Heat tolerance of reptile embryos in a changing world: Physiological mechanisms and ecological effects

by

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ABSTRACT

Aspects of global change (e.g. climate change, urbanization) create stressful thermal environments that threaten biodiversity. Oviparous, non-avian reptiles have received considerable attention because eggs are left to incubate under prevailing conditions, leaving developing embryos vulnerable to increases in temperature. Though many studies assess embryo responses to long-term (i.e. chronic) incubation temperatures, few assess responses to acute exposures which are more relevant for many species and may become more common due to global change. Because warming temperatures cause increases in both mean and variance of nest temperatures, it is crucial to consider embryo responses to both chronic and acute heat stress. Currently, there are no standard metrics or terminology for determining heat stress of embryos. This impedes comparisons across studies and species and hinders our ability to predict how species will respond to warming temperatures. In Chapter 1, I compare various methods that have been used to assess embryonic heat tolerance in reptiles and provide new terminology and metrics for quantifying embryo responses to both chronic and acute heat stress. I apply these recommendations to data from the literature to assess chronic heat tolerance in 16 squamates, 16 turtles, 5 crocodilians, and the tuatara and acute heat tolerance for 9 squamates and 1 turtle. My results indicate there is relatively large variation in chronic and acute heat tolerance across species, and I outline directions for future research, calling for more studies that assess embryo responses to acute thermal stress and identify mechanisms that determine heat tolerance. In Chapters 2, 3 and 4, I make progress toward both these goals.

In Chapter 2, to better understand the effects of acute thermal stress on development, I subjected brown anole (*Anolis sagrei*) eggs to heat shocks, thermal ramps, and extreme diurnal fluctuations to determine the lethal temperature of embryos, measure the thermal sensitivity of

embryo heart rate and metabolism, and quantify the effects of sub-lethal but stressful temperatures on development and hatchling phenotypes and survival. Most embryos died at heat shocks of 45 or 46 °C, which is ~12 °C warmer than the highest constant temperatures suitable for successful development (i.e. chronic heat stress). Heart rate and O_2 consumption increased with temperature; however, as embryos approached the lethal temperature, heart rate and CO_2 production continued rising while O_2 consumption plateaued. These data indicate a mismatch between oxygen supply and demand at high temperatures. Exposure to extreme, diurnal fluctuations depressed embryo developmental rates and heart rates, and resulted in hatchlings with smaller body size, reduced growth rates, and lower survival in the laboratory. Thus, even brief exposure to extreme temperatures can have important effects on embryo development, and Chapter 2 highlights the role of both immediate and cumulative effects of high temperatures on egg survival.

In Chapter 3, I consider how increased nest temperatures due to urbanization (i.e. the urban heat island effect) influence embryo development. In reptiles, relatively warm incubation temperatures increase developmental rate and often enhance fitness-relevant phenotypes, but extremely high temperatures cause death. Human-altered habitats (i.e., cities) potentially create unusually warm nest conditions that differ from adjacent natural areas in both mean and extreme temperatures. Such variation may exert selection pressures on embryos. To address this, I measured soil temperatures in places where the Puerto Rican crested anole lizard (*A. cristatellus*) nests in both city and forest habitats. I bred anoles in the laboratory and subjected their eggs to 5 incubation treatments that mimicked temperature regimes from the field, three of which included brief exposure to extremely high temperatures (i.e. thermal spikes) measured in the city. I monitored growth and survival of hatchlings in the laboratory for three months and found that

warmer, city temperatures increase developmental rate, but brief, thermal spikes reduce survival. Hatchling growth and survival were unaffected by incubation treatment. Thus, the urban landscape can potentially create selection pressures that influence organisms at early life stages.

Finally, studies that examine thermal tolerance of embryos rarely assess the potential for tolerance to change with ontogeny or how effects differ among sympatric species, and often utilize unrealistic temperature treatments. In Chapter 4, I used thermal fluctuations from nests within the urban-heat island to determine how thermal tolerance of embryos changes across development and differs between two sympatric lizards (*A. sagrei* and *A. cristatellus*). I applied fluctuations that varied in frequency and magnitude at different times during development and measured effects on embryo physiology, egg survival, and hatchling morphology, growth, and survival. Thermal tolerance differed between the species by ~ 2 °C: embryos of *A. sagrei*, a lizard that prefers warmer, open-canopy microhabitats, were more robust to thermal stress than embryos of *A. cristatellus*, which prefers cooler, closed-canopy microhabitats. Moreover, thermal tolerance changed through development; however, the nature of this change differed between the species. For *A. cristatellus*, thermal tolerance was greatest mid-development. For *A. sagrei* the relationship was not statistically clear. The greatest effects of thermal stress were on embryo and hatchling survival and embryo physiology. Hatchling morphology and growth were less affected.

Collectively, the chapters of this dissertation demonstrate that inter-specific responses and the timing of stochastic thermal events with respect to development have important effects on egg survival. Thus, research that integrates responses to both acute and chronic thermal stress using ecologically-meaningful thermal treatments and examines interspecific responses will be critical to make robust predictions of the impacts of global change on wildlife.

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BACKGROUND INFORMATION

The following research considers the heat tolerance of lizard embryos against the backdrop of novel thermal environments induced by global change. Therefore, in this opening segment, I introduce two concepts that are vital to understand this research: warming temperatures due to global change (climate change and urbanization) and thermal developmental plasticity. I also describe the two primary goals of this dissertation and their justifications with respect to these concepts. Finally, as a physiological ecologist, I believe it is critical for the reader to connect physiology to organisms and their environments to understand and appreciate the research. Thus, I provide background information regarding the study species used in this dissertation. Additional background information is provided in each chapter.

Warming temperatures due to global change

Two important aspects of global change result in increased ambient temperatures: global climate change and urbanization. Climate change is primarily the result of increased carbon emissions into the atmosphere (i.e. the greenhouse effect; Pachauri et al., 2014), while warming temperatures due to urbanization are caused by the urban heat island effect. The urban heat island is a phenomenon by which a reduction in canopy cover and an abundance of heat absorbing artificial surfaces (e.g. concrete, metal) result in cities and suburban areas having higher temperatures than surrounding rural or natural habitats (Arnfield, 2003; Mohajerani et al., 2017). The conversion of natural habitats to urban areas is accelerating as the human population grows and more people move to urban vs rural communities (DeFries et al., 2010). Both climate change and urbanization impose heat stress on ecosystems, threatening the survival of

biodiversity across the globe (Kingsolver et al., 2013; Chown and Duffy, 2015). Therefore, there has never been a greater need to understand the ecological and physiological effects of heat stress on wildlife. Much research has considered the effects of heat stress due to climate change, but stress induced by the urban heat island effect has been less studied (Chown and Duffy, 2015; Brans et al., 2017). Increases in temperature along rural to urban gradients may afford great opportunity to understand how species respond to novel thermal regimes, like those induced by future climate change (i.e. spatial vs temporal increases in temperature; Youngsteadt et al., 2015). Therefore, a clearer understanding of species responses to the urban heat island will help us understand how species respond to urbanization, specifically, but also to novel thermal environments more generally.

Thermal developmental plasticity

Most complex organisms start life as a single cell and traverse multiple life-stages to reach sexual maturity (i.e. develop). Early-life stages (e.g. embryos, larvae) are often highly sensitive to environmental conditions. For example, the temperature at which lizard eggs develop can influence an array of hatchling phenotypes like body size, running speed (Pearson and Warner, 2018), and even learning ability (Dayananda and Webb, 2017). This potential for a given genotype to produce different phenotypes due to temperature is known as thermal developmental plasticity. Oviparous, non-avian reptiles (i.e. turtles, crocodilians, lizards and snakes; henceforth “reptiles”) have served as models for studies of thermal developmental plasticity for many decades (reviewed by Warner et al., 2018; While et al., 2018). Most species deposit eggs in nests (usually holes in the ground) and provide little or no parental care

thereafter. As a result, eggs often incubate at a wide range of temperatures and embryos must be able to deal with, sometimes extreme, thermal heterogeneity.

Therefore, unlike mammals and birds, reptile embryos are usually able to complete development across a wide range of temperatures. There are, however, thermal limits for reptilian development, and this has caused researchers to consider how rising temperatures due to global change may influence nest temperatures and the persistence of populations across the planet (Janzen, 1994; Telemeco et al., 2009; Carlo et al., 2018). Much of this research has been focused on species with temperature-dependent sex determination for fear that rising temperatures will skew population sex ratios in harmful ways (Janzen, 1994; Valenzuela et al., 2019); however, incubation temperature influences nearly every phenotype (Noble et al., 2018a) and research on species without temperature-dependent sex determination is also important. Historically, most studies have considered how pervasive nest temperatures (e.g. mean temperatures) influence developmental and hatchling phenotypes and survival, but less studied are the effects of temporary exposure to extreme temperatures (e.g. heat waves, daily spikes in nest temperature) (Bentley et al., 2017; Carter et al., 2018; Gunderson et al., 2020). Such acute exposures to heat stress are important to consider because urbanization and climate change result in increases in extreme temperatures in addition to greater mean temperatures (Field et al., 2012; Mohajerani et al., 2017).

Goals of this dissertation

There are two major gaps in our current understanding of reptile thermal developmental plasticity. First, because most studies have considered pervasive (e.g. mean) nest temperatures, we know comparatively little about the effects of brief exposure to extreme temperatures. For

many species, nest temperatures exhibit relatively large diurnal fluctuations and occasionally reach stressfully hot temperatures for a few minutes or hours during the warmest time of day (Angilletta et al., 2013; Sanger et al., 2018). We know very little about how development is influenced by the magnitude and frequency of such thermal events. Such knowledge will be important to understand the relationships between natural nest temperatures and phenotypes in the wild as well as responses to rising temperature due to global change. Second, because heat stress studies are usually concerned with climate change, we know much less about the ways that urban nest temperatures influence development (Tiatragul et al., 2017; Kent et al., 2019). Studying climate change is important, but urbanization is an immediate, global threat to biodiversity. Moreover, should we halt or reverse warming trends due to climate change, the threat of urbanization will remain. Thus, it is essential to understand the impact of the urban heat island effect on wildlife. Therefore, the major goals of this dissertation are to 1) assess the effects of acute thermal stress on embryo development using ecologically-relevant incubation treatments and 2) consider embryo responses to acute thermal stress in urban habitats. More specific goals and their justifications are presented in the introduction to each chapter.

Study species

Anoles are a radiation of tropical lizards composed of nearly 400 species. They inhabit the Caribbean islands as well as the mainland of North, Central, and South America. Most species are arboreal and use toepads with lamellae to cling to smooth surfaces (convergent with geckos). Unlike nearly all other lizards, females lay a single egg clutch once every 4-14 days. Many species (including those used in this dissertation) deposit eggs in shallow nests in the ground (< 5 cm) or on the soil surface beneath cover objects (e.g. leaf litter, rocks; Rand, 1967;

Tiatragul et al., 2019; Pruett et al., 2020; Dees et al., 2020). Therefore, nest temperatures fluctuate widely across the day, but temperature does not influence sex (i.e. chromosomal sex determination). Two species have emerged as models for studying thermal developmental plasticity and urbanization: the Puerto Rican crested anole (*Anolis cristatellus*) and the Cuban brown anole (*Anolis sagrei*) (Figure 1). These species are native to Puerto Rico and Cuba, respectively, but both are established in Florida, USA, where they often co-occur across the landscape, particularly in urban and suburban areas (Tiatragul et al., 2019; Battles and

Figure 1. Study species used in this dissertation. Male (A) and female (B; Photo Credit: CJ Thawley) Puerto Rican Crested Anoles (*Anolis cristatellus*). Female (C) and male (D) Cuban brown anoles (*Anolis sagrei*). Panel (E) shows a brown anole hatching from an egg after lab incubation.

Kolbe, 2019). Regardless, they prefer somewhat different habitats: brown anoles prefer warmer, open canopy environments while crested anoles prefer cooler, dense canopy areas (Figure 2). These preferences are typically maintained in the urban matrix of South Florida (Battles and Kolbe, 2019). Figure 3 shows an example nest site of the brown anole in Florida.

Figure 2. Example habitats of (A) Puerto Rican crested anoles (*A. cristatellus*) and (B) Cuban brown anoles (*A. sagrei*) in Florida. Both species thrive in urbanized habitats (as in C). Panel (D) shows a pair of crested anoles mating in an urban area.

Each species has many qualities that make them great models for both field and laboratory research. They are highly abundant, relatively easy to capture, hardy in captivity, and highly fecund. These qualities allow for robust sample sizes for field and laboratory studies.

They are relatively small lizards (females, 2-4 grams; males 4-10 grams), which makes housing large numbers of breeding pairs in the laboratory feasible. Moreover, they lay relatively small eggs (0.15 – 0.25 grams; Figure 1E), which is preferable for studies of development since large numbers of eggs and hatchlings can be maintained in a small space. Additional information about the benefits of these species in research is provided in each chapter.

Figure 3. Typical nest site for the brown anole (*Anolis sagrei*) in Florida. Panel A shows a cluster of rocks on a small spoil island in the Intra-coastal Waterway. One rock (outlined in white) has an egg beneath it. Panel B shows the focal rock removed, revealing a brown anole egg on the soil surface (enclosed by a white circle).

CHAPTER 1

Heat tolerance of reptile embryos: current knowledge, methodological considerations, and future directions

INTRODUCTION

Warming temperatures due to global change threaten biodiversity across the planet. Eggs of non-avian, oviparous reptiles (henceforth "reptiles") are particularly vulnerable to heat stress due to a lack of parental care during incubation and the inability to behaviorally thermoregulate (Telemeco et al., 2009). Consequently, the biotic impacts of global change have motivated a surge in research devoted to understanding the effects of warming nest temperatures on reptile development. Two aspects of global change have been center stage: global climate change (Levy et al., 2015; Carlo et al., 2018) and habitat alteration (Kolbe and Janzen, 2002; Tiatragul et al., 2017). Both can increase nest temperatures in detrimental ways (Chapter 3; Dayananda and Webb, 2017; Hall and Warner, 2018). Historically, reptiles have served as a primary model in studies of thermal developmental plasticity (Warner et al., 2018; While et al. 2018), which has resulted in a large body of literature (reviewed by Pezaro et al., 2017; Noble et al., 2018a; While et al., 2018; Warner et al., 2018; Refsnider et al., 2019; González et al., 2019) upon which researchers can draw to predict species responses to rising temperatures; however, there are currently no standard assays for measuring heat stress of reptile embryos (unlike post-hatching stages; Angilletta et al., 2013). Such methods are critical to understand the evolution and ecology of embryo heat tolerance and predict responses to global change. Given the threat of rising temperatures, the available data, and this recent surge in interest, now is an ideal time to discuss what is known about the upper thermal limits of reptile embryos, define basic terminology that

will enable efficient communication among researchers, and consider the pros and cons of various protocols for measuring embryo heat tolerance.

Although standard assays exist for determining heat tolerance of post-hatching stages (reviewed by Taylor et al., 2020), these may not be applicable to embryos for biological and methodological reasons. For example, a common measure of heat tolerance is the critical thermal maximum (CT_{max}; see Table 1 for terms and abbreviations), which is the upper temperature at which an individual loses motor control and is measured by heating individuals until they are immobile (Huey and Kingsolver, 1989). Since eggs do not move, the failure of the cardiovascular system has been used to determine CT_{max} (Angilletta et al., 2013). These endpoints, however, are not comparable because one results in a breakdown of performance and the other results in death. Additionally, adults and juveniles often navigate a thermally heterogenous landscape and are able, even in extreme or novel thermal environments, to maintain preferred body temperatures via behavior (Battles and Kolbe, 2019). Indeed, behavioral thermoregulation is likely the primary way that ectotherms will maintain a thermal safety margin in the face rising temperatures (Sunday et al., 2014). Embryos of oviparous reptiles, however, are generally left to develop in prevailing conditions with limited opportunities to thermoregulate (but see Li et al., 2014). Therefore, they are subjected to large changes in mean and variance of body temperature and both chronic and acute thermal stress must be considered to describe heat tolerance. Thus, embryo thermal ecology requires a set of definitions, methods, and interpretations that differ from post-hatching stages.

In this review, I consider several questions. How should we measure and express the upper thermal limits of reptile embryos? How do these upper limits differ across species? Furthermore, how should we interpret measurements of upper thermal limits with respect to

ecology? Finally, how can such data be used to make predictions about the future? First, I compare existing methods for measuring the upper thermal limits of reptile embryos and propose metrics that can be used to categorize existing studies and make comparisons across species. Second, I use data for the brown anole lizard (*Anolis sagrei*) as a case study to demonstrate the importance and ecological relevance of various measures of embryo heat tolerance. Third, I present data from the literature to summarize what is currently known about the upper thermal limits of reptile embryos. Finally, I outline directions for future research, calling for 1) more complete thermal reaction norms in studies of developmental plasticity, 2) more studies of embryo responses to acute thermal stress, and 3) studies that identify mechanisms that determine acute heat tolerance. Chapters 2, 3, and 4 of this dissertation make progress toward goals 2 and 3.

MEASURING HEAT TOLERANCE OF REPTILE EMBRYOS

Chronic vs acute heat stress

Andrews and Schwarzkopf (2012) were first to broadly assess thermal physiology of reptile embryos with metrics that could be compared across a wide array of species. For 40 squamate species, they calculated a slope of developmental rate (i.e. 1/incubation period) across constant temperatures within the optimal temperature range (OTR). The OTR is the range of constant temperatures across which hatching success is relatively high. With this regression (see Figure 1B in Andrews and Schwarzkopf, 2012), they estimated a developmental rate index (DRI) as the slope of developmental rate vs temperature, the lowest temperature for development (i.e. T_0) as the x-intercept, and the optimal temperature for development (T_{opt}) as the highest

Table 1. List of terms and abbreviations.

Abbreviation	Term	Definition
CT_{max}	Critical thermal maximum	Upper body temperature causing loss of motor function (Huey and Kingsolver, 1989)
DRI	Developmental rate index	Slope from regressing developmental rate (1/incubation period) on temperature using values within the OTR. High values are associated with high absolute developmental rates (Andrews and Schwarzkopf, 2012)
EAHT	Embryo acute heat tolerance	Mean acute temperature resulting in embryo mortality. Can be determined using methods in Table 2.
ECHT	Embryo chronic heat tolerance	Constant incubation temperature that reduces hatching success within the OTR by 50%. Can be determined using a dose-response model (e.g. log logistic model).
OTR	Optimal temperature range	Range of constant incubation temperatures resulting in high hatching success (Andrews and Schwarzkopf, 2012)
T_0	Minimum developmental temperature	Lowest temperature that supports development. Estimated as the x intercept in the regression to calculate DRI (Andrews and Schwarzkopf, 2012)
T_{opt}	Optimal temperature for development	The warmest temperature within the OTR. Assumed optimal because it maximizes developmental rate without reducing hatching success (Andrews and Schwarzkopf, 2012)

temperature within the OTR. One problem with their method is that it cannot estimate the upper thermal limits of development. Although T_0, T_{opt}, and DRI are important traits to understand how historical factors (e.g. phylogeny, historical climate) have shaped embryo thermal ecology, the upper thermal limit is necessary to understand how species will respond to warming temperatures in the future. For example, to calculate a thermal safety margin, one needs to know the typical operative temperatures (i.e. pervasive nest temperatures) and a temperature that induces thermal stress (e.g. CT_{max}) (Sunday et al., 2014). Given the limits on embryo

thermoregulatory behavior, multiple parameters may be required to describe their thermal tolerance. For example, when nest temperatures fluctuate widely throughout the day, embryos may become damaged or die due to a single, brief exposure to a high temperature (Chapter 4). Conversely, chronic exposure to sublethal temperatures may also result in damage and death (Carlo et al., 2018). Thus, we need standardized methods and terminology to assess the upper thermal limits of reptile embryos and these should reflect vulnerability to both chronic and acute heat stress.

Most estimates of embryo heat tolerance have been in response to constant temperatures, even though nest temperatures typically fluctuate in the wild (Booth, 2018). However, Angilletta et al. (2013) were first to measure CT_{max} of embryos by heating eggs of *Sceloporus undulatus* at a constant rate until embryos underwent cardiac arrest. This measure of heat tolerance (~ 46.5 °C) was much greater than the warmest constant temperature that results in viable hatchlings (~ 35 °C; Angilletta et al., 2000). Since publication of this groundbreaking study, several researchers have measured CT_{max} of reptile embryos (e.g. Gao et al., 2014; Smith et al., 2015). However, I propose that when researchers measure CT_{max} using acute exposures to temperature, they are measuring a different phenotype than assessing heat tolerance with constant temperatures. Although each assay assesses egg survival, the mechanisms resulting in death may differ: acute heat tolerance may result from cardiac arrest or oxygen limitation (Chapter 2; Angilletta et al., 2013; Smith et al., 2015; Hall and Warner, 2020a), while chronic heat stress results in morphological abnormalities (Sanger et al., 2018). I propose that researchers should use the term *embryo chronic heat tolerance* (ECHT) when referring to constant temperatures that induce high mortality. Thus, the ECHT is the constant temperature at which typical rates of hatching success (i.e rates within the OTR) are reduced by 50% (i.e. lethal temperature; LT_{50}).

Moreover, I suggest the term CT_{max} invites confusion given the differences between embryos and post-hatching stages discussed above. I recommend the term *embryo acute heat tolerance* (EAHT) when measuring responses to acute temperatures (as in Angilletta et al., 2013). The EAHT is the mean acute temperature that causes embryo mortality.

The EAHT should be considered a measure of the total amount of heat stress that an embryo can withstand at a given moment, while the ECHT represents thermal damage that is accumulated across time. These two measures potentially have different uses and importance depending on ecological context. For example, some species nest relatively deep in the ground and temperatures are mostly constant through development (e.g. *Chelonia*, Booth and Astill, 2001; *Varanus*, Doody et al., 2015; *Chameleo*, Andrews, 2018). For such species, we might expect EAHT to be relatively low. Moreover, when assessing potential thermal stress, EAHT may have little relevance compared to ECHT. For other groups (e.g. *Sceloporus*, Angilletta et al., 2013; *Emydura*, Booth, 2018; *Anolis*, Chapter 2), nest temperatures fluctuate widely, and EAHT may be relatively high and serve as the more important phenotype when considering thermal stress. I propose that researchers begin using the terms EAHT and ECHT. This will 1) encourage researchers to consider which phenotype is most appropriate for their system and research questions, 2) facilitate comparisons across the literature, and 3) enable a more accurate use of thermal tolerance phenotypes when generating predictive models.

