-Suárez et al. 2015). For invasive herpetofauna, there is evidence of eurythermality enabling them to achieve near optimal physiological performance over a wider range of  $T_{b,s}$  compared to native species (Kelley 2014, Coates et al. 2017, Kosmala et al. 2018, McCann et al. 2018). Adaptation to wide climatic conditions is likely of particular importance for invasive herpetofauna because physiological function and performance of ectotherms is temperature-dependent (Angilletta Jr 2009). Examining the temperature-dependence of various physiological traits could thus provide insight into how invasive herpetofauna exploit eurythermy to colonize thermally variable, suboptimal conditions.

While sprint speed is perhaps the most commonly tested thermal performance trait in ectotherms (Huey et al. 1989, Martin and Huey 2008), immune function is another trait critical to survival that can exhibit temperature sensitivity. However, immune thermal performance is understudied especially in the context of invasiveness and field thermoregulatory patterns. For example, Butler et al. (2013) showed that complement-mediated immune function exhibits temperature-dependency that is associated with species-specific thermal biology in vertebrates, suggesting adaptation to thermal regimes. However, temperature dependence of varied immune functions remains poorly tested, especially *in vivo* and within the context of thermal performance curves (TPCs) (Sacchi et al. 2014, Palackdharry et al. 2017). Both immunity and sprinting link cellular and tissue level function to organismal-level traits associated with survival (Miles 2004),

and measuring their performance across an ecologically relevant thermal range can provide insight for understanding how temperature influences whole body performance and interpopulation differences (Huey et al. 1989, Du et al. 2000, Gaby et al. 2011, Watson and Formanowicz 2012).

In this study, we tested the temperature-dependence of sprint performance and immune responses to phytohemagglutinin (PHA) injection in relation to thermoregulatory performance in the field of a population of Mediterranean house geckos (*Hemidactylus turcicus* [Linnaeus 1758]) at its northern invasion front. In the field, we measured  $T_b$  in relation to wall, air, and potential refuge temperatures to detail thermoregulatory patterns. We then collected individuals for lab-based assessment of thermal performance of sprinting and swelling responses to PHA injection. Testing the thermal performance of sprinting and immune function could elucidate how this invasive, nocturnal ectotherm can effectively colonize cooler climates. We predicted that both sprint performance and immunity of this lizard would exhibit similar temperature-dependence, and that individuals in the field would select thermal habitats that enable them to reach and maintain  $T_b$ s that optimized performance for these traits.

## Materials and Methods

### *Study Species*

*Hemidactylus turcicus* is a nocturnal, small-bodied (adult snout-vent length = 50–60 mm) member of Gekkonidae that is native to the countries bordering the Mediterranean Sea (Rödger and Lötters 2009). This species was first documented in the United States in Key West, Florida in 1910 (Fowler 1915) and quickly spread throughout the Gulf Coast states and now is found in > 10 states (Locey and Stone 2006, Meshaka Jr et al. 2006) including more northern states such as Oklahoma (Locey and Stone 2006), Virginia (Harris 2009), and Tennessee (Wessels et al.



2018). The rapid, long-distance dispersal of this species between geographically isolated locations has been suggested, and in some cases documented (McCallum et al. 2008), to be the result of vehicular transport (Davis 1974, Short and Petren 2011). In the United States, the habitat of *H. turcicus* is, with near exclusivity, buildings and other anthropogenic structures (Rose and Barbour 1968, Selcer 1986, Meshaka Jr 1995, Punzo 2001, Gomez-Zlata et al. 2006, Hitchcock and MacBrayer 2006, Meshaka Jr et al. 2006, Stabler et al. 2012, Wessels et al. 2018), and its diet is generalized, consisting of a variety of arthropods (Klawinski et al. 1994, Saenz 1996).

### *Study Design*

Nighttime visual surveys and collections were conducted across ten buildings in downtown Carbondale, IL (N 37.72°, W 89.22°) where the population has persisted since at least 2012 (Whiles et al. 2013). A total of nine surveys were conducted between 2000–2200 hours from mid-September to early-October 2018, during which *H. turcicus* were hand captured for temperature and body measurements, in conjunction with habitat characterization.

Upon collection, we recorded the time of capture and measured  $T_b$ , snout-vent length (SVL), wall temperature ( $T_{wall}$ ), and the perch height at the location where each individual was initially observed. Building and cardinal direction of the wall they were observed on (orientation) were also recorded. Perch height was measured using a measuring tape to the point a *H. turcicus* was observed.  $T_b$  was measured using a Raytek® laser infrared thermometer and was only included in the dataset if collections occurred within 30 seconds following capture ( $n = 81$ ). We excluded  $T_b$  measures from 83 geckos (50.6% of collected individuals) from the analysis because their handling time exceeded 30 seconds and therefore likely reflected handling effects (Hitchcock and MacBrayer 2006). Individuals were marked on their dorsal surface with a

waterproof marker to minimize repeated measures on individuals. Because ecdysis would have resulted in this mark being lost over subsequent days, it is likely individuals were recaptured without our knowledge. Since the focus of this study was on habitat use, the lack of recapture data did not negatively affect our goals. Age classes were based on SVL measurements from previous publications on *H. turcicus* (Adults > 43 mm, Juveniles > 30 mm, Hatchlings < 30 mm; (Selcer 1986, Punzo 2001) with juveniles and hatchlings grouped together. Hourly measures of air temperatures were sourced from a weather station at the Southern Illinois Airport, 9.82 km from the study site. Thermal refugia were also characterized using iButtons (Maxim Integrated®) that were placed in cracks distributed across the sampled buildings (varied across heights and cardinal direction) and were representative of the refugia frequented by *H. turcicus*. These iButtons (n = 18) sealed in plastic bags were set in refugia from September 28, 2018 to October 6, 2018 and recorded temperatures every 30 minutes.

Due to their elusive nature, numerous observed *H. turcicus* escaped capture or were uncatchable. For these individuals, perch height and  $T_{\text{wall}}$  were recorded in an identical fashion to captured individuals along with the building and cardinal direction. Individuals that were observed to utilize perches  $\geq 3.2$  m were all described as having a perch height of 3.2 m as it was not possible to obtain accurate perch height measurements above this point. Age class was estimated visually and, as with captured individuals, both juveniles and hatchlings were grouped together.

To assess the thermal performance of *H. turcicus*, a total of 26 adults (SVL =  $52.14 \pm 0.88$  [39–58], Mass =  $3.49 \pm 0.16$  [1.43–4.73]) were collected in early-October and brought to the lab (Southern Illinois University Carbondale). Sex was not determined for captured individuals. Individuals were housed in groups of 2–3 in 16 l plastic tubs lined with paper towels

and PVC pipes as refugia. The tubs were kept in environmentally controlled rooms at 25°C, and a 12:12 light:dark schedule. Water was provided *ad libitum* both through a spray bottle and plastic dish and crickets sprayed with Zilla<sup>®</sup> calcium supplement were provided twice a week.

Sprint speed of each *H. turcicus* (N = 26) was measured at five temperatures ( $T_e$ ) in the following order: 28, 20, 24, 32, and 36°C. Following the protocol of Huey et al. (1989), each gecko was maintained in a chamber for one hour at each temperature to equilibrate to the target  $T_b$  before sprint trials began. Individuals were run on a cardboard racetrack (100 x 10 x 6 cm) with markings in 10 cm intervals. A DBPower<sup>®</sup> Action Camera was positioned above the track recording at 120 frames per second (fps). Following the hour-long equilibration period, the gecko was removed from the chamber and placed at one end of the track and persuaded to move by waving a hand in close proximity. Two sprint trials were conducted in succession, with most being completed in 30–45 seconds, after which the geckos were returned to the temperature chamber for another hour. Geckos were then run another two times, for a total of four recordings, and then returned to their standard housing conditions. Once completed, geckos were given four–five days before the next sprint trial. Geckos that lost their tail during handling (n=3) were excluded for the remainder of sprint trials to prevent any possible effects of tail loss on measures of sprint speed. Maximum sprint speed was calculated by counting the number of frames recorded between the 10 cm intervals.

Immune performance was assessed by measuring the swelling response to PHA, a common compound used in vertebrate immunity studies to test for inflammatory responses coupled with lymphocyte proliferation (Martin et al. 2006, Tella et al. 2008, Brown et al. 2011). After sprint tests, the same geckos were randomly assigned (N = 6–7) to one of four different temperature treatments: 20, 25, 30, 35°C. Individuals were not tested at multiple temperatures to

avoid repeated injections of PHA within a short time period. PHA also elicits an adaptive response that includes immunological memory, which prevents repeated measures across temperatures. We also reduced the temperature treatments from five (in sprint tests) to four temperatures to maximize sample sizes per treatment. These reduced temperature treatments were also selected to align with the sprint tests as well as a separate experiment not included in this paper. The groups were placed into a chamber at their respective treatment temperature and given an hour to equilibrate. Prior to injections, individuals were weighed and the thickness of rear feet was measured to the nearest 0.01 mm using a Mitutoyo® micrometer. Any individuals weighing less than 2.5 g (n=2) were excluded from trials due to small foot size and increased chance of injury. Measurements of foot thickness were made four times to account for variability in measurements. Individuals were then injected with 10  $\mu\text{L}$  of PHA (0.2 mg x 10  $\mu\text{L}$  PBS<sup>-1</sup>) in their rear right foot and 10  $\mu\text{L}$  PBS in their rear left foot, as a control, using a 0.3 ml syringe. After an individual was injected, the gecko was immediately returned to the temperature chamber. If PHA leaked from the injection site, that individual was excluded from further measurements (n=2). After 24 and 48 hours post injection, swelling of rear feet thickness was measured in both PBS and PHA injected feet, with each foot measured four times. Geckos were then returned to the temperature chambers. Following the 48 hour measurements, geckos were returned to their standard housing conditions. Foot swelling was calculated as the mean of the four measurements for each rear foot, and immune response was calculated as the change in foot thickness between pre-injection and 24 and 48 hours post-injection.

### *Statistical Analyses*

### Field Collection

We also used a generalized linear model (GLM) to test factors that were associated with

habitat use or height from the ground where geckos were observed or captured. In this model, height from the ground was the response variable with age class, time of capture,  $T_{\text{wall}}$ , wall orientation, and an interaction term for  $T_{\text{wall}} \times$  orientation as fixed effects. Note both captured and observed individuals were included in this model ( $n = 264$ ), and a GLM was used because the response variable and transformations were not normally distributed.

The environmental factors determining  $T_b$  of field collected geckos ( $n = 81$ ) were also tested using a GLM with  $T_b$  as the response variable and  $T_{\text{wall}}$ , height from the ground, time of capture, wall orientation (cardinal direction the wall faced), and a  $T_{\text{wall}} \times$  orientation interaction as fixed effects. We used a GLM because  $T_b$  and transformed  $T_b$  were not normally distributed. Comparison of  $T_b$  patterns between age classes (adult and juvenile) was conducted through linear fits of  $T_b$  in relation to  $T_{\text{wall}}$  at the point of collection. To characterize potential environmental heat sources in relation to  $T_b$ , we calculated the median and range of temperatures for wall, air, and refugia (measured by iButtons) during the nocturnal active period (2000 to 2200 hours).

### Thermal Experiments

The effect of temperature on sprint performance was analyzed with a generalized additive mixed model (GAMM; R-package 'mgcv' v 1.8-28), which is used to assess nonlinear relationships between the predictor and response variables and produce smooth response curves through non-parametric smoothing functions (Zajitschek et al. 2012, Gilbert et al. 2016). As with previous research on sprint speed (Huey et al. 1989, Angilletta Jr et al. 2002a, Gilbert et al. 2016), maximum sprint speed at each temperature was used as the response variable with temperature treatment as the fixed effect and gecko ID as a random effect to account for repeated measures. The model was produced under a Gaussian error distribution. Swelling responses to PHA and PBS were recorded as the difference in average foot thickness between the

measurements at 24 and 48 hours in respect to those collected prior to the injections. All analyses were conducted in JMP® Pro Ver. 13 (SAS, 2016) and R v 3.6.1 (R Development Core Team, 2019). All measurement results are reported as mean  $\pm$  SEM.

## Results

A total of 165 *H. turcicus* were collected over the nine sampling nights with an additional 174 observed but not captured. Of those collected, 44% were adults and 56% were juveniles, while missed individuals had a 1:1 ratio. The mean SVL of captured adults was  $54.1 \pm 0.6$  mm, juveniles  $31.9 \pm 0.5$  mm. Activity was unimodal for all individuals with a peak between 2100–2200 and there was little difference among the age classes, although adults and juveniles that were observed but eluded capture appeared to be more active between 2200–2230.

Including all captured and observed geckos ( $n = 264$ ) perch height differed between both age classes (Fig. 2.1;  $\chi^2 = 21.7$ ,  $df = 1$ ,  $P < 0.0001$ ), with adults selecting higher locations ( $2.1 \pm 0.09$  m,  $n = 125$ ) than juveniles ( $1.6 \pm 0.09$  m,  $n = 139$ ) ( $P < 0.001$ ). Although it is possible that geckos utilizing higher perches were underestimated due to decreased detectability during surveys, this was assumed to be minimal because the buildings were  $\leq 5$  m in height. Wall orientation was also associated with perch height ( $\chi^2 = 12.8$ ,  $df = 3$ ,  $P = 0.005$ ), and while  $T_{\text{wall}}$  did not predict perch height (Fig. 2.1), there was an interaction between  $T_{\text{wall}}$  and orientation ( $\chi^2 = 8.6$ ,  $df = 3$ ,  $P = 0.04$ ). There was also a time of capture effect ( $\chi^2 = 3.7$ ,  $df = 3$ ,  $P = 0.05$ ). It should be noted that wall orientation influenced  $T_{\text{wall}}$  ( $\chi^2 = 16$ ,  $df = 3$ ,  $P = 0.001$ ), whereby north-facing walls were cooler ( $25.1 \pm 0.2^\circ\text{C}$ ) than the other directions (east:  $26.2 \pm 0.3^\circ\text{C}$ , south:  $26.2 \pm 0.2^\circ\text{C}$ , west:  $26.3 \pm 0.5^\circ\text{C}$ ). Accordingly, individuals occupied south- and west-facing walls (85 and 73 observations, respectively) more often, compared to fewer observations for north- and east-facing walls (55 and 51, respectively).

Body temperatures of captured *H. turcicus* ( $28.4 \pm 0.4^\circ\text{C}$ ) were within their reported  $T_{\text{pref}}$  range ( $26.4\text{--}31.6^\circ\text{C}$ ) (Huey et al. 1989, Angilletta Jr et al. 1999, Hitchcock and MacBrayer 2006).  $T_b$  was influenced by  $T_{\text{wall}}$  ( $\chi^2 = 12.7$ ,  $df = 1$ ,  $P = 0.004$ ), and wall orientation ( $\chi^2 = 14.4$ ,  $df = 3$ ,  $P = 0.002$ ), however, perch height had no effect on  $T_b$  and there was no interaction. Wall temperature predicted  $T_b$  ( $F_{1,76} = 77.12$ ,  $P < 0.0001$ ) and while age class was not associated with  $T_b$ , the interaction between  $T_{\text{wall}}$  and age class approached significance ( $F_{1,76} = 3.04$ ,  $P = 0.085$ ). This interaction suggests juveniles with  $T_b$  that more closely aligns with  $T_{\text{wall}}$  (Fig. 2.2, slope =  $0.95$ ,  $F_{1,19} = 42$ ,  $P < 0.0001$ ,  $r^2 = 0.39$ ) are stronger thermoconformers compared to adults (slope =  $0.63$ ,  $F_{1,57} = 33.7$ ,  $P < 0.0001$ ,  $r^2 = 0.37$ ). In relation to environmental heat sources,  $T_b$  of all captured geckos was elevated (Fig. 2.3A; median =  $28.7^\circ\text{C}$ , range =  $20.4$  to  $34.0^\circ\text{C}$ ) compared to  $T_{\text{wall}}$  (median =  $26.2^\circ\text{C}$ , range =  $17.8$  to  $32.4^\circ\text{C}$ ), and air (median =  $23.06^\circ\text{C}$ , range  $13.9$  to  $23.9^\circ\text{C}$ ). Temperatures recorded in refugia or cracks in the sampled buildings during the nocturnal active period had a median of  $25.0^\circ\text{C}$  (Fig. 2.3B; range  $18$  to  $31.5^\circ\text{C}$ ).

Sprint speed increased as temperature increased ( $F_{1,124} = 24.87$ ,  $P < 0.0001$ ) from  $20$  ( $1.03 \pm 0.03$  m/s),  $24$  ( $1.23 \pm 0.04$  m/s), and  $28$  ( $1.38 \pm 0.05$  m/s) $^\circ\text{C}$  but then plateaued between  $28\text{--}36^\circ\text{C}$  (Fig. 2.4). There was no single optimal temperature because maximal performance was maintained across the three highest temperature treatments ( $28^\circ\text{C} = 1.38 \pm 0.05$  m/s,  $32^\circ\text{C} = 1.38 \pm 0.04$  m/s,  $36^\circ\text{C} = 1.41 \pm 0.03$  m/s). Variation in sprint speed was not associated with individuals ( $P > 0.40$ ).

The swelling response to PHA injections did not differ between 24 and 48 hours post injection ( $P > 0.68$ ), therefore only the 24-hour values were used for analyses. Additionally, swelling to PBS injections was not different from zero across the temperature treatments ( $P > 0.14$ ) indicating it was an adequate control, and that PHA induced the expected swelling

response. Elevated temperatures increased the swelling response to PHA (Fig. 2.5;  $F_{3,14} = 3.82$ ,  $P = 0.035$ ) but body mass prior to injections had no effect ( $F_{1,14} = 0.80$ ,  $P = 0.381$ ) and there was no interaction ( $F_{3,14} = 0.089$ ,  $P = 0.97$ ). Swelling was lowest at 20°C ( $0.22 \pm 0.044$  mm,  $P < 0.032$ ; Tukey's HSD test) and increased with temperature and plateaued between 30°C ( $0.39 \pm 0.028$  mm) and 35°C ( $0.38 \pm 0.023$ ).

## Discussion

Invasive species are often thought to have a wide tolerance and capacity to cope with novel environmental conditions that underlie their persistence and successful establishment in new habitats (Kelley 2014, Cortes et al. 2016), and thermal performance of multiple fitness-related traits is expected to exhibit coevolved temperature-dependent responses with wide thermal tolerances (Angilletta Jr et al. 2006). We tested these expectations through coupling field assessments of thermoregulation with thermal performance tests among a population of invasive geckos (*H. turcicus*) at their northern invasion front. Wild caught geckos exhibited a wide distribution of  $T_b$  that correlated with  $T_{wall}$ , consistent with a thermoconformer strategy, and that was most pronounced among juvenile geckos. In the lab, sprinting and an immune swelling response to PHA injection both displayed thermal performance responses that increased with temperature, and both exhibited wide, overlapping thermal breadths. The wide thermal performance values of these traits suggest eurythermia contributes to the colonization success of *H. turcicus* in suboptimal thermal climates. Below we explore how coping mechanisms and capacity of invasive species to maintain functional performance across wide conditions likely contribute to their ability to invade new regions.

The  $T_b$ s of wild caught geckos were strongly correlated with  $T_{wall}$  at the point of capture, indicative of a thermoconformer, but the degree of thermoconformity varied between adults and



juveniles (Fig. 2.2). Juveniles had  $T_b$ s that were more tightly correlated with  $T_{wall}$  (slope = 0.95) than adults (slope = 0.63). The differences in thermoconformity could be a result of intraspecific interactions, habitat use, and/or body size effects. *Hemidactylus* geckos are known to exhibit high levels of intraspecific aggression (Frankenberg 1982, 1984, Regalado 2003), and juveniles will commonly avoid adult conspecifics (Briggs 2012) and select perches further from adults or disperse to uncolonized buildings (Locey and Stone 2008, Paulissen et al. 2013). Indeed, we found juveniles were consistently at lower perch heights on walls compared to adults (Fig. 2.1). While  $T_{wall}$  was independent of perch height in this study, adults could have restricted juvenile access to refugia that could be important for behavioral thermoregulation (Schlesinger and Shine 1994) as nocturnal lizards select warmer retreat sites over cooler options (Downes and Shine 1998, Kearney 2002, Shah et al. 2004, Lettink et al. 2009). The differences in the thermoconformity could have also been related to thermal inertia as a result of differences in body sizes between adults and juveniles (Bell 1980). Further work should examine if juveniles, in the absence of adults, reduce their thermoconformity to identify if intraspecific interactions drove the observed differences.

Field  $T_b$  generally overlapped with maximal thermal performance of sprint speed and immune function. Both traits exhibited increasing performance with temperature that were generally highest between 28 – 36°C. As temperature-dependent function of these traits overlapped with the field  $T_b$  measured in this study and the reported  $T_{pref}$  for *H. turcicus* (26.4–31.6°C) (Huey et al. 1989, Hitchcock and MacBrayer 2006), our data support the prediction of Martin and Huey (2008) that  $T_{pref}$  exists below the  $T_b$  for optimal performance. Additionally, the relatively low field and reported  $T_{pref}$  could also reflect maintenance of temperatures that maximize other functional performance traits not measured in this study (e.g., digestion, growth

rate). We suggest that the eurythermality of the measured physiological traits allows the geckos to be active at these lower temperatures without significantly compromising performance.

The relationship between lizard  $T_b$  and sprint speed in particular is well studied, and our results followed the expected TPC (Huey and Stevenson 1979, Angilletta Jr et al. 2002a, Angilletta Jr et al. 2006, Angilletta Jr et al. 2009) but with a plateau at higher temperatures that defined a wide functional range. These data and our conclusions thus come with the caveat that we did not subject the geckos to sufficiently high temperatures to detail the entire thermal performance curve. However, in a follow up experiment using higher temperatures we found that the pejus range for sprint speed of this population occurred at 38°C (Dallas et al. unpub. data). With this caveat in mind, we suggest the sprint performance and thermal breadth of this population was not different from a Texas population (Huey et al. 1989), despite being at a higher latitude and cooler climate, suggesting an innate eurythermality that likely pre-adapts this species to invading a wide array of climates.

PHA-induced swelling similarly increased with temperature and exhibited a wide performance breadth at higher temperatures. These data, however, also come with the caveat that higher temperatures were necessary to reach the pejus and to accurately quantify the TPC. Despite this shortcoming, these data are consistent with previously published work. For example, *in vitro* PHA responses in blood from the common wall lizard (*Podarcis muralis*) exhibited TPC for which lymphocyte proliferation was maximized at 32°C, which corresponded to their  $T_{pref}$  (Sacchi et al. 2014). In our study, the *in vivo* immune swelling response to PHA most likely induced both an innate inflammatory response, followed by a lymphocyte, cell-mediated adaptive immune response (Brown et al. 2011). Although aspects of immune function can clearly exhibit temperature dependence, the relationship is complex and likely varies with differing

immune components. For example, Palackdharry et al. (2017) found that temperature had contrasting effects on two aspects of B-cell immunity in red-eared slider turtles (*Trachemys scripta*). Butler et al. (2013) also found the temperature dependence of innate immune responses across vertebrate clades was variable, while other researchers showed the herpetofaunal complement system typically exhibited stereotypical TPC (e.g., Baker and Merchant 2018, Baker et al. 2019, Moretti et al. 2019). Our results are in agreement with those that identified immune performance increasing positively with temperature in a non-linear fashion, and suggest that higher temperatures would likely induce a typical TPC for the PHA swelling response. These results contribute to a growing body of research suggesting that animals can thermoregulate (behaviorally and physiologically) for body temperatures that optimize immunity when combating infections (Butler et al. 2013, Palackdharry et al. 2017, Baker and Merchant 2018, Baker et al. 2019).

Our work contributes to growing research detailing that eurythermality likely contributes to successful invasion and establishment of herpetofauna in novel environments. While our study was limited to a single population, the similarity in TPCs of these fitness related traits and their overlap with field  $T_b$  are also consistent with the coadaptation hypothesis (Mayr 1963), which postulates that TPCs coevolved with the preferred temperature ( $T_{pref}$ ) of an ectotherm (Angilletta Jr et al. 2006). Considering that the maximal performance and the similarly wide thermal tolerance of these traits overlapped with  $T_b$  of geckos in the field, suggests that selection of thermally optimal microhabitats is associated with maintenance of  $T_b$  that supports the functional performance of these fitness related traits. A comparative approach testing physiological performance of multiple such traits across a wider range of  $T_b$  and differing populations across a latitudinal gradient could provide insight into how thermally dependent physiological

performance can determine range expansion of invasive species. Additionally, our study provides insight into the thermal dependence of immunity, which is poorly examined among herpetofauna and vertebrates in general. Ultimately, our research highlights the need to incorporate multiple aspects of physiology when examining temperature-dependent performance, and how these may influence the ability of invasive species to expand their range and shift energy allocation in the face of novel environments.