Measuring embryo chronic heat tolerance (ECHT)

Measuring ECHT is logistically simple. Eggs should be incubated at various constant temperatures under normoxic conditions (Figure 1a). The water potential of the incubation

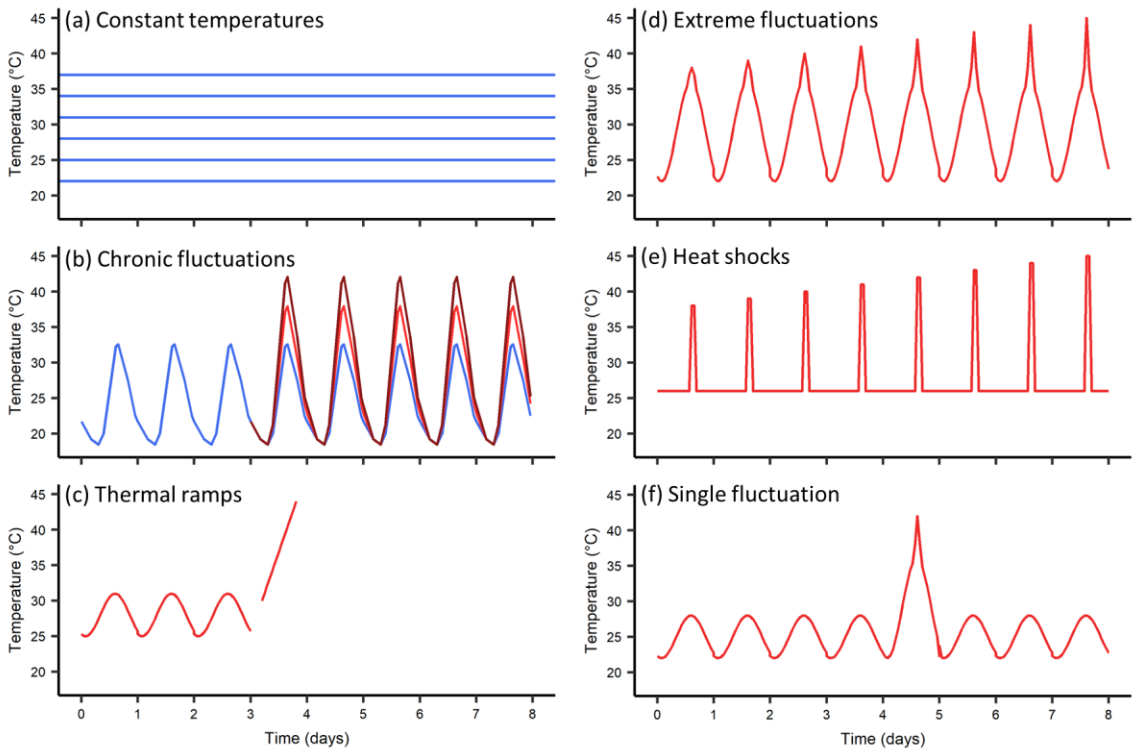

Figure 1. Methods for measuring heat tolerance of reptile embryos. Blue and red colors denote methods for measuring embryo chronic heat tolerance (ECHT) and acute heat tolerance (EAHT), respectively. The constant temperature approach (a) is used for measuring ECHT, and the other approaches (b-f) can be used to measure EAHT. The chronic fluctuations method (b) combines exposure of both chronic and acute thermal stress (see text). See text and Table 2 for explanations of each method.

medium should support high hatching success. Ideally, at least 5 temperatures should be used with the two warmest temperatures reducing survival within the OTR by at least 50%. Each egg should be categorized as 1 (hatched) or 0 (did not hatch) and a dose-response model can be used to estimate ECHT. Figure 2 demonstrates a hypothetical example, where 100 eggs were divided among 5 temperatures. Multiple dose-response curves were fit to the data using the 'drc' package in R (Ritz et al., 2015) and the best model was used to estimate ECHT. A bootstrap was then applied to generate a 95% confidence interval for ECHT. Although many studies report hatching

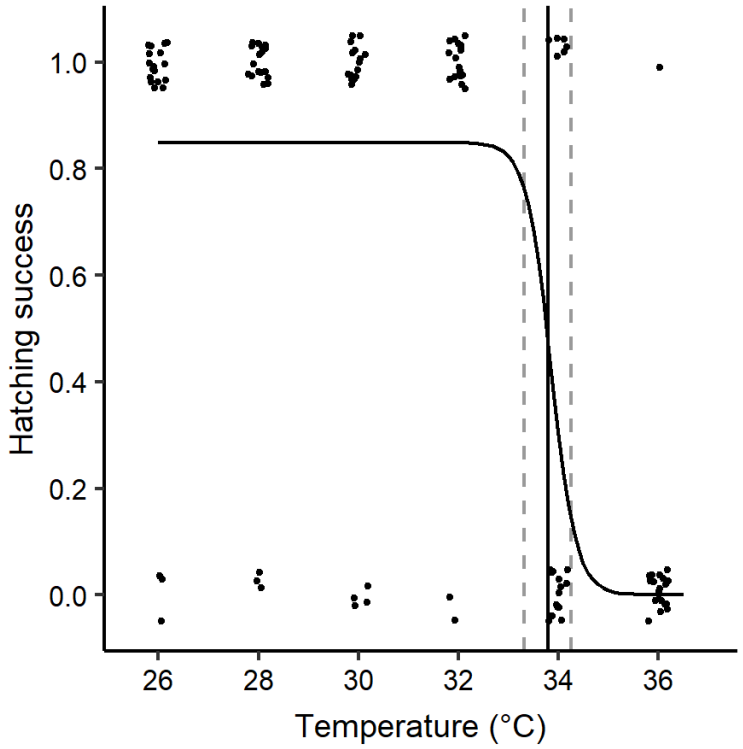

Figure 2. Dose-response model applied to hypothetical egg survival data. In this example, 100 eggs were incubated at 5 constant temperatures (n=20 per temperature) and hatching success was recorded for each egg (1=hatched, 0= did not hatch). Closed circles denote the raw data (jittered around 0 and 1 to reduce overplotting). Several dose-response curves were applied to the data and assessed via AIC using the 'drc' package in R (best model was a log-logistic model: "fct = LL.3()"). The solid black vertical line denotes the embryo chronic heat tolerance (ECHT; 33.8 °C) and vertical dashed lines denote the 95% CI obtained by applying a bootstrap with 1000 replicates.

success (often as a percentage) across a wide-range of constant temperatures, a standard metric (i.e. ECHT) is required to make comparisons across studies and species.

I recommend that hatching success be the proper measure for determining ECHT. Studies that dissect eggs prior to hatching may overestimate the ECHT because late-stage embryos may be particularly vulnerable to heat stress due to their relatively high oxygen demand (Chapter 4; Kobayashi et al., 2017; Hall and Warner, 2019). Moreover, death at high constant temperatures

is likely due to factors that compound across development (Sanger et al., 2018). The advantage of measuring ECHT is that it is logistically simple, only requiring several relatively inexpensive constant temperature incubators. Moreover, results are easily compared across studies since the methods are simple to repeat and there is a wealth of literature describing embryo responses to constant temperatures (While et al., 2018; Noble et al., 2018a). However, because many, perhaps most, eggs do not incubate at constant temperatures in the wild and because responses to constant temperatures differ from responses to fluctuating temperatures (reviewed by Booth, 2018); the ecological relevance of the ECHT is questionable. This is the primary limitation to measuring this phenotype.

Measuring embryo acute heat tolerance (EAHT)

Measuring EAHT is more challenging compared to ECHT. Most methods require the use of programmable incubators which may be prohibitively costly (but see Greenspan et al., 2016). To my knowledge, there are five ways that researchers have measured the EAHT (Figure 1b-f), and each method has strengths and limitations. Importantly, unlike ECHT, EAHT is measured at a single point during development. Because embryos change dramatically across development with respect to size and physiology, EAHT likely varies with ontogeny (see Chapter 4), and it is important to control for and/or report the embryo stage at which EAHT is measured.

Levy et al. (2015) were first to incorporate acute measures of heat tolerance into species distribution models. They incubated eggs of the eastern fence lizard (*S. undulatus*) at fluctuating temperatures that were suitable for successful development (i.e. blue line, days 1-3; Figure 1b) and then allocated eggs to incubation treatments that varied in the peak temperature of the daily

thermal cycle (i.e. red lines, days 4-8; Figure 1b). Some eggs remained at the standard fluctuation to serve as controls (i.e. blue line, days 4-8; Figure 1b). At the end of each day, eggs were placed in a heart rate monitor (Buddy, Avitronics Inc) to determine survival. Eggs remained in these treatments until hatching. The primary advantage of this method is that it is highly ecologically relevant, since thermal stress is experienced as it would be in natural nests, which exhibit wide daily thermal fluctuations. There are, however, a few disadvantages. First, this method requires as many programmable incubators as treatments. Second, it confounds the effects of chronic and acute thermal stress since treatments differ in both maximum and mean temperature. Therefore, whether this method produces an estimate of ECHT or EAHT is not clear. Finally, Levy et al. (2015) subjected eggs to chronic fluctuations at a relatively late developmental stage (from 70-95% development completed). Because heat tolerance can change with ontogeny, results may vary depending on the timing of treatment allocation with respect to development.

The thermal ramp (Figure 1c) is the most used method to estimate EAHT and was first used by Angilletta et al. (2013). Eggs incubate at temperatures suitable for development (e.g. repeated sine wave shown on days 1-3, Figure 1c) and then, on a particular day, each egg is placed in a heart rate monitor (Buddy, Avitronics Inc) and heated at a fixed rate until cardiac arrest (shown on day 3 of Figure 1c). A thermocouple probe can be attached to the egg or, for large eggs, can be inserted inside the egg, to monitor egg temperature during the assay. The egg temperature at which the heart stops beating is the EAHT (e.g. Angilletta et al., 2013; Gao et al., 2014). One major benefit of this method is its high degree of ecological relevance: eggs can be heated at a rate similar to diurnal temperature increase of natural nests (e.g. 3 °C per hour; Angilletta et al., 2013). Conversely, this method is logistically challenging to perform since

researchers must have specific equipment (e.g. heart rate monitor, programmable incubators), and, unless a lab is equipped with multiple heart rate monitors and programmable incubators (and multiple personnel to run the assays), only one egg can be measured at a time. Moreover, controlling the warming rate of eggs makes the assay strictly time sensitive. Thus, this method is complicated and may not be feasible for some study questions, particularly those that require measuring EAHT on many individuals of multiple species or populations (e.g. Chapter 4).

An alternative method was first used by Smith et al. (2015). Eggs incubate at temperatures suitable for development. On a chosen day during development, eggs are subjected to thermal fluctuations that increase in peak temperature each day (Figure 1d). At the end of each day, eggs are placed in the heart rate monitor to assess survival. The peak temperature that kills the embryo is the EAHT. This method has high ecological relevance – the assay exposes eggs to daily nest fluctuations as in the wild. Moreover, all eggs are treated simultaneously, which is logistically favorable. However, there are three major drawbacks. First, it requires specific equipment (at least one programmable incubator and a heart rate monitor). Second, previous exposure to extreme, non-lethal temperatures can reduce measures of EAHT (see Chapter 4); thus, damage may accumulate during the first days of the experiment, resulting in lower estimates of EAHT than would be measured by other methods (e.g. thermal ramps). Finally, EAHT likely changes with ontogeny (Chapter 4), and the peak temperature is confounded with embryo age.

A modified version of the Smith et al (2015) method can be used if programmable incubators are unavailable. Heat shocks of ½ or 1 hour can be applied. This involves incubating eggs at a constant temperature within the OTR and then placing eggs in an incubator set to an extreme temperature for a short time, removing the eggs and then assessing survival using a heart

rate monitor. Survivors can then be heat shocked at a higher temperature the next day (Figure 1e). The lethal temperature is recorded as the EAHT. Logistically, this is the simplest of all methods to measure EAHT since only constant temperature incubators are required. Moreover, all eggs can be tested simultaneously. However, this assay lacks ecological relevance since eggs would not experience such rapid changes in temperature in the wild. Additionally, previous exposure to extreme temperatures may influence the final measure of EAHT and temperature is confounded with embryo age (as in Smith et al., 2015).

A modification of the Smith et al. (2015) protocol has been used by Hall and Warner (Chapter 4). In this assay, eggs are randomly allocated to be exposed to a single extreme temperature fluctuation with a pre-determined peak temperature (Figure 1f). These peak temperatures range from below to above an estimated EAHT (based on preliminary data). After exposure, each egg incubates until hatching. A logistic binomial regression (1= hatch, 0= did not hatch) is used to estimate EAHT. If programmable incubators are unavailable, this method could be modified to use heat shocks rather than extreme fluctuations. The benefits of this assay are that it is ecologically relevant and relatively simple to perform (e.g. does not require measuring heart rates). Moreover, it eliminates the potential for previous exposures to extreme temperatures to influence the EAHT, and peak temperature and embryo age are not confounded. However, the disadvantages are that it requires large sample sizes and an estimate of EAHT is not made for each egg. This limits interpretations, particularly about how EAHT might vary across individuals.

Finally, reproducibility is vital. Constant temperatures, thermal ramps, and heat shocks are easy to reproduce, allowing for comparisons across studies and species. Fluctuating treatments (Figure 1b, d, f) are difficult to reproduce because the breadth of the thermal

fluctuation will influence embryo survival in addition to the peak temperature. Therefore, temperatures across the entire thermal fluctuation, not just the peak temperature, are required to reproduce results. Due to the strengths and limitations of each method to measure EAHT (summarized in Table 2), I do not recommend a preferred method but encourage researchers to consider their model species, available equipment, and study question when selecting a method. Moreover, researchers should consider these limitations and confounding variables when comparing estimates of EAHT across studies. Finally, assessing multiple methods simultaneously may be useful to select a preferred method for a given study system (Chapter 2).

A case study of embryo heat tolerance: the brown anole (*Anolis sagrei*)

The brown anole (*Anolis sagrei*) is becoming an important model for developmental ecophysiology because it is hardy in captivity, has relatively high fecundity, protocols are established for egg and embryo collection, and its developmental staging series is described (Sanger et al., 2008a,b; Hall et al., 2018; Hall et al., 2020). Females construct shallow nests (< 5 cm depth) across a diversity of habitats; thus, in the wild, embryos experience relatively large thermal variation during development (Sanger et al., 2018; Gunderson et al., 2020). Daily fluctuations in nest temperature often reach stressfully warm temperatures (i.e. > 40 °C; Sanger et al., 2018), indicating that embryos have physiological mechanisms for ameliorating the adverse effects of acute heat stress. Past studies demonstrate that temperature has important effects on embryo development, egg survival, and hatchling phenotypes (Pearson and Warner, 2018), and to my knowledge, *A. sagrei* is the only species for which EAHT has been measured using 3 of the methods described above and data exist to estimate ECHT. Thus, this species is an excellent

Table 2. Comparison of methods used to measure reptile embryo heat tolerance.

Method; Figure	Description	Data recorded	Estimates	Example studies	Advantages	Disadvantages
Constant temperatures; Figure 1a	Eggs are incubated at multiple constant temperatures in a split-clutch design. Warmest temperature should induce at least 50 % mortality compared to OTR.	Hatching success for each egg expressed as a binary (0,1).	DRI ECHT OTR T_{opt} T_0	Mueller et al., 2019 Telemeco, 2015 Ligon and Lovern, 2009	Logistically simple. Need only constant temperature incubators. Assesses embryo responses to chronic thermal stress. Easy to reproduce.	Constant temperatures are not ecologically relevant for many species. Does not consider embryo responses to acute thermal stress. Does not provide estimate of heat tolerance for each egg.
Chronic fluctuations; Figure 1b	Eggs are first incubated at fluctuating temperatures suitable for development. Later, eggs are allocated to one of multiple repeated fluctuations which differ in peak temperature.	Hatching success for each egg expressed as a binary (0,1).	??? (see text)	Levy et al., 2015	High degree of ecological relevance. Exposes embryos to increasing chronic and acute temperatures as would be experienced due to global change.	Requires many programmable incubators. Confounds the mean and peak temperature of thermal treatments. Does not provide estimate of heat tolerance for each egg.

Thermal ramp; Figure 1c	Eggs are placed (individually) in a heart rate monitor, temperature is increased at a steady, ecologically relevant rate, heart rate is monitored across temperature until cardiac arrest.	Temperature at which heart rate is zero (i.e. cardiac arrest) for each egg.	EAHT	Angilletta et al., 2013 Gao et al., 2014 Sun et al., in revision	Ecologically relevant measure of EAHT. Allows for estimation of thermal sensitivity of heart rate. Reproducible.	Logistically difficult since eggs must be measured one at a time. Requires specific equipment (water baths, programable incubator, heart rate monitor). May overestimate EAHT (see text)
Extreme fluctuations; Figure 1d	Eggs are exposed to extreme thermal fluctuations that increase in peak temperature each day. Survival is assessed via heart rate at the end of each day.	Peak temperature that causes death for each egg.	EAHT	Smith et al., 2015	High level of ecological relevance for species that exhibit wide fluctuations in nest temperature. All eggs can be treated simultaneously.	Potentially underestimates EAHT due to compounding effects of sublethal exposures to thermal stress. Confounds embryo age with peak temperature. Difficult to reproduce.
Heat shocks; Figure 1e	Eggs are exposed to 1-hour heat shocks that increase in temperature each day. Survival is assessed via heart rate each day.	Heat shock temperature resulting in death for each egg.	EAHT	Chapter 2	Logistically simplest way to measure EAHT. Does not require programmable incubators. Easy to reproduce.	Low ecological relevance. Similar disadvantages as described for extreme fluctuations method.

Single fluctuation; Figure 1f	Eggs are exposed to a single extreme fluctuation with a randomly selected peak temperature. Eggs then incubate until hatching.	Hatching success for each egg expressed as a binary (0,1).	EAHT	Chapter 4	Ecologically relevant measure of EAHT. Decouples embryo age and peak temperature. Avoids effects of previous, sublethal exposures.	Requires multiple programmable incubators and large sample sizes. Does not provide estimate of EAHT for each egg. Difficult to reproduce.

model to consider the importance of ECHT and EAHT with respect to egg survival and physiology

I used estimates of EAHT from Chapters 2 and 4 (below) and used unpublished data (Pruett and Warner) of hatching success at 8 constant temperatures (21, 23, 25, 27, 29, 31, 33, 35 °C) to estimate T_{opt} (as in Andrews and Schwarzkopf, 2012) and ECHT. To estimate ECHT, I analyzed mean survival at each temperature with multiple dose-response models (2-, 3-, 4-, and 5-parameter log-logistic models, Weibull I and II, log-normal, gaussian, quadratic) using the drc package in R (Ritz et al., 2015). A 3-parameter logistic regression (i.e. lower bound fixed at zero with a symmetrical inflection) was the best model according to AIC. I used mean survival at each temperature (rather than raw data as in Figure 2) to illustrate how ECHT can be calculated when raw data are not available (as in my literature review below). Estimates were made according to this equation:

$$y = \frac{a}{\left(1 + \exp\left(b(\log(x) - \log(c))\right)\right)}$$

Where *a* is hatching success in the OTR (i.e. upper asymptote), *b* describes the steepness of the curve (i.e. Hill's slope), *c* is the ECHT (i.e. effective dose 50), *x* is temperature, and *y* is hatching success expressed as a ratio from 0 to 1. Finally, I used temperatures from a relatively warm nest in an urbanized area (taken from Tiatragul et al., 2020) to calculate acute and chronic thermal safety margins by subtracting the mean daily maximum nest temperature from the lowest estimate of EAHT and subtracting the mean nest temperature from the ECHT, respectively.

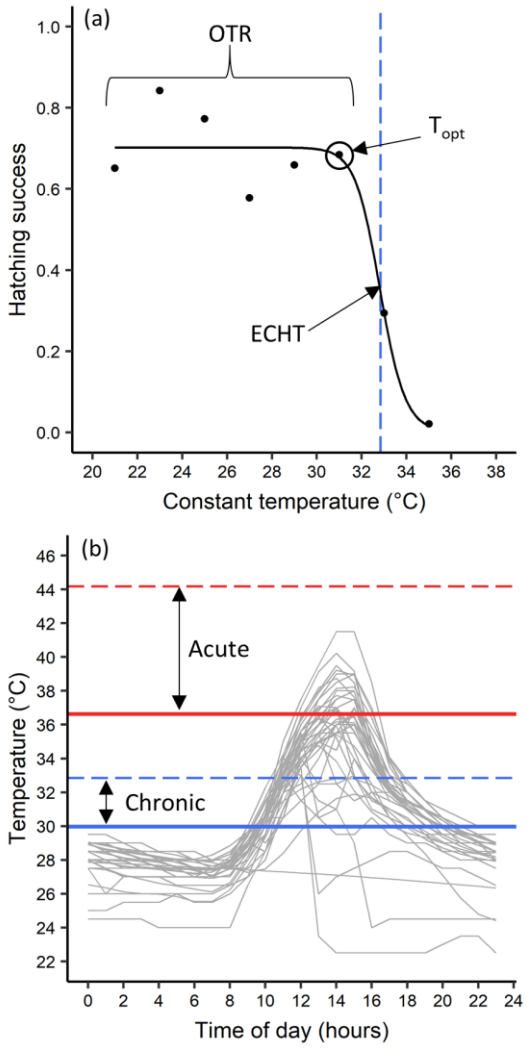

Figure 3. *Anolis sagrei* (a) egg survival across constant temperatures and (b) nest temperatures from an urbanized habitat. In panel (a), closed circles are the raw data, the black line is the model fit, and the vertical blue dashed line denotes the ECHT. In panel (b) the dashed blue and red lines show the ECHT and EAHT, respectively. Gray lines show daily temperatures collected from a single nest in Pinecrest, FL (see Tiatragul et al., 2020). The solid blue and red lines show the mean nest temperature and the mean daily maximum temperature, respectively. Arrows denote the acute and chronic thermal safety margins.

Figure 3a demonstrates the log-logistic curve and the parameters of interest. Here, T_{opt} and ECHT are 31 and 32.8 °C, respectively. The EAHT of *A. sagrei* is 44.17 (43.92–44.43°C, 95% CI), 45.3 (45.15 - 45.45; 95% CI), and 46.15 (45.87 - 46.44; 95% CI) °C for extreme

fluctuations, heat shocks, and thermal ramps, respectively. Thus, extreme fluctuations provided the lowest estimate of EAHT, thermal ramps provided the highest estimate, and heat shocks resulted in an intermediate estimate (95% confidence intervals do not overlap). Finally, I observed safety margins of 7.7 and 2.9 °C for EAHT and ECHT, respectively (Figure 3b).

There are biological and methodological explanations for the observed differences in EAHT. First, when exposed to extreme fluctuations (e.g. Figure 1d), additional thermal damage may occur after the embryo has reached the peak temperature and is cooling down. Heat shocks and thermal ramps do not require embryos to "come down" from the test temperature; thus, estimates of EAHT taken from thermal fluctuations may be lower than heat shocks or thermal ramps. Second, thermal fluctuations and heat shocks may generate lower estimates of EAHT compared with thermal ramps because these methods use different metrics to assess survival. Thermal fluctuations and heat shocks assess embryo survival, *per se*, while thermal ramps assess cardiac function (i.e. cardiac arrest). There is some evidence that acute exposure to sublethal temperatures damages the cardiovascular system (Chapter 2); thus, embryos may suffer potentially lethal damage at temperatures below the point of cardiac arrest, resulting in higher estimates of EAHT from thermal ramps compared to other methods. Finally, measures of EAHT were assessed on two different populations and in different years, and we do not know how EAHT changes across space and time for *A. sagrei*.

These data demonstrate the potential ecological importance of various thermal parameters. For example, *A. sagrei* is considered a highly successful urban colonizer (Stroud et al., 2019; Hulbert et al., 2020), and Tiatragul et al., (2017) propose this is because relatively warm urban nest temperatures enhance embryo development. In the example nest (Figure 3b), the mean temperature (29.9 °C) is close to T_{opt}, but below ECHT, and peak nest temperatures do

not reach EAHT. In general, *A. sagrei* occupy relatively warm, open canopy environments (Battles and Kolbe, 2019), and their embryo thermal physiology may have evolved to maximize fitness in relatively warm nests that exhibit wide thermal fluctuations. Urban environments replicate such conditions, and embryo thermal physiology may explain, in part, their success in urban habitats (see Chapter 4). Species like *A. sagrei* that have adapted to warm, variable nest temperatures may exhibit wide divergence in EAHT and ECHT. However, species that nest in relatively cool, thermostable microenvironments may exhibit little difference between ECHT and EAHT. This must be considered when modeling responses to climate change because nest temperatures will likely increase in both mean and variance in the future.

A QUANTITATIVE REVIEW OF REPTILE EMBRYO HEAT TOLERANCE

To summarize what is known about reptile heat tolerance and identify existing knowledge gaps, I conducted a quantitative review. This review sets the stage for this dissertation by demonstrating that few researchers have assessed embryo responses to acute thermal stress. Therefore, our knowledge of mechanisms that dictate such responses and the ecological effects of those responses are poorly understood.

Literature review

I combined data from the Reptile Development Database (www.repdevo.com; Noble et al., 2018b) with literature collected from my own Web of Science search to estimate ECHT for as many species as possible. I used the same search terms reported in Noble et al (2018a) to add

literature published since the last update of the Reptile Development Database. I selected species for which hatching success is reported for at least 4 different constant temperatures. Of these, I excluded species that did not have at least one warm temperature that reduced hatching success by 50% of hatching success in the OTR. For each species remaining, I searched using "incubat" and the species name to try and find additional studies reporting hatching success at extremely warm temperatures. I excluded some studies that exhibited unusually low hatching success within the OTR (compared to other studies of the same species). Because crocodilians were underrepresented in my final dataset, I used literature reviewed by González et al. (2019) to add additional studies. For two species (*Alligator mississippiensis* and *Caiman crocodilus*), hatching success was not provided for each temperature within the OTR but was described generally (e.g. hatching success was "greater than 90%" at all temperatures). I assigned each temperature a hatching success above this threshold (e.g. 0.91) because I wanted to include as many crocodilian species as possible. My final dataset included 16 squamates, 16 turtles, 5 crocodilians, and the tuatara (Table S1).

To my knowledge, only the studies listed in Table 2 have measured EAHT of reptiles (n = 5 squamates and 1 turtle). To increase sample size, I added unpublished data (n= 4 squamates; assessed via thermal ramp) provided by B Sun (Table S2).

Analyses

For each species, I estimated T_{opt} as described previously. Because I did not have the raw data for each species, I analyzed mean survival at each constant temperature (as in Figure 3a). Moreover, sample sizes were necessarily low (i.e. usually 1 survival estimate per temperature),

and studies differ with respect to the intervals between incubation temperatures (e.g. every 2 vs every 3 °C). For these reasons, had I applied a model selection process for each species, among-species differences in final models would probably represent methodological rather than biological variation. Therefore, I applied a 3-parameter log-logistic model to each species to estimate ECHT (as above). This function is biologically appropriate because it aligns with theory concerning ectotherm embryo survival (i.e. high survival in the OTR and then a sharp decline; van der Have, 2002). Moreover, it is often used to describe sex ratios in studies of TSD (e.g. Carter et al., 2018); therefore, ecologists interested in reptile developmental plasticity are familiar with its application. I subset T_{opt} and ECHT by reptile order and performed a bootstrap to obtain 95% confidence intervals from 10,000 replicates. For EAHT, I combined estimates across orders (due to low sample sizes for most orders) and performed the bootstrap. I assessed the overlap of confidence intervals to consider statistically significant differences among groups.

Results

T_{opt} was greatest for crocodilians and lowest for squamates. Confidence intervals did not overlap for crocodilians and squamates, but other pairwise comparisons exhibit overlap of confidence intervals (Table 3). ECHT was greatest for crocodilians and lowest for squamates; however, due to overlap between the confidence intervals, I doubt these estimates are meaningfully different (Table 3; Figure 4). The highest estimate of ECHT is 40.2 °C (desert iguana, *Dipsosaurus dorsalis*), and the lowest was 24.6 °C (Tuatara, *Sphenodon punctatus*). The highest EAHT is 47 °C (Chinese softshell turtle; *Pelodiscus sinensis*) and the lowest is 35.8 °C (Southern grass lizard; *Takydromus sexlineatus*). Overall, the estimate of the EAHT is much

higher than the ECHT of crocodilians, turtles, and squamates (~ 9°C), indicating that reptiles can withstand brief exposures to temperatures much greater than ECHT (Figure 4).

Table 3. Estimates of optimal developmental temperature (T_{opt}), embryo chronic heat tolerance (ECHT), and embryo acute heat tolerance (EAHT) for major reptile clades. Data are combined for EAHT due to low sample size of most orders.

	n	Estimate (°C)	Lower 95% CI	Upper 95% CI	Range (°C)
Squamata T_{opt}	16	30.5	29.3	31.7	28.0 – 38.0
Sphenodontia T_{opt}	1	24.0	-	-	-
Testudines T_{opt}	16	31.7	30.9	32.4	29.0 – 34.0
Crocodilia T_{opt}	5	32.9	32.0	33.8	31.0 – 33.5
Squamata ECHT	16	32.7	31.4	33.9	29.2 – 40.2
Sphenodontia ECHT	1	24.6	-	-	-
Testudines ECHT	16	33.1	32.3	33.9	30.6 – 36.3
Crocodilia ECHT	5	33.9	33.5	34.3	33.2 – 34.4
EAHT	10	42.0	39.65	44.37	35.8 – 47.0

Discussion

The ECHT is similar among crocodilians, squamates, and turtles; however, my sample vastly underrepresents the diversity within reptiles. This is best exemplified by the extreme difference in ECHT between the desert iguana (*D. dorsalis*) and tuatara (*S. punctatus*) (~ 15 °C). Differences in ECHT could be due to lineage-specific thermal adaptation (Andrews and Schwarzkopf, 2012; Du et al., 2019). Indeed, nest temperatures of *D. dorsalis* often exceed 40 °C (Muth, 1980) while those of *S. punctatus* are between 16 and 20 °C in mean temperature

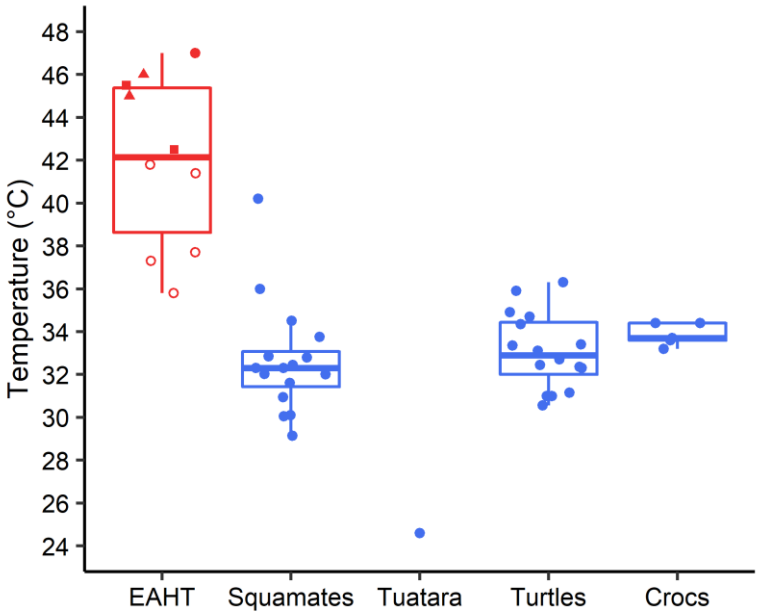

Figure 4. Estimates of ECHT (blue) of squamates (n=16), tuatara (n=1), turtles (n=16) and crocodilians (n=5) and EAHT (red). EAHT includes nine squamates (*Takydromus* (open circles), *Anolis* (squares), *Sceloporus* (triangles)) and one turtle (*Pelodiscus* (closed circle)).

(Thompson et al., 1996). These extreme examples of ECHT aside, there is still considerable variation in ECHT. Additional analyses, which are beyond the scope of this dissertation, are required to explain this variation. Importantly, there is even greater variation in EAHT (Figure 4). For example, just within the genus *Takydromus*, EAHT ranges across 6 °C, which is nearly equal to the total variation in ECHT of turtles and squamates (without considering *D. dorsalis* – an outlier). Finally, there is some overlap between EAHT and ECHT across reptiles as demonstrated by the relatively high ECHT of the desert iguana (*D. dorsalis*, 40.2 °C) and Bibron's agama (*Agama impalearis*, 36.0 °C) and the relatively low EAHT of some *Takydromus* lizards (35.8 - 37.7 °C). These anecdotes, collectively, indicate great potential for lineage-specific adaptation of both ECHT and EAHT to abiotic conditions (e.g. climate).

Both urbanization and climate change can potentially increase nest temperatures by 1-2 °C, even after accounting for maternal adjustments in nesting behavior (Telemeco et al., 2009; Tiatragul et al., 2020). T_{opt} for crocodilians, squamates, and turtles are only 1.0, 2.2, and 1.4 °C lower than ECHT, respectively. Therefore, if species are currently nesting at temperatures that optimize development, future warming will result in increased mortality in the absence of embryo adaptation or compensatory adjustments of nesting behavior (Telemeco et al., 2009; Carlo et al., 2018). We need more predictive models that consider embryo responses to global change (e.g. Levy et al. 2015; Carlo et al. 2018); however, these models will be hindered by only considering responses of embryos to chronic conditions (i.e. ECHT). Measuring EAHT can increase our understanding of embryo thermal physiology. For example, it is often assumed, based on constant temperature incubation, that embryos have a narrower thermal tolerance breadth than adults. Clusella-Trullas et al. (2010) estimated the mean CT_{max} of adult squamates to be ~42 °C, which is nearly 10 °C greater than squamate ECHT but essentially equal to EAHT. The EAHT is not perfectly compatible with CT_{max} because the former results in death and the latter results in loss of motor function; however, the large difference between EAHT and ECHT (~ 9 °C) should compel us to abandon comparisons between chronic incubation conditions of embryos and the CT_{max} of post-hatching stages and induce skepticism concerning the assumption that the thermal tolerance breadth of embryos is far less than that of later life stages (van der Have, 2002).

There are some caveats to this study. First, because studies are often limited with respect to sample size, most researchers incubate eggs within the OTR and at widely-spaced intervals of temperatures (e.g. 26, 30, 34 °C). Thus, sample sizes were small for estimating ECHT, and there are many "gaps" among treatments, which may reduce the accuracy of my estimates. Second,

although hatching success is relatively constant across a broad range of temperatures, many fitness-relevant traits have a thermal optimum (e.g. performance, body size); thus, considering only hatching success with respect to temperature may obscure the true relationship between temperature and fitness. Finally, most studies have used constant incubation temperatures; thus, we used constant temperatures to estimate the ECHT. Thermal variation, however, typifies most nests and alters the relationship between temperature and hatchling phenotypes (Les et al., 2007). Future work could incorporate fluctuating temperatures (e.g. repeated sine waves) into calculations of ECHT.

FUTURE DIRECTIONS

There is now a preponderance of data concerning thermal developmental plasticity in reptiles. Consequently, researchers have an abundance of knowledge and tools to answer new and exciting questions. However, measuring thermal limits in addition to responses to optimal temperatures is vital to understand thermal ecology and adaptation and make predictions about responses to global change. To construct a framework for studying embryo thermal ecology, we need meaningful, consistent terminology and methodology. My criticisms and suggestions make progress toward these goals; however, there are many gaps in our understanding, and I make several recommendations about where researchers can focus their attention in the future.

First, we need more studies that characterize embryo responses across a wide range of temperatures, including extreme temperatures. The relationship between incubation temperature and survival/phenotypes is often curvilinear (Noble et al., 2018a); therefore, complete reaction norms may be necessary to understand relationships between temperature, physiology, and

fitness. Moreover, most studies have examined effects of temperatures within the OTR, but we need a better understanding of development at extreme temperatures to predict responses to global change. When possible, researchers should quantify embryo responses to the full range of constant temperatures from the lower to upper lethal limits as a foundational part of their research program. Both the upper and lower limits for development are vital to describe responses to rising temperatures (Levy et al., 2015). For perspective, only 8 species in my dataset have measures of both the upper and lower limits for development. Moreover, I could only estimate ECHT for 38 species, representing 24.5% of species in the Reptile Development Database (n=155; Noble et al., 2018b). My estimates span a wide range of families across lepidosaurs, testudines, and crocodilians; however, there are many groups for which no estimates are available (Figures S1, S2, S3). Importantly, studies should calculate and report ECHT. Current studies report survival rates across temperature, but ECHT will allow for comparisons across studies and species.

Second, we need more studies that quantify EAHT across a range of species. Current estimates are few and highly clustered with respect to phylogeny (Figure S1, S2). Given the relatively large variation in EAHT within some genera (e.g. *Anolis, Takydromus*; Figure 4), there is likely great variation across reptiles which may relate to lineage-specific ecology and physiology. For example, we predict EAHT will exhibit latitudinal or altitudinal trends and trends associated with the relative thermostability of nest temperatures (e.g. shallow- vs deep-nesting species). Only two studies have considered geographic variation in EAHT, but they found conflicting results: geographic variation was detected for *T. septentrionalis* (Sun et al., in revision) but not *S. undulatus* (Angilletta et al., 2013). Moreover, there may be interesting relationships between EAHT and ECHT (e.g coevolution). Many more studies are required to

understand the ecology and evolution of EAHT. Chapters 2, 3, and 4 of this dissertation make progress toward filling this knowledge gap by considering the ecological physiology of EAHT using *Anolis* lizards. In chapter 2, I estimate EAHT for the brown anole (*Anolis sagrei*) using multiple methods described above. Moreover, I quantify the effects of acute thermal stress on embryo and hatchling phenotypes and survival and use nest temperature data to consider the potential for embryos to experience thermal stress in the wild. In chapter 3, I consider how embryos of the Puerto Rican crested anole (*A. cristatellus*) respond to elevated nest temperatures in urban habitats (i.e. the urban heat island effect). Such responses have consequences for how populations will respond to urbanization, a pervasive aspect of global change. Additionally, I test the potential for EAHT to adapt or acclimate to local conditions. Finally, in chapter 4, I compare EAHT for both species and consider how and why EAHT may differ among species and across development in reptiles.

Third, we need a better understanding of the ecological and physiological factors that determine embryo heat tolerance. Indeed, the mechanisms that determine the thermal limits of complex life are debated and may result from complications at cellular (van der Have, 2002) or organ-system levels (Pörtner et al., 2017), or both (Gangloff and Telemeco, 2018). Several studies have demonstrated a strong link between oxygen supply and thermal tolerance in reptile embryos for both the ECHT (Liang et al., 2015; Parker et al., 2019) and the EAHT (e.g. Smith et al., 2015); however, we need more studies that assess embryo physiology at near-lethal temperatures (as in Chapter 2). Studies that incubate eggs at hypoxic and normoxic conditions (e.g. Liang et al., 2015; Smith et al., 2015) have identified oxygen availability as an important factor determining heat tolerance, but they cannot identify the mechanisms that mediate the relationship between oxygen, temperature, and survival. I recommend more studies that expose

embryos to acute thermal stress (i.e. measure EAHT) under normoxic conditions and measure physiology at near-lethal temperatures (e.g. oxygen consumption; expression of heat shock proteins; anaerobic respiration). Indeed, studies that estimate pejus and critical temperatures by measuring aerobic vs anaerobic metabolism at high temperatures would be most helpful (Wittman et al., 2008). Such studies will move beyond our current "black box" understanding of the relationships among temperature, oxygen, and survival. Chapters 2 and 4 of this dissertation make progress toward filling this knowledge gap by exploring ecological and physiological factors that influence survival under acute heat stress. Chapter 2 considers the role of oxygen availability and cardiac performance in determining EAHT. Moreover, it explores the effects of repeated exposure of embryos to sublethal levels of acute heat stress (which has not previously been considered). Chapter 4 determines the relationship between EAHT and ontogeny.

CONCLUSIONS

Understanding how embryos respond to thermal stress is vital when predicting responses to global change. Embryos differ from later life stages in many ways and our methods and terminology should reflect these disparities. Researchers should consider whether chronic or acute heat tolerance is more relevant based on the developmental ecology of their study species and their research questions. The upper thermal tolerance of reptile embryos in response to both acute and chronic temperature treatments varies across species, but more data are required to understand how these responses evolve with respect to one another and with respect to important ecological variables. Future studies should focus on assessing embryo responses to both chronic and acute thermal stress and incorporating these measures into predictive models regarding

global change. Such work will provide great insight into the evolutionary, ecological, and physiological mechanisms that determine heat tolerance in reptile embryos.

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CHAPTER 1 SUPPLEMENTAL MATERIAL

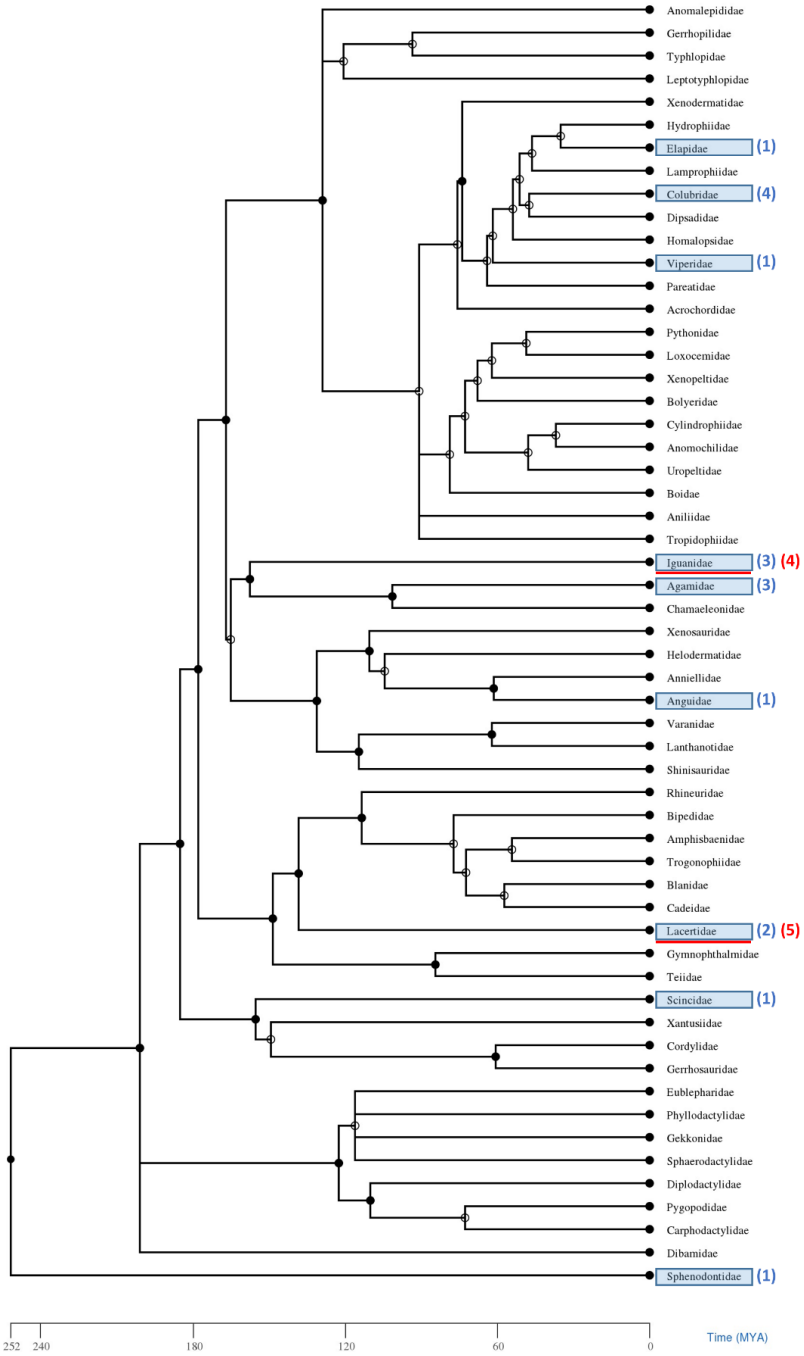

Figure S1. Lepidosaur families. Blue boxes and red bars denote families with at least one species for which ECHT and EAHT is measured, respectively. Samples sizes (i.e. number of species) for ECHT and EAHT are listed in blue and red, respectively. The tree was made using www.timetree.org.

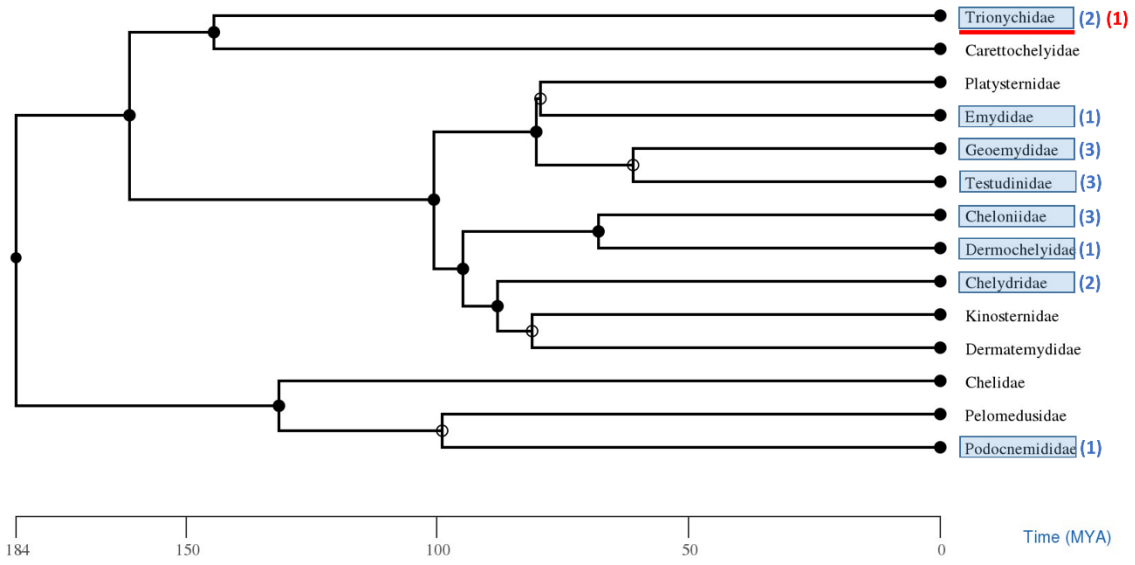

Figure S2. Turtle families. Blue boxes and red bars denote families with at least one species for which ECHT and EAHT is measured, respectively. Samples sizes (i.e. number of species) for ECHT and EAHT are listed in blue and red, respectively. The tree was made using www.timetree.org.