## CHAPTER 3

### ECOLOGY OF THE SYNANTHROPIC MEDITERRANEAN HOUSE GECKO (*HEMIDACTYLUS TURCICUS*) AT THEIR NORTHERN INVASION FRONT

#### Introduction

Biological invasions pose a significant threat to native species as a recent review found invasive species to play a role in 33% of animal extinctions (Blackburn et al. 2019). Their role in relation to conservation biology signifies the need to monitor invasive populations as they become established. While there has been a great deal of research into the commonalities amongst successful invasive vertebrate species (Lockwood et al. 2005, Hayes and Barry 2007, Blackburn et al. 2009, Allen et al. 2013, Capellini et al. 2015), invasive ectotherms, such as herpetofauna, require either specific thermal habitat quality or rapid thermal acclimation/adaptation in order to become established in novel, oftentimes cooler climates (Mahoney et al. 2015, Winwood-Smith et al. 2015, Cortes et al. 2016, Card et al. 2018). Despite their introduction into cooler regions, many herpetofauna are able to rapidly colonize and become established in these areas (Meshaka Jr 2011) particularly through exploitation of urban habitats and anthropogenic structures (Battles et al. 2018, Battles and Kolbe 2019, Hulbert et al. 2020, Thawley and Kolbe 2020). As invasive herpetofauna expand their geographic ranges, examining the ecology of these recently colonized populations may provide insights into how they successfully gain a foothold in these new areas.

As the thermal environment is critical to physiological performance of herpetofauna (Taylor et al. 2020), long-term exposure to cooler thermal habitats is expected to result in increased dependence on behavioral thermoregulation to regulate their body temperature ( $T_b$ ) to offset any potential loss of performance (Huey et al. 2003). However, for nocturnal lizards, there

is little opportunity for behavioral thermoregulation during active periods and they instead exhibit ‘thermoconformity’ (Hitchcock and MacBrayer 2006, Tan and Schwanz 2015).

Thermoconforming lizards have  $T_b$ s that closely match their thermal environment ( $T_e$ ), which typically results in nocturnal lizards having a relatively larger mismatch between their  $T_b$  and both optimal performance temperature ( $T_{opt}$ ) and preferred temperature ( $T_{pref}$ ) relative to diurnal lizards (Huey et al. 1989, Hitchcock and MacBrayer 2006, Wessels et al. 2018). As physiological traits exhibit a thermal reaction norm with respect to  $T_b$  (e.g., Huey and Kingsolver 1989), this mismatch should result in reduced performance, particularly for those in temperate regions that must endure seasonal shifts in  $T_e$  and, in turn,  $T_b$ . Therefore, as invasive thermoconformer lizards colonize higher latitudes, they must be able to cope with greater fluctuations in  $T_b$  or exhibit some degree of behavioral thermoregulation (e.g., Angilletta Jr et al. 1999) and sources within) to minimize the inherent thermal variability.

Mediterranean House Geckos (*Hemidactylus turcicus*) are native to southern Europe, northern Africa, and the Arabian Peninsula (McCoy 1970) and were first documented in the United States in Key West, Florida in 1910 (Fowler 1915). They quickly spread throughout the Gulf Coast states and now are found in > 15 states (Meshaka Jr et al. 2006, Powell et al. 2016, Stebbins and McGinnis 2018) including those at higher latitudes. Despite poor dispersal ability (Trout and Schwaner 1994, Locey and Stone 2006, Paulissen et al. 2013), they exploit jump dispersal to colonize new areas many kilometers away (Davis 1974, McCallum et al. 2008, Short and Petren 2011) as their eggs are highly desiccation-tolerant (Dunson 1982). Rödder and Lötters (2009) found mixed support for both habitat conservatism and shifts in the invasive Mediterranean House Gecko range when compared to their native range, indicative of potential expansion of their environmental niche accompanying invasion. In the United States,

Mediterranean House Geckos are synanthropic, occupying buildings and other anthropogenic structures with near exclusivity (Rose and Barbour 1968, Selcer 1986, Meshaka Jr 1995, Punzo 2001, Gomez-Zlata et al. 2006, Meshaka Jr et al. 2006, Stabler et al. 2012, Wessels et al. 2018). As with other invasive lizards (e.g., Tiatragul et al. 2017), being human commensals may aid Mediterranean House Geckos in rapid colonization of temperate areas through a climate buffering effect of the urban heat island effect. Additionally, they are eurythermic (Huey et al. 1989, Dallas et al. 2021) capable of broad thermal reaction norms that may further their ability to cope with the seasonal thermal conditions they experience during northward colonization.

While Mediterranean House Geckos have been well-studied in the southern core of their invasive range (e.g., Rose and Barbour 1968, Selcer 1986), there remains only limited insight into their natural history along the northern invasion front (Norden and Norden 1991, Paulissen and Buchanan 1991, Stabler et al. 2012, Wessels et al. 2018), indicating a need to better understand how these populations persist in cooler, more seasonal climates. We sought to expand upon the current information of Mediterranean House Geckos by examining a recently colonized population at their northern invasion front. Through a mark-recapture study over seven months across two years, we examined aspects of Mediterranean House Gecko ecology, including body length, population demography, reproduction, and growth rate, and broadly compared our findings to other populations. As tail shedding is accompanied by a variety of physiological costs (e.g., Bateman and Fleming 2009, Higham et al. 2013, McElroy and Bergmann 2013), we also categorized tail shedding rates. Furthermore, due to the thermoconformer nature of Mediterranean House Geckos, we measured aspects of their thermal biology to determine any potential reduction in physiological performance they may experience based on previous thermal performance studies (Huey et al. 1989, Dallas et al. 2021).

## Materials and Methods

We conducted nighttime visual surveys for Mediterranean House Geckos in downtown Carbondale, Illinois (N 37.72°, W 89.22°) where this species was initially observed on a single building in 2012 (Whiles et al. 2013) and has since dispersed to buildings ~150 m away within six years (Dallas et al. 2021). The climate of Carbondale is characterized by cooler temperatures and greater seasonality than most native and invasive populations (Fig. 3.1). Monthly surveys were conducted across eight buildings in September 2019 and May – October 2020 with two – three visits over the course of seven days. As Mediterranean House Geckos become active after sunset (Rose and Barbour 1968, Selcer 1986, Punzo 2001), start times varied across the year (2100 hours in April and May 2020, 2130 hours for June – August 2020, and 2000 hours for September and October 2020) and surveys lasted between two – five hours to ensure each building was sampled once. All surveys were conducted on nights without precipitation. Animal collection was approved by the Southern Illinois University Carbondale Animal Care and Use Committee (protocol 18-043).

Mediterranean House Geckos were identified with flashlights on the walls of buildings and captured by hand. The  $T_b$  of Mediterranean House Geckos was collected within 30 seconds of capture by gently inserting a thermocouple probe (Physitemp BAT-12, Clifton, New Jersey) into the cloaca. However, the  $T_b$  of hatchlings ( $\leq 30$  mm) was measured by placing the thermocouple between the thigh and cloaca; this method has previously been used to accurately record gekkonid  $T_b$  (e.g., Aparicio Ramirez et al. 2020). Snout-vent length (SVL) was measured with a ruler and sex was recorded by the presence/absence of pre-cloacal pores and hemipenes in adults only. We only provided juvenile sex when individuals were recaptured as adults and sex could be determined. Gravid females were identified by the presence of eggs that can be seen



through the thin, ventral surface. We observed tail status as intact (no observable damage or regeneration) or damaged (those that were broken, regenerating, or fully regenerated). All individuals  $> 35$  mm SVL were given a unique ID through toe clipping by removing a single toe per foot and no more than three toes total as this has been shown to not affect climbing performance (Paulissen and Meyer 2000). The smaller individuals were marked as cohorts, with only a single toe removed from the rear foot as their size precluded us from safely removing multiple toes. Individuals then received a mark on their dorsal surface with a waterproof marker to ensure they were not recaptured again during that night or month and were released at site of capture after being held for less than five minutes. For those captured in 2019, we only measured SVL and marked them.

During the 2020 surveys, we recorded the  $T_e$  by measuring the temperature of the wall at the spot where the gecko was first observed by using an infrared thermometer (Etekcity Lasergrip 774, Anaheim, California) approximately 10 – 20 cm away from the wall. Similar to Hitchcock and MacBrayer (2006), we measured random  $T_e$  ( $T_{\text{Random}}$ ) approximately 1 m away from the site of capture to determine if  $T_e$  selection was nonrandom. We did not record the  $T_e$  of individuals captured on the ground as we likely collected them in the process of dispersal rather than a representation of the thermal environment they use (Locey and Stone 2008). Perch height of the initial gecko observation was measured via a tape measure. We also recorded the time of capture, the building where the gecko was captured, and the direction of the wall face.

### *Statistical Analyses*

We separated captures into three discrete age classes (hatchlings  $\leq 30$  mm, juveniles = 31 – 43 mm, adults  $> 43$  mm SVL) based on previous published reports for Mediterranean House Geckos (Rose and Barbour 1968, Selcer 1986, Punzo 2001). Potential differences in population

demography across the sampling months were assessed via  $\chi^2$  Goodness of Fit tests. For assessing age ratios, we combined juveniles and hatchlings into a single, young-of-the-year (YOY) category. Adult sex ratios were only compared for 2020 as sex was not determined for individuals captured in 2019. Tail status was only examined for the initial capture of individuals to reduce the confounding effects of those whose tails broke during capture. We also examined how tail status varied by adult sex and the relationship between SVL and tail status was assessed via Pearson's correlation coefficient. We examined potential differences in perch height among the age classes with a one-way ANOVA.

Individuals that were held for  $> 30$  seconds before  $T_b$  was collected ( $N = 20$ ) were dropped from the temperature analyses to reduce the potential risks associated with heat transfer during prolonged handling. Pearson's correlation coefficient was calculated to examine the relationship between  $T_b$  and  $T_e$ , a proxy for thermoconformity, for all 2020 individuals. Linear models assessed how  $T_b$  was influenced by 1)  $T_e$  and age class along with the interactions and 2) month and age class along with the interaction. We compared  $T_e$ s used by Mediterranean House Geckos against  $T_{Random}$  with a paired t-test separated by age class. We then assigned  $T_{Random}$  warmer than occupied  $T_e$ s a 1 and all other  $T_{Random}$  a 0 to fit a binomial general linear model to identify if Mediterranean House Gecko selection of  $T_e$  was nonrandom with respect to available  $T_e$ s and age class.

We assessed recaptures for those caught  $\geq$  one month apart. The effect of sex and tail status (intact v. damaged) on recapture rates were analyzed via separate  $\chi^2$  Goodness of Fit tests. Growth rate was calculated as a daily estimate by taking the difference in SVL between capture events and dividing it by the number of days between capture. For individuals recaptured multiple times, we estimated daily growth rate by the differences in SVL and days between the

first and final capture. We assumed that individuals initially captured in 2019 and then recaptured in 2020 had a period of limited-to-no growth during the winter that extended from 1 November to 1 April (153 days). In calculating daily growth rate, individuals that exhibited declines in SVL between recapture events ( $N = 4$ ) were removed from the analyses as they likely represent researcher error rather than a true reduction in body size. We averaged the SVL of cohorts captured in August 2020 and subtracted that from the average SVL of recaptured cohorts in September 2020 to determine daily growth rate of hatchlings. The remaining cohorts from either 2019 or 2020 were not included in daily growth rate analyses as we could not specify unique individuals from one another. We used Pearson's correlation coefficient to examine how initial SVL was related to daily growth rate as seen in other studies (Selcer 1986, Punzo 2001, Paulissen et al. 2014). Additionally, a two-sample t-test compared daily growth rate between sexes. All analyses were conducted in R v 3.6.1 (Team 2021).

## Results

Over 19 sampling days across seven months, we collected 444 Mediterranean House Geckos a total of 555 times. Adults represented a plurality of the population ( $N = 202$ ) followed by juveniles (129) and hatchlings (113). Age ratios varied significantly across the months ( $\chi^2_6 = 255.62$ ,  $P < 0.0001$ ) with YOY absent in July and more abundant in September and October (Table 3.1). Adult sex ratios never differed from 1:1 across the months of 2020 ( $P > 0.17$ ; Table 3.1). Furthermore, there was no evidence of sexual size dimorphism in the population ( $P > 0.97$ ) as adult males ( $53.5 \pm 0.5$  mm,  $N = 95$ ) were identical in size as females ( $53.5 \pm 0.5$ , 86).

There was strong evidence that Mediterranean House Geckos exhibited thermoconformity in southern Illinois (Fig. 3.2). Most of the variation in  $T_b$  (84%) was primarily – and expectedly – due to  $T_e$  ( $F_{1,413} = 2053.97$ ,  $P < 0.0001$ ), age class was only marginally

significant ( $F_{2,413} = 2.91$ ,  $P = 0.055$ ) and the interaction had no effect ( $P > 0.78$ ). Across 2020 (Fig. 3.3),  $T_b$  varied by month ( $F_{4,405} = 276.77$ ,  $P < 0.0001$ ) as mean  $T_b$  remained  $> 25.0^\circ\text{C}$  from May – August but was  $< 21.5^\circ\text{C}$  for September and October. There was no effect of age class on monthly  $T_b$  ( $F_{2,405} = 0.31$ ,  $P = 0.73$ )

We have previously identified  $T_{\text{pref}}$  of this population to range from  $28.3 - 31.3^\circ\text{C}$  (J. W. Dallas, M. Deutsch, and R. W. Warne, unpublished data) which is slightly narrower than other published data ( $26.4 - 31.6^\circ\text{C}$ ; (Huey et al. 1989, Angilletta Jr et al. 1999, Hitchcock and MacBrayer 2006). From June to August, the mean  $T_b$ s of all age classes were within the published  $T_{\text{pref}}$ . However, the  $T_b$ s of adults in May approached  $T_{\text{pref}}$ , and mean  $T_b$ s during September and October were  $\sim 6^\circ\text{C}$  below the lower range of  $T_{\text{pref}}$  (Fig. 3.3).

Perch height varied among the three age classes (Fig. 3.4;  $F_{2,548} = 9.31$ ,  $P < 0.001$ ) with a Tukey post-hoc test identifying that adults selected higher perches relative to hatchlings ( $P < 0.001$ ) and juveniles, although the latter's only approached significance ( $P = 0.053$ ). There was no effect of sex on perch height ( $t_{257} = 1.55$ ,  $P = 0.12$ ).

The paired t-tests indicated that mean  $T_{\text{eS}}$  selected by Mediterranean House Geckos were warmer than  $T_{\text{Random}}$  (Fig. 3.5a) for adults ( $t_{232} = 3.55$ ,  $P < 0.001$ ), but this difference only approached significance for both juveniles ( $t_{109} = 1.89$ ,  $P = 0.062$ ) and hatchlings ( $t_{75} = 1.93$ ,  $P = 0.058$ ). However, the logistic regression model found the selection of warmer microhabitats compared to  $T_{\text{Random}}$  was random for any age class across all selected  $T_{\text{eS}}$ , perch heights, and the interactions (Fig. 3.5b;  $P > 0.56$ ).

There were 29 gravid females captured over the 2020 season (Table 3.1). Gravid females were observed from May – August with June and July having the highest relative proportion of gravid individuals with 66.7% and 46.4% of females, respectively, containing visible eggs. Most

females (82.8%) contained two visible eggs while only 13.8% contained a single egg and we could not accurately determine the number of eggs in one individual. While no nests were observed, hatchlings first appeared in August but were also encountered in September and October indicative of Mediterranean House Geckos producing two clutches per year in southern Illinois.

The overwhelming majority of Mediterranean House Geckos had their tails intact (79.0%). The risk of tail shedding was negatively related to SVL (Fig. 3.6;  $r = -0.34$ ,  $t_{437} = 7.51$ ,  $P < 0.0001$ ). Adult sex did not influence tail status ( $\chi^2_1 = 2.43$ ,  $P = 0.12$ ) although there was a larger proportion of males with damaged tails.

We recaptured 62 non-cohort-marked individuals a total of 141 times. When compared to those only captured once, neither sex nor tail status influenced recapture success ( $P > 0.20$ ) although females and tailed individuals had slightly higher recapture rates. Only four individuals were recaptured on different buildings than their original capture site, signifying limited dispersal. The daily growth rate of 58 individuals with non-negative changes in growth ranged from 0.0 – 0.22 mm/day ( $0.059 \pm 0.0067$  mm/day) for a monthly average of 1.77 mm/month. Variation in growth rate was strongly driven by initial SVL ( $F_{1,52} = 61.49$ ,  $P < 0.0001$ ) with smaller individuals growing faster than adults (Fig. 3.7), but neither sex nor the interaction had any effect ( $P > 0.46$ ). The average SVL growth rate of six recaptured cohort individuals in September 2020 representing hatchlings from the previous month was 0.14 mm/day, ranging from four – five mm of total SVL growth. Examining the SVL of all captures in each month shows growth rates comparable to what we calculated from the recaptured individuals (Fig. 3.8).

Discussion

Mediterranean House Geckos have been a successful invasive species in North America as they have dispersed from southern Florida and are now established in urban areas across the southern half of the United States (Meshaka Jr et al. 2006, Powell et al. 2016). Our research exemplifies the ability of this species to rapidly colonize a novel environment in under eight years and a similar number of generations. Therefore, despite having to cope with relatively long winters compared to their native and introduced southern United States geographic range core (Fig. 3.1), Mediterranean House Geckos can readily persist with little observable negative population costs as has been shown for this species (Norden and Norden 1991, Paulissen and Buchanan 1991, Stabler et al. 2012, Wessels et al. 2018). Our focal population underwent significant seasonal changes in  $T_b$  (Fig. 3.2) that resulted in them remaining active below their  $T_{pref}$  during the autumn which may have potential consequences for physiological performance. Additionally, while Mediterranean House Geckos occupied warmer  $T_e$  than  $T_{Random}$ , this selection was random indicating that the thermal microhabitat is not an essential characteristic of microhabitat selection for this species.

The strong predictive effect of  $T_e$  on  $T_b$  demonstrates the thermoconformer nature of Mediterranean House Geckos (Fig. 3.2). This has been previously identified in this species (Hitchcock and MacBrayer 2006, Dallas et al. 2021) but may be of particular importance for physiological performance across the year. Ectotherms generally maintain their  $T_b$  within their  $T_{pref}$  range to ensure near-peak performance without risk of approaching their critical thermal limits (Martin and Huey 2008), and accomplish this primarily through behavioral thermoregulation (Angilletta Jr 2009). However, thermoconformer  $T_b$ s reflect the available  $T_e$ s meaning they have lax thermoregulatory control and are often outside of their  $T_{pref}$  (Hertz et al. 1993). Our results indicated that the majority of Mediterranean House Geckos could attain  $T_{pref}$

across the summer months but failed to do so in autumn (Fig. 3.3). These suboptimal thermal conditions suggest that Mediterranean House Geckos exhibit reduced whole-body and immune performance based on laboratory studies (Huey et al. 1989, Dallas et al. 2021). Data from this population show that at 20°C, maximum sprint speed and immune performance would be reduced ~70% and 55%, respectively, compared to performance in their  $T_{pref}$ . This could place greater costs upon this species at their northern invasion front as they may exhibit reduced foraging success and may be ill-equipped to mount a proper immune response. However, the costs of maintaining activity for several months at relatively cool temperatures remains unknown as it is clear the Mediterranean House Gecko is thriving in southern Illinois. It may be that the lack of any sustained predatory pressure and abundance of arthropod prey in urban centers enables them to remain active despite reduced performance. Their eurythermic reaction norms (Huey et al. 1989, Dallas et al. 2021) may also minimize the risks of low  $T_b$ s *H. turcicus* experience across the early- and latter-periods of their annual activity. Therefore, while the thermoconformer nature of Mediterranean House Geckos results in seasonal variation in their ability to achieve their  $T_{pref}$ , the cool conditions during autumn do not seem to have any significant costs for their population persistence.

We identified that age-dependent habitat partitioning occurred in the vertical axis (Fig. 3.4). Variation in perch height with adults occupying higher perches than juveniles and hatchlings has previously been recognized for Mediterranean House Geckos (Saenz 1996, Locey and Stone 2008, Paulissen et al. 2013, Dallas et al. 2021). As juvenile Mediterranean House Geckos exhibit submissive behaviors towards adults (Briggs 2012), it is likely that juveniles avoid adult-established microhabitats to reduce potential conflicts. Additionally, dietary partitioning (Saenz 1996) and juvenile dispersion (Locey and Stone 2008) may further account

for ontogenetic habitat differentiation. Further exploration of whether needed to determine if these potential mechanisms act synergistically to restrict younger conspecifics to lower perches could which elucidate dispersal dynamics and ontogenetic microhabitat selection.

While Mediterranean House Geckos were found to occupy warmer thermal microhabitats than random sites within their home range, the differences were minimal suggesting this result may be a statistical artifact rather than true thermal microhabitat selection. This was supported as the selection of warmer  $T_{es}$  was random with respect to  $T_{Random}$  and perch height (Fig. 3.5). We must acknowledge that this methodology has two limitations. First, since the home range of Mediterranean House Geckos is up to five meters (Klawinski 1991, Paulissen et al. 2013), sampling approximately one meter away from the capture location may have underestimated the true variation in  $T_{es}$  available within their home range. Second, as we only sampled  $T_{Random}$  from walls on which Mediterranean House Geckos occupied,  $T_{es}$  from unoccupied walls may be substantially different, resulting in more broad-scale habitat selection based on  $T_e$  resulting in the observed occupancy pattern (reviewed in Hertz et al. 1993). Regardless of these limitations, it is unlikely that available  $T_{es}$  significantly influence microhabitat selection for Mediterranean House Geckos and other, unmeasured features (e.g., artificial lighting; Capula and Luiselli 1994, Williams and McBrayer 2007, Harris 2009; availability of refugia; Paulissen and Buchanan 1991, Nelson and Carey 1993, Williams and McBrayer 2007), may play more important roles.

Our results provide insight into the structure of this population and revealed a general similarity with those from other invasive locales. The relatively evenness of adult sex ratios has been found for other populations (Selcer 1986, Meshaka Jr 1995, Punzo 2001, Wessels et al. 2018). Monthly changes in age classes resembled published findings particularly with YOY being relatively more abundant in the autumn (Rose and Barbour 1968, Selcer 1986, Meshaka Jr



1995, Wessels et al. 2018) but this was not found previously for this population (Dallas et al. 2021). The timing of gravid females (May – August) is similar to that seen in Florida (Meshaka Jr 1995, Punzo 2001) but shorter than that in Louisiana (Rose and Barbour 1968) and Texas (Selcer 1986). Our findings indicate that Mediterranean House Geckos in southern Illinois are likely producing two clutches a year, similar to other populations (Rose and Barbour 1968, Paulissen and Buchanan 1991, Punzo 2001), but that there is enough time for them to triple clutch, which has been previously observed in this species (Meshaka Jr 1995, Sever et al. 2009). Hatchlings were first observed in August and were found in each of the following months, which may indicate their ability to triple clutch or variable hatching of two clutches. Examination of ovarian follicles is needed to develop definitive conclusions of annual clutch production. Meshaka Jr et al. (2006) suggested that as Mediterranean House Geckos expand into higher latitudes, the longer winter period of inactivity would likely reduce the breeding season necessary to develop > two clutches, but this may not be the case in southern Illinois. We speculate that the ability of Mediterranean House Geckos to produce two or three clutches annually likely explains their rapid increase in population size in recently colonized regions.

The high percentage of individuals with intact tails (79.0%) reflects little pressure on individuals to exhibit autotomy. In their native range, Itescu et al. (2017) found that intraspecific competition, not predation, was the primary factor behind high rates of autotomy with approximately 29% of island populations retaining intact tails. We would expect a similar result as predation for our population is likely to be minimal, and Mediterranean House Geckos – particularly males – exhibit high degrees of intraspecific aggression (Frankenberg 1984, Briggs 2012). However, the lack of a sex-effect amongst adults suggests that, despite occupying isolated buildings, there may not be as many direct agonistic interactions among individuals as expected.

Alternatively, the density of geckos in southern Illinois may be too low to facilitate a high degree of agonistic interactions. We speculate that the relationship between tail status and body size may simply be that adults have more time than juveniles for autotomy to occur, although no such body size effect was found previously (Itescu et al. 2017). Assessing tail status across the introduced range of Mediterranean House Geckos, especially in areas with greater predation risks (e.g., Meshaka Jr et al. 2004), would provide substantial insight into the roles of predation, intraspecific interactions, or a yet unidentified drivers of tail loss.

Growth rates of adult Mediterranean House Geckos in southern Illinois were between those reported in Texas (1.49 mm/month; Selcer 1986) and Florida (2.03 mm/month; Punzo 2001) but higher than seen in a Louisiana population (0.94 mm/month; Paulissen et al. 2014). In agreement with the data from Texas and Florida, sex had no effect on growth rate and, there was a strong, negative effect of initial SVL on growth rate, similar to data from Louisiana, indicating that growth rate dramatically slows as Mediterranean House Geckos attain larger body sizes. From a limited number of YOY recaptures, we estimate a monthly growth rate of between 4 – 5 mm and that sexual maturity is reached during their first summer, approximately 9 – 10 months post-hatching – which is the upper range limit for more southern populations (Selcer 1986, Meshaka Jr 1995, Punzo 2001). However, as we only assessed SVL growth rate over a single year and grouped all buildings sampled together, we may have missed interannual and spatial variation in the growth rate of Mediterranean House Geckos. Paulissen et al. (2014) found that SVL growth rate varied across years, while Hibbs et al. (2004) observed a change in body mass growth between buildings sampled while SVL growth rate did not differ. The authors of both studies suggested differences in arthropod abundance drove concurrent changes in growth rates. Therefore, it may be necessary to further examine growth rate across both temporal and spatial

scales in conjunction with assessing arthropod abundance to identify what factors can influence Mediterranean House Gecko growth rate in southern Illinois.