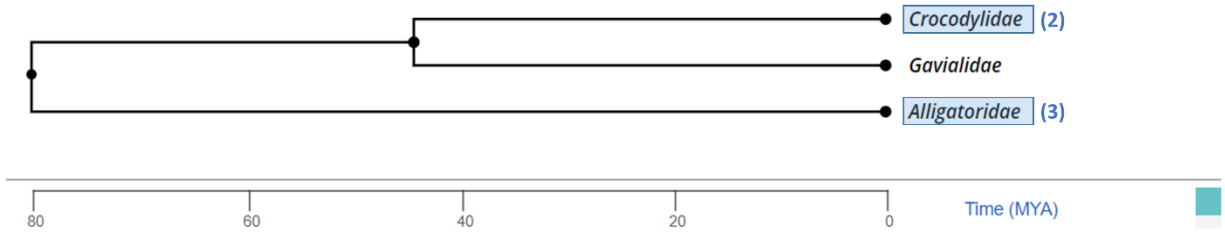

Figure S3. Crocodilian families. Blue boxes denote families with at least one species for which ECHT is measured. Samples sizes (i.e. number of species) for ECHT are listed in blue. There are no estimates of EAHT for any crocodilian. The tree was made using www.timetree.org.

Table S1. All species for which ECHT could be estimated.

Family	Species	Source
Squamata		
Agamidae	*Agama impalearis*	El Mouden et al., 2001
Agamidae	*Calotes versicolor*	Ji et al., 2002
Agamidae	*Phrynocephalus versicolor*	Qu et al., 2011; Tang et al., 2012
Anguidae	*Elgaria multicarinata*	Telemeco, 2015
Colubridae	*Elaphe taeniura*	Du and Ji, 2008
Colubridae	*Ptyas dhumnades*	Lin et al., 2010
Colubridae	*Rhabdophis tigrinus*	Chen and Ji, 2002
Colubridae	*Xenochrophis piscator*	Ji et al., 2001; Lu et al., 2009
Dactyloidae	*Anolis sagrei*	Pruett, pers. comm.
Elapidae	*Naja atra*	Ji and Du, 2001
Iguanidae	*Dipsosaurus dorsalis*	Muth, 1980
Lacertidae	*Podarcis muralis*	Van damme et al., 1992; Ji and Brana, 1999; Le Henanff et al., 2013
Lacertidae	*Takydromus septentrionalis*	Du and Ji, 2006; Du and Feng, 2008
Phrynosomatidae	*Sceloporus undulatus*	Sexton and Marion, 1974; Angilletta et al., 2000; Andrews et al., 2000
Scincidae	*Scincella modesta*	Lu et al., 2006; Li et al., 2012
Viperidae	*Deinagkistrodon acutus*	Lin et al., 2005
Rhynchocephalia		
Sphenodontidae	*Sphenodon punctatus*	Thompson, 1990; Nelson et al., 2004; Besson et al., 2012
Testudines		
Cheloniidae	*Caretta caretta*	Mrosovsky et al., 2002; Reid et al., 2009; Howard et al., 2014

Cheloniidae	*Chelonia mydas*	Howard et al., 2014; Rafferty and Reina, 2014; Stubbs and Mitchell, 2018
Cheloniidae	*Lepidochelys olivacea*	Mueller et al., 2019
Chelydridae	*Chelydra serpentina*	Yntema, 1976; Packard et al., 1985
Chelydridae	*Macrochelys temminckii*	Ligon and Lovern, 2009
Dermochelyidae	*Dermochelys coriacea*	Binckley et al., 1998
Emydidae	*Chrysemys picta*	Schwarzkopf and Brooks, 1985; Les et al., 2009; Bodensteiner et al., 2019
Geoemydidae	*Mauremys mutica*	Zhu et al., 2006; Du et al., 2010a; Guo et al., 2010; Zhao et al., 2015
Geoemydidae	*Mauremys reevesii*	Du et al., 2006; Du et al., 2007
Geoemydidae	*Mauremys sinensis*	Du et al., 2010a
Podocnemididae	*Podacnemis expansa*	Lubiana and Ferreira Júnior, 2009
Testudinidae	*Actinemys marmorata*	Geist et al., 2015
Testudinidae	*Gopherus agassizii*	Spotila et al., 1994; Lewis-Winokur and Winokur, 1995
Testudinidae	*Gopherus polyphemus*	Demuth, 2001
Trionychidae	*Apalone mutica*	Janzen, 1993; Plummer et al., 1994; Doody, 1999; Mullins and Janzen, 2006
Trionychidae	*Pelodiscus sinensis*	Choo and Chou, 1987; Du and Ji, 2003; Ji et al., 2003
Crocodylia		
Alligatoridae	*Alligator mississippiensis*	Lang and Andrews, 1994
Alligatoridae	*Caiman crocodilus*	Lang and Andrews, 1994
Alligatoridae	*Caiman latirostris*	Pina et al., 2003
Crocodylidae	*Crocodylus acutus*	Charruau et al., 2017
Crocodylidae	*Crocodylus palustris*	Lang et al., 1989; Lang and Andrews, 1994

Table S2. All species for which EAHT estimates were available.

Family	Species	Source
Dactyloidae	*Anolis cristatellus*	Chapter 4
Dactyloidae	*Anolis sagrei*	Chapter 4
Lacertidae	*Takydromus septentrionalis*	Gao et al., 2014; Sun, unpublish.
Lacertidae	*Takydromus woleri*	Sun, unpublish.
Lacertidae	*Takydromus sexlineatus*	Sun, unpublish.
Lacertidae	*Takydromus kuenhei*	Sun, unpublish.
Lacertidae	*Takydromus amurensis*	Sun, unpublish.
Phrynosomatidae	*Sceloporus tristichus*	Smith et al., 2015
Phrynosomatidae	*Sceloporus undulatus*	Angilletta et al., 2013
Trionychidae	*Pelodiscus sinensis*	Gao et al., 2014

CHAPTER 2

Thermal sensitivity of lizard embryos indicates a mismatch between oxygen supply and demand at near-lethal temperatures

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INTRODUCTION

Multiple aspects of global change (e.g. urbanization, climate change) create novel, stressful thermal environments that threaten biodiversity across the planet (McDonald et al., 2008; Sinervo et al., 2010). Much research on this topic quantifies how adult organisms respond to high temperatures (e.g. Sinervo et al., 2010; Battles and Kolbe, 2019); however, the effect of global change on developing offspring (e.g. embryos) is less studied, but also important (Ma et al., 2018; Burggren, 2018). Embryos are particularly sensitive to thermal stress due to a relatively narrow thermal tolerance compared to adults (van der Have, 2002) and little to no capacity to behaviorally thermoregulate (Cordero et al., 2018). Additionally, because many important, thermally sensitive processes occur during development (e.g. organogenesis), thermal stress at this stage can induce life-long negative effects (Shine et al., 2005; Kaiser et al., 2016). Finally, egg mortality can drive population cycles (Chalcraft and Andrews, 1999); therefore, the thermal sensitivity of embryos can influence species distributions and population persistence in the face of global change (Ma et al., 2018; Carlo et al., 2018). Thus, to understand how biodiversity will respond to novel thermal conditions, it is critical to quantify embryo responses to extreme thermal variation in natural habitats (Burggren, 2018).

Studies of non-avian reptiles have contributed greatly to our understanding of embryo thermal ecology (Noble et al., 2018a; Refsnider et al., 2019), and many species are threatened by

global change (Sinervo et al., 2010; Santidrián Tomillo et al., 2015). A recent flurry of work has synthesized existing egg incubation studies (While et al., 2018; Warner et al., 2018; Booth, 2018; Noble et al., 2018a), demonstrating that most researchers use a series of constant incubation temperatures to define the upper thermal limits for development (Andrews and Schwarzkopf, 2012). Constant temperatures may be appropriate for species that construct relatively deep nests that experience little thermal variation; however, most eggs incubate in nests that exhibit daily fluctuations in temperature (Booth, 2018). The effects of fluctuating temperatures differ widely from those of constant temperatures for a diversity of phenotypes (Bowden et al., 2014; Warner and Shine, 2011; Noble et al., 2018a); therefore, constant temperature treatments are insufficient to assess the effects of thermal stress on many wild populations. Moreover, most studies have used incubation treatments that persist throughout development (i.e. chronic exposure), but we know very little about the immediate or cumulative effects of brief (i.e. acute) exposure(s) to stressful temperatures (Angilletta et al., 2013). This knowledge gap should be filled for two reasons. First, this gap limits our ability to conduct broad, comparative analyses of embryo responses to ecologically relevant incubation temperatures. Indeed, such analyses currently depend on constant temperature incubation studies and ignore the effects of acute exposure to thermal extremes (e.g. Andrews and Schwarzkopf, 2012). Second, in some contexts, maximum nest temperatures can drive the evolution of life-history traits more so than mean temperatures (Shine et al., 2003). Given that nest temperatures often fluctuate above the critical thermal maximum for some species (e.g. Angilletta et al., 2013; Sanger et al., 2018) and that global change will cause nest temperatures to rise in both mean and variance, more studies of acute exposure to thermal stress are needed.

The brown anole lizard (*Anolis sagrei*), is an excellent model to understand the effects of extreme thermal variation on development (see Chapter 1). Therefore, we used eggs of the brown anole to quantify the immediate and cumulative effects of acute thermal stress on development and obtain novel, baseline information on physiology and thermal tolerance for this species. Our objectives were to 1) determine the embryo acute heat tolerance (EAHT) for *A. sagrei*, 2) quantify the thermal sensitivity of embryo physiology across nest temperatures and near-lethal temperatures, 3) and assess the effects of repeated exposure to sublethal, but stressful temperatures on embryo development and hatchling phenotypes and survival.

To determine EAHT, we exposed eggs to 1-hour heat shocks of increasingly high temperatures (see Figure 1e from Chapter 1). To quantify embryo physiology, we measured embryo heart rates (f_H) and oxygen consumption (VO_2) across temperatures from 22 to 47 °C, which encompasses the range of nest temperatures commonly reported for this species (Sanger et al., 2018; Gunderson et al., 2020; Pruett et al., 2020). To understand the effect of repeated exposures to sublethal temperatures, we subjected eggs to four exposures of an extreme fluctuation in nest temperature and measured aspects of embryo physiology, hatchling morphology, growth, and survival in the laboratory. Given that the frequency and magnitude of extreme temperatures is predicted to increase due to climate change, these thermal treatments provide an ecologically relevant evaluation of the relationship between thermal stress and embryo development under current and future environments.

METHODS

Determining EAHT via heat shock

We collected adult lizards (n=60 females and n=12 males) from Palm Coast, FL (coordinates: 29.602199, -81.196211) from 22 to 24 March 2019. Lizards were transported to Auburn University and housed in screen cages (45 x 45 x 92 cm; Repti-Breeze, Zoo Med Inc.) in a 5:1 female:male ratio, and maintained at 27 °C with a 12:12 hr light:dark cycle using Reptisun 5.0 UVB bulbs (Zoo Med Inc.) and plant grow bulbs (model F40; General Electric Co.). We fed lizards 3 crickets each, dusted with vitamins and calcium twice per week and misted cages with water daily. Nest pots were provided with a mixture of peat moss and potting soil. We collected eggs twice per week from 7 to 20 June and placed all eggs in 60 mm petri dishes half-filled with moist vermiculite (-150 kPa) and wrapped with parafilm to prevent desiccation. Eggs were incubated at temperatures that fluctuated in a daily sine wave with amplitude of 2.4 °C and a mean of 26.3 °C, which is like nest temperatures at our field site (Pearson and Warner, 2018).

We subjected all eggs (n=72) to a series of 1-hour heat shocks starting at 44 °C because eggs are robust to brief exposures to 43 °C (Chapter 4). Eggs that survived were given 3-4 days to recover and were heat-shocked at 45 °C. We repeated this process, increasing the heat shock by 1 °C, until all eggs were dead. For each egg, we recorded the temperature at which it died (i.e. the lethal temperature). To apply heat shocks, we placed eggs in glass jars (59 mL FLINT s/s) that were ¾ filled with moist vermiculite (-150 kPa) and covered with half of a 60 mm petri dish to reduce water loss but allow gas exchange. Jars were kept in an incubator set to the heat shock temperature for 1 hour prior to receiving eggs and were immediately returned to the incubator once eggs were added. After the heat shock, we checked each egg for a f_H using the Buddy® heart rate monitor (Hulbert et al., 2017). If no f_H was detected, we repositioned the egg on the

monitor multiple times over a period of 5 minutes to ensure no f_H could be detected. Eggs with no f_H were considered dead and were returned to the fluctuating incubator and monitored daily for additional signs of mortality (e.g. fungal growth). All eggs without a f_H eventually shriveled and molded; thus, using heart rate to determine survival was effective.

Due to variation in egg-laying date among females, eggs ranged in age from 4 to 17 days post oviposition (i.e. 11 to 47% development completed). To estimate EAHT while controlling for variation in egg size and age, we performed a linear regression with the lethal temperature as the response variable and egg age (i.e. days since oviposition) and egg mass (which was measured prior to treatment) as fixed effects. Egg mass and age were centred at zero prior to analysis.

Thermal sensitivity of embryo physiology

We collected additional eggs from a different breeding colony (n=38 female; n=12 males) that was captured from 18-19 March 2018 from Pinecrest, FL (coordinates: 25.678125, -80.287655). Husbandry was as previously described; however, we housed females individually in cages (29 × 26 × 39 cm; height × width × depth) and rotated males among females. We used 11 randomly selected eggs collected 25 May to 1 June 2018 to determine the thermal sensitivity of embryo f_H. Eggs were incubated in petri dishes (as previously described) at a constant 28 °C for 1 week prior to measurements. A constant temperature was used to avoid potential circadian rhythms in f_H. Thus, embryos varied in age from 7-14 days post oviposition (i.e. 23 to 46% development completed); however, embryo f_H does not covary with age in the first few weeks of development (Hulbert et al., 2017). For logistical reasons, f_H measurements occurred over the course of 3 days. On day 1, eggs were kept at room temperature (~ 22 °C) for 24 hours to ensure

they were at the appropriate starting temperature for the assay. On day 2, eggs were slowly (3°C per hour) raised from 22 to 39 °C inside a Memmert brand IPP 55 Plus incubator. We programmed the incubator to stop increasing temperature for approximately ½ hour at various target temperatures (22, 26, 29, 31, 34, 37, and 39 °C). During these intervals, we measured f_H. Eggs were quickly removed (one at a time) from the incubator and placed in the heart rate monitor which was housed in another incubator set to the target temperature. Eggs remained in the monitor for 45 to 60 s before we recorded a f_H. We recorded the air temperature inside the monitor with a thermocouple along with each f_H. After f_H measurements, eggs were returned to the Memmert incubator to increase to the next target temperature. All eggs were returned to the 28 °C incubator at the end of day 2. Due to time constraints, eggs could not be measured at all temperatures on the same day. Thus, on day 3, we measured f_H from 40 to 47 °C. Heart rates were measured at 40 °C (after bringing them from 28 to 40 °C by 3 °C per hour), and we increased the temperature of eggs at a steady rate (~ 3 °C per hour) while measuring f_H periodically (at approximately 1 °C intervals) until each egg was dead (i.e. no f_H; i.e. thermal ramp method – see Figure1c from Chapter 1). Brief exposure to 42 °C has no detectable effect on *A. sagrei* embryo development or survival (Chapter 4); thus, f_H measured on day 3 were likely not affected by the thermal ramp on day 2.

To determine the thermal sensitivity of f_H, we used the temperatures recorded inside the heart rate monitor at the time each f_H was measured (rather than the nominal target temperature). We performed two linear mixed effects models with f_H as the response variable and temperature as the independent variable. One model assumed the relationship was linear and the other assumed it was curvilinear (i.e. linear plus quadratic term). Egg ID was a random effect. Best fit models were determined with likelihood ratio tests. Two data points were removed from the

analysis because they were extreme outliers and likely represent eggs near death (see results). Preliminary analysis revealed that embryo age did not covary with f_H ($p = 0.32$).

A different subset of eggs was used to determine the thermal sensitivity of embryo VO_2 (n=45 eggs; i.e. all eggs collected from 23 – 30 July 2018). These eggs were incubated in petri dishes (as previously described) at a constant 28 °C for 1 week prior to measurements. A constant temperature was used to avoid potential circadian rhythms in metabolic rate. Thus, embryos were between 7-14 days since oviposition at time of measurement (i.e. 23 to 46% development completed). These eggs were randomly allocated to 1 of 9 temperature treatments (21, 25, 29, 33, 35, 39, 41, 44, 47 °C; n=5 per treatment). Eggs were brought to the target temperature as previously described.

We used a Qubit Q-box RP1LP respirometer (Qubit Biology Inc., Kingston, ON) and applied a dynamic injection analysis method (Lighton 2018). Each egg was placed in a 10 ml syringe with Luer-Lok tip attached to a 3-way stopcock (Becton, Dickinson and Company, Franklin Lakes, NJ 07417). A 5 µl drop of tap water was placed inside the syringe via a micropipette (to prevent desiccation of eggs). The syringe was flushed with CO_2-free room air for 2 minutes at a rate of 100 ml/min. Two previously drilled holes (between the 5- and 6-ml marks) allowed air to exit the syringe. This air was drawn through several meters of coiled tubing that was inside an incubator set to the target temperature. A dummy syringe with a thermocouple was used to verify that the air stream was at the target temperature. After flushing, the egg was sealed in the syringe in a 4 ml volume of air. Eggs were placed in a constant temperature incubator set to the target temperature for 30 minutes. We injected a 2 ml sample of air into a stream of dry, CO_2-free air flowing at 50 ml/min. After measurements, each egg was placed in the heart rate monitor to determine survival. CO_2 production was simultaneously

measured so we could calculate respiratory quotients (CO_2 produced/O_2 consumed, i.e. RQ) at each temperature.

To analyse VO_2, we performed two general linear models: one was an asymptotic model, and the other was a second-degree polynomial. The best fit model was determined by likelihood ratio test. We excluded eggs incubated at 47 °C because they died during treatment which prevented us from reliably calculating VO_2. We included egg mass in the model to control for variation in the size of eggs.

To compare changes in f_H and VO_2 across temperature, we estimated Q_{10} values (i.e. rate of change for a 10 °C increase in temperature) between each target temperature and generated 95% confidence intervals by bootstrapping the data with 10,000 replicates.

Repeated exposure to sublethal temperatures

On 4 March 2017, adult lizards (n=30 females; n=15 males) were captured from Pinecrest, FL and housed as described in section 2.2 above. We collected eggs three times per week and used all eggs produced from 3 to 17 July (n= 71). For each egg, we recorded the mass, date of oviposition, and maternal identity. Eggs were individually placed in petri dishes as previously described and randomly assigned to one of two incubation treatments (described below) and placed in an incubator that repeated a daily thermal fluctuation that was suitable for successful development and created from field nest temperatures (Figure S1). Because anoles lay a single egg every one or two weeks, we randomly assigned each female's first egg to a treatment and alternated subsequent eggs between the two treatments. Moreover, eggs varied in age from 3 to 17 days at time of treatment (i.e. 10 to 57% development completed). Treatments subjected eggs to either zero (i.e. control) or four (i.e. experimental) exposures to an extreme

fluctuation in temperature (henceforth, “thermal spike”) measured from the field (see Chapter 3). The peak temperature of this thermal spike was 43 °C. A previous study showed that 1 or 2 exposures to this fluctuation result in moderate reductions in egg survival and hatchling body size, but most effects were not statistically clear (Chapter 4). For example, hatching success was 82 and 78% for 1 and 2 spikes, respectively, compared to 93% for controls. Here, we use 4 exposures to induce a stronger effect. Moreover, due to spatial and temporal autocorrelation of nest temperatures, embryos are often exposed to stressful temperatures on multiple days in a row (2-5 days) during development (see results). Thus, 4 exposures are a relevant treatment. Figure 1 provides an overview of the experimental design and Figure S2 provides more details of temperature treatments.

To quantify latent effects of thermal spikes on embryo physiology, we measured embryo f_H using the Buddy® egg monitor. For each egg, we measured f_H at 28 °C on the day before and on each of two days after treatments. For all hatchlings (n=61), we measured snout-vent length (SVL; to nearest 0.01 mm) and tail length (to nearest 0.01 mm) with a digital calliper, and hatchling mass (to nearest 0.0001 g). We kept hatchlings in cages that were identical to those described for adults in section 2.2. We aimed to keep 6 hatchlings per cage (3 from each treatment); however, due to differences in egg survival per treatment (see results) there were n=1 cage with 5 lizards and n=8 cages with 6 lizards. To minimize large age discrepancies among cage-mates, we filled cages in the order that lizards hatched; thus, the last 8 control lizards that hatched were not assigned to a cage but were euthanized because there were no more experimental hatchlings to serve as cage mates. We fed hatchlings fruit flies, *ad lib*, dusted with vitamins and calcium and misted cages with water each day. Once hatchlings reached approximately 2 months of age, we measured the final SVL and body mass of all survivors.

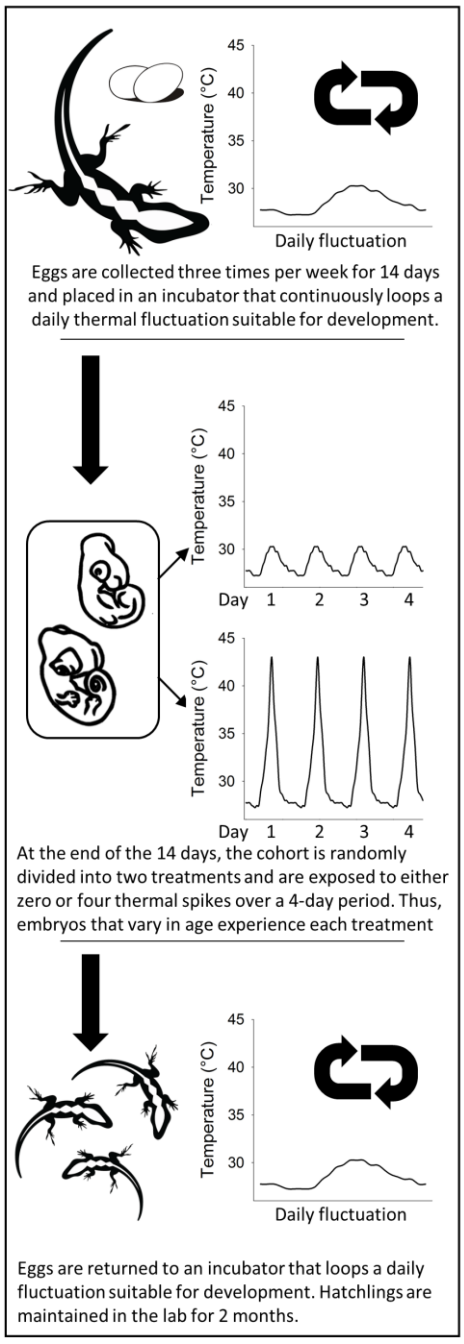

Figure 1. Overview of experimental design to assess repeated exposure to sublethal temperatures.

To assess the effects of thermal spikes on developmental rates, hatchling morphology (SVL, mass, tail length), and hatchling growth rates, we performed linear mixed effects models (LMMs) with initial mass (egg mass at oviposition for developmental rate and hatchling

morphology; body mass at hatching for hatchling growth), embryo age at time of treatment, treatment (0 vs 4 spikes), and an age-by-treatment interaction as fixed effects. Maternal ID was the random effect for all analyses except cage ID was the random effect for hatchling growth rates. The interaction term was not statistically significant in any model, so it was omitted (all p-values > 0.13). Embryo age was the first day of treatment minus the day the egg was collected. To convert incubation periods to developmental rates, we divided 1 by the incubation period (days from oviposition to hatching) (Andrews and Schwarzkopf 2012). Hatchling growth rate was the final mass of each hatchling minus its body mass at hatching divided by the total number of days it was in captivity.

Only one egg died in the control group (vs 9 in the experimental group); so, we split the data by treatment and analyzed the experimental group with a generalized linear mixed effects model (GLM) with a binomial distribution. Embryo age and egg mass were fixed effects. Due to variation in hatchling survival across cages (6 cages = 0.50, 1 cage =0.67, 2 cages =0.83), we initially included cage as a random effect to analyze hatchling survival; however, due to model convergence issues, we changed it to a fixed effect to control for among-cage variation in survival. Including cage did not improve model fit (assessed via likelihood ratio test; X^2=2.62; p = 0.96), so we omitted it from the final model.

To analyze f_H before and after thermal spikes, we performed a LMM with f_H as the dependent variable and day (day before treatment, one day post-treatment, or two days post-treatment), treatment (0 vs 4 spikes), and a treatment-by-day interaction as fixed effects. The exact temperature in the heart rate monitor and embryo age were covariates. All data analyses were performed in R (ver. 3.5.1; R Core Team 2018).

Temperatures of nest sites in the field

We used nest temperatures collected by Pearson and Warner (2018) (n = 22 nest sites) and Pruett et al. (2020) (n = 47 nest sites) to assess the potential for embryos to experience thermal stress in the wild. Nest temperatures were collected from spoil islands in the Intracoastal Waterway in Palm Coast, Florida (i.e. same populations used for sections 2.1 and 2.2 above). See Pearson and Warner (2018) and Pruett et al. (2020) for more details concerning the placement of temperature loggers. Briefly, Pearson and Warner (2018) deployed iButtons in potential nest sites (i.e. microhabitats commonly used for nesting but eggs not necessarily present) across multiple islands and recorded temperatures every 2.5 hours during May, June, and July of 2013. Pruett et al. (2020) deployed iButtons in actual nests (i.e. at least one egg present) on a single island and recorded hourly temperatures during June and July of 2018. These are the months when most eggs are incubating in the wild (Pruett et al., 2020). To assess potential thermal stress, we calculated the percentage of nest sites with temperatures that exceed 34 °C and 40 °C, which we consider the upper pejus temperature and upper critical temperature, respectively. The pejus temperature is the point where aerobic performance begins declining (Wittman et al., 2008). We selected this temperature because oxygen consumption begins to plateau at this temperature (i.e. approaching capacity; see results) and because constant incubation temperatures above 33 °C result in high rates of embryo mortality and developmental abnormalities (Sanger et al., 2018). The critical temperature is the point when aerobic scope has collapsed, and animals respire via anaerobic respiration (Wittman et al., 2008). We selected 40 °C based on results from this study (see below). Indeed, brief daily exposure to similar temperatures (33.4 and 39.7 °C) induces high rates of egg mortality (42.9 and 60.3%, respectively; Gunderson et al., 2020).

RESULTS

Determining EAHT via heat shock

Most embryos died at 45 or 46 °C (Figure 2). There was no statistically clear effect of egg age (0.006 ± 0.02 SE; $t_{1,69}$ = 0.23; p=0.80) or initial egg mass (0.00004 ± 0.003 SE; $t_{1,69}$ = 0.01; p=0.99) on the lethal temperature. Thus, we consider the intercept of the linear model, 45.3 °C (45.14 - 45.44; 95% CI), to be EAHT (i.e. mean lethal temperature).

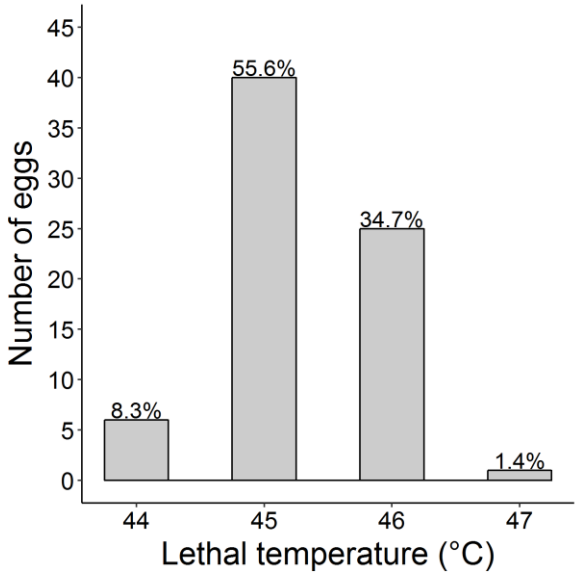

Figure 2. Histogram of lethal temperatures for *A. sagrei* eggs exposed to 1-hour heat shocks. Values above each bar are the percentage of total eggs (n=72) that died at each temperature.

Thermal sensitivity of embryo physiology

The relationship between embryo f_H and temperature was curvilinear (Table S1; Figure 3): the linear component of the regression was 3.40 (± 1.30 SE; $t_{1,97}$ =2.62; p=0.01) and the quadratic term was 0.071 (± 0.019 SE; $t_{1,97}$ =3.71; p=0.0003). The mean temperature at which f_H was no longer detectable (i.e. EAHT) was 46.15 °C (45.87 - 46.44; 95% CI). This estimate of EAHT differs from that of the heat shock experiment (i.e. confidence intervals do not overlap).

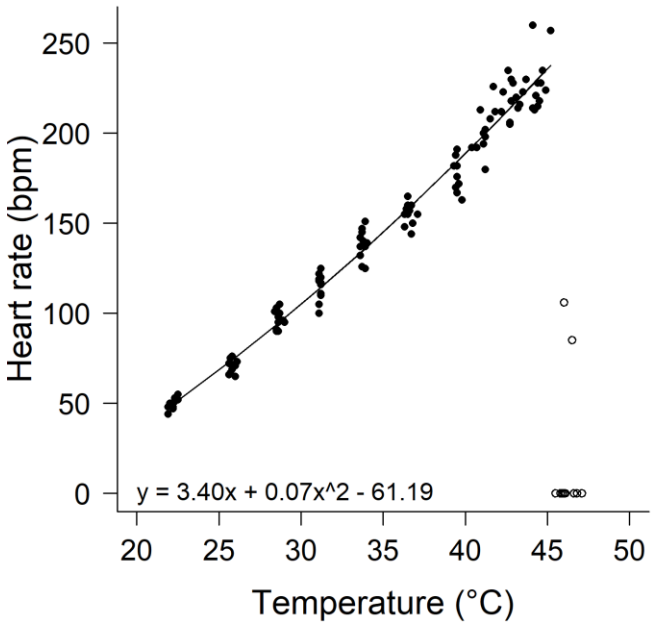

Figure 3. Heart rates of *A. sagrei* embryos across temperature. Closed and open circles show data that are included or excluded from the regression, respectively. The equation for the regression is given in the panel.

VO_2 of embryos was better explained by a second-degree polynomial than an asymptotic model (Table S1): VO_2 increased steadily up to 34 °C (i.e. pejus temperature), remained relatively constant from 37 to 42 °C and then declined slightly at 44 °C (Figure 4a). The linear component of VO_2 by temperature was 3.92 (± 0.52 SE; $t_{1,36}$ =7.55; p<0.0001) and the quadratic term was -0.048 (± 0.0079 SE; $t_{1,36}$ =-6.06; p<0.0001). VO_2 increased with egg size but this relationship was not statistically clear (15.14 ± 13.16 SE; $t_{1,36}$ = 1.15; p=0.26). RQ was stable from 22 to 39 °C, but steadily increased as embryos approached the lethal temperature (Figure 4b). Thus, we consider 40 °C to be the critical temperature. Q_{10}s of f_H and VO_2 were similar at lower temperatures but diverged as embryos approached the lethal temperature. Importantly, confidence intervals for VO_2 overlap with 1 (i.e. no sensitivity to temperature) for most temperatures of 34 °C and above. For f_H, confidence intervals never overlapped with 1 (Figure 5).

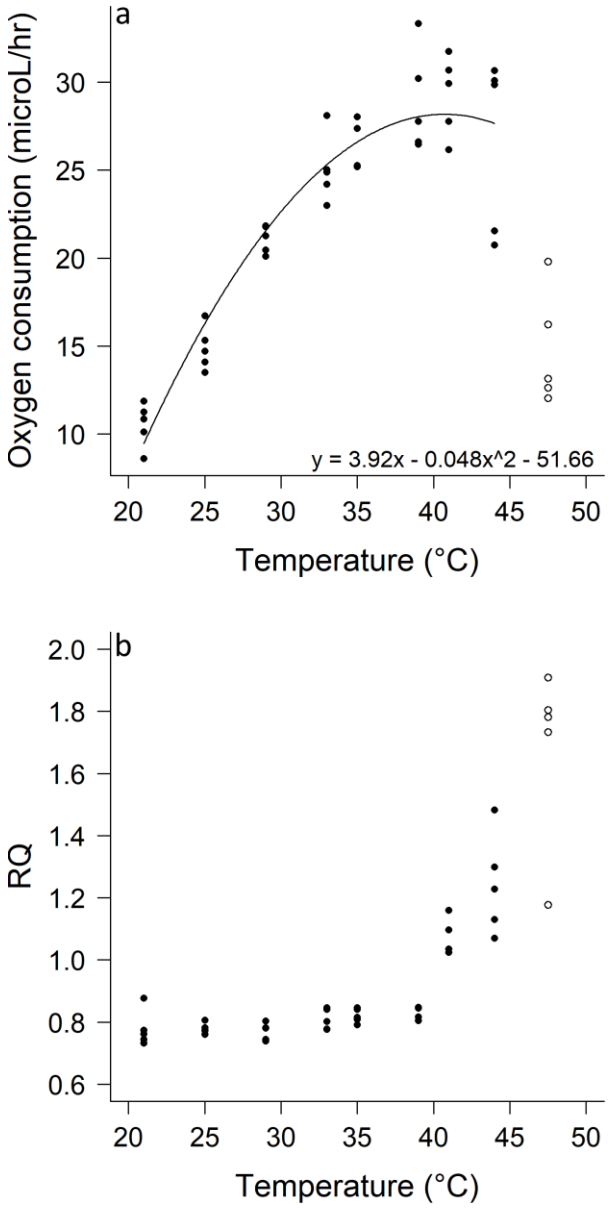

Figure 4. Oxygen consumption (a) and respiratory quotient (b) of *A. sagrei* embryos across temperature. Closed and open circles show raw data for eggs that did and did not survive measurements, respectively. Only surviving eggs were used to analyze oxygen consumption. The solid line in panel (a) shows the fit of a second-degree polynomial model.

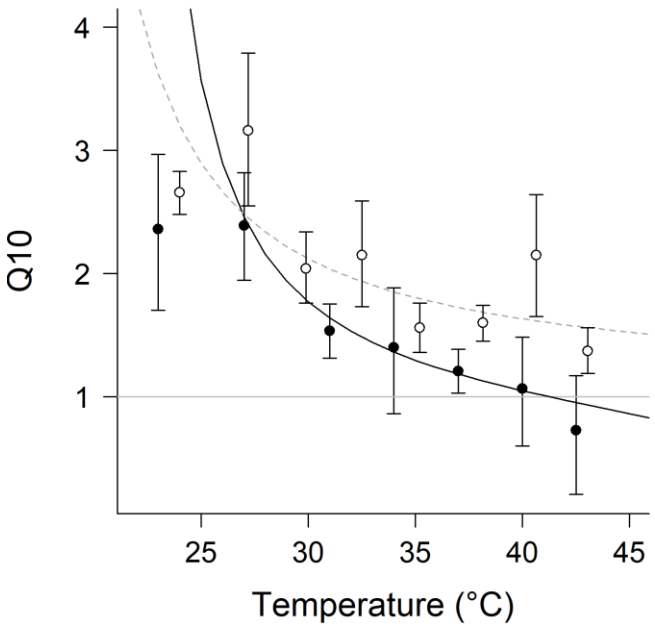

Figure 5. Temperature coefficient (i.e. Q_{10}) of heart rate (open circles; dashed gray line) and oxygen consumption (closed circles; solid black line) across temperature. A horizontal gray line shows the point at which reactions are insensitive to temperature (i.e. $Q_{10} = 1$). Regression lines were calculated using the equations in Figures 3 and 4a. Vertical bars are 95% confidence intervals obtained by bootstrapping the data. The temperature value for each estimate of Q_{10} represents the median temperature (e.g. a Q_{10} between 21 and 25 °C is plotted at 23 °C).

Repeated exposures to sublethal temperatures

Raw mean egg survival was 97% and 75% for the control and experimental groups, respectively. Egg survival during thermal spikes was largely influenced by embryo age at time of treatment (Table 1): embryos were 1.42 (± 1.14 SE) times as likely to die with each 1 day increase in age (Figure 6a). For eggs that survived to hatching, thermal spikes reduced developmental rate by 4.96% (Table 1; Figure 6b).

We observed a statistically clear effect of the day by treatment interaction for embryo f_H ($F_{2,123} = 11.73$; $p<0.0001$): f_H of experimental embryos were 10.3 bpm (± 2.0 SE; $p<0.0001$) and 9.0 bpm (± 1.9 SE; $p<0.0001$) lower than controls on one and two days after experiencing the

Table 1. Results for final models testing the effects of treatment (0 or 4 thermal spikes at 43 °C peak temperature), embryo age at time of treatment and interactions and covariates on *A. sagrei* embryo and hatchling phenotypes and survival. Interactions with no statistics provided were omitted from the final model due to lack of statistical significance. See supplementary material for sample size, raw mean, and standard deviation of all response variables. For egg survival and developmental rates, initial mass was egg mass at oviposition. For hatchling morphology, growth, and survival, initial mass was hatchling mass at time of hatching. Results for egg survival are only for the experimental group (see statistical methods). Treatment estimates are the experimental group minus the control. Bold type denotes statistical significance.

Response variable	Initial mass		Age		Treatment	
	Estimate (SE)		Estimate (SE)		Estimate (SE)	
Egg survival**	5.87 (29.44)	χ^2_1=0.04; p=0.84	**-0.35 (0.13)**	**χ^2_1=10.97; p=0.001**	-	-
Developmental rate (days^{-1})	-0.003 (0.009)	$F_{1,34}$=0.13; p=0.72	0.00001 (0.00003)	$F_{1,34}$=0.05; p=0.82	**-0.0017 (0.00028)**	**$F_{1,34}$=35.05; p<0.0001**
Hatchling initial SVL (mm)	7.51 (5.00)	$F_{1,34}$=2.3; p=0.14	0.02 (0.02)	$F_{1,34}$=1.2; p=0.29	-0.25 (0.18)	$F_{1,34}$=2.0; p=0.16
Hatchling initial mass (mg)	**357.5 (107.8)**	**$F_{1,34}$=11.0; p=0.002**	0.6 (0.4)	$F_{1,34}$=2.5; p=0.13	**-8.0 (3.2)**	**$F_{1,34}$=6.2; p=0.02**
Hatchling initial tail length (mm)	22.60 (12.00)	$F_{1,34}$=3.6; p=0.07	0.044 (0.039)	$F_{1,34}$=1.3; p=0.27	**-0.93 (0.34)**	**$F_{1,34}$=7.5; p=0.01**
Hatchling survival	0.44 (0.34)	χ^2_1=1.8; p=0.18	-0.46 (0.34)	χ^2_1=2.0; p=0.16	**-1.31 (0.68)**	**χ^2_1=4.0; p=0.045**
Hatchling growth rate (mg/day)	-23.6 (26.8)	$F_{1,20}$=0.78; p=0.37	-0.001 (0.08)	$F_{1,20}$=0.001; p=0.99	-1.5 (0.7)	$F_{1,20}$=4.2; p=0.053

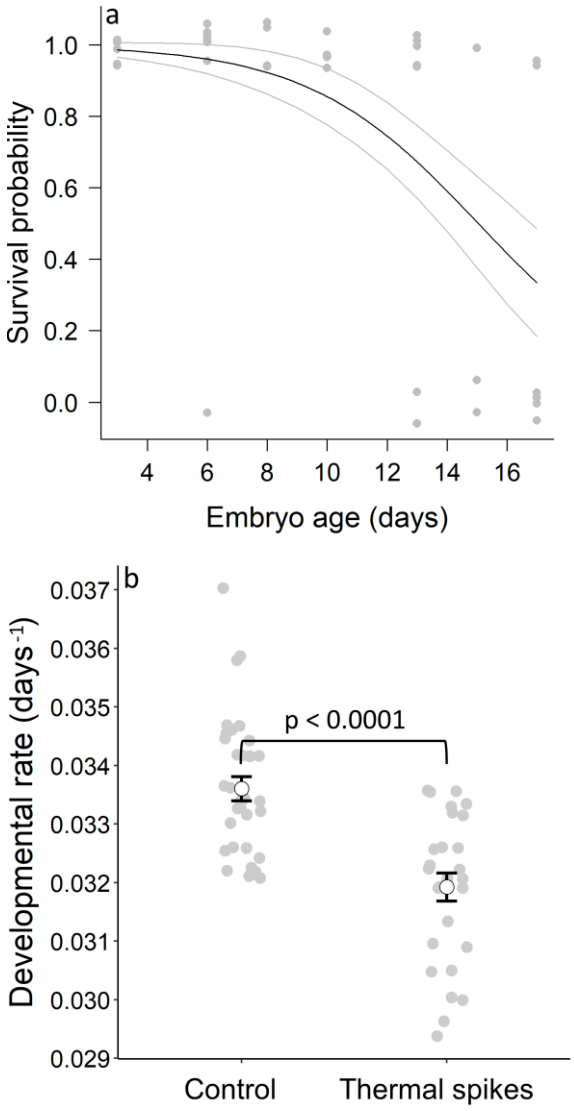

Figure 6. Effects of zero ("Control") or four exposures ("Thermal spikes") to thermal spikes on *A. sagrei* embryos. Panel a shows how survival probability of embryos in the experimental treatment declines with age. The solid line shows a regression of the raw data and gray circles are the raw data, jittered around 0 and 1 to avoid over-plotting. Panel b shows the effects of each treatment on developmental rate. Open circles are the raw mean, bars show standard error, gray circles show raw data.

thermal spike, respectively (Figure 7). These equate to 11.0 and 9.4 % reductions in f_H, respectively.

Experimental hatchlings were shorter in SVL than those from the control, but this effect was not statistically clear (Table 1); however, experimental hatchlings weighed 8.0 (± 3.2 SE)

mg less than controls (Figure 8a) and their tails were 0.93 mm (± 0.34 SE) shorter (Figure 8b; Table 1). This equates to a 5.9% reduction in body mass and a 3.1% reduction in tail length. Experimental hatchlings were 3.72 (± 1.97 SE) times as likely to die as those from the control treatment (Figure 8c; Table 1), and their growth rates were 1.5 (± 0.7 SE) mg per day lower than controls (Figure 8d). This equates to a 44% reduction in daily growth rate; this effect, however, was only marginally statistically clear (Table 1). See Supporting tables S2, S3, S4, and S5 for raw means, sample sizes, and standard deviations of all variables.

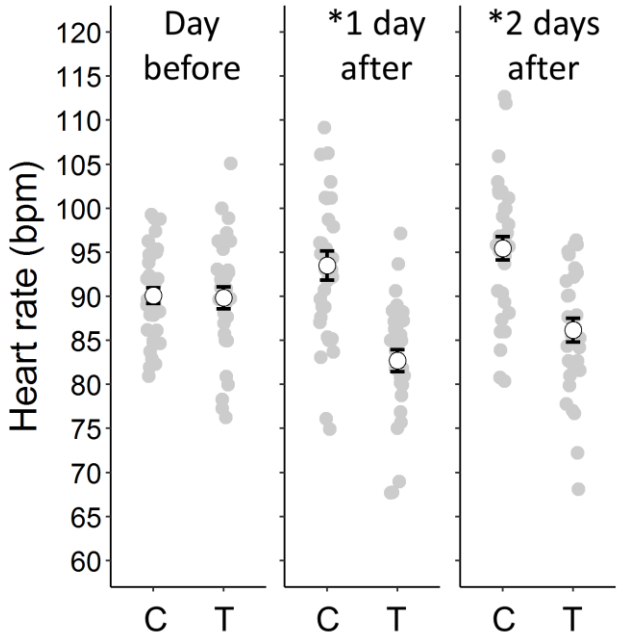

Figure 7. Heart rates of *A. sagrei* embryos exposed to 0 (C for "Control") or 4 (T for "Thermal spikes") thermal spikes. Heart rates were measured at 28 °C on the day before exposure and on one and two days after exposure. Open circles show the raw means for each group, bars show standard error, and gray circles show the raw data. Asterisks signify a statistically significant difference in heart rate between groups.