The Mediterranean House Gecko is a successful colonizer in North America establishing populations in urban centers across the United States (Meshaka Jr et al. 2006). The high degree of seasonality for our focal population resulted in monthly  $T_{bs}$  fluctuations that lowered the capacity for Mediterranean House Geckos to maintain  $T_{bs}$  within their  $T_{pref}$  during the summer, and often to exhibit  $T_{bs}$  that were 6°C below  $T_{pref}$  in autumn. While we know physiological performance for this population is hindered at cooler  $T_{bs}$  like those in autumn months (Dallas et al. 2021), we do not know the fitness costs associated with months of reduced performance for this and other high latitude Mediterranean House Gecko populations. Our results suggest that prolonged periods of cold have minimal effect on the population as a whole since population demographics, reproductive patterns, and growth rates were similar to those in their southern, geographic core. Therefore, our previous suggestion (Dallas et al. 2021) that Mediterranean House Geckos exploit their eurythermic physiology to successfully colonize higher latitudes seems appropriate but requires further exploration. In conjunction, their association with urban structures likely buffers against cold conditions in higher latitudes, minimizing potential costs associated with these temperatures. Continued monitoring of this population through a combination of field and laboratory methods would enable us to better understand the behavioral or physiological mechanisms that enable Mediterranean House Geckos to rapidly colonize and establish large populations in suboptimal thermal environments.

## CHAPTER 4

### HEAT HARDENING OF A LARVAL AMPHIBIAN IS DEPENDENT ON ACCLIMATION PERIOD AND TEMPERATURE

#### Introduction

Environmental temperature is one of the most important abiotic drivers of organismal physiology (Angilletta Jr 2009). As mean temperatures and heat wave frequencies are expected to increase due to climate change (IPCC 2021), ectotherms will be under greater risk of approaching their upper thermal limit. This could lead to shifts in species distributions, altered biological interactions, and reduced activity periods, all of which can result in extinction (Somero 2010, Bellard et al. 2012, Blois et al. 2013, Cox et al. 2022). Global declines in amphibians have been linked to climate change (e.g., Blaustein et al. 2010, Rollins-Smith 2017, Campbell Grant et al. 2020, Lowe et al. 2021), highlighting the need for continued research on how they respond to warming and thermal extremes.

As thermal traits generally evolve slowly in herpetofauna (e.g., narrow-sense heritability < 15%; Bodensteiner et al. 2020), phenotypic plasticity is likely a primary response to climate change and increasing thermal stress. Thermal acclimation represents reversible plasticity in basal heat tolerance and develops over days to weeks of chronic exposure to altered environmental temperatures (e.g., Cupp Jr 1980, Li et al. 2009, Sgro et al. 2010, Rohr et al. 2018, Lapwong et al. 2021b). However, acclimation does not necessarily protect organisms against acute exposure to short-term heat events such as heat waves, which are projected to increase in frequency (Seneviratne et al. 2021). The related heat hardening response is another form of thermal plasticity that, by contrast, develops rapidly over minutes to hours of exposure to acute heat stress (Bowler 2005). Heat hardening is generated by exposing organisms to temperatures

near or at their upper thermal limit. While hardening rapidly increases heat tolerance, these increases are transient and disappear within 36 hours (Maness and Hutchison 1980, Rutledge et al. 1987, Phillips et al. 2016, Deery et al. 2021; but see Moyen et al. 2020), highlighting its role as a short-term protection mechanism. Therefore, plasticity in heat tolerance occurs at two different levels: basal thermal tolerance, measured as the limits of thermal performance curves (Huey and Stevenson 1979) following an acclimation period, and hardening, which temporarily increases basal thermal tolerance following an acute heat stress.

Under an ideal scenario, both high basal thermal tolerance and hardening would improve ectotherm persistence under climate change. However, there appears to be a physiological limitation such that elevated basal thermal tolerance constrains the capacity of an organism to further increase their heat tolerance. For example, Stillman (2003) found a negative relationship between basal thermal tolerance and acclimation capacity in *Petrolisthes* crab populations across a latitudinal gradient. Building upon this, van Heerwaarden and Kellermann (2020) identified that this negative link was widespread across ectothermic clades and named this pattern the tolerance–plasticity trade-off hypothesis. Heat shock proteins (HSPs) may underlie the trade-off hypothesis because of the central role they play in maintaining homeostasis during extreme temperatures (Feder and Hofmann 1999, Sørensen et al. 2003) and improving basal thermal tolerance (Krebs and Feder 1998, Bahrndorff et al. 2009, Gao et al. 2014, Blair and Glover 2019; but see Easton et al. 1987, Jensen et al. 2010). Because HSPs are energetically expensive to produce and maintain (e.g., Hoekstra and Montooth 2013), populations from warm environments may be ‘preadapted’ to favor relatively high constitutive HSP expression to elevate basal thermal tolerance but exhibit less flexibility in upregulation following an acute heat shock compared to cool environment populations (Gleason and Burton 2015). Therefore, under the trade-off

hypothesis, hardening may be more useful to species that are less likely to experience chronic heat stress but receive greater benefits in combating acute stress (van Heerwaarden and Kellermann 2020; but see Sgro et al. 2010). Thus, acute upper thermal limits that are near or pushed near adapted thermal maxima restrict additional plasticity for further increased thermal tolerance through acclimation (Somero 2010). While a meta-analysis on ectotherms by Barley et al. (2021) provided support for the trade-off hypothesis, there is mixed evidence in larval amphibians (e.g., Menke and Claussen 1982, Simon et al. 2015, Turriago et al. 2022) suggesting a need for further exploration.

The role of heat hardening in adult (Maness and Hutchison 1980) and larval amphibians (Sherman and Levitis 2003, Sørensen et al. 2009) is understudied. We aimed to investigate the trade-off hypothesis by testing how acclimation temperatures (low or high) and duration (short or long acclimation periods) affect interactions between heat hardening and basal thermal tolerance – estimated via critical thermal maximum ( $CT_{max}$ ). These tests were conducted on larval wood frogs, *Lithobates sylvaticus* (LeConte 1825). Because larval anurans display a positive relationship between acclimation temperature and  $CT_{max}$  (e.g., Cupp Jr 1980, Ruthsatz et al. 2022), we predicted that longer acclimation to warmer temperatures would increase basal heat tolerance compared to those acclimated to cooler temperatures. In line with the trade-off hypothesis, we also expected the hardening effect would be most pronounced in larvae with the lowest  $CT_{max}$  suggesting greater acute thermal plasticity under these environments.

## Materials and Methods

### *Field Collection and Husbandry*

Freshly laid (< 36 hours old) wood frog egg masses were collected from wetlands in Jackson Co., IL on 23 February 2022, with air temperatures ~ 0°C, under an Illinois Department

of Natural Resources permit (HSCP 19-03). The egg masses were maintained in 60 L plastic containers with aerated, carbon-filtered water. After hatching, larvae were initially fed autoclaved algal flakes (Bug Bites Spirulina Flakes, Fluval Aquatics, Mansfield, MA, USA), followed by crushed alfalfa pellets at two weeks after hatching. Animals were fed twice weekly, and water was changed weekly. All experimental procedures were approved by the Southern Illinois University Institutional Animal Care and Use Committee (22–008).

#### *Critical Thermal Maximum Assay*

After larvae reached early pro-metamorphic stages, 64 individuals were randomly selected and staged, weighed, and transferred to individual 750 mL plastic containers filled with 600 mL of aged (>24 hours) aerated, carbon-filtered water. Larvae were split (N=32/treatment) into low ( $15^{\circ}\text{C} \pm 0.2$ ) and high ( $25^{\circ}\text{C} \pm 0.3$ ) acclimation temperatures. The former represents a temperature commonly experienced late in larval development in southern Illinois (Pers. Obs), and  $25^{\circ}\text{C}$  is the temperature associated with maximum larval performance (Watkins and Vraspir 2006). There were no differences in initial Gosner (1960) stage (range = 27 – 35) or mass (0.25 – 0.55 g) between these groups ( $P > 0.3$ ). The larvae were further randomly split into four groups (n=8 per group) that differed in acclimation period and heat hardening treatment: 1) 3-day control, 2) 3-day hardened, 3) 7-day control, and 4) 7-day hardened. Larvae were acclimated to low or high temperatures for either 3 or 7 days, which can stimulate an acclimation response (Rohr et al. 2018) without those at day 7 being significantly more developed than those at day 3. On the last day of acclimation, the  $\text{CT}_{\text{max}}$  of control groups was measured. The hardened groups were heated for 10 minutes at  $2\text{--}4^{\circ}\text{C}$  below the  $\text{CT}_{\text{max}}$  of control groups, following the protocol of Sherman and Levitis (2003). After this heat hardening treatment, the animals were returned to their acclimation temperatures for 2 hours, after which their  $\text{CT}_{\text{max}}$  was measured. Due to

unexpected mortality, sample sizes were reduced to seven for the 7-day hardened low and high temperatures groups, and the 7-day control low temperature group.

$CT_{max}$  was measured between 1000 – 1600 hrs to minimize potential diel effects on heat tolerance (Maness and Hutchison 1980, Healy and Schulte 2012). Larvae were staged, weighed, and then placed in individual 125 mL flasks filled with 75 mL of aged, aerated, carbon-filtered water and submerged in a hot water bath (Isotemp 220, Fischer Scientific) and given 5 minutes to acclimate prior to beginning the assay. Water temperatures increased  $0.6 \pm 0.01^{\circ}\text{C}$  per minute from a starting temperature of  $19.9 \pm 0.2^{\circ}\text{C}$ . Beginning at  $\sim 34^{\circ}\text{C}$ , larvae were prodded with a spatula every 30 seconds until they did not respond to the stimulus. At this point, a thermocouple probe (Physitemp BAT-12) was placed in the flask, water temperature was recorded which represented the larval  $CT_{max}$ . Flasks were then placed in a water bath at room temperature to facilitate larval recovery, and all larvae recovered  $\leq 5$  minutes. Upon completion of  $CT_{max}$  measurements, all larvae were euthanized via snap-freezing in  $-80^{\circ}\text{C}$  ethanol.

### *Statistical Analyses*

We assessed how larval  $CT_{max}$  shifted in response to our various treatments using a general linear model. While Gosner stage recorded prior to the  $CT_{max}$  measurement was normally distributed, mass was log-transformed to achieve normality, and both were included as covariates in the model. Fixed effects included acclimation period (3 or 7 days), acclimation temperature (low or high), hardening treatment (control or hardened), and their interactions. Post-hoc analyses were conducted using Tukey tests. All analyses were conducted in R Studio v. 2022.02.3 (<https://www.Rstudio.com/>) and significance values were set as  $\alpha = 0.05$ .

### *Results*



Across all treatments, wood frog larvae displayed a moderate degree of variation in their  $CT_{max}$  (range = 35.8° – 39.6°C; Fig. 4.1). Two individuals were dropped from analyses due to abnormally low  $CT_{max}$  values ( $\leq 34.9^\circ\text{C}$ ) in relation to their group mean. Of the main effects, both acclimation period ( $F_{1,49} = 20.92$ ,  $P < 0.0001$ ) and temperature ( $F_{1,49} = 6.52$ ,  $P = 0.014$ ) had significant effects on  $CT_{max}$  (Table 4.1), with those in the high acclimation temperature treatment and assayed on day 7 exhibiting the highest heat tolerance (Fig. 4.1). In contrast, the heat hardening treatment did not have a significant effect ( $F_{1,49} = 0.088$ ,  $P = 0.77$ ). There was an acclimation period by acclimation temperature interaction ( $F_{1,49} = 18.71$ ,  $P < 0.0001$ ), as there was a pronounced increase in  $CT_{max}$  among larvae in the high acclimation treatment on day 7 (Fig. 4.1). Lastly, a significant three-way interaction was found for acclimation period, acclimation temperature, and hardening treatment ( $F_{1,49} = 4.47$ ,  $P = 0.040$ ). Larvae in the low acclimation treatment on day 7 showed the largest hardening effect of 0.9°C, which was more than double the hardening effect of any other group (Fig. 4.1). Larval mass and Gosner stage were unrelated to  $CT_{max}$  ( $P \geq 0.29$ ).

## Discussion

Phenotypic plasticity of heat tolerance provides ectotherms the ability to counter the threat of overheating due to temperature extremes associated with climate change. Heat hardening, a form of thermal plasticity, represents the “first line of defense” against heat stress (Deery et al. 2021) through rapid upregulation of HSPs and/or changes to cellular structure in response to an acute thermal shock that can increase short-term heat tolerance (Bowler 2005). However, the tolerance–plasticity trade-off hypothesis (van Heerwaarden and Kellermann 2020) proposes that basal heat tolerance and thermal plasticity are negatively correlated; such that individuals with high  $CT_{max}$  have limited hardening (Gilbert and Miles 2019). While numerous

studies have demonstrated that amphibians exhibit plastic basal heat tolerance (e.g., Cupp Jr 1980, Ruthsatz et al. 2022), hardening remains understudied.

In our study, we found evidence in support of the trade-off hypothesis for larval wood frogs, although the effect was minor (Fig. 4.1), potentially due to low sample sizes. The group with the lowest mean  $CT_{max}$  (36.5°C) had the greatest hardening effect (0.9°C), while the group with the highest mean  $CT_{max}$  (39.0°C) had a minimal hardening effect (0.1°C). While the 0.9°C hardening effect was comparable to larval American toads (*Anaxyrus americanus*) and African clawed frogs (*Xenopus laevis*) (Sherman and Levitis 2003), the remaining groups had a minor hardening response ( $\leq 0.4^\circ\text{C}$ ) that was similar with values reported for larval bullfrogs (*L. catesbeianus*) (Menke and Claussen 1982). Additionally, the bullfrogs in the earlier study showed no evidence of the trade-off hypothesis as  $CT_{max}$  increased positively with acclimation temperatures while the hardening effect was unchanged. Hardening effects in lizard, salamander, and fish species are variable ranging from  $-0.4^\circ\text{C}$  (*Anolis sagrei*) to  $2.1^\circ\text{C}$  (*A. carolinensis*) (Maness and Hutchison 1980, Rutledge et al. 1987, Phillips et al. 2016, Deery et al. 2021, Lapwong et al. 2021a). In relation to other species, larval wood frogs acclimated to cooler conditions had a relatively strong hardening effect indicating significant plasticity in heat tolerance to improve their tolerance of overheating. This may benefit wood frogs as ephemeral pond breeding species are threatened by climate change (Blaustein et al. 2010) during the larval stage (Enriquez-Urzelai et al. 2019).

We can only speculate on the mechanism that drove the observed results, but we propose that HSPs represent an intriguing answer. This is because they are intimately tied to environmental temperature (Dalvi et al. 2012, Gu et al. 2019, Jin et al. 2019) and basal thermotolerance (Bahrndorff et al. 2009, Blair and Glover 2019). Warm-tolerant ectotherms

often express higher constitutive levels of *hsp70* relative to less-tolerant populations, but that an acute heat-stress results in greater *hsp70* expression in those with lower basal thermal tolerance (Zatsepina et al. 2000, Zatsepina et al. 2001, Gleason and Burton 2015). Zatsepina et al. (2000) proposed that this provided temperate populations the capability to rapidly and intensely synthesize HSPs after brief exposure to heat shock that was absent in low latitude populations. We propose a similar pattern in the wood frog larvae, such that higher constitutive HSP levels in warm-acclimated larvae provided increased basal heat tolerance compared to cold-acclimated larvae, yet hardened larvae from the latter group greatly upregulated HSP expression following a heat shock, enhancing their hardening response. This is in line with *Drosophila* acclimated to cooler temperatures (Bettencourt et al. 1999), which exhibited pronounced hardening plasticity that was absent in the warm-acclimated group. Quantifying constitutive and heat-shocked *hsp70* mRNA of larval liver and gill tissues would offer support to this conclusion. Additionally, many ectotherms appear to have hard upper-limits to thermal tolerance after which their pejus range constraints any further plastic responses (Denny and Dowd 2012). Thus, the warm acclimated larvae in our study could have approached their physiologically and evolutionarily determined upper limit that constrained any further plastic responses. Future tests are required to understand 1) if there is a degree of plasticity to hard upper limits of thermal acclimation, 2) the cellular and physiological mechanisms underlying these limits, 3) how these mechanisms determine the trade-offs between hardening and acclimation to chronic heat stress, and 4) how these mechanistic interactions are shaped by evolution in comparative studies.

Wood frog larvae with low basal heat tolerance demonstrated a large hardening effect suggesting a trade-off between the two traits. There is an inherent link between  $CT_{max}$  and hardening which could bias detection of the trade-off hypothesis (van Heerwaarden and

Kellermann 2020), and Deery et al. (2021) proposed that correlative evidence of the trade-off hypothesis is a statistical artifact. However, we believe our methodology of using different individuals for  $CT_{max}$  and hardening removed the risks of spurious correlation and strengthened our analyses. We acknowledge the use of acclimation to constant temperatures may have influenced our results as acclimation to fluctuating temperatures induces greater  $CT_{max}$  plasticity in larval anurans (Kern et al. 2015, Turriago et al. 2022). What role fluctuating acclimation temperatures play in the tolerance–plasticity trade-off hypothesis should be explored in future studies. Based on our results, we propose that larval wood frogs support the trade-off hypothesis after a relatively short acclimation period. Hardening benefits cool-acclimated populations in response to acute heat stress but plasticity in basal heat tolerance in response to prolonged warming are likely to be more beneficial in reducing overheating risk.

## CHAPTER 5

# RANAVIRUS INFECTION DOES NOT REDUCE HEAT TOLERANCE IN A LARVAL AMPHIBIAN

## INTRODUCTION

Biodiversity loss is an ever-increasing risk that jeopardizes the stability and function of ecosystems. Two widespread threats, climate change (Deutsch et al. 2008) and emergent infectious diseases, endanger both wildlife and humans (Blehert et al. 2009, Lorch et al. 2016, Zhou et al. 2020, Hoyt et al. 2021, Woodburn et al. 2021). One particularly imperiled taxonomic group are amphibians with 41% of species threatened with extinction (IUCN 2021). Individually, climate change and disease have led to documented declines in amphibians (e.g., Miller et al. 2018, Fisher and Garner 2020, Arietta and Skelly 2021), but how pathogens influence their host's heat tolerance (e.g., critical thermal maximum [ $CT_{max}$ ]) remains underexplored. Since the number of extreme heat events are projected to rise (Seneviratne et al. 2021), any factors that reduce  $CT_{max}$  would increase the frequency amphibians are exposed to temperatures that could result in heat-induced mortality (e.g., Huey et al. 2009, Dahlke et al. 2020). This highlights the importance of documenting this relationship for a greater diversity of host-pathogen systems.

Pathogenic infections often reduce the  $CT_{max}$  of ectothermic hosts (Sherman 2008, Hector et al. 2019, Laidlaw et al. 2020, Porras et al. 2021) and this has been observed in anurans infected with parasites including *Batrachochytrium dendrobatidis* (*Bd*) (Greenspan et al. 2017, Fernandez-Loras et al. 2019), although these results can vary with respect to host and pathogen genotype (Hector et al. 2019) and ontogeny (Fernandez-Loras et al. 2019). While many of these studies have not examined a mechanism underlying these outcomes, there have been some suggestions put forth. Sherman (2008) found that newts (*Notophthalmus viridescens*) infected by

an *Ichthyophonus*-like parasite had a lower  $CT_{max}$  compared to uninfected newts despite maintaining higher preferred body temperatures, a sign of behavioral fever. They suggested greater morbidity in the parasitized newts which impaired their heat tolerance without limiting their ability to perform a behavioral fever response. Hector et al. (2020) proposed a potential tradeoff between the heat stress and immune responses in infected hosts, but this may be limited to arthropods. As global temperatures warm, the thermal safety margin of animals decreases, forcing animals to behaviorally avoid extreme heat or suffer from heat stress that could result in mortality (Sunday et al. 2014). If pathogenic infections consistently reduce host  $CT_{max}$ , disease outbreaks can place affected populations under a greater threat of heat stress that leads to the combined risks of overheating and disease (Neely et al. 2020, Rollins-Smith 2020).

Here, we compared the  $CT_{max}$  of healthy larval wood frogs (*Lithobates sylvaticus*) to those infected with ranavirus. Ranaviruses are a global pathogen that infects vertebrate ectotherms and lead to large-scale die-offs of many species, particularly wood frogs (Hoverman et al. 2011, Warne et al. 2011), signifying the threat ranaviruses have on an already imperiled vertebrate group. Ranaviral infection typically induces inflammatory and adaptive immune responses in amphibians (Gantress et al. 2003, Morales et al. 2010), that notably vary with life stage (De Jesus Andino et al. 2012). Thermal tolerance can be dependent on a variety of factors including molecular damage resulting from greater oxidative stress (Kassahn et al. 2009, Christen et al. 2018) and membrane leakage (Schmidt-Nielsen 1997, Ørsted et al. 2022), both of which are exacerbated under increased inflammatory responses (Sears et al. 2011, de Groot and Burgas 2015, Chatterjee 2016, Camini et al. 2017). This suggests that a trade-off between an immune response and the physiological factors determining thermal tolerance could be possible (Hector et al. 2020). A previous study observed that post-metamorphic southern toads (*Anaxyrus*

*terrestris*) exposed to ranavirus selected warmer temperatures which reduced viral loads (Sauer et al. 2019), but they did not examine if heat tolerance was affected.

We tested the relationship between infection and  $CT_{max}$  at two timepoints following infection to identify if there existed a relationship between viral loads and heat tolerance. While ectotherms typically employ behavioral measures to avoid temperatures approaching their  $CT_{max}$  (Munoz 2022), acute heat stress is highly relevant to larval anurans since their aquatic habitats have greater heat capacity and reduced thermal heterogeneity which hinders their ability to behaviorally thermoregulate and escape high temperatures (Huey et al. 2012, Gunderson and Stillman 2015). While larval amphibians from temperate habitats, such as wood frogs, exhibit a high degree of warming tolerance making them unlikely to experience their  $CT_{max}$  (Duarte et al. 2012, Katzenberger et al. 2018), examining  $CT_{max}$  is a commonly used metric in larval anurans (e.g., Sørensen et al. 2009, Turriago et al. 2015, Katzenberger et al. 2021, Fontaine et al. 2022, Ruthsatz et al. 2022) and represents a proxy for studying organismal homeostasis under an extreme stress event (Kregel 2002, Madeira et al. 2013). In line with similar studies on pathogens, we predicted that ranavirus would negatively affect larval wood frog heat tolerance. This is the first study to explore how ranavirus affects host heat tolerance and, therefore, is relevant to how this virus could exacerbate the deleterious effects of climate change.

## METHODS

### *Animal Collection and Husbandry*

Wood frog egg masses were collected from Jackson Co., IL and placed in a 30 L plastic container filled with water from the collection site. Hatched larvae were initially fed algal flakes (Bug Bites Spirulina Flakes, Fluval Aquatics, Mansfield, MA, USA) followed by crushed alfalfa

pellets twice weekly. Larvae were reared at ~15°C with 12:12 light scheduling and biweekly water changes for >50 days prior to this experiment.

### *Ranavirus Inoculation*

We moved 48 larvae to a 30 L container with aerated, carbon-filtered water at 20°C for three days to acclimate to the experimental temperature. Afterwards, all larvae were staged (Gosner 1960), weighed, and divided into two treatments: 1) uninfected controls (N = 24) and 2) ranavirus infected (N = 24). There was no difference in mean larval stage (controls = 34.5 infected = 34.5;  $t_{46} = 0.13$  P = 0.89) or mean body mass (controls = 0.51 g, infected = 0.49 g;  $t_{46} = 0.87$ , P = 0.39) between the treatments. The ranavirus used for inoculation was a frog-virus 3-like (FV3) isolate cultured in baby hamster kidney cells (BHK-21) to a concentration of  $1.3 \times 10^8$  pfu ml<sup>-1</sup>. Larvae were inoculated with FV3 following the protocol of Warne et al. (2011). Briefly, individual larvae were placed in 750 mL plastic containers with 200 mL of water containing an LD<sub>100</sub> concentration of  $10^{4.25}$  pfu ml<sup>-1</sup> FV3. After 24 hours, 400 mL of water was added along with crushed alfalfa pellets. Uninfected controls underwent a similar protocol except they were only exposed to water.

### *Experimental Protocol*

At 2 and 4-days post infection, the CT<sub>max</sub> of 12 randomly selected uninfected and infected larvae was recorded. We selected these days for three reasons: 1) FV3 can cause an upregulation of proinflammatory cytokines and histological signs of inflammation occur within 1 – 3 days post infection (Morales et al. 2010, De Jesus Andino et al. 2012, Forzan et al. 2017), 2) a separate experiment using our FV3 identified mortality in infected wood frog larvae at 5 days post infection (Dallas et al. Unpublished Results), and 3) changes in anuran CT<sub>max</sub> can occur within 2 – 4 days (Brattstrom and Lawrence 1962, Brattstrom 1968, Turriago et al. 2022, Dallas



and Warne 2023). Individual larvae were placed in a 125 mL flask with 75 mL of water, submerged in a hot water bath (Isotemp 220, Fischer Scientific), and given 5 minutes to acclimate. Starting temperatures of  $CT_{max}$  assays averaged  $18.8^{\circ}C$  with a heating rate of  $0.6^{\circ}C$   $minute^{-1}$ . Beginning at  $\sim 34^{\circ}C$ , larvae were prodded with a spatula every minute until they failed to respond to the stimulus, and water temperature was recorded which represented the larval  $CT_{max}$ . Flasks were then placed in a water bath at  $\sim 19^{\circ}C$  to facilitate recovery. Individuals that failed to recover were removed from all analyses ( $n = 10$  total, 6 ranavirus and 4 controls). Larvae were returned to their respective containers for 24 hours and then subsequently euthanized via snap freezing with  $-80^{\circ}C$  EtOH.

#### *Ranavirus DNA Extraction and Quantification*

The liver was removed from larvae using flame-sterilized forceps and DNA was extracted using GenCatch™ Plasmid DNA Mini-Prep Kit (Epoch Life Science, Missouri City, TX, USA) following the manufacturer's protocol and stored at  $-80^{\circ}C$ . Extracted DNA was quantified using an Epoch Microplate Spectrophotometer (Agilent, Santa Clara, CA, USA). Viral loads were assessed by real-time qPCR on a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) using the primers, probe, and thermal cycling protocol designed by Stilwell et al. (2018). For each run, all samples were run in duplicate with positive and negative controls along with ten-fold serial dilutions of our FV3 isolate from  $10$  to  $10^6$  PFU  $mL^{-1}$  as standards for sample interpolation. A subset of uninfected controls ( $N = 6$ ) was analyzed and all tested negative for FV3.

#### *Statistical Analyses*

All analyses were conducted in R v. 4.1 (Team 2021) and significance values were set as  $\alpha = 0.05$ . The effect of ranavirus on  $CT_{max}$  was determined using a linear mixed model in the

lmer package (Kuznetsova et al. 2017). Larval  $CT_{max}$  was the response variable with infection treatment, days post infection, and their interaction as predictive variables, with larval stage included as a covariate and water bath ID as a random effect. Furthermore, we used Pearson's correlation analyses to determine the relationships between  $CT_{max}$  and 1) Gosner stage and 2) ranavirus load (PFU/ml). Ranavirus DNA quantities were log10-transformed to achieve normality. Summary values are given as mean  $\pm$  1 standard error followed by sample size.

## RESULTS

Larval Gosner stage ranged from 31 to 38 and there was no difference in mass or stage between control and ranavirus treatments ( $P > 0.77$ ). A single larva from the ranavirus treatment on 4 days post infection was dropped from all analyses due to abnormally low  $CT_{max}$  values ( $33.6^{\circ}C$ ) in relation to their group mean. We found that larval wood frog  $CT_{max}$  was unrelated to ranavirus exposure (Table 5.1). There were no significant effects of the ranavirus treatment ( $F_{1,31.3} = 0.30$ ,  $P = 0.59$ ), days post infection ( $F_{1,31.0} = 2.84$ ,  $P = 0.10$ ), and interaction between these variables ( $F_{1,31.2} = 0.15$ ,  $P = 0.70$ ) (Fig. 5.1). Infected larvae had a slightly lower  $CT_{max}$  at 2 days post infection ( $36.9^{\circ} \pm 0.34^{\circ}C$ ,  $n = 8$ ) than uninfected controls ( $37.6^{\circ} \pm 0.13^{\circ}C$ ,  $n = 9$ ), but this difference was absent at 4 days post infection (infected =  $36.6^{\circ} \pm 0.29^{\circ}C$ ,  $n = 9$ ; control =  $36.7^{\circ} \pm 0.26^{\circ}C$ ,  $n = 11$ ). There was a significant negative effect of larval stage on  $CT_{max}$  ( $F_{1,31.3} = 4.77$ ,  $P = 0.037$ ) that explained 11% of the observed variance. We observed positive correlations between  $CT_{max}$  and ranavirus loads at both 2- and 4-days post infection, but this relationship was only statistically significant for day 2 days post infection (2 days post infection:  $r = 0.84$ ,  $P = 0.0093$ ; 4 days post infection:  $r = 0.64$ ,  $P = 0.086$ ) (Fig. 5.2). These correlations were independent of larval stage as ranaviral loads were unrelated to stage ( $t_{14} = 0.91$ ,  $P = 0.38$ ).

## DISCUSSION

Heat tolerance is a critical facet of organismal physiology and underlies the environments they occupy, but pathogens commonly reduce heat tolerance (Hector et al. 2021) increasing an organism's susceptibility to heat stress (Neely et al. 2020). In contrast to this pattern, we found that ranavirus-infected wood frog larvae exhibited no decline in  $CT_{max}$  relative to uninfected controls (Fig. 5.1). Remarkably, there was a strong positive correlation between larval  $CT_{max}$  and ranaviral loads (Fig. 5.2). Our results suggest ranavirus infection in larval wood frogs, a typical ephemeral pool-breeding anuran species, does not affect their thermal tolerance, or their capacity to tolerate thermal extremes.

### *Amphibian Pathogens and Thermal Tolerances*

Our observation that ranavirus-infected wood frog larvae exhibited no decline in  $CT_{max}$  relative to uninfected controls is in contrast to reductions in thermal tolerance among *Bd* infected amphibians, which suggests pathogen-specific effects. For example, *Bd*-infected terrestrial anurans (Greenspan et al. 2017, Siddons and Searle 2021) typically experience reduced thermal tolerance. Greenspan et al. (2017) speculated that *Bd*-induced disruption in skin barrier function of terrestrial frogs could account for the observed  $CT_{max}$  reduction. In frogs, *Bd* infection disrupts cutaneous ion osmoregulation that can induce mortality by cardiac arrest due to loss of sodium (Voyles et al. 2009). Ranavirus virulence, by contrast, is systemic with substantial cellular and widespread tissue damage (Miller et al. 2015) that could be assumed to alter host thermal tolerance, and which makes our findings rather surprising. While the mechanisms that underlie upper thermal limits remain poorly understood, the hypothesized and few known mechanisms include oxygen limitation, mitochondrial disruption, enzyme dysregulation (e.g. lactase dehydrogenase), and changes to cell membrane integrity (Somero 2010, Pörtner et al. 2017,

Bowler 2018, Chung and Schulte 2020). It is possible that ranavirus infection does not significantly affect these respiratory and cardiovascular systems, at least during early stages of infection, and thus does not impact thermal tolerance, an issue worthy of further study.

Acclimation to thermal conditions by contrast, could have played a role in our results, as a few studies have shown that differing acclimation regimes influence the effects of pathogens or immune activation on amphibian  $CT_{max}$ . When acclimated to elevated temperatures (32°C), immune activation by injection of lipopolysaccharide in cane toads (*Rhinella marina*) induced significantly higher  $CT_{max}$  compared to control toads, however, immune activation had no effect on thermal tolerance in cooler acclimated (25°C) toads (Sherman et al. 1991). Additionally, Greenspan et al. (2017) found that even though *Bd* infection lowered the  $CT_{max}$  of *Litoria spenceri*, acclimation to a daily heat pulse rather than constant temperatures attenuated this interaction with a much weaker reduction in  $CT_{max}$ . Similarly, larvae in our study were acclimated to a constant and relatively mild temperature (~20°C) which could have attenuated such effects of ranavirus infection on heat tolerance. However, considering that ranavirus mortality is greater at warmer temperatures (Brand et al. 2016), future studies exploring interactions between thermal acclimation, immune activation, infection, and the mechanisms underlying thermal tolerance could provide valuable insight into the conditions driving disease outbreaks and mortality in amphibians.

We also acknowledge that the lack of an effect of ranavirus infection on  $CT_{max}$  could have resulted from this metric being less biologically relevant to larval wood frogs than other thermal physiology traits. Temperate larval amphibians display high warming tolerances relative to tropical species (Duarte et al. 2012, Katzenberger et al. 2018), which indicates their reduced risk of experiencing temperatures associated with  $CT_{max}$ . Other assays of thermal tolerance might

have revealed different outcomes than we observed. For example, comparing the thermal performance curve of ranavirus infected to uninfected larvae could reveal differences in shape and height of the curves in response to infection. Alternatively, as highlighted by (Jørgensen et al. 2022), examining the heat failure rate could indicate that prolonged exposure to sublethal temperatures increase accrued damage in infected larvae relative to uninfected larvae accelerating their time to death. However, the simplicity and ubiquitous nature of  $CT_{max}$  analyses across taxa make them a useful metric to study, and our use of  $CT_{max}$  is appropriate within a comparative assessment of how ranavirus influences larval anuran thermal physiology.

#### *Ranaviral Loads and $CT_{max}$*

There was a surprising positive correlation between  $CT_{max}$  and viral loads, especially at two days post infection. This relationship could suggest that the mechanisms that allow for higher temperature tolerance also enable individuals to tolerate greater infections, such as cellular responses that have overlapping effects on thermal tolerance and immune function (i.e., heat shock proteins, Wallin et al. 2002, Pockley and Henderson 2018). However, our experimental design could have caused a heat stress-induced ranavirus proliferation and limits our capacity to infer the factors driving our results. Viral loads were measured the day following  $CT_{max}$  assays, rather than immediately after the trials. The positive correlation could thus have occurred because larvae with higher  $CT_{max}$  experienced greater heat stress-associated cellular damage or immune suppression (e.g., Liu et al. 2018, Sokolova 2023), which could have promoted more ranavirus replication over the subsequent 24 hour period. Similarly, environmental stressors that induce cellular stress appear to promote ranavirus proliferation. For example, Hall et al. (2020) found that larval wood frogs exposed to increased water salinity experienced increased ranavirus infections potentially due to salt-induced oxidative stress and

inflammation (Burraco and Gomez-Mestre 2016). Additionally, exposure to experimental heat waves over several days have been linked with reduced immune performance and greater disease susceptibility (Seppala and Jokela 2011, Dittmar et al. 2014, Stahlschmidt et al. 2017). However, the effects of a brief thermal shock as in our  $CT_{max}$  assay (~30 minutes) might not be long enough to affect pathogen loads (Fernandez-Loras et al. 2019, Hector et al. 2023). Assessing cellular stress (i.e., heat shock proteins), proinflammatory cytokines (i.e., IL-4), and immune performance (i.e., plasma bactericidal ability) in relation to differing heat shock regimes would greatly improve our understanding for the potential interactions between immune function and heat tolerance. Additionally, future research should compare pathogen loads at differing times points following  $CT_{max}$  measurements to test if the positive correlation we observed is an experimental artifact or biologically related to host-pathogen interactions.

#### *Gosner Stage and $CT_{max}$*

Finally, we observed a minor negative relationship between  $CT_{max}$  and larval Gosner stage. While a previous study on wood frogs found no change in  $CT_{max}$  across Gosner stage (Cupp Jr 1980), other studies have suggested this could be a relatively common pattern among larval anurans especially those nearing metamorphosis (Floyd 1983). As anurans near metamorphic climax (Gosner stages > 40),  $CT_{max}$  appear to decline (e.g., Agudelo-Cantero and Navas 2019, Ruthsatz et al. 2022), which could be linked to tissue disruption and physiological stress associated with tissue remodeling during metamorphosis (Sherman and Levitis 2003, Kirschman et al. 2017).

#### *Conclusions*

While larval amphibians are susceptible to climate change and emerging diseases, we found that the  $CT_{max}$  of FV3-infected larval wood frogs was unchanged relative to uninfected

controls. This result stands in stark contrast to the typical pattern observed in diseased ectotherms. Additionally, the observed positive correlation between ranaviral loads and  $CT_{max}$  is an interesting finding that demands further study. Since ranaviral loads in anurans decline with prolonged exposure to warm temperatures (Brunner et al. 2019, Sauer et al. 2019, but see Brand et al. 2016), our results suggest maintenance of their  $CT_{max}$  could promote selection of high temperatures associated with behavioral fever. As immune performance of herpetofauna follows the typical thermal performance curve (Graham et al. 2017, Baker and Merchant 2018, Moretti et al. 2019, Dallas et al. 2021), larvae maintaining their  $CT_{max}$  would be able to select higher temperatures to combat infection while reducing the risk of heat related mortality. However, more exploration of behavioral fever in ranavirus-infected larvae is also needed. Being the first study to examine how ranavirus infection impacts the  $CT_{max}$  of infected hosts, there is substantial room to expand on this subject and explore the cellular mechanisms, such as ROS and heat shock proteins, that attribute to the interaction between ranavirus and thermal tolerance.

## CHAPTER 6

### HEAT TOLERANCE IS AFFECTED BY GUT MICROBIOTA IN A VERTEBRATE ECTOTHERM

#### Introduction

The host microbiota represents a complex biological community consisting of bacteria, fungi, and viruses that has received increased focus over recent decades as its highly influential role on host physiology has become elucidated (McFall-Ngai et al. 2013, Kohl and Carey 2016, Warne and Dallas 2022). Resultantly, how the host microbiota responds to changes in environmental variables that are intimately tied to organismal biology, such as temperature, is of great interest. There is correlative evidence that environmental temperatures alters both the gut microbiota compositional and functional diversity in vertebrates and invertebrates (Kohl and Yahn 2016, Moghadam et al. 2018, Wang et al. 2018, Li et al. 2020, Onyango et al. 2020, Woodhams et al. 2020, Hassenrück et al. 2021, Jaramillo and Castaneda 2021). In their review, Sepulveda and Moeller (2020) found that warming temperatures are associated with increasing the relative abundance of Proteobacteria in invertebrates and a reduction in Firmicutes in vertebrates, and these temperature-dependent changes in the microbiota community affect varying aspects of host fitness and performance (Prado et al. 2010, Kikuchi et al. 2016, Fontaine et al. 2018, Horváthová et al. 2019). Therefore, the risks of climate change, which are expected to worsen over the coming decades (IPCC 2021), extend to the host microbiota and represents an important topic of study.

A fundamental aspect of an ectotherm's biology is their thermal tolerance, as it limits the temporal and spatial range they can occupy (Huey 1982, Adolph and Porter 1993, Hoffmann et al. 2013). Thermal tolerance of ectotherms is typically estimated via critical thermal (CT) limits



that denote the endpoints of their thermal performance curve (TPC) where organismal performance reaches zero (Huey and Stevenson 1979). Species with higher CT maxima (CT<sub>max</sub>) are expected to be less susceptible to warmer environments, enabling them to persist in the projected climates (Huey et al. 2012, Gilbert and Miles 2017, Roeder et al. 2021). Therefore, we suggest that a warmer CT<sub>max</sub> is beneficial to many populations going forward. The factors that underlie organismal CT<sub>max</sub>, and to a broader extent, heat death in ectotherms, remain debated. Several proposed mechanisms include perturbations in the plasma membrane (Bowler 2018), a decline in aerobic scope (Pörtner 2002), loss of cardiovascular function (Somero 2010), and disruption in mitochondrial activity (Chung and Schulte 2020). Due to the role of CT<sub>max</sub> in promoting species persistence, there is great importance to exploring the different aspects of an individual's environment and biology that can elevate CT<sub>max</sub>.

One potential factor of organismal biology that can influence host heat tolerance is the gut microbiota. Across several invertebrate and vertebrate species, there are correlative links between gut microbiota and changes in CT<sub>max</sub> (Moeller et al. 2020, Doering et al. 2021, Baldassarre et al. 2022, Fontaine et al. 2022). For example, an increasing prevalence of the *Anaerotignum* genus increased the CT<sub>max</sub> of western fence lizards *Sceloporus occidentalis* (Moeller et al. 2020). Additionally, Doering et al. (2021) showed that transplanting the microbiota of coral populations from thermally fluctuating waters to conspecifics from thermally stable environments enhanced their bleaching resistance, indicative of greater heat tolerance. There are several potential pathways through which the gut microbiota can elevate host CT<sub>max</sub>. For instance, mitochondrial enzyme activity was reduced in larval anurans with an experimentally-depleted microbiota (Fontaine et al. 2022), which can decrease aerobic scope at high temperatures and limit CT<sub>max</sub> (Pörtner 2002). Furthermore, warm-acclimated microbiota

communities exhibit higher expression of reactive oxygen scavengers (Ziegler et al. 2017, Fontaine and Kohl 2023) that can limit the damage reactive oxygen species impart on mitochondrial mechanisms at high temperatures (Christen et al. 2018, Sokolova 2023). Beyond enhanced  $CT_{max}$ , members of the gut microbiota can promote enhanced host fitness in warmer temperatures as demonstrated in pea aphids *Acyrtosiphon pisum* hosting an essential bacterial symbiont (*Buchnera aphidicola*) (Montllor et al. 2002, Zhang et al. 2019). These examples show that the gut microbiota confers benefits to heat tolerance in the host that could facilitate survival in warmer environments.

Amphibians are imperiled by climate change as increases in temperature and unpredictable shifts in precipitation will restrict activity periods and place species at risk of experiencing heat stress (Sunday et al. 2014, Campbell Grant et al. 2020, Greenberg and Palen 2021, Hoffmann et al. 2021). Based on the risks associated with warming temperatures, we sought to examine how early-life manipulation of the gut microbiota in a geographically widespread anuran influenced its heat tolerance. Specifically, we used a cross-species microbiota transplant to demonstrate that species-specific differences in thermal tolerance is mediated, at least partially, by the gut microbiota. While cross-species microbiota transplants have not been examined in terms of heat tolerance, Warne et al. (2019) showed that egg masses of wood frogs (*Lithobates sylvaticus*) inoculated with the gut microbiota of larval bullfrogs (*L. catesbeianus*) displayed higher growth and developmental rates as well as enhanced disease resistance compared to those inoculated with a conventional wood frog microbiota.

To test this, we stripped the resident microbiota of field-collected wood frog egg masses using antibiotics and separated these “sterilized” eggs into three microbiota treatments. Following the antibiotic wash, eggs were either inoculated with pond water from the collection

site, the gut microbiota of field-collected larval green frogs (*L. clamitans*), or received no inoculation. As green frogs breed in warmer temperatures than wood frogs and their larvae are active over the summer months while wood frogs metamorphose prior to the summer (Hulse et al. 2001), green frog larvae have been shown to exhibit a  $CT_{max}$  more than 1.5°C higher than wood frog larvae (Katzenberger et al. 2021). Resultantly, we hypothesized that inoculating wood frog eggs with the gut microbiota of larval green frogs would increase the heat tolerance of the recipient. We also tested if a short-term acclimation period would alter the gut microbiota. Most studies measuring the effects of different acclimation temperatures on the gut microbiota exceed 10 days (e.g., Moeller et al. 2020, Zhu et al. 2021, Fontaine et al. 2022), and the impact of a short-term thermal acclimation period has not been thoroughly explored. Therefore, we sought to identify how the gut microbiota changes in response to a brief acclimation period of three days and if there was an interactive effect between acclimation temperature and microbiota treatment on wood frog  $CT_{max}$ .

## Materials and Methods

### *Field Collection*

Four freshly laid (< 36 hours old) wood frog egg masses were collected from wetlands in Jackson Co., IL, under an Illinois Department of Natural Resources permit (HSCP 19-03). Additionally, 10 larval green frogs were collected from Southern Illinois University Carbondale research ponds in Jackson Co., IL. All experimental procedures were approved by the Southern Illinois University Institutional Animal Care and Use Committee (22–008).

### *Gut microbiome treatments*

On the day of collection, eggs were rinsed in antibiotics following a previously published protocol (Warne et al. 2017, 2019). Briefly, the egg masses were separated into sterile 50 mL

tubes (~ 20 eggs/tube), rinsed three times with 40 mL of autoclaved, aerated carbon-filtered water. The eggs were then sterilized by exposure to 500  $\mu$ L penicillin-streptomycin (10,000 U/mL; Life Technologies #15140-122), 200  $\mu$ L of kanamycin sulphate (25  $\mu$ g/mL; Life Technologies #11815-032) and 50  $\mu$ L of amphotericin B solution (250  $\mu$ g/mL; Sigma-Aldrich #A2942) for 4 hours on a nutator. The sterilized eggs were then triple rinsed with sterile water and placed into sterilized 6 L plastic containers containing 3 L of carbon-filtered sterile water and aerated with a HEPA inline filter disc (0.3  $\mu$ m pore, Whatman Inc.) overnight. Additionally, we retained eggs from all four masses that were not exposed to any antibiotics.

The following day, the antibiotic-rinsed eggs were collected and separated into three different treatments: 1) wood frog (WF), 2) no-inoculum (NI), and 3) green frog (GF). The WF treatment eggs were placed in sterile 50 mL tubes and received 40 mL of pond water that was previously mixed with the unmanipulated eggs with the intention of re-inoculating them with their natural microbiota. The NI treatment received no further manipulation, and all microbial colonization of this group was assumed to be environmentally acquired post-hatching. For the GF treatment, intestinal tracts were dissected from the larval green frogs (Gosner stage 27–30; (Gosner 1960), homogenized in 40  $\mu$ L of autoclaved water, and added to sterile 50 mL tubes with the sterilized wood frog eggs. All treatments were mixed with their respective inoculum for 30 minutes on a nutator and were then returned to their respective containers (N = 16 per treatment).

Larvae hatched within four days of the microbiota treatments and were allowed to feed on the inoculated egg jelly for two days. Bubblers were removed one week after hatching. Initial feedings consisted of autoclaved algal flakes (Bug Bites Spirulina Flakes, Fluval Aquatics,

Mansfield, MA, USA) after which they were fed crushed alfalfa pellets twice weekly. Water was changed weekly with aerated, carbon-filtered water.

#### *Critical Thermal Maximum Assay*

Over a four-day period, we randomly collected 40 larvae from each microbiota treatment. All larvae were staged, weighed, and transferred to individual 750 mL plastic containers filled with 600 mL of aged (>24 hours) aerated, carbon-filtered water. To reduce the effect of individual containers, only one to three larvae were collected from each container per day. Across all individuals, larval stages ranged from 27 – 36, and there was no difference among the treatments in terms of stage ( $F_{2,107} = 0.92$ ,  $P = 0.40$ ) or log-transformed mass ( $F_{2,107} = 0.75$ ,  $P = 0.48$ ), but collection day influenced larval stage ( $F_{3,107} = 4.44$ ,  $P = 0.006$ ) but not mass ( $F_{3,107} = 1.96$ ,  $P = 0.13$ ) and there was no interaction. Lastly, larvae ( $N = 20$  per microbiota treatment) were then split into two acclimation temperatures, low ( $15^{\circ}\text{C} \pm 0.2$ ) and high ( $23^{\circ}\text{C} \pm 0.3$ ), for a three-day acclimation period during which individuals were fasted. Following an attempt to measure the CT minimum of larvae from the low acclimation temperature, the sample size was reduced to  $N = 18$ .

After the acclimation period, larvae were staged and weighed then placed in individual 125 mL flasks filled with 75 mL of aged, aerated, carbon-filtered water. Larvae were then submerged in a hot water bath (Isotemp 220, Fischer Scientific) and given 5 minutes to acclimate prior to beginning the assay. In each bath, there were eight flasks containing two individuals from each treatment. To record  $\text{CT}_{\text{max}}$ , temperatures increased  $\sim 0.7^{\circ}\text{C}$  per minute from a starting temperature (mean  $\pm 1$  standard error) of  $19.4 \pm 0.2^{\circ}\text{C}$ . Beginning at  $33^{\circ}\text{C}$ , larvae were prodded with a spatula every 30 seconds until they failed to respond to the stimulus. At this point, a thermocouple probe (Physitemp BAT-12) was placed adjacent to the larvae and the water

temperature was recorded, which represented larval CT<sub>max</sub>. Flasks were then placed in a bath of room temperature water to facilitate larval recovery. While most larvae recovered within 3 minutes, 6 larvae died from the heat shock and there was no treatment effect ( $\chi^2 = 3.31$ ,  $df = 2$ ,  $P = 0.19$ ), and two individuals died prior to the assay; all were removed from further analyses.

To test for acclimation effects on gut microbiomes, four larvae were selected from each of the six groups (three microbiota treatments x two acclimation temperatures) after three days of temperature acclimation for microbial sequencing. We did not measure the CT<sub>max</sub> of these 24 larvae to eliminate potential confounding effects of heat shock on the microbiota community. All larvae were euthanized via snap-freezing in -80°C ethanol and stored at -80°C until intestinal extraction.

#### *Gut Microbiota DNA Extraction*

The entire intestinal tract was removed and placed into an autoclaved 1.5 mL microcentrifuge tube. To reduce cross-contamination, forceps were rinsed in 70% ethanol and then flame-sterilized prior to intestine removal. Additionally, intestines were briefly rinsed with autoclaved water to remove any transient bacteria. To improve DNA extraction, the intestines were homogenized using sterilized forceps and then stored at -80°C. Microbial DNA was extracted using the GenCatch™ Plasmid DNA Mini-Prep Kit (Epoch Life Science) following manufacturer instructions with minor modifications: The volume of Proteinase K was increased to 25  $\mu$ L and the initial incubation at 60°C was increased to 3 h to improve cellular digestion. Lab controls were used to identify any microbial DNA present in the extraction kit reagents. All extracted DNA samples were stored at -80°C prior to sequencing. The extracted microbial DNA were sequenced on the Illumina MiSeq, with the 16S rRNA V4 region amplified using the

primers 515F and 806R with barcodes at the Kansas State University Integrated Genomics Facility (Caporaso et al. 2012).