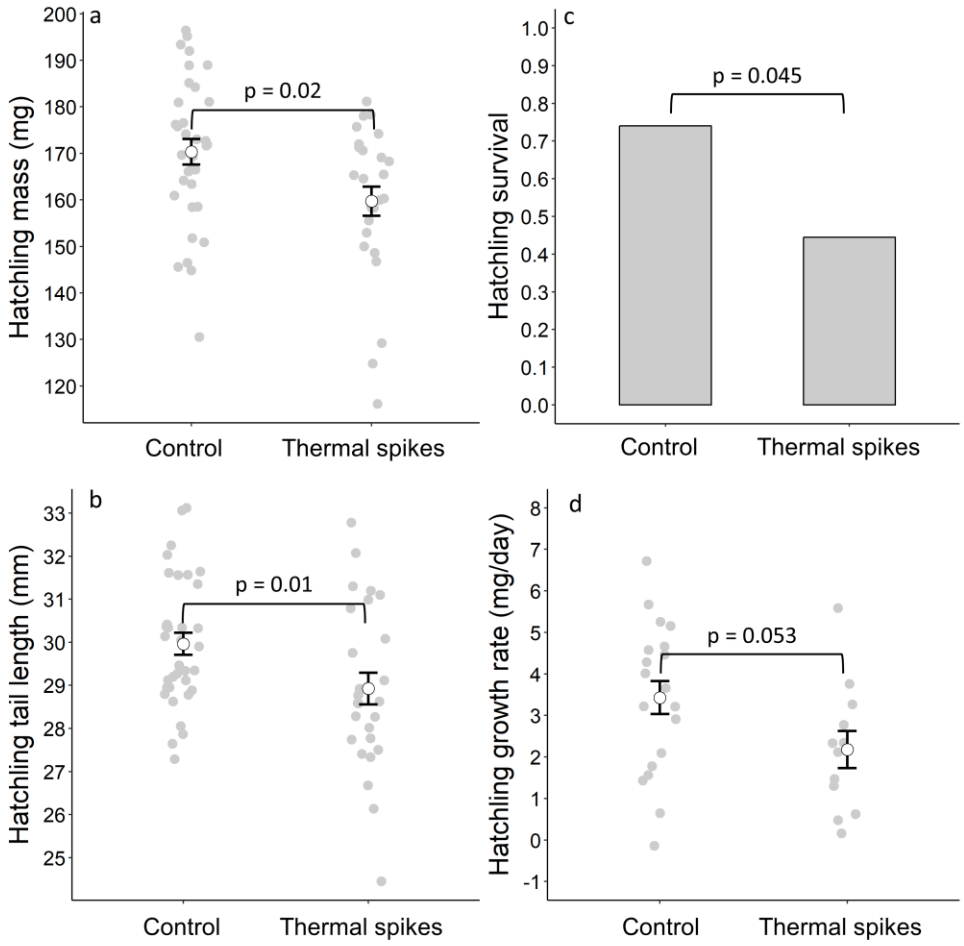

Figure 8. Effects of zero ("Control") or four exposures ("Thermal spikes") to extreme thermal fluctuations (43 °C peak temperature) during development on hatchling mass (a) and tail length (b) at time of hatchling and hatchling survival (c) and growth rate (d) over a two-month period in the laboratory. In panels a, b, and d, open circles show the raw mean, bars show the standard error, and gray circles show the raw data.

Temperatures of nest sites in the field

Examples of the hottest nests are shown in Figures S3 and S4. Maximum nest temperatures during 2013 and 2018 were 45.0 and 45.5°C, respectively (Figure 9). Twelve nests in 2013 (55%) and 26 in 2018 (55%) exhibited peak temperatures greater than 34 °C on at least one day. In 2013 and 2018, six (27%) and 7 (15%) nest sites, respectively, exhibited peak temperatures of 40 °C or higher on at least one day. Four (18%) and 5 (11%) nesting sites

exhibited temperatures > 40 °C on at least 3 days in 2013 and 2018, respectively. Only 1 (5%) and 2 (4%) nesting sites exhibited temperatures of 43 °C or greater on ≥4 days during 2013 and 2018, respectively (i.e. like our thermal spikes treatment; Figures S3a and S4d). The relatively cool nest temperatures (i.e. < 20 °C) in 2013 (vs 2018) were recorded during May (See Figure S3). Nest temperatures were not monitored in May of 2018.

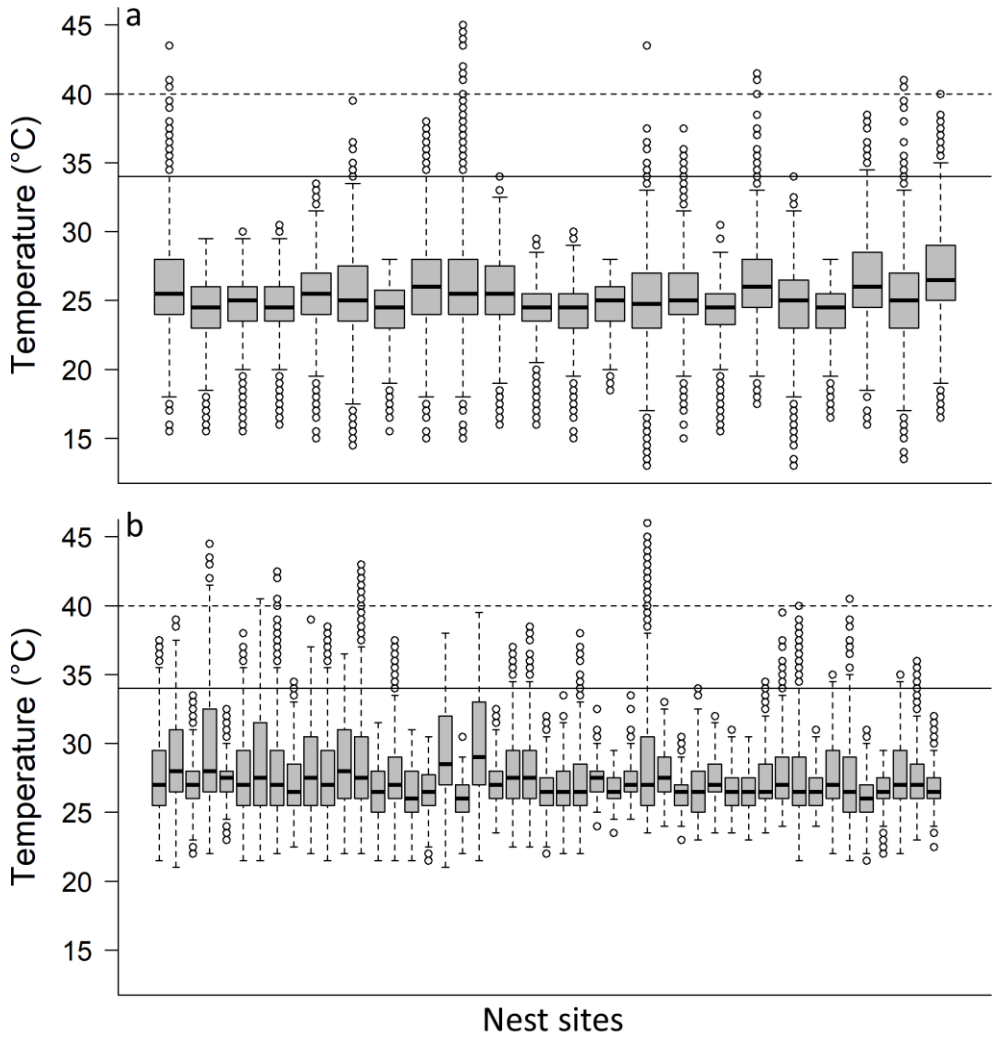

Figure 9. Temperatures from potential *A. sagrei* nest sites in Palm Coast, Florida collected during May, June, and July of 2013 (a) and from actual nest sites June and July of 2018 (b). Solid and dashed horizontal lines denote the pejus (34 °C) and critical temperatures (40 °C), respectively.

DISCUSSION

Most studies of reptile embryo thermal tolerance utilize chronic, constant temperature treatments, and we know comparatively little about the effects of acute thermal stress on development. We subjected eggs of the brown anole lizard to brief exposures of high temperature to determine EAHT, quantify the thermal sensitivity of embryo physiology, and assess the cumulative effects of sub-lethal but stressful temperatures. Most embryos died at 45 or 46 °C, which is similar to that reported for other species (see below). Heart rate and VO_2 increased across temperatures; however, as temperatures approached EAHT, f_H and CO_2 production increased while VO_2 did not. Exposure to extreme fluctuations in nest temperature depressed developmental rates and embryo f_H and resulted in hatchlings with smaller body size, reduced growth rates, and lower survival. Thus, even brief exposure to extreme temperatures can have important effects on embryo development.

Determining EAHT via heat shock

For reptile embryos, extreme (i.e. stressful) temperatures are generally considered those outside the range of constant temperatures that result in high hatching success (i.e. the optimal temperature range "OTR"; Andrews and Schwarzkopf, 2012). The OTR for *A. sagrei* is probably between 20 and 32 °C (see Chapter 1). Eggs incubated from 26 to 30 °C have high hatching success (~93%; Warner et al., 2012); however, Sanger et al., (2018) found that survival was high (90%) at 33 °C but low (39%) at 36 °C. Thus, the pejus temperature is certainly between 34 and 36 °C. In the latter study, eggs were dissected at day 12 and hatching success, *per se*, was not quantified. Assessing egg survival via dissection, rather than hatching success, may overestimate the OTR (Chapter 1). Other data indicate that hatching success begins declining at 29°C and

reaches zero at 35 °C under constant temperature incubation (see Figure 3a from Chapter 1). Thus, *A. sagrei* embryos can survive acute exposure to temperatures as much as 12 °C higher than the upper limit of the OTR. This should make us question the widely held assumption that embryo thermal tolerance breadths are less than that of adults, since this assumption is based on chronic incubation conditions (van der Have, 2002). Ultimately, inferences about the thermal tolerance of species will differ depending on the use of chronic vs acute exposures to thermal stress. For example, embryos of the eastern fence lizard (*Sceloporus undulatus*) have adapted to pervasive (i.e. chronic) nest temperatures across a broad geographic range (Oufiero and Angilletta, 2006) such that northern populations develop more quickly than southern populations when incubated at a common temperature. However, embryo tolerance of acute thermal stress does not differ among populations (Angilletta et al., 2013). This is probably because even the most northerly populations experience nest temperatures above EAHT, potentially maximizing EAHT across the range. Thus, population-specific thermal adaptation differs with respect to chronic vs acute temperatures.

Although there are established protocols for quantifying the critical thermal maximum of adults, no common protocols exist for estimating EAHT of reptile embryos (discussed in Chapter 1). We recommend that researchers consider the developmental ecology of their study species to determine the best method (e.g. chronic vs acute temperatures for deep vs shallow nesting species, respectively). For shallow-nesting species, like anoles, heat shock experiments may be appropriate; however, we did find some discrepancies among estimates of EAHT. Our f_H study gave an estimate that was nearly 1 °C higher than the heat shock experiment (i.e. 46.2 vs 45.3 °C). The differences in EAHT indicate that methods may influence estimates of thermal tolerance. This must be considered when designing experiments and making comparisons across

the literature. One caveat is that we used different populations, which could account for variation in thermal sensitivity due to local adaptation (Oufiero and Angilletta, 2006); however, the only existing study to address this issue found no population-specific responses of lizard embryos to acute thermal stress (Angilletta et al., 2013).

In reptiles (including birds), lethal temperature positively correlates with the optimal incubation temperature, which correlates with mean nest temperatures (Nechaeva, 2011; Goa et al., 2014). This should generate a positive relationship between pervasive nest temperatures and thermal tolerance (Ma et al., 2018). Because *A. sagrei* nests can reach extremely warm temperatures (> 43 °C) during the hottest hours of the day, we expect the upper thermal limit of *A. sagrei* embryos to be high compared to species that develop in cooler nests (e.g. *A. cristatellus*; Tiatragul et al., 2019; see Chapter 4). However, few studies have determined the upper thermal limit of reptile embryos using acute exposures, which prevents broad comparisons among species and precludes our ability to relate acute thermal tolerance to ecological factors (e.g. nest temperatures). These studies have found estimates of EAHT similar to what we observed: embryos of the eastern fence lizard (*S. undulatus*), the plateau fence lizard (S. *tristichus*), the Chinese softshell turtle (*Pelodiscus sinensis*), and the Chinese grass lizard (*Takydromus septentrionalis*) die at 46, 45, 47, and 41 °C, respectively (Angilletta et al., 2013; Goa et al., 2014; Smith et al., 2015). Thus, it is reasonable to think that acute thermal tolerance may be similar across many species, but there is not enough data to draw this conclusion. Given that many reptile species are threatened by climate change in part because mean temperatures and thermal variation of nests are increasing (Telemeco et al., 2017), a broad scale analysis of thermal tolerance of reptile embryos using acute exposures is warranted.

Thermal sensitivity of embryo physiology

The effect of temperature on embryo f_H has been studied extensively in birds; however, much less attention has been given to non-avian reptiles (Nechaeva, 2011). Our results are consistent with existing studies. For example, Q_{10} of f_H across nest temperatures was 2.18 for *A. sagrei* (Q_{10} of 2 to 3 for other reptiles; Du et al., 2011; Nechaeva, 2011). Q_{10} typically declines as temperature increases (Du et al., 2011) and we found this was true even up to the point of death (Figure 5); however, at no point did f_H become insensitive to temperature (i.e $Q_{10} = 1$). Q_{10} for VO_2 and f_H were similar at lower temperatures (23 to 30 °C); however, as temperatures approached T_{LETHAL}, VO_2 became less responsive to temperature compared with f_H (Figure 5).

To our knowledge, no other study has measured the thermal sensitivity of reptile embryo f_H and VO_2 across the full range of nest temperatures, including those near EAHT. At high temperatures, VO_2 should plateau (i.e. maximum oxygen capacity, see Gangloff and Telemeco 2018), causing f_H to decline or plateau due to low oxygen supply to cardiac muscle (Crossley and Altimiras, 2005). Thus, we expected a similar relationship between f_H and VO_2 as temperatures approached EAHT. Rather, when approaching EAHT, f_H continued rising. Moreover, RQ values increased near EAHT, indicating the use of anaerobic respiration since lizard embryos derive energy from lipids and protein (RQs between 0.7 and 0.9, Thompson et al., 2001). These data implicate a mismatch between oxygen demand and supply as a cause for death at high temperatures (Wittman et al., 2008; Gangloff and Telemeco, 2018). Indeed, other studies find that hypoxic and hyperoxic incubation conditions decrease or increase, respectively, the thermal tolerance of reptile embryos (e.g. Smith et al., 2015; Liang et al., 2015; Vimmerstedt et al., 2019); however, these have used chronic oxygen and/or temperature conditions. Thus, they demonstrate the positive correlation between oxygen supply and the lethal temperature but fail to

unearth mechanisms that link the two. For example, measuring embryo physiology (in addition to survival, as in other studies) allows us to estimate important breakpoints in temperature (e.g. pejus and critical temperatures) and consider how these might relate to survival in an ecological context (i.e. across different nest sites).

Contrary to our results, Angilletta et al. (2013) found that f_H stabilized prior to death, which is the expected relationship for physiological performance curves. The difference between their results and ours could be due to inter-specific variation in the thermal sensitivity of embryos. Indeed, reptile embryo f_H varies widely according to phylogeny (Du et al., 2011). Moreover, due to ecological factors (e.g. shallow vs deep nests) embryo physiology may have adapted to respond to extreme temperatures in species-specific ways (Ma et al., 2018). Alternatively, we may not have measured f_H across temperature at a fine enough scale to detect this stabilization phase. For example, f_H substantially declined just prior to death for two individuals (open circles in Figure 3), but we did not detect this for the other eggs. Regardless, our data indicate that the thermal sensitivity of f_H and VO_2 diverge at near-lethal temperatures, implicating a mismatch between oxygen supply and demand as a cause of death.

Repeated exposure to sublethal temperatures

In a separate study (Chapter 4), we subjected eggs to one or two thermal spikes with a peak of 43 °C. That study was conducted simultaneously with this one and utilized the same breeding colony, incubators, hatchling housing conditions, and incubation treatments. Therefore, results from these studies are comparable. For each response variable that was influenced by thermal spikes (egg survival, hatchling body size, hatchling growth and survival), the effect of 4 spikes was greater than that of 1 or 2. Thus, the negative effects of thermal spikes observed in

this study represent an accumulation of damage rather than immediate effects of high temperature. Younger embryos were more robust to the treatment (Figure 6a), possibly due to the relatively lower oxygen demand of early- vs late-stage embryos (Thompson and Stewart, 1997). Due to differences in embryo ages and the incubation temperatures used, the oldest embryos in the thermal spike assay had completed 57% of development, but those in the heat shock experiment had completed only 47% of development. This may, in part, explain why survival correlated with embryo age in the thermal spike assay but not the heat shock experiment. Indeed, the second half of squamate development is characterized by rapid growth of the embryo and a concomitant increase in oxygen demand, while the first half of development is characterized by organogenesis. Death at high temperatures likely results from multiple factors at different levels of biological complexity (Gangloff and Telemeco, 2018) and additional research is required to understand how these effects combine to set the thermal limits of complex life.

Exposure to thermal spikes had substantial effects on physiology since both developmental rate and f_H were suppressed by the treatment. Reduced developmental rates may have resulted directly from the reduction in f_H since the total number of heart beats determines the incubation period in reptiles (Du et al., 2009). However, thermal spikes reduced developmental rates by 5%, but the observed decrease in f_H (10% reduction for 2 days) can only account for 0.7% of this reduction. To fully account for the difference, f_H would need to be depressed for the remainder of development, but we do not know how long this f_H depression lasts. Depressed developmental rates can also result from diapause at extreme temperatures (Du et al., 2009); however, this is not likely since f_H and VO_2 remain relatively high across temperatures. Alternatively, high temperatures can induce cellular damage and subsequent repair (Sanger et al., 2018), which may slow developmental rates. Because hatchlings from the

experimental treatment were smaller in body size, they may have diverted energy away from somatic growth and toward repair and maintenance.

Slower developmental rates equate to longer incubation periods; thus, eggs are potentially exposed to adverse conditions for a longer time (e.g. egg depredation, extreme temperatures; Doody and Paul, 2013). However, the effect we observed equates to a 1- or 2-day increase in the incubation period, which may not be biologically important. Hatchling body size and growth rates were reduced by thermal spikes, which may be responsible for the decreased rate of hatchling survival we observed. Indeed, larger hatchling body size can enhance survival probability in lizards (Sinervo et al., 1992); however, other factors, like the timing of hatching, may be much more important (Pearson and Warner, 2018). Our study design (i.e. housing hatchlings communally) was ecologically meaningful since intraspecific competition is an important determinant of survival and growth for anoles (Calsbeek and Cox, 2010); however, it prevents us from precisely identifying the cause of the detrimental effects on hatchlings. Reduced survival and growth may have resulted from the treatment *per se* (i.e. physiological effects) or from a diminished ability for experimental hatchlings to compete with those from the control group (i.e. ecological effects). Such effects could also combine or interact.

Temperatures of nest sites in the field

The mean incubation temperatures for our control and experimental groups were 28.7 and 29.0 °C, respectively. If eggs were incubated at these two constant temperatures or at uniform, repeated fluctuations around them (e.g. sine wave), we would observe virtually no difference among treatments (Warner et al., 2012; Pearson and Warner, 2018). Yet, the inclusion of a few extreme fluctuations resulted in significant depression of embryo physiology and reductions in

egg and hatchling survival, hatchling body size and growth. These data indicate that studies that solely utilize chronic incubation conditions poorly predict the effects of natural developmental environments. This is particularly true when natural nest temperatures fluctuate widely (Figure S3).

Few studies have examined nest temperatures for *A. sagrei* in the field; however, each indicates that embryos are commonly exposed to pejus and critical temperatures. Gunderson et al (2020) report at least one nest peaking at or above 40 °C on 10 out of 16 days that temperatures were monitored. Sanger et al (2018) report average day-time temperatures of 36.6 °C and maximum temperatures of 44.5 °C. Pruett et al (2020) found that despite *A. sagrei* females' tendency to nest in sites that are relatively cool compared to what is generally available, mean daily maximum temperatures were 36.4 and 33.3 °C during June and July, respectively. Not surprisingly, egg survival was extremely low during the month of June (2 %). Our nest site temperatures demonstrate large variation in maximum nest temperatures across space and time (means of 28.6 °C vs 36.4 °C for 2013 and 2018, respectively). Thus, there is great potential for embryos to experience greater thermal stress in some years and locations than others. Although nest temperatures commonly reach the pejus temperature and occasionally reach the critical temperature, relatively few nests (< 5%) reach lethal temperatures (i.e. > 43 °C).

Stressful thermal events exhibit a high degree of spatial and temporal autocorrelation (Figures S3 and S4); thus, in the wild, embryos that experience one exposure to acute thermal stress are likely to experience several. Moreover, every field study thus far records maximum nest temperatures of ~44 °C, indicating that eggs experience near-lethal temperatures, despite evolved responses of females to nest in relatively cool areas (Tiatragul et al., 2020; Pruett et al., 2020). Therefore, future warming will certainly increase incidences of embryo thermal stress for

A. sagrei in the absence of embryo adaptation or adjustments in nesting behavior. This is potentially true for other species that nest in similar microhabitats.

CONCLUSIONS

Given the projected increases in the mean and variance of global temperatures, more research should be dedicated to understanding the effects of brief exposure of embryos to thermal stress (Burggren, 2018). Indeed, when such exposures induce mortality, they may have more influence on the evolution of thermally sensitive traits than mean temperatures (Buckley and Huey, 2016). Our results indicate that brown anole embryos have an EAHT that is much higher than the upper limit of the OTR. Moreover, we show that at near lethal temperatures there is a mismatch between oxygen demand and supply, which may contribute to death. However, when embryos are repeatedly exposed to temperatures below EAHT, thermal damage can accumulate, resulting in death or long-term effects on physiology that result in reduced survival of hatchlings. Thus, our study highlights the roles of both immediate and cumulative effects of high temperatures on embryo development, which can provide important insight into thermal adaptation and population response to predicted increases in local and global temperature.