#### *Gut Microbiota Analyses*

We used QIIME 2 (v. 2019.7) to process the sequence data and to profile the microbial communities (Bolyen et al. 2019). We used QIIME 2 plugin cutadapt to remove the primer sequences; reads with no primer were discarded (Martin 2011). Additionally, we used DADA2 for quality control with the same parameters across different runs, and truncated the reads to length where the 25th percentile of the reads had a quality score below 15 (Callahan et al. 2016). The pre-trained classifier offered by QIIME 2, using SILVA database (v. 132) was used for taxonomic assignment for bacteria. *Statistical Analyses*

All statistical analyses were conducted using R version 4.1 (Team 2021). Larval heat tolerance was assessed via a linear mixed model using the lmer package (Kuznetsova et al. 2017) with  $CT_{\max}$  as the response variable and both microbiota treatment and acclimation temperature as fixed effects. We included larval mass, which was square root transformed to improve normality, as a covariate and the location of the 125 mL flask in the hot water bath as a random effect to account for potential differences in heating rates. In the results,  $CT_{\max}$  is reported as mean  $\pm$  one standard error along with sample size.

Gut microbiota alpha and beta diversity metrics were analyzed using the vegan package (Oksanen et al. 2022). We compared three alpha diversity metrics (ASV richness, Shannon index, and Simpson index) using two-way ANOVAs with microbiota treatment, acclimation temperature, and their interaction as predictive variables and Gosner stage as a covariate. We used the adonis2 function to perform PERMANOVAs, with 999 permutations, using Bray-Curtis and Jaccard distance matrices based on microbiota treatment, acclimation temperature, and their

interaction along with Gosner stage as a covariate. Using the same distance indices, we compared intragroup heterogeneity via permutation tests with 999 permutations and the betadispr function.

We determined significant differences in bacterial phyla and families across the microbiota treatments and acclimation temperatures using the MaAsLin2 package (Mallick et al. 2020). We used an arcsine-square root transformation to improve normality of the relative abundance data. To reduce the risks of false positives, we corrected P-values using the Benjamini-Hochberg false discovery rate method.

## Results

### *Heat Tolerance*

We found that larval wood frog  $CT_{max}$  was affected by gut microbiota treatment and acclimation temperature, although the differences in heat tolerance were small (Fig. 6.1). In line with our prediction, GF larvae had the highest  $CT_{max}$ , controlling for body mass and acclimation temperature, followed by WF and NI larvae (Table 6.1; GLMM,  $\chi^2 = 14.86$ ,  $P < 0.001$ ). However, when examining post-hoc pairwise comparisons within acclimation temperatures, the difference between GF ( $38.3^\circ\text{C} \pm 0.2$ ,  $N = 13$ ) and NI ( $37.7^\circ\text{C} \pm 0.1$ ,  $N = 12$ ) larvae was marginally significant ( $P = 0.051$ ). When controlling for body mass and microbiota treatment, acclimation temperature had a pronounced effect on  $CT_{max}$  in the expected direction with those acclimated to warmer temperatures exhibiting higher heat tolerance (Table 6.1; GLMM,  $\chi^2 = 19.98$ ,  $P < 0.0001$ ). Of the microbiota treatments, only NI showed a significant increase in  $CT_{max}$  between the low and high ( $38.3^\circ\text{C} \pm 0.1$ ,  $N = 16$ ) acclimation temperatures ( $P = 0.037$ ). There was no interaction between gut microbiota treatment and acclimation temperature, and the differences in  $CT_{max}$  were independent of body mass (Table 6.1).



### *Gut Microbiota Alpha Diversity*

There was a total of 145 unique ASVs identified across the 24 intestinal samples. Across the three gut microbiota treatments, ASV richness was lowest in the WF group and highest in the GF group, but there was no significant treatment or acclimation temperature effect (Fig. 6.2A). Using Shannon's and Simpson's indices, alpha diversity was significantly different across microbiota treatments, acclimation temperature, and larval stage. The WF larvae had significantly lower diversity metrics than both GF and NI larvae, and those acclimated to the high temperature had higher diversity, although the difference was minor (Figs. 2B and 2C). Across both indices, diversity increased with larval stage, but Shannon's index model had a near-significant interaction of stage and treatment as NI larvae showed no change in diversity across different stages (Fig. 6.2B).

### *Gut Microbiota Beta Diversity*

The community composition and membership of the wood frog gut microbiota was varied with respect to microbiota treatment, acclimation temperature, and larval stage (Fig. 6.3). Adonis PERMANOVAs identified significant effects of the microbiota treatments (Bray-Curtis and Jaccard distances, respectively:  $F_{2,14} = 4.22$ ,  $P < 0.001$ ;  $F_{2,14} = 2.98$ ,  $P < 0.001$ ), temperature ( $F_{1,14} = 4.41$ ,  $P < 0.001$ ;  $F_{1,14} = 3.27$ ,  $P < 0.001$ ), and Gosner stage ( $F_{1,14} = 2.90$ ,  $P = 0.009$ ;  $F_{1,14} = 2.09$ ,  $P = 0.013$ ). For both distance methods, there was no significant interactions. The PCoA plots of Bray-Curtis and Jaccard distances showed moderate overlap among the microbiota treatments and acclimation temperatures (Fig. 6.3). While the NI larvae at the low acclimation temperature was the most variable, within-group dispersion was similar for both Bray-Curtis ( $F_{5,18} = 1.15$ ,  $P = 0.36$ ) and Jaccard ( $F_{5,18} = 1.11$ ,  $P = 0.41$ ) distance analyses. Among the most prominent phyla, variation along PC1 of both distance methods was negatively related to

Bacteroidetes abundance while Firmicutes and Proteobacteria abundances had positive effects. Actinobacteria accounted for most of the variation along PC2 being negatively related in the Bray-Curtis distance PCoA and positively related in the Jaccard distance PCoA.

Across all wood frog larvae, gut bacterial communities were dominated by three phyla: Bacteroidetes, Firmicutes, and Proteobacteria with relatively lower abundances of Actinobacteria, Desulfobacterota, and Verrucomicrobiota (Fig. 6.4). Across both acclimation temperatures, the relative abundance of Bacteroidetes was lower in NI larvae (mean: 38.7%) compared to the GF (53.4%) and WF larvae (66.0%), but NI larvae had a higher relative abundance of Proteobacteria (38.7%) with respect to GF (22.1%) and WF larvae (16.8%). However, these differences were not statistically significant (MaAsLin2, corrected  $P > 0.09$ ; Table 6.2). The relative abundance of these major phyla was unaffected by the acclimation temperature, indicating their resistance to the short-term acclimation period (MaAsLin2, corrected  $P > 0.72$ ). Actinobacteria was enriched under the low acclimation treatment (1.1%) relative to the high temperature (0.45%), although the difference only approached statistical significance (MaAsLin2, corrected  $P = 0.067$ ).

When examining the relative abundance of bacterial families, only a single family differed between the acclimation temperatures with Beijerinckiaceae being more abundant across all treatment groups at the high temperature (MaAsLin2, corrected  $P < 0.0001$ ; Table 6.2). In contrast, there were 14 bacterial families that were significantly different among the three microbiota treatments (MaAsLin2, corrected  $P < 0.05$ ; Table 6.2). Specifically, the GF larvae were enriched in Rikenellaceae relative to the other treatments while NI larvae were enriched in Aeromonadaceae (Fig. 6.5).

Discussion

Increased heat tolerance in organisms is predicted to be beneficial in the future through minimizing the mortality risk to acute heat extremes (Huey et al. 2012). In this study, we build upon current evidence that the gut microbiota influences host heat tolerance. By manipulation of the larval wood frog gut microbiota, we observed that transferring the gut microbiota of a more heat-tolerant larval anuran (green frogs) to wood frogs resulted in elevated heat tolerance, indicating that the microbiota is partially tied to host thermal physiology. These larvae had a higher relative abundance of Rikenellaceae (a SCFA-producing bacterial family) and a lower relative abundance of Aeromonadaceae (a potential pathogen) when compared to NI larvae. We found a significant, albeit small, effect of short-term acclimation on gut microbiota community composition and diversity suggesting the gut microbiota is susceptible to changes following a brief change in environmental temperature. While our results are correlative, the significance of a cross-species microbiota transplant on increasing the recipient's  $CT_{max}$  underscores the role of the gut microbiota in host thermal tolerance, and suggests that this method could be useful in species conservation efforts.

Our study is the first to identify that cross-species gut microbiota transplantations influenced the heat tolerance of the recipients in a predictive manner. In line with our prediction, inoculating wood frog eggs with the gut microbiota of larval green frogs, a species with greater heat tolerance than the former (Katzenberger et al. 2021), resulted in higher  $CT_{max}$  compared to the WF and NI treatments (Fig. 6.1). Previous studies in invertebrates have shown that, within species, microbiota transplants of individuals that were acclimated to warmer temperatures promoted enhanced heat tolerance in the recipient (Moghadam et al. 2018, Doering et al. 2021, Baldassarre et al. 2022). Such findings indicate that temperature-based restructuring of the gut microbiota selects for bacterial taxa and/or functions that improve survival under heat stress. For

example, Fontaine and Kohl (2023) found that the gut microbiome of larval green frogs exposed to a 24 hr period of heat stress significantly upregulated genes associated with carbohydrate metabolism, transcription, and translation. Surprisingly, they failed to find that the heat stress altered bacterial expression of heat shock proteins (HSPs) within the gut microbiota, which are commonly upregulated in response to thermally stressful environments to maintain protein structure integrity (Feder and Hofmann 1999). This suggests that the gut microbiota improves host heat tolerance through increased metabolic function and DNA processes rather than bacterial HSP expression, but they did not measure intestinal HSP expression, which has been shown to be modulated by the gut microbiota (Arnal and Lalles 2016). We lack transcriptomic data to explore how the GF treatment promoted greater  $CT_{max}$  relative to the other treatments, therefore our conclusions are limited to taxonomic differences we observed rather than altered metabolite and gene expression. Inclusion of metagenomic and transcriptomic analyses of the gut microbiota, along with expression of intestinal HSPs, on studies assessing heat tolerance would be the advisable next step in assessing the functional role the gut microbiota has in influencing host  $CT_{max}$ . Regardless, our findings are in line with other studies showing benefits of cross-species microbiota transplants in species conservation (Dallas and Warne 2022) and could improve survival for threatened species under warming conditions.

There were several bacterial taxonomic groups that differed among the microbiota treatments. Most prominently, the Bacteroidetes family Rikenellaceae, primarily *Mucinivorans hirudinis*, was significantly enriched in GF larvae. This species was initially described in leeches (Nelson et al. 2015) and produces the short chain fatty acid (SCFA) acetate as a byproduct of metabolizing host mucin glycans (Bomar et al. 2011). Its prevalence in other taxa has not received much attention, but *Mucinivorans* species have been previously described in late-stage

Asiatic toads (*Bufo gargarizans*) (Chai et al. 2018), suggesting this species is not limited to leeches. Other members of Rikenellaceae promote beneficial phenotypes such as enhanced heat tolerance in cows (Wang et al. 2022), improved health of late-stage giant spiny frogs (*Paa spinosa*) (Long et al. 2020), and lower obesity rates in humans (Pessoa et al. 2021). One potential mechanism through which *M. hirudinis* could promote heat tolerance is through upregulation of antioxidants as a byproduct of acetate production (Gonzalez-Bosch et al. 2021). Heat stress is associated with an increased production of reactive oxygen species (ROS) in vertebrates that induce oxidative damage which negatively affect cellular function, host survival, and can limit host CT<sub>max</sub> (reviewed in Ritchie and Friesen 2022). Acetate was shown to significantly reduce ROS production in human and mouse cells *in vitro*, while promoting greater mitochondrial respiration rates (Huang et al. 2017, Hu et al. 2020). The beneficial role of acetate in promoting antioxidant concentrations has also been demonstrated *in vivo* using piglets (Pang et al. 2021), but direct links between *M. hirudinis* and antioxidant production or ROS reductions are not present. While the link between Rikenellaceae (*M. hirudinis*) and greater heat tolerance is not definitive, its production of acetate could represent a pathway by which GF larvae benefited from its increased relative abundance.

While the WF and NI larvae had similar relative abundances of bacterial families, the latter displayed a higher relative abundance of Aeromonadaceae, specifically those in the *Aeromonas* genus. Members of these family are Gram-negative bacteria that are potential pathogens of amphibians and fish (Janda and Abbott 2010) and degrade the mucosal barrier of the intestines enabling bacteria to enter systemic circulation of the host (Dong et al. 2018). Additionally, *Aeromonas* can reach relatively high abundances in the intestines of wild (Hird et al. 1983) and lab-reared anuran larvae (Chai et al. 2018). In a study on wild-collected

Dybowski's frog (*Rana dybowskii*), Tong et al. (2020) found that the abundance of *Aeromonas* was enriched in diarrheic adults compared to healthy adults and they posited the presence of this genus lead to the emergence of a pathogenic phenotype. While we did not observe direct evidence of such a phenotype, a reduction in the heat tolerance of NI larvae may be related to higher *Aeromonas* abundance. A potential mechanism for this outcome is through *Aeromonas*-induced systemic-inflammation. As *Aeromonas* metabolites degrade the intestinal barrier (Feng et al. 2022), this could enable Gram-negative bacteria with their lipopolysaccharide (LPS) membrane to enter the bloodstream of the host. LPS promotes inflammation through increased production of pro-inflammatory cytokines and leukocyte activity (Kamada et al. 2013, Boulange et al. 2016, Wang et al. 2020). As an activated immune response can cause a decline in  $CT_{max}$  (Hector et al. 2020), the increased Aeromonadaceae abundance in NI larvae may have indicated increased immune costs. This may be indicative that the host could incur negative costs if Aeromonadaceae are enriched in the larval intestines, but further exploration of the topic is required to address this potential relationship.

In addition to increased Aeromonadaceae abundance, Warne et al. (2019) and Fontaine et al. (2022) offer a potential explanation why NI larvae had the lowest  $CT_{max}$ . These studies demonstrated that larvae experiencing early-life bacterial depletion had lower mass-specific metabolic rates and reduced mitochondrial enzymatic activity, respectively. Fontaine et al. (2022) predicted that larvae with a diminished aerobic scope would have a constrained heat tolerance (Pörtner et al. 2017, Chung and Schulte 2020), although the importance of such a link has been debated (e.g., Ern et al. 2016, Semsar-Kazerouni and Verberk 2018, Claunch et al. 2021). Microbial-derived SCFAs are also associated with increased mitochondrial activity (Schonfeld and Wojtczak 2016), which may constrain the host's ability to maintain ATP

production at high temperatures resulting in a lower  $CT_{max}$  (Sokolova 2023). Compared to GF larvae, NI larvae had a lower, although not statistically significant, relative abundance of Firmicutes and Bacteroidetes, two phyla associated with SCFA production (den Besten et al. 2013). This could account for our observed  $CT_{max}$  pattern. Future studies should examine this link through direct supplementation of SCFAs and then measure the host's heat tolerance, or by measuring  $CT_{max}$  and SCFA concentrations in the intestines. In addition, identifying if changes in the gut microbiota composition affects a host's aerobic scope would determine if changes in  $CT_{max}$  are related to whole-body changes in metabolic rates.

While prolonged acclimation to different thermal regimes alters gut microbiota  $\alpha$ - and  $\beta$ -diversity (Fontaine et al. 2018, Moeller et al. 2020, Zhu et al. 2021, Baldassarre et al. 2022, Theys et al. 2023), our short-term acclimation exposure had mixed effects on these metrics. While both bacterial membership and structure were dependent upon acclimation temperature (Fig. 6.3), only two metrics of  $\alpha$ -diversity (Shannon and Simpson indices) displayed a small, temperature-dependent increase (Fig. 6.2B and 2C). A similar pattern was observed in larval *Ischnura elegans* damselflies exposed to a simulated seven-day heatwave (Theys et al. 2023). The temperature effect in the damselflies was primarily driven by increases in Proteobacteria, a pattern commonly observed in arthropods experiencing warmer temperatures (Sepulveda and Moeller 2020). In a study on green frog larvae, Fontaine et al. (2022) showed a minor decline in  $\alpha$ -diversity with increasing acclimation temperature, but bacterial composition did diverge with the relative abundance of Proteobacteria negatively related to increasing temperatures, although the effect was non-significant. This demonstrates the complex relationship between environmental temperature and the gut microbiota. In line with the predictions of Lozupone et al. (2012) and concept of disturbance ecology (Christian et al. 2015), we propose that exposure to

different environmental temperatures represents a disturbance to the gut microbiota that causes a shift in bacterial diversity, composition, and transcriptomics. While a rapid temperature shift can drastically alter gut microbiota transcriptomics of ectothermic vertebrates (Zhou et al. 2022, Fontaine and Kohl 2023), community-wide changes are likely more pronounced following prolonged exposure to new temperatures enabling a new community to become established. Longitudinal studies measuring gut microbiota diversity metrics exposed to different acclimation temperatures (Baldassarre et al. 2022) would be beneficial in addressing this prediction.

We found that high temperature-acclimated wood frog larvae were depleted in the Actinobacteria phylum while the Proteobacteria family Beijerinckiaceae (*Methylobacterium-Methylorubrum* sp.) was enriched. The relative abundance of Actinobacteria in the gut microbiota, members of which are associated with antibiotic production (Bérdy 2012), has been shown to be negatively impacted by exposure to warm temperatures in arthropods (Horváthová et al. 2019) and Mongolia racerunners (*Eremias argus*) (Zhang et al. 2022). However, the opposite was found in lab-reared northern leopard frog larvae (*L. pipiens*) (Kohl and Yahn 2016) and Chinese giant salamanders (*Andrias davidianus*) (Zhu et al. 2021). The pronounced increase in Beijerinckiaceae under the warm temperature was surprising as members of this family have the capacity to grow across a broad thermal breadth in natural environments (Sharp et al. 2014). In previous studies on free-ranging Nile tilapia (*Oreochromis niloticus*) and northern leopard frog larvae, the relative abundance of Beijerinckiaceae (*Methylobacterium* spp.) was enriched at cool temperatures (Kohl and Yahn 2016, Bereded et al. 2022), while pinfish (*Lagodon rhomboides*) had higher relative abundances at warmer temperatures (Givens 2012). Many Beijerinckiaceae members are associated with nitrogen fixing (Marin and Arahal 2014) which can provide their host with additional energy (Russell et al. 2009), although how this influences



larval anuran performance or physiology requires further assessment. Our results show that complex relationships exist between environmental temperature and the relative abundance of Actinobacteria and Beijerinckiaceae that are likely host specific.

A limitation of our study design is that we did not have an unmanipulated microbiota treatment that represents wildtype wood frog larvae. The WF treatment was meant to restore the wildtype microbiota following antibiotic exposure, but a subsequent study showed that unmanipulated wildtype larvae significantly diverged in both alpha and beta diversity indices from WF larvae (Dallas et al., In Prep.). Therefore, the WF larvae did not accurately represent the wildtype condition. While GF larvae exhibited the highest  $CT_{max}$  among our microbiota treatments, their  $CT_{max}$  were comparable to less-developed wildtype larvae. This indicates that transplanting the green frog microbiota improved heat tolerance of recipient larvae relative to the other antibiotic-treated larvae but not unmanipulated individuals. Early-life antibiotic exposure in anurans leads to phenotypic costs including higher mortality risk, delayed metamorphosis, and increased tail deformities (Peltzer et al. 2017, Warne et al. 2019, Zhu et al. 2022), suggesting that the green frog transplantation rescued antibiotic-treated larvae from the lower heat tolerance evident in the NI and WF larvae. A follow-up study incorporating a more successful within-species microbiota inoculation would provide greater support for linking cross-species gut microbiota transplants to warmer  $CT_{max}$ .

## Conclusion

As the risk of extreme heat events becomes more prevalent under climate change, how the gut microbiota modulates host thermal physiology represents an intriguing research question. Our results represent an important step in linking this relationship by demonstrating that inoculating antibiotic-treated wood frog larvae, a heat-sensitive species, with the gut microbiota

of green frog larvae, a more heat-tolerant species, promoted higher  $CT_{max}$  relative to other antibiotic-treated larvae. This suggests that interspecific differences in heat tolerance are partially driven by the gut microbiota, and that this difference can be transferred between species simply through microbial transplants. The green frog transplant resulted in large increases in the Bacteroidetes family Rikenellaceae, a producer of the SCFA acetate, which is linked to increased antioxidant activity (Gonzalez-Bosch et al. 2021). As heat shock increases the production of ROS that disrupt cellular and mitochondrial activities which restrict heat tolerance, a greater abundance of antioxidants can maintain cell function at high temperatures. However, we acknowledge that our results are correlative but the inclusion of metagenomics to assess a mechanistic link between the gut microbiota and heat tolerance represents a future direction to explore. Microbial transplants represent a species-rescue technique against multiple threats, and this study represents that cross-species transplants can rapidly induce greater heat tolerance which promotes survival under warming temperatures.

## CHAPTER 7

### CONCLUSIONS AND SYNTHESIS

The study of thermal biology in herpetofauna is a highly relevant topic demanding more attention in the face of these taxa suffering from largescale population declines (Beebee and Griffiths 2005, Cox et al. 2022). Across my studies, I demonstrated that the invasive Mediterranean House Gecko is a eurythermic species which contributes to its ability to rapidly colonize new environments along the edge of their invasion edge. This is in agreement with a previous meta-analysis by Kelley (2014) suggesting that broad thermal performance breadths are necessary for successful, largescale invaders. I also highlighted that Mediterranean House Geckos are strict thermoconformers while active, which is a likely outcome of their nocturnality that limits their thermoregulatory capabilities (Gunderson and Stillman 2015, Tan and Schwanz 2015). This further supports their eurythmic thermal physiology, as species with greater behavioral thermoregulation opportunities have greater control over their body temperature (Hertz et al. 1993, Angilletta Jr et al. 2002a). Despite their broad performance breadth, the geckos were active at temperatures that constrained performance for multiple months, but this did not appear to place any significant negative costs on the population as they maintained growth rates and reproductive potential on par with populations from their invasive range core (Rose and Barbour 1968, Selcer 1986, Paulissen et al. 2014). Therefore, it is likely that the geckos exploit their urban habitat during the day to offset the cool temperatures experienced during their nighttime activity and maintain population persistence.

My research also demonstrated that plasticity in heat tolerance of larval wood frogs was influenced by heat hardening and gut microbiota but not viral infection. For aquatic species that have limited behavioral thermoregulation capabilities, such as larval amphibians, plasticity in

heat tolerance is more relevant (Gunderson and Stillman 2015, Pottier et al. 2022). My heat hardening experiment provided support for the tolerance–plasticity tradeoff hypothesis proposed by van Heerwaarden and Kellermann (2020). This has consequences regarding climate change as acclimation to warmer temperatures enhanced basal heat tolerance at the cost of a restricted heat hardening response. As heat hardening is often related to heat shock protein production (Sørensen et al. 2003, Bowler 2005), this suggests that increases in basal heat tolerance either restricts the ability to further upregulate heat shock protein expression or approaches a hard cap on further plasticity in response to acute heat events projected to increase under climate change. The role of the gut microbiota in heat tolerance suggests that the many integral aspects this diverse microbial community has to the host extends to thermal biology. Furthermore, my results demonstrate that cross-species gut microbiota transplants may be a viable option in species conservation to improve heat tolerance, which has currently been limited to within-species transplants (Moghadam et al. 2018, Doering et al. 2021, Baldassarre et al. 2022). There is a need to explore the mechanisms behind this effect to determine through which means the gut microbiota can modulate host heat tolerance. I suspect that the gut microbiota provides heat tolerance through upregulated intestinal production of antioxidants (Huang et al. 2017, Wang et al. 2021), heat shock proteins (Arnal and Lalles 2016), and improved protein synthesis (Fontaine and Kohl 2023); all of which can maintain intestinal integrity under heat stress. Since the gut microbiota’s metabolites can modulate more distal organs, including the brain (Cryan and O’Mahony 2011, Dalile et al. 2019, Williams et al. 2020) and liver (Morrison and Preston 2016, Kokou et al. 2018), there is potential that microbiota manipulations can improve heat tolerance within these organs as well. Lastly, since larval wood frogs were able to maintain their heat tolerance while infected with ranavirus, it suggests that they would be able to select warmer

temperatures for behavioral fever. Such a febrile response is associated with a decline in ranaviral loads in terrestrial anurans (Sauer et al. 2019) and improved immune performance in other ectotherms (Rakus et al. 2017, Barrile et al. 2021). This maintenance of heat tolerance may come at a cost to the host as increased heat-induced stress could elevate viral loads resulting in greater risk of succumbing to the pathogen.

My findings provide more support for the importance of eurythermality in the success of invasive species colonizing cooler climates and that heat tolerance plasticity is related to the gut microbiota but not ranaviral infection. As both invasive species and heat tolerance represent two highly relevant topics to herpetofaunal biodiversity in the 21<sup>st</sup> century (Blaustein et al. 2011, Bellard et al. 2012, Falaschi et al. 2020, Meyer et al. 2022), my research provides useful baselines for additional exploration to build upon. Since both topics are integrated with climate change, the need to detail how different ecophysiological factors shift the thermal biology of herpetofauna will continue to be an active field of research with numerous outstanding questions to answer.

## EXHIBITS

Month	Age Classes			Sex		Gravid
	Adults	Juveniles	Hatchlings	Males	Females	
September 2019	38	34	29	19	18	0
May 2020	48	33	1	35	27	1
June 2020	45	14	0	31	24	12
July 2020	51	1	0	24	28	13
August 2020	39	0	33	20	19	3
September 2020	49	48	24	22	26	0
October 2020	13	25	30	5	8	0

Table 3.1 – A summary of Mediterranean House Gecko population demographics. Age classes are based upon SVL measurements from Rose and Barbour (1968). Sex was determined in 2020 via the presence/absence of pre-cloacal pores and hemipenes in males. Gravid females only included those with calcareous eggs present in the abdominal cavity.

Source of Variation	S. S.	d. f.	F	<i>P</i>
Log-Transformed Body Mass	0.14	1	0.73	0.40
Gosner Stage	0.22	1	1.15	0.29
Hardening	0.36	1	1.88	0.18
Acclimation Period	3.99	1	20.92	<b>&lt; 0.001</b>
Acclimation Temperature	1.25	1	6.52	<b>0.014</b>
Hardening x Acclimation Period	0.057	1	0.30	0.59
Hardening x Acclimation Temperature	0.094	1	0.49	0.49
Acclimation Period x Acclimation Temperature	3.57	1	18.71	<b>&lt; 0.001</b>
Hardening x Acclimation Period x Acclimation Temperature	0.85	1	4.47	<b>0.040</b>
Residuals	9.35	49		

Table 4.1 – Effects of body mass, Gosner stage, hardening treatment, acclimation period, and acclimation temperature on larval wood frog critical thermal maximum from a generalized linear model. Values in bold indicate significant differences,  $p < 0.05$ .

<b>Fixed Effects</b>	<b>S.S.</b>	<b>d.f.</b>	<b>F</b>	<b>P</b>
Gosner Stage	2.34	1, 31.3	4.77	<b>0.037</b>
Ranavirus Treatment	0.15	1, 31.4	0.30	0.59
Days Post Infection	1.39	1, 31.0	2.84	0.10
Ranavirus Treatment x Days Post Infection	0.08	1, 31.2	0.15	0.70
<b>Random Effects</b>	<b>Standard Deviation</b>	<b>% Variance Explained</b>		
Bath ID	0.51	25.8		

Table 5.1 – Model parameters from the fitted general linear mixed effects model for the  $CT_{max}$  of larval wood frogs. Significant results are bolded.



Variable	$\chi^2$	df	<i>P</i>
√Mass	0.16	1	0.69
Microbiota Treatment	14.86	2	< <b>0.001</b>
Acclimation Temperature	19.98	1	< <b>0.0001</b>
√Mass x Microbiota Treatment	1.04	2	0.6
√Mass x Acclimation Temperature	0.0032	1	0.96
Microbiota Treatment x Acclimation Temperature	0.94	2	0.62
√Mass x Microbiota Treatment x Acclimation Temperature	0.92	2	0.66

Table 6.1 – Effects of square root transformed body mass, microbiota treatment, acclimation temperature, and their interactions on larval wood frog critical thermal maximum from a generalized linear model. Significant results are bolded.

	Relative Abundance (%)			BH FDR P-value	Coefficient
<b>Temperature Effects</b>	<b>Low</b>	<b>High</b>			
<b>Phyla</b>					
Actinobacteria	<b>1.12 ± 0.30</b>	0.45 ± 0.17		0.07	-0.96
<b>Family</b>					
Beijerinckiaceae	0.38 ± 0.15	<b>16.64 ± 2.42</b>		< 0.001	3.06
	Relative Abundance (%)			BH FDR P-value	Coefficient
<b>Microbiota Treatment Effects</b>	<b>Green Frog</b>	<b>No Inoculum</b>	<b>Wood Frog</b>		
<b>Family</b>					
[Clostridium] methylpentosum group	<b>0.52 ± 0.20</b>	N.O.	N.O.	< 0.001	-1.55
Clostridiaceae	< 0.01	<b>2.25 ± 0.87</b>	0.20 ± 0.08	< 0.03	
Rikenellaceae	<b>14.54 ± 2.65</b>	0.75 ± 0.50	0.06 ± 0.03	< 0.001	-4.21
Yersiniaceae	N.O.	<b>2.79 ± 1.26</b>	N.O.	< 0.001	2.93
Gracilibacteraceae	<b>1.00 ± 0.52</b>	N.O.	<b>0.67 ± 0.67</b>	< 0.01	-3.15
Aeromonadaceae	0.05 ± 0.05	<b>9.69 ± 3.06</b>	0.85 ± 0.83	< 0.01	3.45
Pseudomonadaceae	< 0.01	<b>2.20 ± 1.45</b>	0.07 ± 0.01	< 0.01	2.82
Flavobacteriaceae	0.01 ± 0.01	<b>1.61 ± 1.46</b>	<b>3.20 ± 1.42</b>	< 0.01	3.81
Promicromonosporaceae	<b>0.17 ± 0.07</b>	N.O.	<b>0.10 ± 0.07</b>	< 0.01	-1.52
Muribaculaceae	<b>0.08 ± 0.04</b>	N.O.	N.O.	0.01	-1.47
Uncultured Rickettsiales	<b>0.16 ± 0.12</b>	N.O.	N.O.	0.02	-1.24
Eubacteriaceae	<b>0.52 ± 0.38</b>	N.O.	N.O.	0.06	-1.76
Unknown Oscillospirales	<b>1.34 ± 0.57</b>	<b>3.78 ± 1.64</b>	0.06 ± 0.05	< 0.01	-4.24
Comamonadaceae	<b>1.01 ± 0.59</b>	<b>4.44 ± 1.86</b>	0.06 ± 0.04	< 0.01	3.58

Table 6.2: Relative abundances of bacterial phyla and families in tadpole gut microbial communities that were significantly impacted by acclimation temperature and gut microbiota treatment. Relative abundances are displayed as means ± 1 standard error. The group in which

the specific taxa was most abundant is in bold. Statistical testing was conducted using MaAsLin2. N.O. represents groups where the bacterial taxa was not observed. P-values were corrected using the BH FDR method.

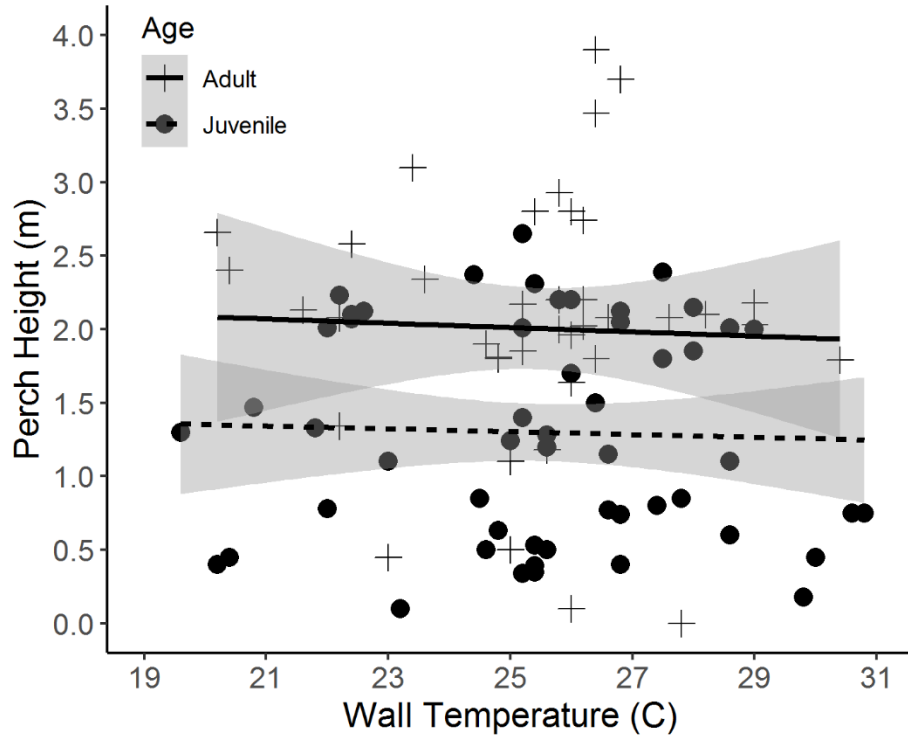


Figure 2.1 – Perch heights and wall temperatures of adult and juvenile *H. turcicus* collected from buildings in Carbondale, IL. Regression lines (adult = solid, juvenile = dashed) represent the generalized linear model with 95% confidence intervals. Neither slope for adults ( $-0.028 \pm 0.055$  [95% confidence interval]) nor juveniles ( $-0.0095 \pm 0.037$ ) differed from zero ( $P > 0.60$ ).

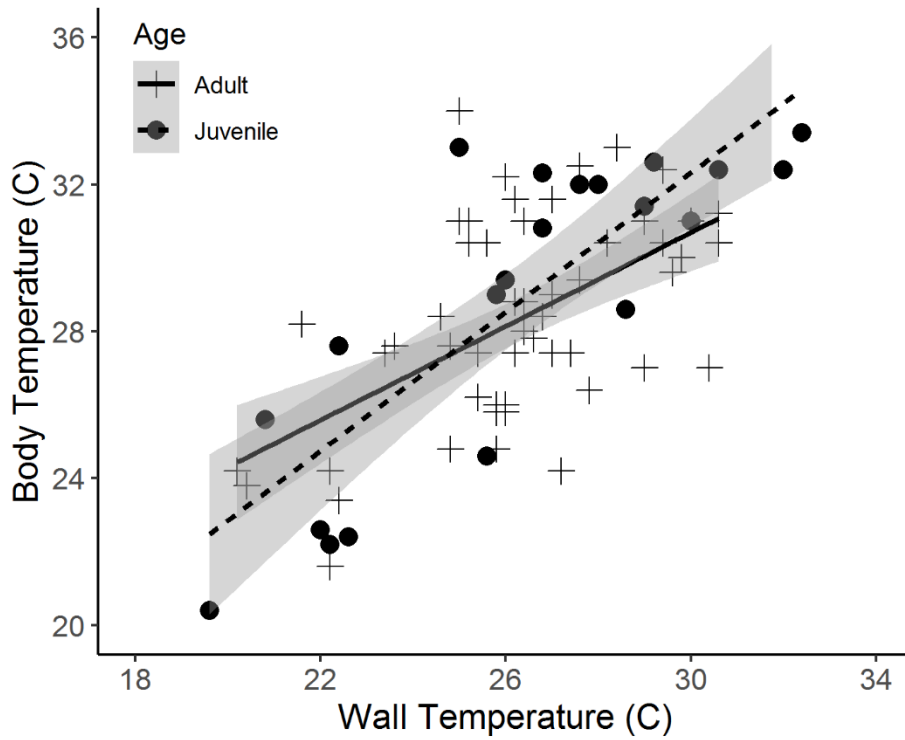


Figure 2.2 – The wall and body temperatures of *H. turcicus* collected from buildings in Carbondale, IL. All body temperatures were recorded within 30 seconds of capture to avoid transferring heat from the hand to captured individuals. Regression lines (adult = solid, juvenile = dashed) represent the generalized linear model with 95% confidence intervals. Slopes for adults ( $0.63 \pm 0.11$ ) and juveniles ( $0.95 \pm 0.15$ ) significantly differed from zero ( $P < 0.0001$ ).

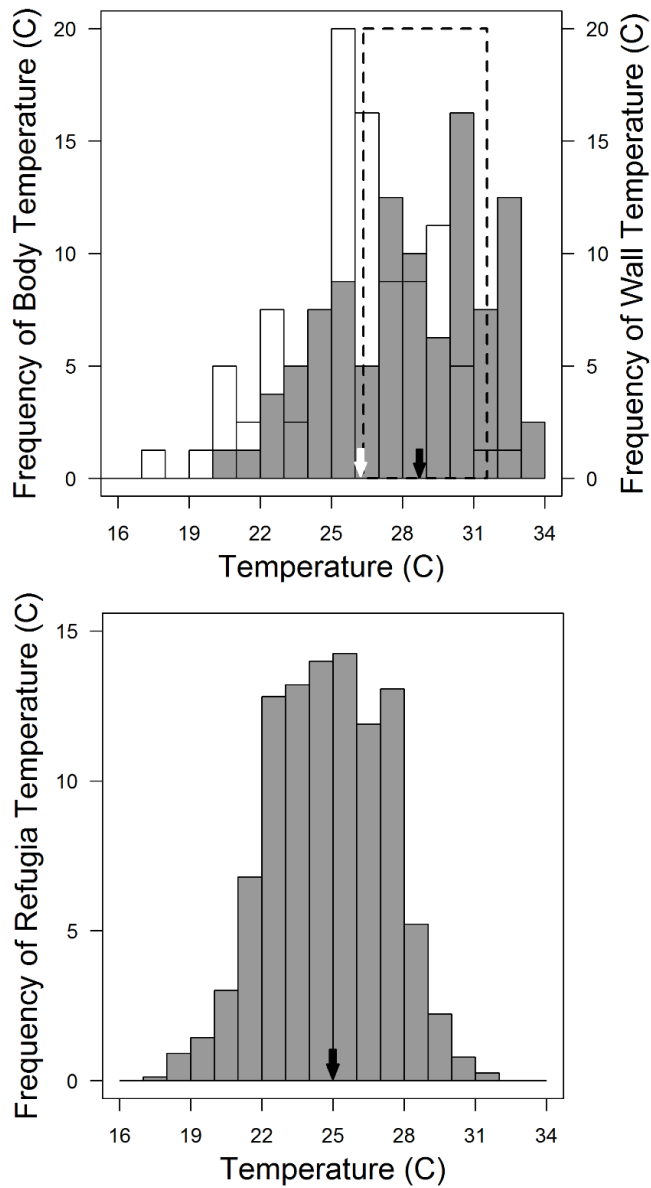


Figure 2.3 – Frequencies of (A) body temperatures (gray bars) and wall temperature (white bars) of all captured *Hemidactylus turcicus*. The dashed rectangle represents the preferred temperature ranges of *H. turcicus* from both Huey et al. (1989) and Hitchcock and MacBrayer (2006). (B) Distribution of refugia temperatures represent those collected during the active period of *H. turcicus* (2000–2200 hours) from late-September to early-October. Arrows indicate median temperatures.

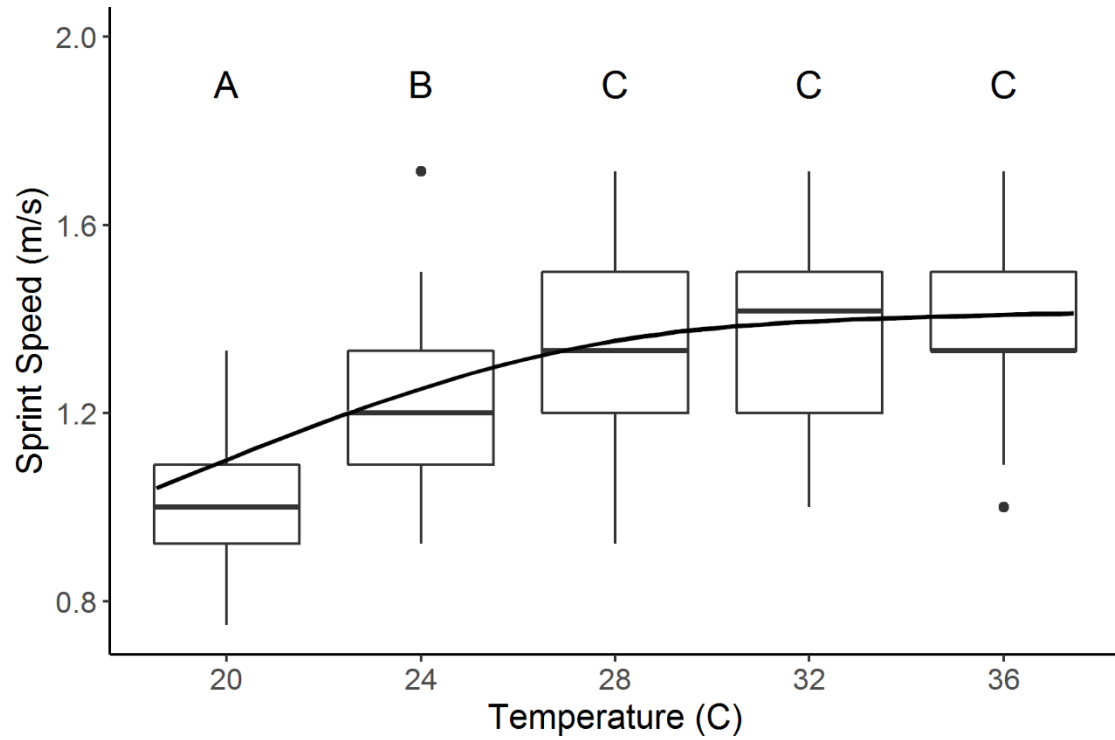


Figure 2.4 – Effect of temperature on maximum sprint performance of *Hemidactylus turcicus* across a 10 cm distance. The curve is an estimate derived from a generalized additive mixed model. Center lines indicate the group median with boxplots denote the interquartile range with whiskers representing 1.5x the upper or lower quartile. Letters indicate significant differences between post hoc pairwise comparisons (Tukey HSD,  $P < 0.05$ ).

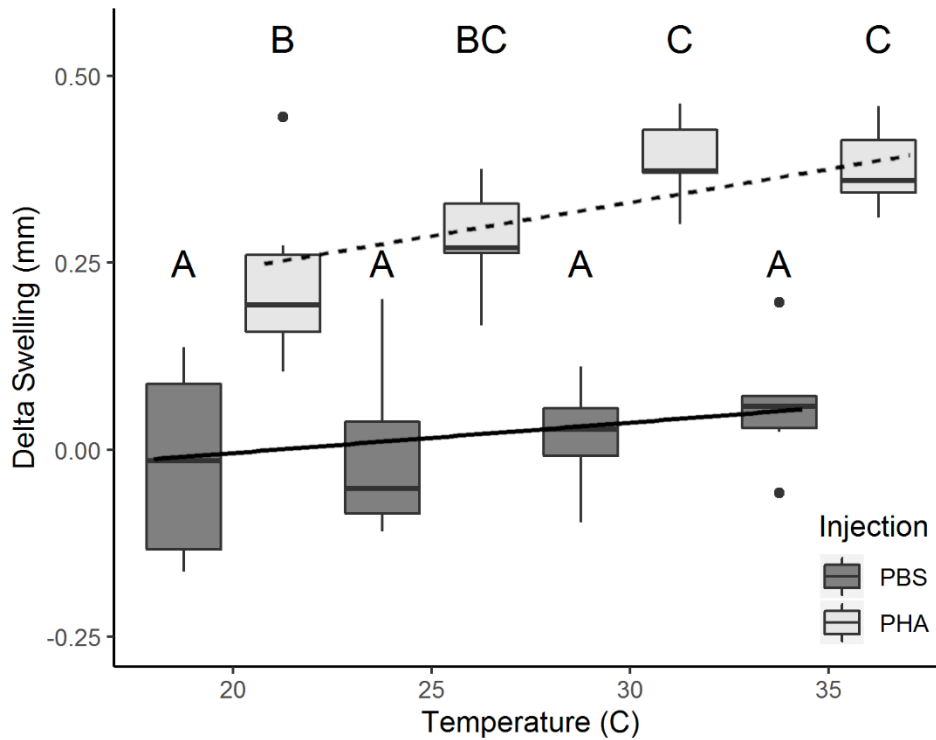


Figure 2.5 – Swelling of *H. turcicus* rear feet in response to PHA or PBS injections across temperature treatments. Measurements represent the difference in foot swelling 24 hours after injections. The plotted lines are estimates derived from a linear model. Center lines indicate the group median with boxplots denote the interquartile range with whiskers representing 1.5x the upper or lower quartile. Letters indicate significant differences between post hoc pairwise comparisons (Tukey HSD,  $P < 0.05$ ).



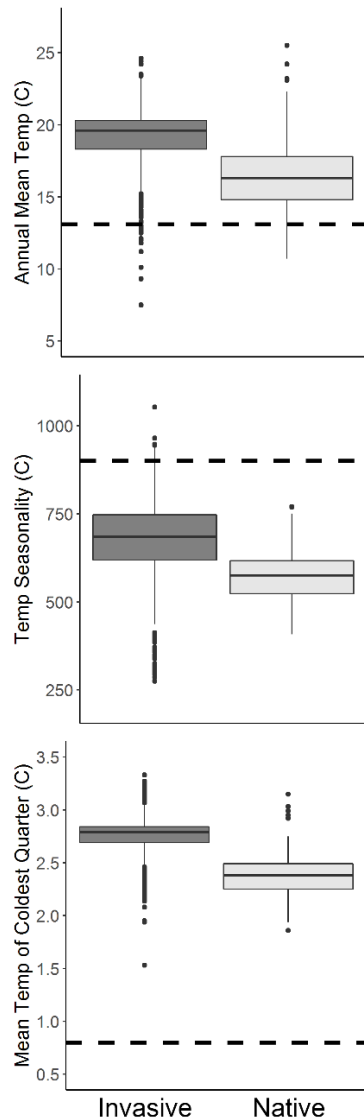


Figure 3.1 – Comparisons of selected bioclimatic variables for Mediterranean House Geckos from both their native (light gray) and invasive (dark gray) populations along with the Carbondale, IL population (dashed horizontal line). The center lines indicate the median and the boxplots denote the interquartile range with whiskers representing 1.5x the upper or lower quartile. Location data for Mediterranean House Geckos were obtained from the Global Biodiversity Information Facility (GBIF.org) and rarefied to reduce the densities of occurrences using SDM Toolbox 2.0 (Brown et al. 2017). Bioclimatic variables were obtained from WorldClim version 2.0 (Fick and Hijmans 2017).

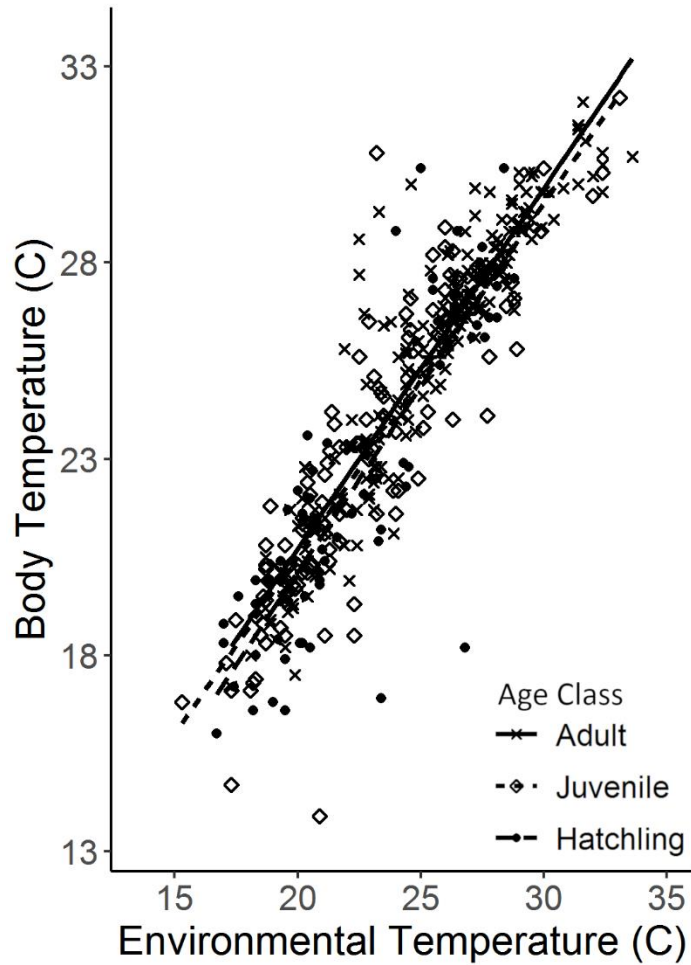


Figure 3.2 – Relationship of  $T_e$  and perch height on the  $T_b$  of Mediterranean House Geckos reflects their strong thermoconformer nature. Additionally, a minor, significant positive effect of perch height on  $T_b$  was also present. Data points are separated by age although no significant age effect on  $T_b$  was found. Best fit linear model is shown by the gray plane.