Acknowledgements

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CHAPTER 2 SUPPLEMENTAL MATERIAL

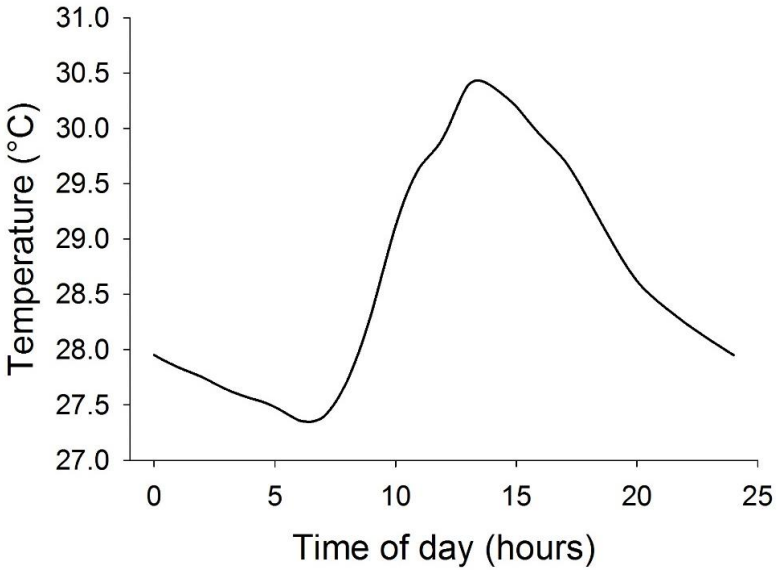

Figure S1. Thermal regime for incubation of most eggs. These temperatures are suitable for development of *A. sagrei* eggs (Tiatragul et al., 2017). Most eggs were incubated at these temperatures for the entirety of development. The only exception is that experimental eggs were removed from this fluctuation for 4 days to experience the thermal spikes. Control eggs were exposed to the fluctuation shown here during that time. Eggs used to determine the thermal sensitivity of heart rate and to measure rates of oxygen consumption were not incubated at these temperatures. They were incubated at a constant 28 °C until measurements of heart rate or metabolism were taken.

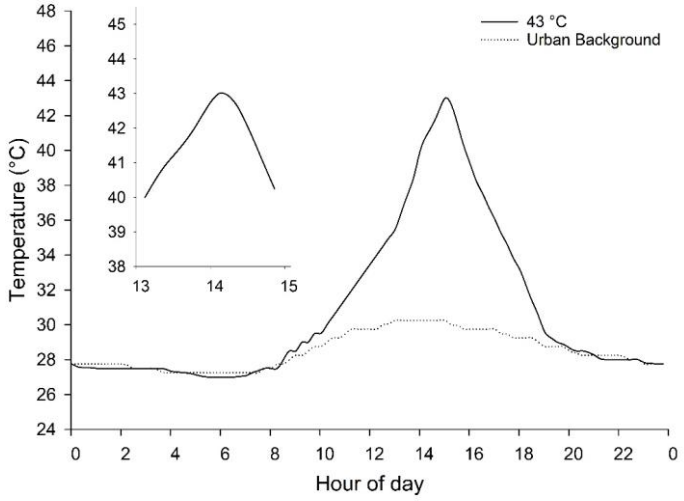

Figure S2. Temperature treatments used in our study. Fluctuations are based on field temperatures. The legend shows the peak temperature of the thermal spike. The inset figure in top left shows the peak at a finer scale. The urban background refers to the daily fluctuation used to incubate eggs before and after exposure to thermal spikes (i.e. Figure S1).

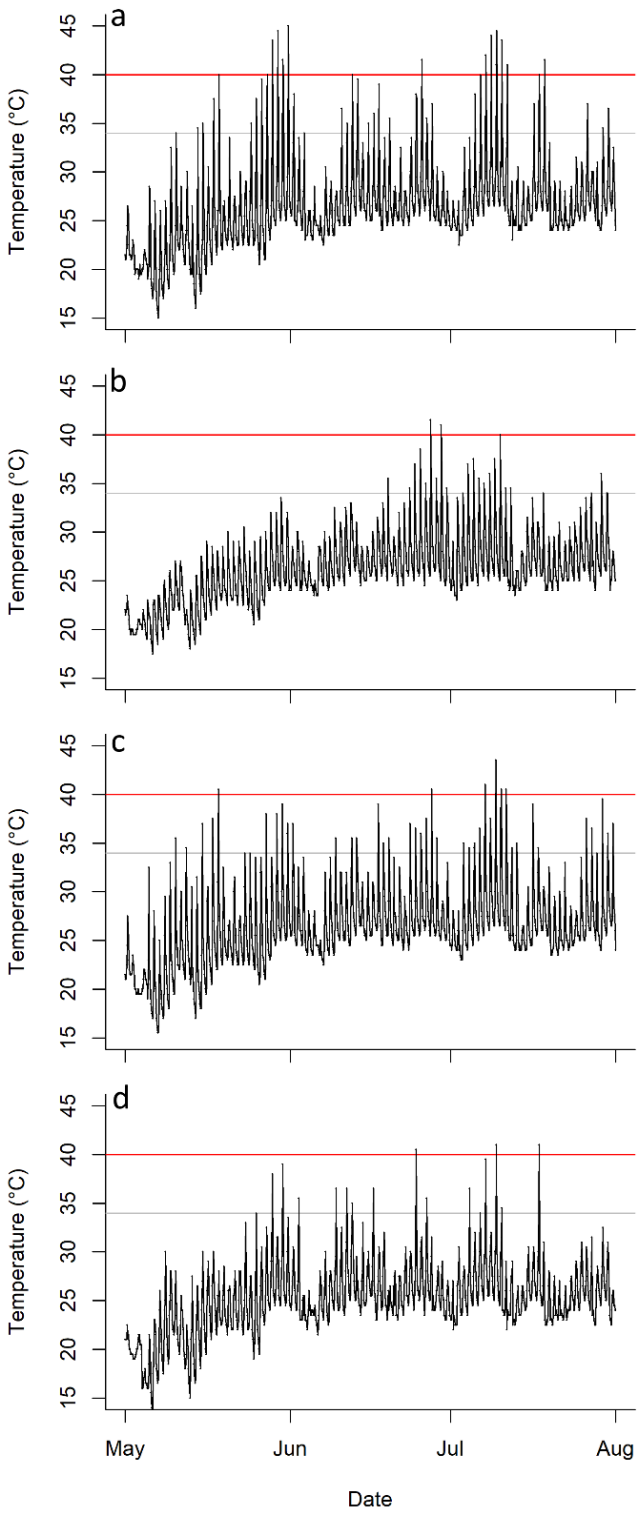

Figure S3. Temperatures from four *A. sagrei* nesting sites that experienced extreme temperatures (i.e. > 40 °C) during multiple days across the reproductive season. Solid black and red horizonal lines denote the pejus (34 °C) and critical temperatures (40 °C), respectively.

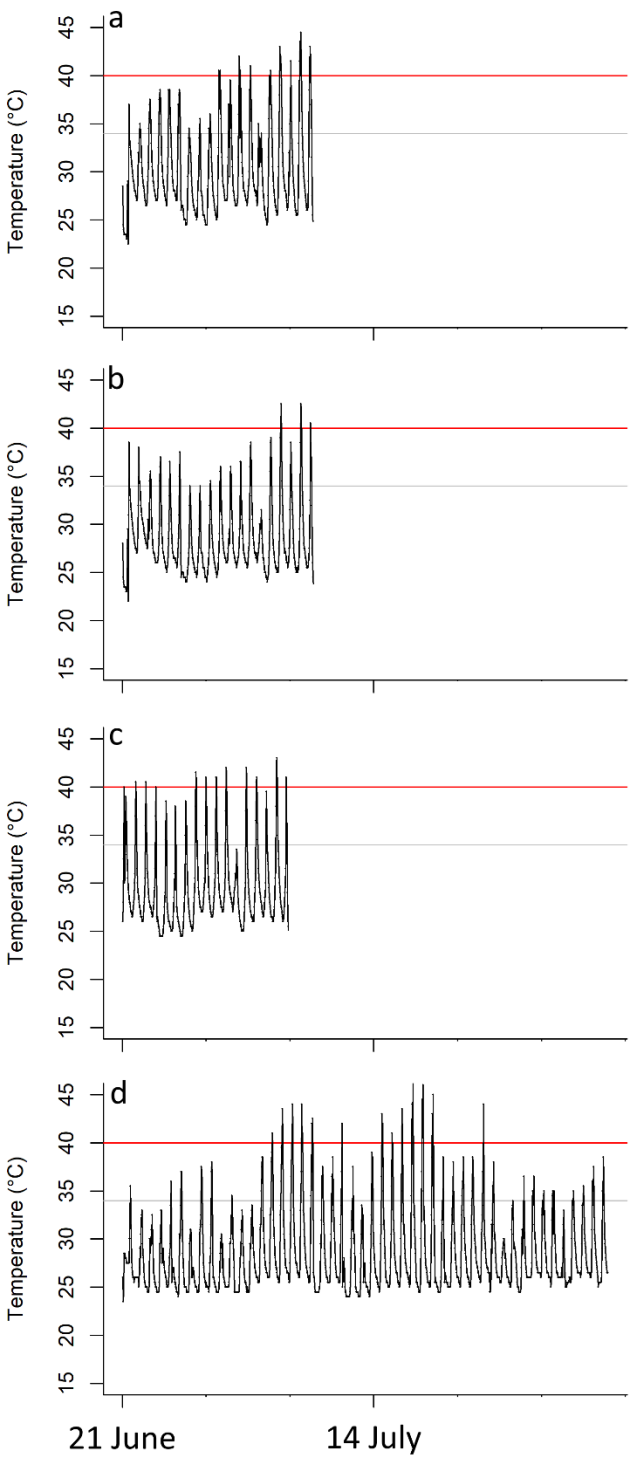

Figure S4. Temperatures from four *A. sagrei* nesting sites that experienced extreme temperatures (i.e. > 40 °C) during multiple days during 2018. Solid black and red horizontal lines denote the pejus (34 °C) and critical temperatures (40 °C), respectively. Panels a-c, show nests that were only monitored at the end of June and beginning of July.

Table S1. Comparison of linear and curvilinear relationship between *A. sagrei* embryo heart rate and temperature. Bold type denotes model chosen for analysis.

	Model	Df	AIC	logLik	p
Heart rate	Linear	1	815.81	-403.90	
	Linear + Quadratic	**2**	**804.59**	**-397.30**	**0.0003**
Respiration rate	Linear + Quadratic	4	190.26	-91.13	
	Asymptotic	**4**	**196.43**	**-94.22**	**0.0001**

Table S2. Sample size (n), raw mean, and standard deviation (sd) for egg incubation period and hatchling phenotypes of *A. sagrei* exposed to zero (control) or four thermal spikes at 43 °C.

	control			four spikes		
Response variable	n	mean	sd	n	mean	sd
incubation period (days)	34	29.79	1.04	27	31.37	1.24
hatchling SVL (mm)	34	18.04	0.73	27	17.73	0.6
hatchling mass (g)	34	0.1703	0.016	27	0.1597	0.0161
hatchling tail length (mm)	34	29.96	1.5	27	28.93	1.91
hatchling growth rate (mg/day)	20	3.43	1.78	12	2.18	1.55

Table S3. Sample sizes (n) and survival frequencies of eggs and hatchling lizards for *A. sagrei* exposed to zero (control) or four thermal spikes. The sample size for each treatment (control, four spikes) is given with the survival frequency for that sample in parenthesis.

Egg survival			Hatchling survival		
n	control	four spikes	n	control	four spikes
71	35 (0.97)	36 (0.75)	53	26 (0.77)	27 (0.44)

Table S4. Mean and standard deviation (sd) for heart rates (f_H) and temperatures (Temp) at which heart rates were measured. Day 1 was the day before treatment (thermal spike), day 2 was the day after treatment, and day 3 was two days after treatment. Sample sizes (n) are listed for each treatment group. This was a repeated measures design and the same individuals were measured across all three days.

		Day 1				Day 2				Day 3			
		f_H (bpm)		Temp (°C)		f_H (bpm)		Temp (°C)		f_H (bpm)		Temp (°C)	
Treat	n	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
Control	34	90.1	5.12	28.21	0.14	93.5	9.67	28.19	0.13	95.47	7.74	27.97	0.16
Four spikes	30	89.83	6.86	28.21	0.15	82.7	6.91	28.17	0.16	86.17	7.4	27.99	0.14

Table S5. Mean and standard deviation (sd) for the thermal sensitivity of embryo heart rates (f_H) of *Anolis sagrei* across nest temperatures.

	Hr (bpm)		Temp (°C)	
n	mean	sd	mean	sd
11	50.00	3.10	22.22	0.23
11	70.73	3.58	25.79	0.17
11	96.73	5.18	28.64	0.18
11	114.82	7.55	31.15	0.05
11	138.27	8.19	33.76	0.14
11	155.18	5.98	36.58	0.24
11	176.78	9.60	39.50	0.14

CHAPTER 3

Thermal spikes from the urban heat island increase mortality and alter physiology of lizard embryos

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INTRODUCTION

Human-modified habitats create novel conditions to which organisms must acclimate or adapt to survive. Urban habitats alter patterns of behavior (Lowry et al., 2013), influence rates of mortality (Koenig et al., 2002), increase population densities (Marzluff, 2001), and alter community assemblages (McIntyre et al., 2001). As such, urban environments have influenced the evolution of diverse taxa (microbes, plants, invertebrates, vertebrates; reviewed by Johnson and Munshi-South, 2017). Moreover, the effects of urban habitats on populations can be similar across the globe because of the relatively homogenous conditions that urbanization creates (McKinney, 2008; but see Niemelä et al., 2002). For example, urban areas are often characterized by a reduction in canopy cover and an increase in heat-absorbing surfaces (asphalt, concrete), which result in higher mean ambient temperatures and higher maximum temperatures in urban landscapes compared to adjacent rural or natural areas (i.e., the urban heat island effect; Arnfield, 2003).

Extreme temperatures in cities can differentially impact species and result in rapid thermal adaptation (Diamond et al., 2017). This is particularly important for ectotherms because their development, growth, and reproduction are dependent upon environmental temperature. Experimenters often quantify the thermal tolerance (e.g. critical thermal maximum) or thermal performance of adults to demonstrate adaptive responses to novel thermal conditions (Angilletta

et al., 2007; Diamond et al., 2017); however, few studies quantify the effects of the urban heat island on embryonic development and early life stages (Kaiser et al., 2016; Tiatragul et al., 2017). These stages are important to consider for three reasons. First, early life stages are extremely sensitive to environmental fluctuations and may have a narrower thermal tolerance than later stages (Pörtner, 2017). As such, abiotic conditions during embryonic development can have lasting effects on fitness-relevant phenotypes (Deeming and Ferguson, 1991; Warner, 2014; Kaiser et al., 2016). Second, because most ectotherms lay eggs which are immobile and cannot behaviorally compensate for adverse conditions, embryos, more so than adults, may be subjected to novel thermal regimes and novel selection pressures in urban environments. Although embryonic development can adapt or acclimate to local conditions (Angilletta et al., 2004; Du et al., 2010b,c), little is known about how embryos respond to extreme, but brief, spikes in incubation temperature like those in urban habitats. Finally, the thermal sensitivity of embryos has the potential to influence population dynamics and species distributions (Carlo et al., 2018), so understanding how embryos respond to extreme thermal stress is essential to predict how aspects of global change (e.g., urbanization, climate change) will impact population or species survival.

Anole lizards are excellent models for studying urban adaptation and acclimation because their development, growth, behavior, and reproduction are correlated with abiotic conditions that vary between urban and natural landscapes (e.g., temperature, moisture, structural complexity; Reedy et al., 2013; Kolbe et al., 2015; Tiatragul et al., 2017). Moreover, they have successfully invaded urban environments both within and outside their native ranges on multiple occasions, and past studies show that urban habitats can alter their behavior, physiology, reproduction, and morphology (Kolbe et al., 2015; Winchell et al., 2016; Chejanovski et al., 2017; Hall and

Warner, 2017; Tiatragul et al., 2017). Existing studies, however, are biased toward adult male lizards because they are relatively easy to locate, observe, and capture due to their larger size, site fidelity, and conspicuous breeding behaviors. Thus, few studies have addressed the influence of urban environments on earlier life stages, like embryonic development or hatchlings (Hall and Warner, 2017; Tiatragul et al., 2017).

Like most reptiles, anoles nest in the ground, so embryos are subjected to substantial and often unpredictable environmental variation during development. Soil temperature and moisture content, which can differ markedly between urban and natural areas, have significant effects on embryo development and, thus, hatchling characteristics (i.e., growth rate, body mass, and sprint speed; Reedy et al., 2013; Pearson and Warner, 2016). Little research, however, has been devoted to understanding how fluctuations or irregularities in incubation temperature contribute to patterns of embryonic development in ways that affect offspring phenotypes (e.g., Du and Ji, 2006; Les et al., 2009; Warner and Shine, 2009). Most of the work in this field has utilized constant temperature incubation (Bowden et al., 2014). This is a considerable drawback because irregular thermal events are characteristic of most nests, affect patterns of development (e.g., Angilletta et al., 2000; Warner and Shine, 2009; Carter et al., 2018), and are more common and extreme in urban areas (Tiatragul et al., 2017). Moreover, recent theoretical work predicts that thermal extremes, even when rare, can have more influence on the evolution of thermal performance than mean conditions (Buckley and Huey, 2016). Thus, studies of extreme temperatures (as opposed to 'typical' variation) are essential to understand the evolution of thermal tolerance (Buckley and Huey, 2016).

To address these issues, we conducted an egg incubation experiment to quantify how embryos from natural and urban areas respond to extreme, but brief, spikes in incubation

temperature. We bred lizards from city and forest populations and subjected their eggs to incubation regimes that included brief, but extremely high thermal spikes measured from the field. We made two, non-mutually exclusive predictions. First, embryos from city populations may have better hatching success and/or higher post-hatching growth and survival than those from forest populations when exposed to brief, thermal spikes (i.e. city embryos are adapted to city conditions). Second, regardless of population of origin (city vs forest), incubation at relatively warm, city temperatures may enhance survival and hatchling traits when subjected to a thermal spike (i.e. embryos acclimate to city conditions). This work provides an ecologically meaningful and novel evaluation of developmental adaptation and plasticity and advances our understanding of their roles in urban invasion and establishment.

METHODS

Lizard collection and husbandry

We collected adult crested anoles (*Anolis cristatellus* (Duméril and Bibron, 1837); females ≥ 36 mm snout-vent length (SVL); males ≥ 45 mm SVL) from one urban habitat (Red Road - henceforth "city") and one adjacent forested habitat (Matheson Hammock Park – henceforth "forest") in Miami, Florida. These sites are approximately 1.5 km apart. Matheson Hammock is a large fragment of dense forest and is structurally and thermally different from adjacent urban areas (Figure S1). From 29 April – 4 May 2016, we collected adult lizards from the forest (48 females, 20 males) and city (40 females, 23 males) by hand or noose. We used data from Tiatragul et al. (2017) to determine our sample sizes (i.e. number of lizards required to produce an adequate sample of eggs). Individuals were visually sexed per body size, dewlap size

and color, and the presence of post-anal scales in males. These animals were transported to Auburn University to form a captive breeding colony.

We housed females in single cages (29 × 26 × 39 cm; height × width × depth) illuminated with Reptisun 5.0 UVB and Tropic Sun 5500K Daylight bulbs (ZooMed Inc.) with a 12:12 hour light/dark cycle and maintained an ambient room temperature of 25.6 °C. Due to light sources, ambient cage temperatures were ~2-3 °C higher than room temperature and, during the day, maximum daily temperatures were 31-33 °C in the warmest part of the cage. Cages included two bamboo perches, an artificial plant, a nesting pot (plant pot filled with a mixture of soil and peat moss) and reptile cage carpet (Zoo Med Inc.) as a floor substrate. We fed lizards three crickets each (dusted with vitamins and calcium) three times per week and misted cages with water daily.

Because we had half as many males as females, each male was shared by two females and was rotated between them approximately once every two weeks. We paired males and females haphazardly, but individuals were not mixed between sites (i.e., males from the city were kept with females from the city).

Egg collection and treatment allocation

From 5 May to 23 September, we collected freshly laid eggs from nest pots three times each week. For each egg (forest population, n=179; city population, n=217), we recorded the mass (to 0.0001 g), date of oviposition, and maternal identity (ID). We placed each egg in individual petri dishes (60 x 15 mm) filled with moist vermiculite (-150 kPa) and wrapped the dish with parafilm to prevent evaporation. This ensured that the ambient humidity available to eggs was controlled across treatments. Eggs were then allocated to one of five incubation treatments that mimicked thermal regimes of potential nest sites in the forest and city. Because

Anolis lizards produce one egg every 7-10 days, each female's first egg was randomly assigned to a treatment and each successive egg she produced was assigned to one of the remaining treatments until she had at least one egg in each treatment. For females that laid more than five eggs (n = 35), the sequence of treatment allocation was repeated. This minimized potential biases from the order of egg production or maternal identity.

Creation of incubation treatments

Although little is known about the nesting ecology of anoles, they usually dig shallow nests in the soil (< 5 cm deep) or place eggs between the soil surface and cover materials (e.g., leaves, logs, pieces of bark; Rand, 1967). In June 2014, we placed temperature loggers (iButtons; n=5 in the forest, n=5 in the city) in locations likely used by anoles for nesting according to our own experience and data reported by Rand (1967). Each was placed at ~ 4 cm depth in the soil. The canopy openness over these locations was 20-90 % (mean=42%) in the city and 1-10% (mean=5%) in the forest. This was representative of the relatively open vs closed canopies of the city and forest, respectively.