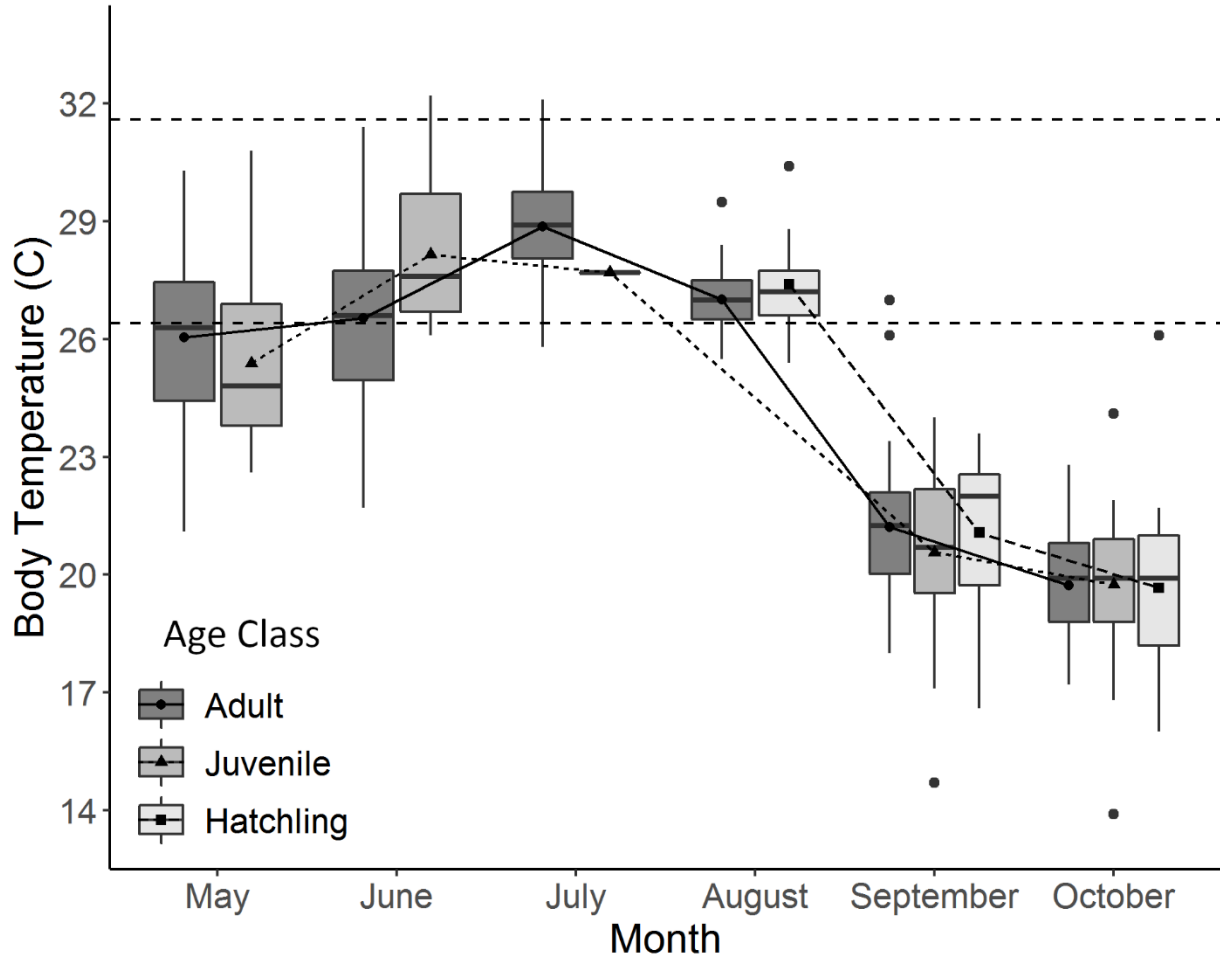


Figure 3.3 – The monthly variation of  $T_b$  in Mediterranean House Geckos from southern Illinois shows limited variation by age class but a strong seasonal shift. During the autumn, Mediterranean House Geckos are unable to achieve  $T_b$ s indicative of  $T_{pref}$  (represented by the dashed horizontal lines; 26.4 – 31.6°C; Angilletta Jr et al. 1999, Hitchcock and MacBrayer 2006; J. W. Dallas, M. Deutsch, and R. W. Warne, unpublished data) and likely suffer from reduced physiological performance. Center lines indicate the group median with boxplots denote the interquartile range with whiskers representing 1.5x the upper or lower quartile. Means are represented by points in the boxplots.

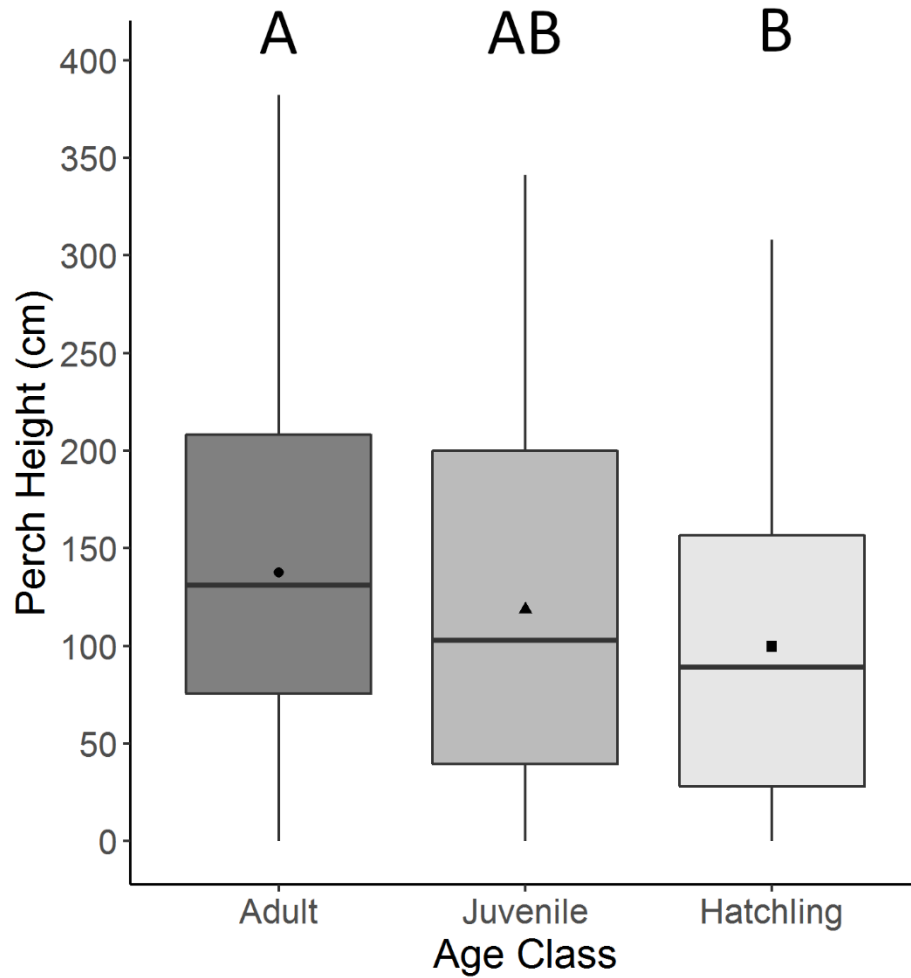


Figure 3.4 – Perch height of Mediterranean House Geckos varied by age class as younger individuals selected lower perch heights. Center lines indicate the group median with boxplots denote the interquartile range with whiskers representing 1.5x the upper or lower quartile. Means are represented by points in the boxplots. Letters indicate significant differences between post hoc pairwise comparisons (Tukey HSD,  $P < 0.05$ ).

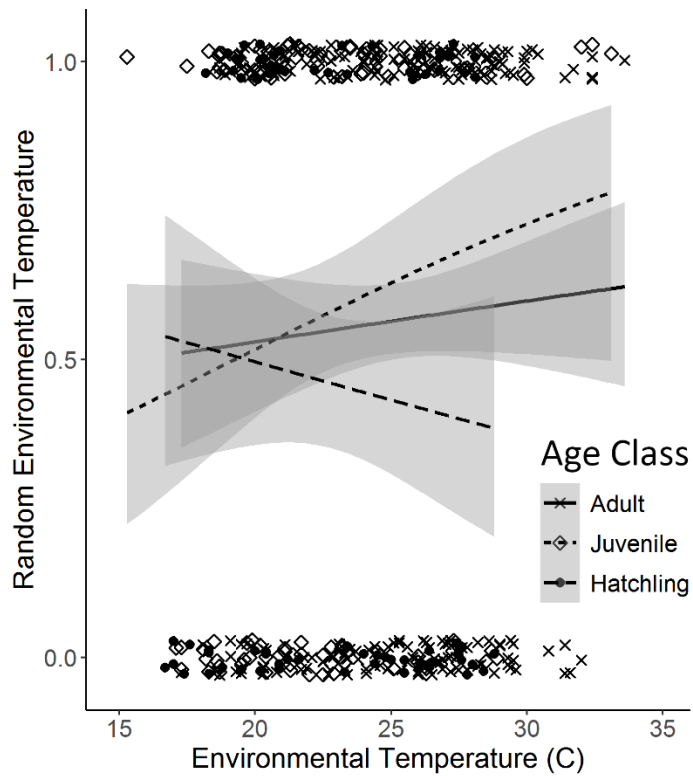
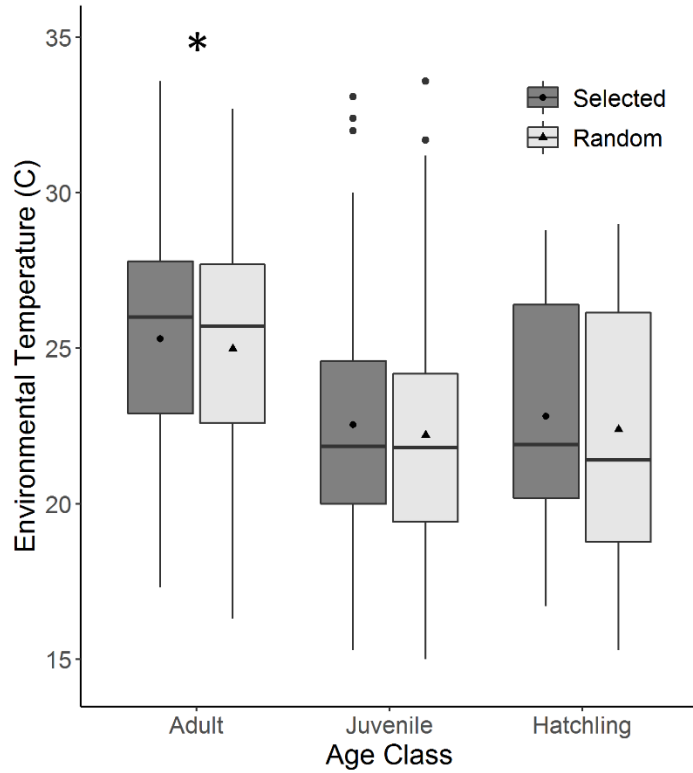


Figure 3.5 – The  $T_{es}$  were compared between those from which Mediterranean House Geckos were found and a random point ~ one meter. A) Across all age classes,  $T_{es}$  that Mediterranean

House Geckos used (Selected) were marginally warmer than  $T_{\text{Random}}$  but this difference was only significant for adults. Center lines indicate the group median with boxplots denote the interquartile range with whiskers representing 1.5x the upper or lower quartile. Means are represented by points in the boxplots. Asterisk indicates significant difference within age classes (Paired t-test,  $P < 0.05$ ). B) However, the selection of  $T_e$  was random with respect to warmer  $T_{\text{Random}}$  (denoted in the figure as a 1) across all age classes. Lines represent a GLM using a binomial family and gray areas represent the 95% confidence interval.

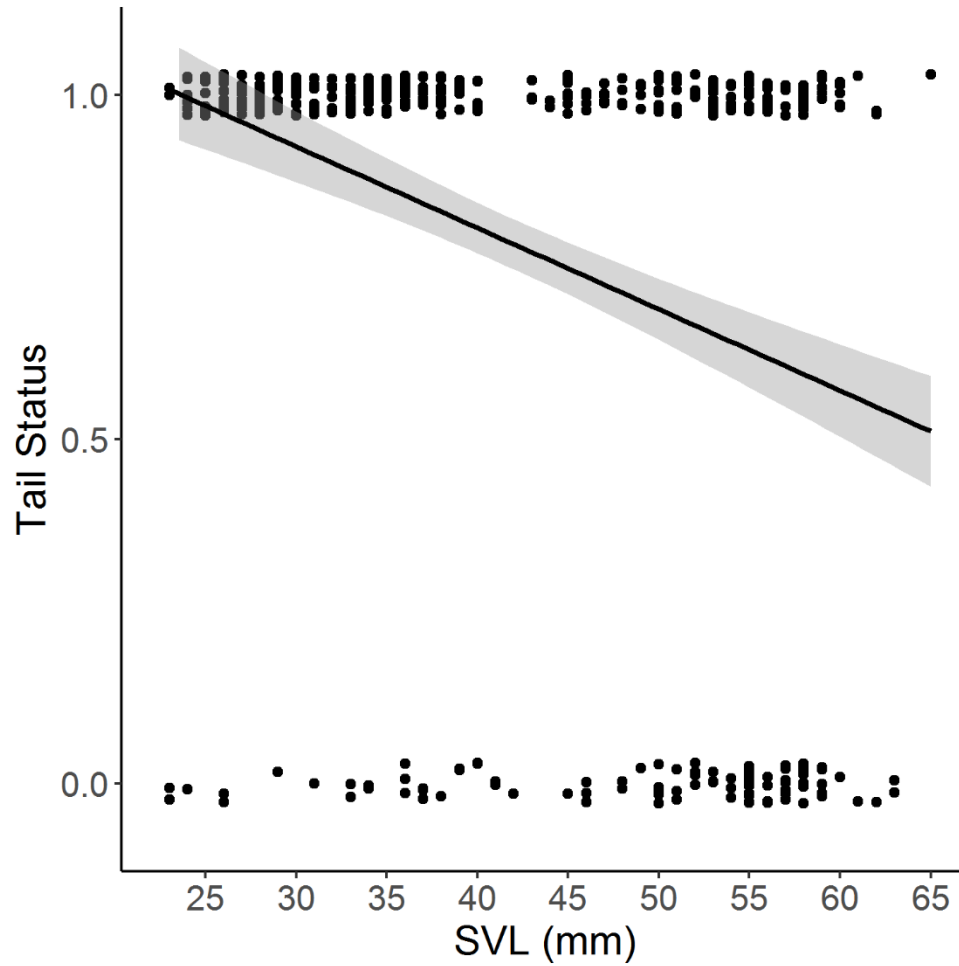


Figure 3.6 – We classified tail status as intact = 1 or damaged = 0 with the latter including complete tail breaks, regenerating tails, and regenerated tails. As individuals grew, there was a greater chance of tails being damaged ( $r = -0.34$ ,  $P < 0.0001$ ). The gray area around the regression line represents the 95% confidence interval.

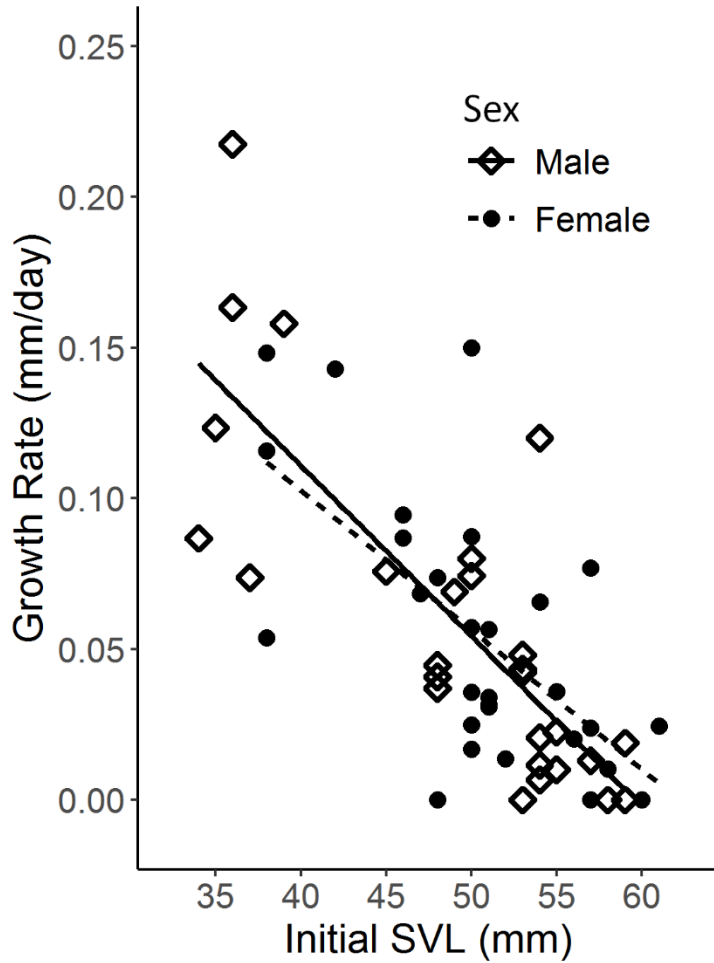


Figure 3.7 – The daily growth rate (mm/day) of Mediterranean House Geckos declined sharply with increasing body length (mean growth rate =  $0.059 \pm 0.0067$  mm/day). There was no effect of sex on daily growth rate ( $P > 0.20$ ).



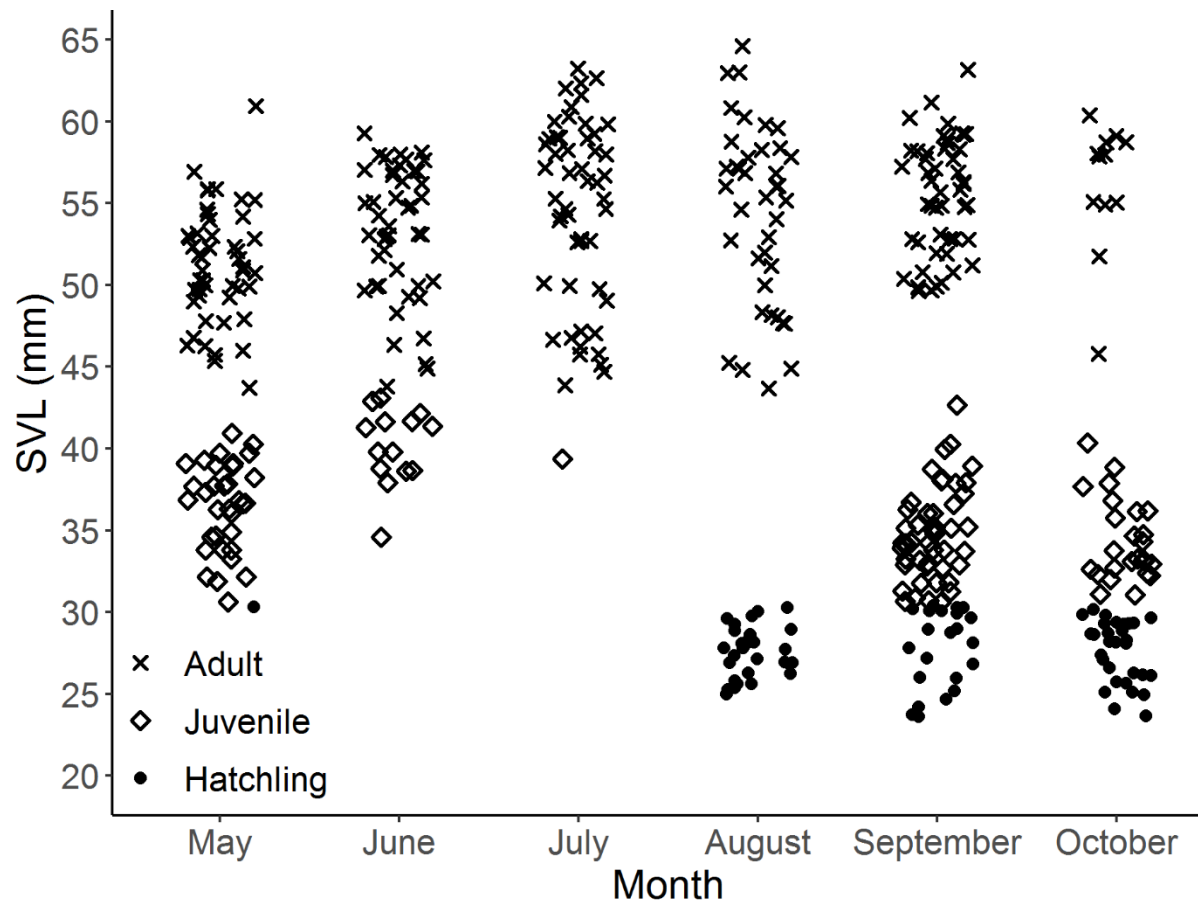


Figure 3.8 – The snout-vent-length (SVL) of Mediterranean House Geckos captured across 2020 is roughly in line with the predicted monthly growth rate (1.77 mm/month for adults and ~ 4 – 5 mm for hatchlings). Sex of adults was not separated as they were of similar length ( $P > 0.97$ ).

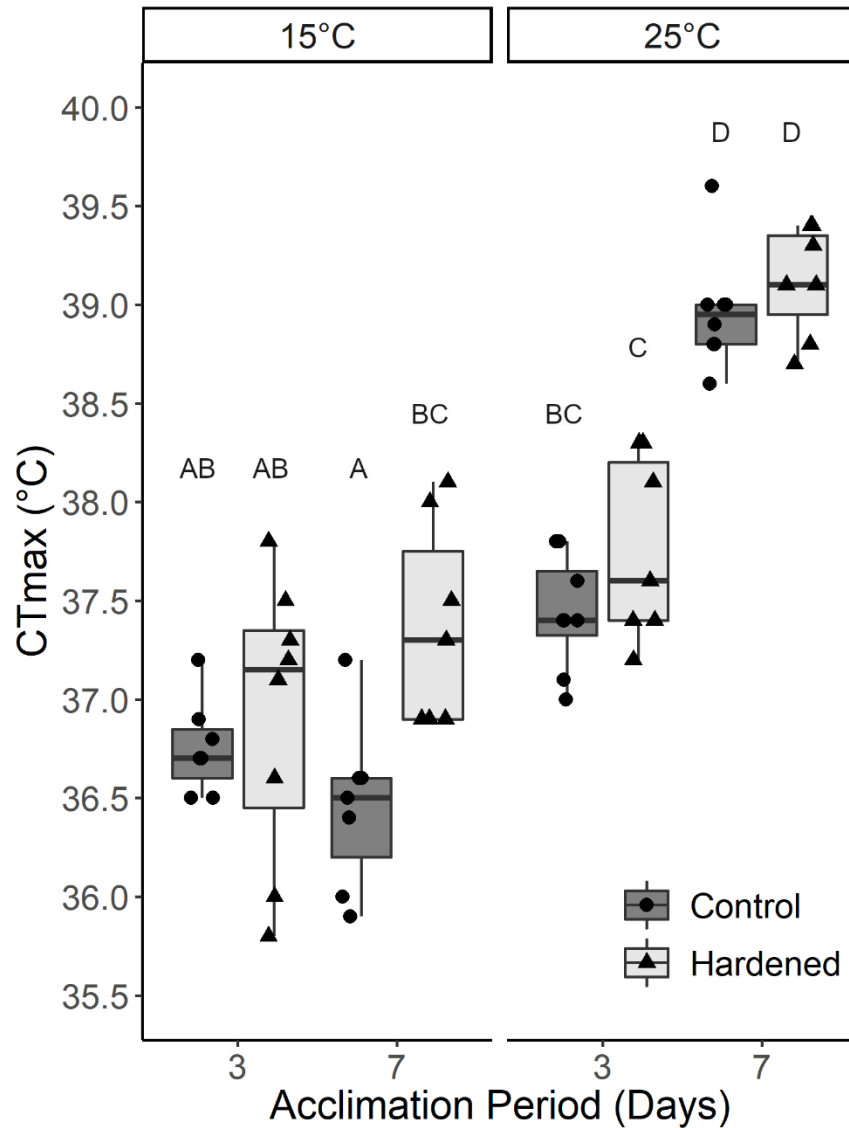


Figure 4.1 – Heat tolerance of larval wood frogs across differing acclimation conditions and hardening. Larval wood frog critical thermal maximum ( $CT_{max}$ ) exposed to two different acclimation temperatures (15° and 25°C), two different acclimation periods (3 and 7 days), and a hardening treatment (control vs. hardened). Points represent individual larvae. Center lines within boxplots represent the median and the boxes denote the interquartile range with whiskers representing 1.5x the upper or lower quartile. Letters indicate significant differences between post hoc pairwise comparisons (Tukey HSD,  $P < 0.05$ ).

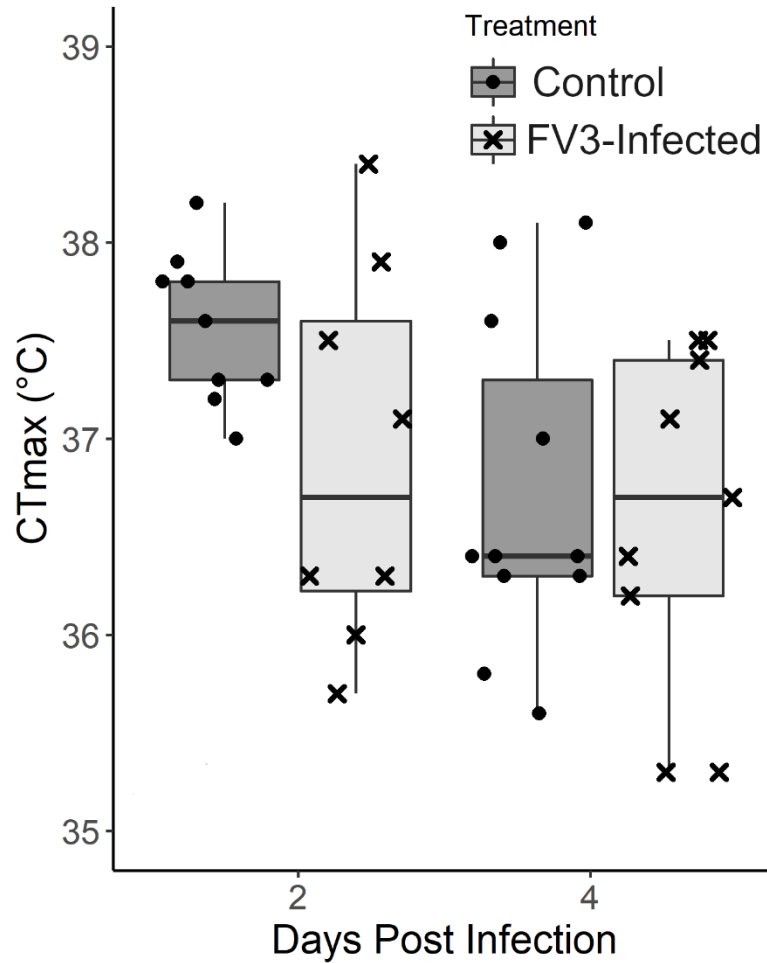


Figure 5.1 –  $CT_{max}$  was similar in FV3-infected and uninfected control wood frog larvae across two sampling dates. Points represent individual larvae. Center lines within boxplots represent the median and the boxes denote the interquartile range with whiskers representing 1.5x the upper or lower quartile.

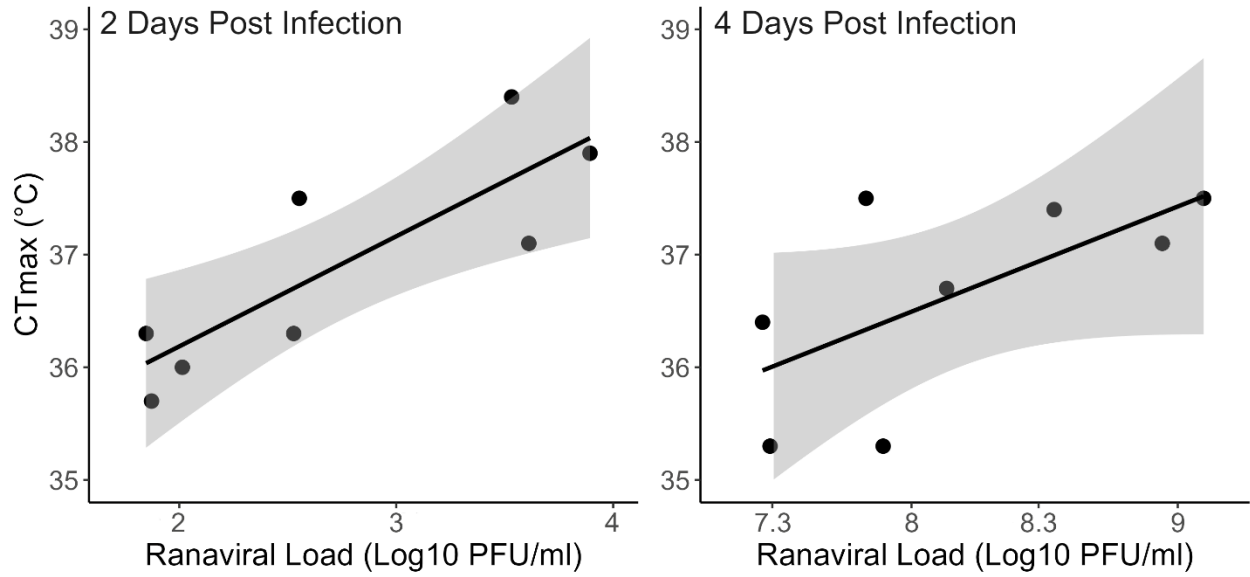


Figure 5.2 – The correlation between ranaviral loads of larval wood frog livers infected with FV3 and  $CT_{max}$  at 2- and 4-days post infection. Strong positive correlations existed at both sampling dates, but the relationship was more pronounced at 2 days post infection ( $r = 0.84$ ) than 4 days post infection ( $r = 0.64$ ). Shaded areas represent 95% confidence interval.

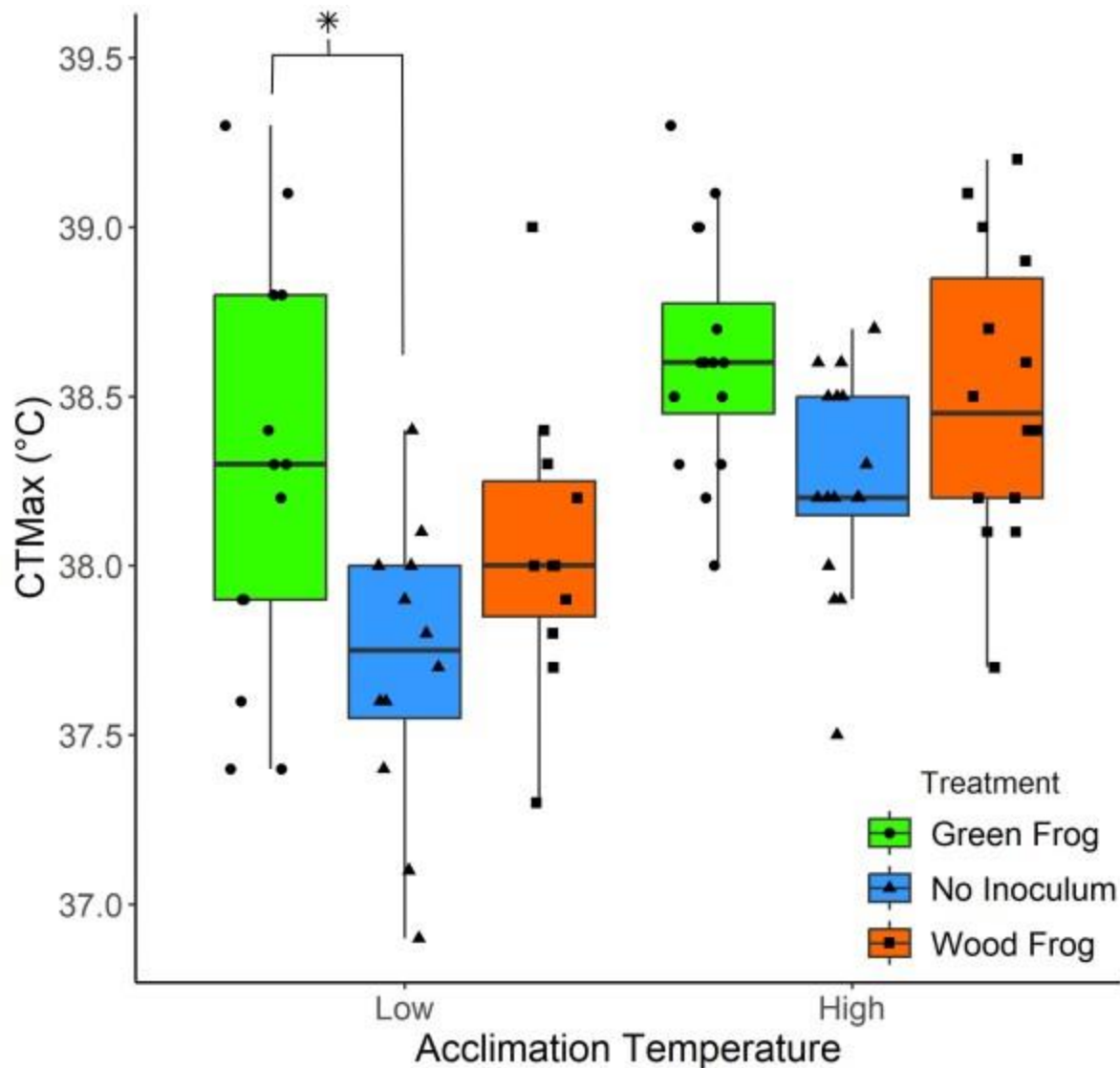


Figure 6.1 – The critical thermal maximum ( $CT_{max}$ ) of larval wood frogs was dependent on their gut microbiota treatment and their acclimation temperature. Larvae acclimated to a higher temperature had a higher  $CT_{max}$  independent of microbiota treatment and the green frog larval treatment had higher  $CT_{max}$ . The asterisk represents a near-significant pairwise difference ( $P = 0.51$ ) within the acclimation temperature. Center lines within boxplots represent the median and the boxes denote the interquartile range with whiskers representing  $1.5\times$  the upper or lower quartile.

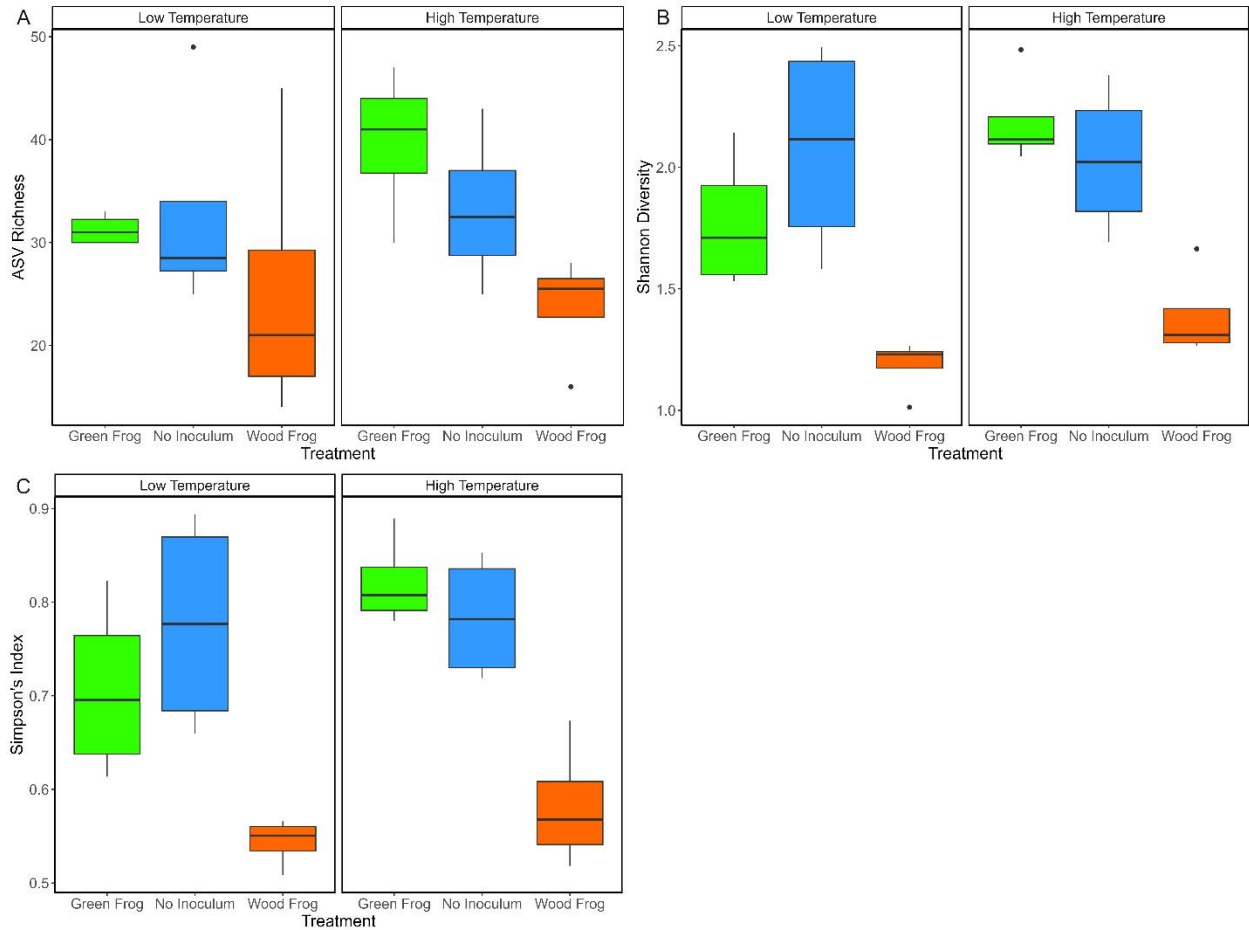


Figure 6.2 – The effect of gut microbiota treatments and acclimation temperature on alpha diversity indices of larval wood frogs: A) ASV Richness, B) Shannon Index, and C) Simpson Index. For both Shannon and Simpson diversity, the wood frog treatment had significantly lower values than the other treatments at both acclimation temperatures (Tukey HSD  $P < 0.05$ ), but approached significance ( $P < 0.08$ ) when compared against the low temperature green frog treatment. Center lines within boxplots represent the median and the boxes denote the interquartile range with whiskers representing  $1.5\times$  the upper or lower quartile.

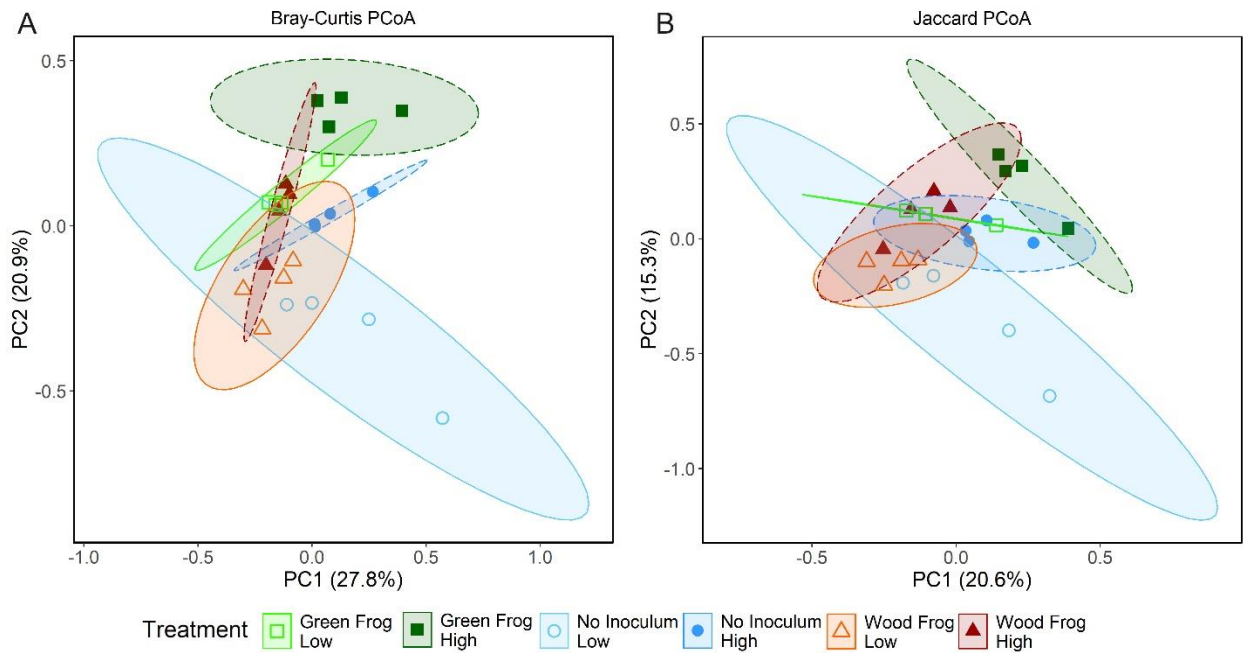


Figure 6.3 – Principal coordinate analyses (PCoA) of the gut microbiota community structure (A) and membership (B) of larval wood frogs that underwent early life microbiota treatments and were acclimated to either low or high temperatures. Larvae from the low acclimation temperature are indicated by hollow symbols and lighter colors relative to high temperature acclimated larvae. PCoAs were based on all ASVs. Percentages on axes titles represent the variance explained by the eigenvector. Each point represents the gut microbiota of an individual wood frog.

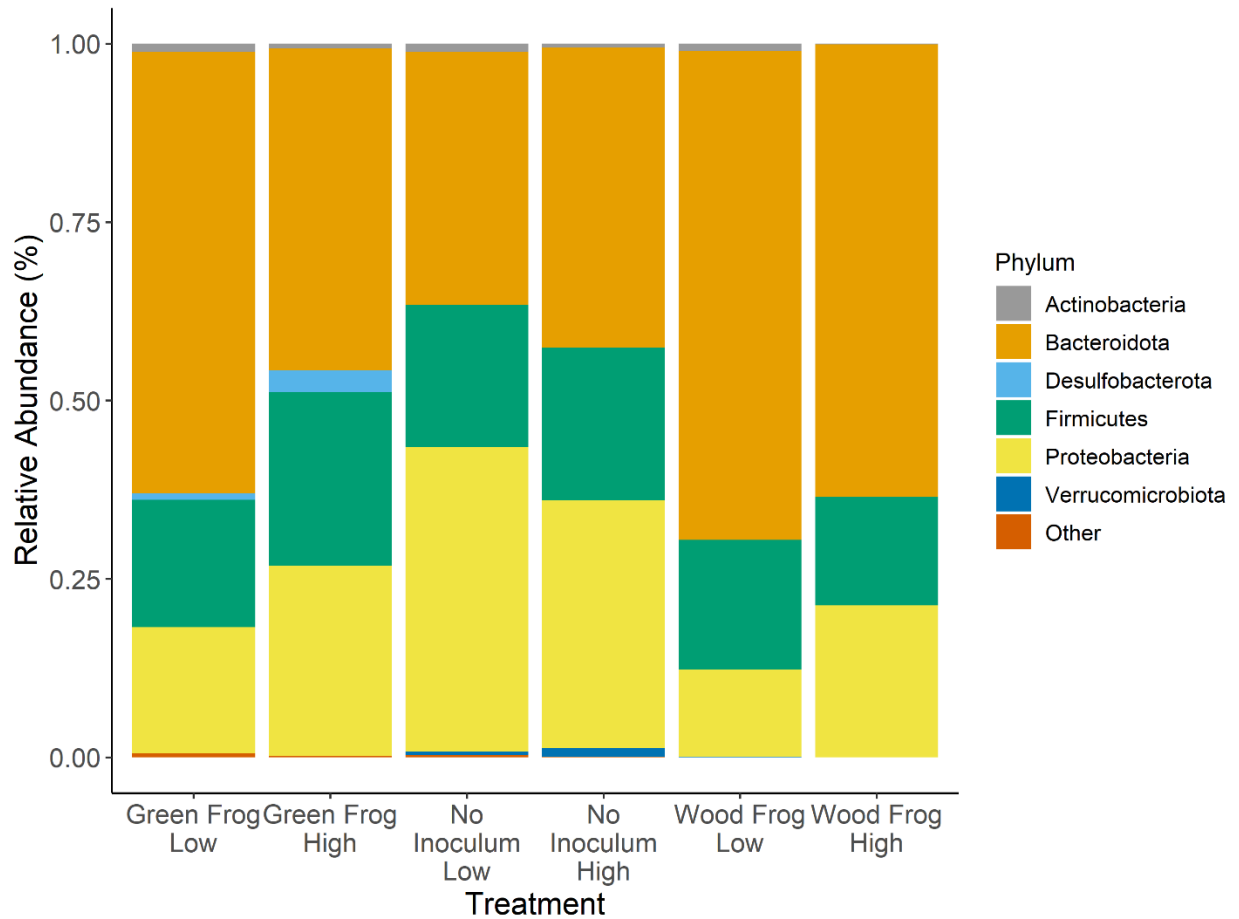
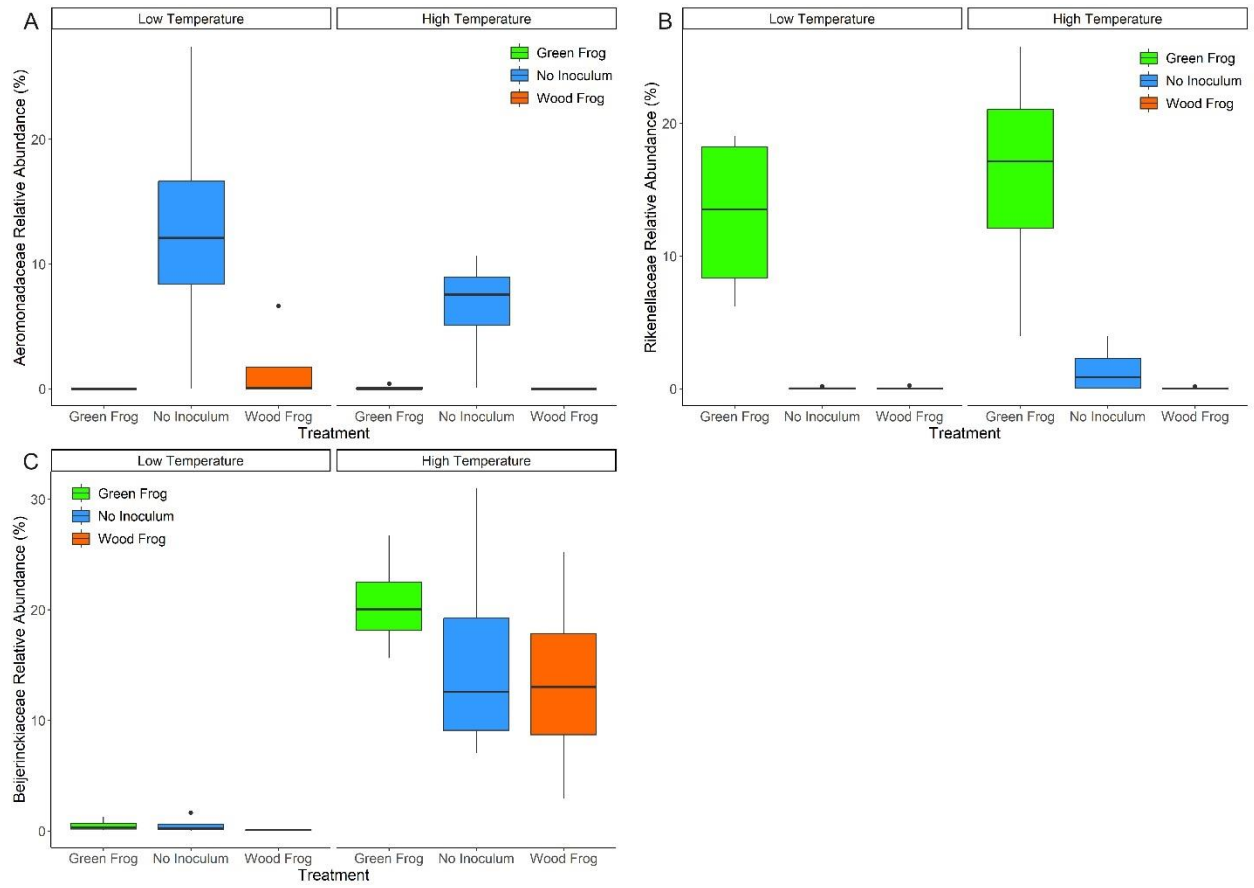


Figure 6.4 – The relative abundance of prominent intestinal bacterial phyla of larval wood frogs that underwent early life microbiota treatments and were acclimated to either low or high temperatures. Larvae that were acclimated to low temperatures are denoted by Low in the X-axis while larvae acclimated to low temperatures are denoted by High.





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Rider University  
Bachelor of Science, Environmental Sciences, May 2014

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Dissertation Paper Title:  
Physiological, Ecological, and Microbial Factors Shaping Thermal Tolerance and Performance in Ectothermic Vertebrates

Major Professor: Robin W. Warne

### Publications:

Dallas, J. W., and R. W. Warne. (2023). Ranavirus Infection does not Reduce Heat Tolerance in a Larval Amphibian. *Journal of Thermal Biology* 114: 103584.

Dallas, J. W., and R. W. Warne. (2023). Heat Hardening of a Larval Amphibian is Dependent on Acclimation Period and Temperature. *Journal of Experimental Zoology – A* 339(4): 339 – 345.

Dallas, J. W., and R. W. Warne. (2022). Captivity and animal microbiomes: Incorporating this emerging field in animal conservation. *Microbial Ecology*: 1 – 19.

Warne, R. W. and J. W. Dallas. (2022). Microbiome mediation of animal life histories via metabolites and insulin-like signaling. *Biological Reviews* 97: 1118 – 1130.

Frick, W.F., E. Johnson, T. Cheng, J. Lankton, R. Warne, J. W. Dallas, K. Parise, J. Foster, J. Boyles, and L. McGuire. (2022). “Experimental inoculation trial to determine the effects of temperature and humidity on white-nose syndrome in hibernating bats”. *Scientific Reports* 12 (1): 971.

Dallas, J. W., W. E. Meshaka Jr., L. Zeglin, and R. W. Warne. (2021). Taxonomy, not locality, influences the cloacal microbiota of two Nearctic colubrids: a preliminary analysis.

Molecular Biology Reports 48(9): 6435 – 6442.

Dallas, J. W., W. E., Meshaka Jr, and P. R. Delis. (2021). The Natural History of the Northern Black Racer (*Coluber constrictor constrictor*) in South-Central Pennsylvania. *Journal of North American Herpetology* 2021(1): 29 – 37.

Dallas, J. W. A. Harris, J. Reinbolt, and R. W. Warne. (2021). Ecology of the Synanthropic Mediterranean House Gecko (*Hemidactylus turcicus*) at their Northern Invasion Front. *Urban Ecosystems* 25: 329 – 340.

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Dallas, J. W. 2020. *Trachemys scripta elegans* (Red-eared Slider). *Disease. Herpetological Review* 51 (3): 585 – 586.

Bowne, D., B. Cosentino, L. Anderson, C. Bloch, S. Cooke, P. Crumrine, J. W. Dallas... and C. Zimmermann. 2018. Effects of urbanization on population structure of freshwater turtles across the United States. *Conservation Biology* 32 (5): 1150 – 1161.