For the city and forest, separately, we calculated the average soil temperature for each hour of the day from May – Sept (when most eggs are produced; Hall and Warner, 2017). These two thermal profiles (henceforth – "city profile" and "forest profile"; Figure 1A) represent average diel temperature cycles for the forest and city and were programmed into incubators (Memmert IPP 55^{Plus}) to loop daily for the duration of the study. These each served as control treatments. Additionally, we selected the warmest diel fluctuation (i.e., thermal spike; 43°C peak) recorded across all city temperature loggers (Figure 1B) and programmed an incubator to

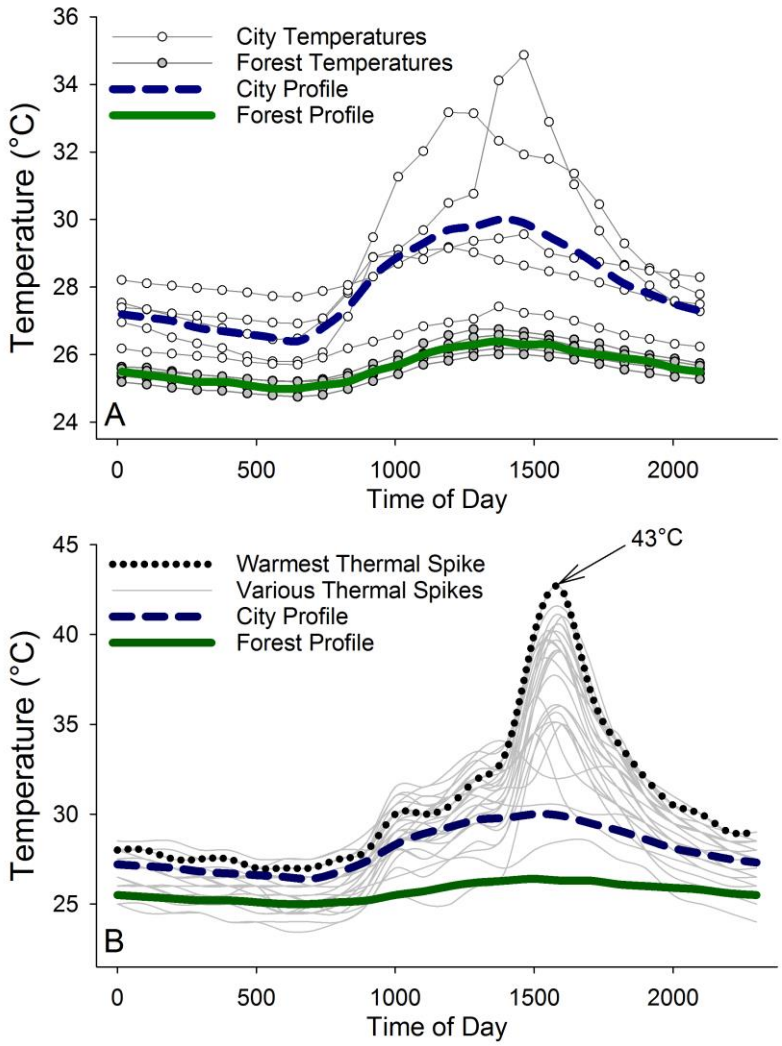

Figure 1. Thermal data from various potential nest sites in city (n=5) and forest (n=5) habitats (collected from 1 June – 15 Sept 2014). Panel (A) shows average hourly temperatures of nests from the city (open circles) and nests from the forest (closed circles) from May to September. Blue and green lines represent the mean of all nests from each habitat and served as our city and forest control incubation profiles, respectively. Panel (B) shows the range of various daily thermal spikes from city nests (light gray lines). Each gray line is a single daily fluctuation from a nest. The highest thermal spike (43°C peak) recorded across all potential nests was used in our study and is denoted with a string of closed circles. The city and forest incubation profiles are shown again in panel B to demonstrate contrast between the typical daily fluctuations and a thermal spike.

repeat this fluctuation daily. For a final incubator, we lowered the peak of this thermal spike by 4°C (the difference between the peak temperatures of the forest and city controls) and looped this fluctuation daily to assess the effect of a lower-magnitude thermal spike (39°C peak). Thus, we

used 4 incubators: 1 looping the city profile, 1 looping the forest profile, 1 looping a thermal spike that peaked at 43°C, and 1 looping a thermal spike that peaked at 39 °C.

A recent study (Tiatragul et al., 2019) confirmed that our incubation regimes represent the thermal conditions of actual anole nests at our sites. In this study, mean daily nest temperatures ranged from 25.3°C to 32.6°C in the city (n=43 nests) and 24.4°C to 28.6°C in the forest (n=43 nests). The warmest temperatures recorded in city and forest nests were 39.5°C and 33 °C, respectively.

Experimental design

With these four incubators, we created five incubation treatments: two controls and three experimental groups. One control group was exposed to the forest profile repeated daily throughout development, and a second was exposed to the city profile repeated daily (i.e., neither controls had a thermal spike; Figure 1A). One experimental group was exposed to the city profile but given a brief thermal spike of 43°C on a single day during development (Figure 2A). A second group was exposed to the forest profile and given a brief thermal spike of 43°C on a single day during development (Figure 2B). These four groups allowed us to determine if habitat-specific incubation temperatures (city vs forest profile) influence an embryo's response to thermal spikes. Though the height of the thermal spike (magnitude; 43°C) was the same for both experimental groups, the differential between the thermal profiles and the peak of the thermal spike was not (Figure 2A,B). Therefore, we created a third experimental group to be incubated at the forest profile and given a brief thermal spike of 39°C (Figure 2C). Comparing this group with the others allowed us to make inferences about the relative effect of a spike's magnitude vs its differential. Each of these five groups (see Table 2 for sample sizes) consisted

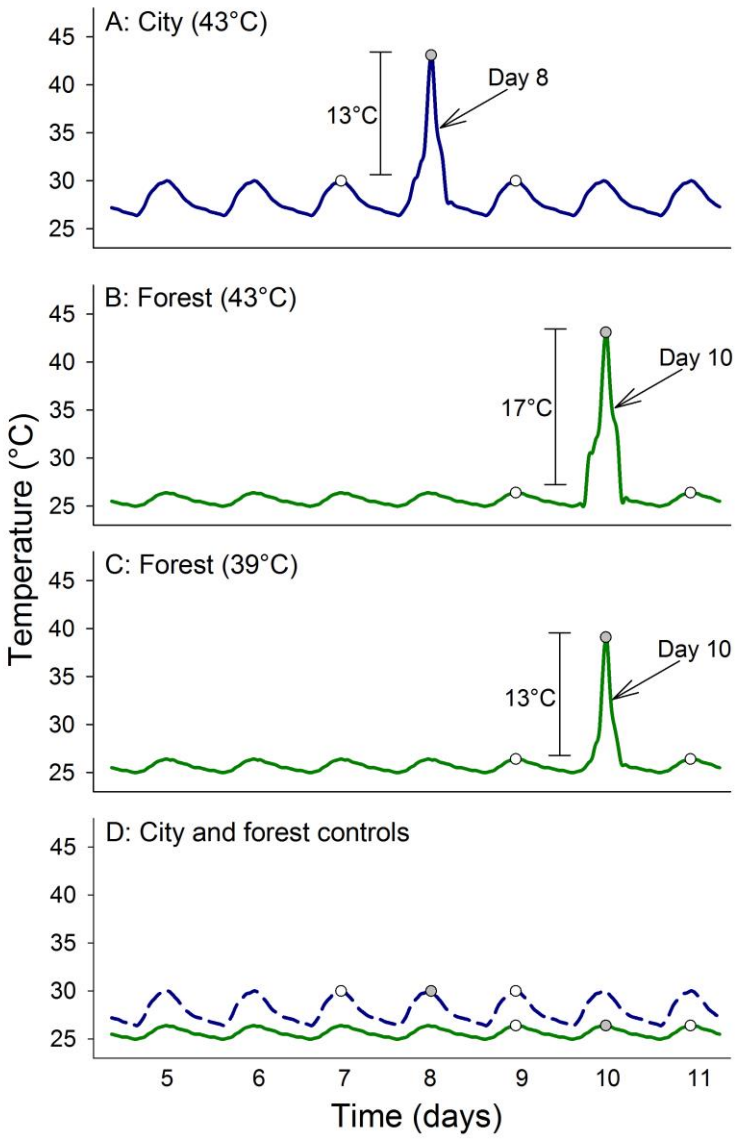

Figure 2. Diagrams representing our five incubation treatments. Blue and green colors denote treatments that utilize the city or forest incubation profile, respectively. The temperatures in parentheses are the peak of each thermal spike. One treatment (A) consisted of the city regime looped daily and a thermal spike delivered on day 8 of development. Two treatments (B,C) consisted of the forest regime looped daily and a thermal spike delivered on day 10 of development. Because developmental rate increases with temperature, thermal spikes were delivered two days later to eggs incubated at the forest temperatures than those incubated at city temperatures. This ensured that all embryos received the treatment at the same stage of development. Bars show the differential between the peak of the thermal regime and the peak of the thermal spike. Embryo heart rates were recorded for one subset of eggs at the peak temperature of each spike (closed gray circles) and for another subset on the day before and the day after a thermal spike (open circles). Two control treatments (D) consisted of either the forest (solid line) or city (broken line) regime repeated daily throughout the entirety of incubation (no thermal spike)

of eggs laid by females from both the city and forest; therefore, this 2 x 5 (population x treatment) factorial design allowed us to evaluate how embryos from different populations respond to habitat-specific incubation conditions and thermal spikes.

All eggs were placed into the incubator that looped their assigned thermal profile (city or forest). On the appropriate day (see below), we moved experimental eggs from these incubators to their assigned treatment incubator (either 39 or 43°C thermal spike). Control eggs were moved within the incubator (i.e., from one shelf to another) to account for any effect of moving eggs. Each egg remained in this position until the following day when eggs were returned to their original positions for the remainder of incubation. These manipulations were made each day prior to 0900 hours when the thermal spike began (Figure 1B). Eggs were incubated at their assigned incubation profile for the remainder of development (see Figure 3 for design summary).

Because developmental rate increases with temperature, thermal spikes were delivered two days later (i.e., day 10 of development) to eggs incubated at the cooler, forest profile than those incubated at the city profile (day 8). This ensured that all embryos experienced the treatment at approximately ¼ of the way through egg incubation. We estimate this is embryo stage 8 or 9 based on published data of developmental rates from Tiatragul et al., (2017) and the *Anolis* embryo staging series of Sanger et al. (2008). We chose this period to provide embryos time to potentially acclimate to their respective incubation regime (i.e., city or forest) yet expose them to the spike relatively early in development. Previous work on lizards shows that this early time is highly sensitive to temperature (Shine and Elphick, 2001; Andrews, 2004).

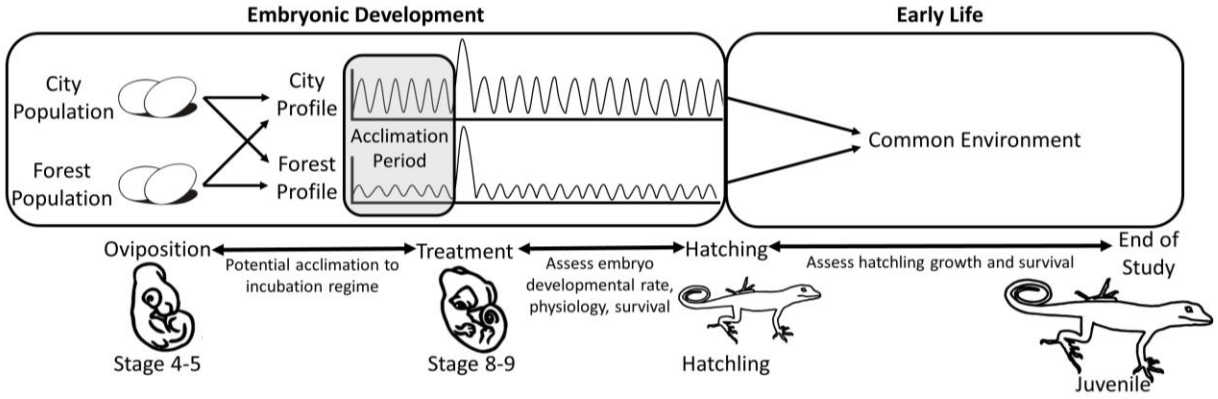

Figure 3. Overview of our design to measure the effects of city and forest thermal regimes on embryonic and early life stages of lizards from both city and forest populations. Thermal treatments were applied approximately ¼ of the way through development. See Figure 2 and methods for more details concerning the thermal treatments. Embryo staging and diagrams were adapted from Sanger et al. (2008).

Heart rate detection

To determine the immediate and lasting effects of thermal spikes on embryo physiology, we noninvasively measured heart rates using the Buddy® egg monitoring system (Du et al., 2010c). This device uses infrared light to detect the heart rate of small embryos. For a subset of eggs (n = 84) we measured heart rates at the peak of each thermal spike (henceforth "stressed heart rate") and for another subset (n = 62), we used a repeated measures design to measure heart rates 24 hours before and 24 hours after a thermal spike (henceforth "resting heart rate"). To measure heart rates, we quickly removed eggs from the incubator and placed them in the Buddy® (at room temperature) and obtained heart rate measures within 20-30 seconds. Some eggs had to be repositioned to obtain a heart rate, so we recorded the number of repositions (0, 1, or 2) and included this as a covariate in our analyses. These measurements have no effect on developmental rate or survival of anole embryos (Hulbert et al., 2017).

Hatchling measurement and husbandry

We recorded the hatch date, snout-vent length (SVL) and tail length (to the nearest 1mm using a ruler), and body mass (to the nearest 0.0001 g) of each hatchling (see Table 2 for sample sizes) and housed them in the lab for approximately 3 months (mean 84.3 days; standard deviation 12.18) to monitor growth and survival. Hatchlings were uniquely toe-clipped for identification and housed in cages with conditions identical to those described for adults except there was no nesting pot, and we provided additional foliage (artificial plants) to increase surface area for perching and hiding. Intraspecific competition is an important determinant of growth and survival in *Anolis* lizards (Calsbeek and Cox, 2010), so we housed hatchlings communally: 6 lizards per cage. Hatchling densities in the field are high (Lee et al., 1989) and comparable to our housing conditions (personal observation). Since eggs incubated at the city profile hatched sooner than those incubated at the forest profile, we segregated hatchlings according to incubation profile to prevent large discrepancies between the oldest and youngest individuals in each cage. Thus, we had two types of cages: some consisted of 3 lizards from the city control group and 3 from the city experimental group (6 total lizards per cage; n = 9 cages) while others consisted of 2 lizards from the forest control, and 2 from each of the 2 forest experimental groups (6 total lizards per cage; n = 20 cages). We assigned hatchlings to cages in order of hatching. Due to differential egg survival among groups, we produced an excess of hatchlings from some treatments, and some lizards (n= 61) were euthanized after hatching. These were systematically chosen throughout the study in a way that minimized age discrepancies among cage-mates. Although our cage assignments are not random, we created ecologically relevant housing for hatchlings that minimized biases due to order of hatching (thus, age and size).

We misted cages with water daily and fed lizards appropriately-sized crickets dusted with calcium and vitamins. We checked each cage for dead lizards prior to every feeding to ensure that, despite variation in the number of surviving lizards across cages, prey density was held constant through the study (4 crickets per lizard, 3 times each week). When all lizards in a cage had reached approximately 3 months of age, we noted survival and remeasured their SVL and body mass. Each lizard was then euthanized.

Statistical Analyses

We used generalized linear mixed effects models to analyze egg and hatchling survival and mixed effects linear models to analyze incubation period, heart rate, and body size (SVL, tail length, body condition, and mass). Initial hatchling body condition was a residual score from a second-degree polynomial regression of log mass vs log SVL (Schulte-Hostedde et al., 2005; but see also Peig and Green, 2010). Final hatchling body condition was calculated without the second-degree term because adding it did not improve fit (F_1=1.11; p=0.3). Each analysis included incubation treatment, population (city vs forest), and their interaction as fixed effects. We also included covariates when appropriate (see Table 1). We built two models for each analysis: one with maternal ID as a random effect and one without. We used likelihood ratio tests or chi square tests to determine which model best fit the data (Table 3). These analyses assessed the potential for maternal effects (e.g. genotype) to influence dependent variables; however, regardless of their outcome, we report the results from the model that included maternal ID (to avoid pseudo-replication of the population variable). For models of hatchling survival and measures of final body size, we also included hatchling cage as a random effect; thus, maternal ID and cage were modeled as crossed random effects. Because hatchlings in each cage varied in

age, we assigned the final lizard added to each cage an age of 1 day and each other lizard in that cage was given an age according to how many days it had been in the cage prior to the addition of the final lizard. We included this relative age in our model. The range of relative ages was 1-31 days and the average was 7.29 days. Most lizards (90.3%) had a relative age of less than 15 days. For stressed heart rate, we performed a Box-Cox transformation to account for heteroskedasticity. To achieve model convergence for egg survival and hatchling survival, we utilized the Nelder Mead optimizer and rescaled and centered all continuous covariates at zero (Bolker et al., 2009). For models with crossed random effects, we used the lme4 package and then generated p-values with the lmerTest package. This package calculates denominator degrees of freedom by Satterthwaite approximation like SAS proc mixed theory. Analyses were performed in R 3.1.3 (R Development Core Team, 2015).

RESULTS

Including maternal ID as a random effect significantly improved model fit for egg survival, initial hatchling mass, initial hatchling tail length, and final hatchling mass and SVL (Table 3); however, we observed no differences between city and forest populations for survival, development, or hatchling traits (Table 1). We also found no interactions between incubation treatment and population: eggs and hatchlings from the city and forest responded similarly to all treatments both before and after hatching (Table 1). The incubation treatment, however, significantly influenced egg survival, incubation period, and embryo heart rate but had no effect on the survival, morphology, or growth of hatchlings (Table 1). Egg survival decreased by as much as 36% when eggs were exposed to our most extreme thermal spike (43°C), but the lower spike (39°C) had no effect (Figure 4A; Table 2). Eggs incubated at cooler forest temperatures

Table 1. Effect of incubation treatment, population (city vs forest), and their interaction on embryonic development, physiology, and hatchling morphology. Symbols signify covariates: *egg mass, **trials, †initial body mass, ‡relative age, ¥ initial SVL, λ initial body condition. Sample sizes, means, and standard deviations are provided in Table 2. Statistics for each covariate are provided in Table S1. Survival test statistics are χ^2, others are F values. Bold type denotes statistical significance.

Dependent Variable	Incubation Treatment			Population			Interaction		
	df	F or χ^2	*P*	df	F or χ^2	*P*	df	F or χ^2	*P*
Egg *Survival	4	10.47	**0.033**	1	1.89	0.17	4	1.56	0.82
*Incubation Period	4,176	119.74	< **0.0001**	1,47	0.83	0.37	4,176	0.44	0.78
**Stressed Heart Rate	4,40	11.31	< **0.0001**	1,33	0.17	0.69	4,40	0.22	0.93
**Resting Heart Rate	4,52	19.42	< **0.0001**	1,52	0.34	0.56	4,52	2.49	0.055
Hatchling †‡Survival	4	3.3	0.51	1	0.23	0.64	4	3.55	0.47
*Initial Mass	4,176	1.95	0.10	1,47	1.02	0.32	4,176	1.84	0.12
*Initial SVL	4,176	0.32	0.86	1,47	0.48	0.49	4,176	0.28	0.89
*Initial Body Condition	4,176	1.09	0.36	1,47	1.83	0.18	4,176	1.28	0.28
*Initial Tail Length	4,176	1.94	0.11	1,47	0.18	0.68	4,176	0.49	0.74
†‡Final Mass	4,42	1.15	0.35	1,27	0.99	0.33	4,49	2.24	0.08
¥‡Final SVL	4,50	2.25	0.08	1,28	2.94	0.10	4,50	2.39	0.06
λ‡Final Body Condition	4,51	1.41	0.24	1,34	2.24	0.14	4,52	1.77	0.15

Table 2. Sample size, mean, and standard deviation of raw data. Because we observed no population (city vs forest) by incubation treatment interactions, we combined data from both populations to generate these means. Resting heart rate is the mean difference between the heart rates measured one day before and one day after a thermal spike (after minus before).

Treatment	Forest		City		Forest (39°C)		Forest (43°C)		City (43°C)	
	n	mean (s.d.)	n	mean (s.d.)	n	mean (s.d.)	n	mean (s.d.)	n	mean (s.d.)
Egg Survival (%)	79	0.67	81	0.67	78	0.68	74	0.55	76	0.43
Incubation Period (days)	54	42.73 (2.23)	54	34.85 (2.12)	53	43.31 (2.37)	41	44.02 (2.44)	33	35.71 (1.82)
Stressed Heart Rate (bpm)	21	94.14 (9.67)	17	101.65 (16.41)	16	149.44 (43.42)	14	166.93 (44.59)	16	175.31 (45.64)
Resting Heart Rate (bpm)	19	-3.68 (7.58)	17	-2.00 (21.85)	7	-8.29 (12.46)	8	-12.75 (7.85)	11	-12.09 (16.97)
Hatchling Survival (%)	40	0.46	27	0.44	40	0.36	40	0.5	27	0.31
Initial Mass (g)	54	0.2288 (0.0283)	54	0.2284 (0.0367)	53	0.2235 (0.0344)	41	0.2315 (0.0312)	33	0.2359 (0.0269)
Initial SVL (mm)	54	19.34 (1.02)	54	19.29 (1.11)	53	19.34 (0.99)	41	19.27 (0.84)	33	19.24 (0.90)
Initial Body Condition	54	-0.0001 (0.10)	54	0.0006 (0.13)	53	-0.03 (0.12)	41	0.008 (0.11)	33	0.035 (0.10)
Initial Tail Length (mm)	54	28.92 (1.6)	54	28.94 (1.63)	53	28.62 (1.98)	41	28.32 (1.6)	33	28.3 (2.26)
Final Mass (g)	19	0.6020 (0.1950)	12	0.5357 (0.1424)	14	0.5439 (0.1494)	20	0.5057 (0.1681)	8	0.5620 (0.1983)
Final SVL (mm)	19	25.95 (2.84)	12	24.83 (2.12)	14	24.43 (1.60)	20	24.25 (2.34)	8	25.13 (2.95)
Final Body Condition	19	-0.032 (0.13)	12	0.002 (0.13)	14	0.054 (0.14)	20	-0.002 (0.15)	8	-0.018 (0.14)

Table 3. Comparisons of models with and without maternal ID as a random effect. Bold type denotes models where including maternal ID significantly improves the fit of the model. Asterisk (*) denotes a chi square test statistic. All others test statistics are a loglikelihood

		With Maternal ID			Without Maternal ID				
		AIC	BIC	logLik	AIC	BIC	logLik	Test Stat	P
Egg	Survival	**510.07**	**557.61**	**-243.04**	519.86	563.43	-248.93	***11.78**	**0.001**
	Incubation period	1011.04	1055.34	-492.52	1010.59	1051.47	-493.29	1.54	0.21
	Stressed heart rate	-454.29	-424.51	240.14	-455.97	-428.49	239.99	0.31	0.57
	Resting heart rate	872.63	923.59	-417.32	870.63	918.91	-417.32	0.01	0.99
Hatchling	Survival	254.83	298.98	-113.42	252.97	293.97	-113.49	*0.14	0.71
	Initial mass	**-930.90**	**-886.60**	**478.45**	-926.69	-885.80	475.35	**6.21**	**0.01**
	Initial SVL	654.44	698.73	-314.22	652.87	693.75	-314.43	0.43	0.51
	Initial body condition	-318.94	-274.65	172.47	-320.18	-279.29	172.09	0.77	0.38
	Initial tail length	**926.27**	**970.57**	**-450.14**	936.02	976.91	-456.01	**11.74**	**0.001**
	Final mass	**-48.1**	**-13.74**	**39.05**	-40.92	-8.85	34.46	***9.18**	**0.002**
	Final SVL	**331.13**	**365.49**	**-150.57**	335.18	367.25	-153.59	***6.05**	**0.014**
	Final body condition	-81.46	47.11	55.73	-80.21	-48.14	54.105	*3.25	0.07

had an incubation period 7.87 days (± 0.39 SE) longer than eggs incubated at city temperatures (Figure 4B; Table 2). Thermal spikes had minimal effect on incubation period; however, eggs from all three experimental groups took longer to hatch than their respective controls (Figure 4B). This was only statistically significant for one group: eggs incubated at the forest profile and given a 43°C spike took 1.37 days (± 0.42 SE) longer to hatch than the control (Table 2). Finally, the incubation treatments altered embryo physiology (Table 1). Heart rate increased substantially during a thermal spike (Figure 5A); however, on the day following treatment, embryos that experienced a thermal spike had lower resting heart rates than controls and lower heart rates than their own pre-exposure baselines (Figure 5B; Table 2).

DISCUSSION

Urban environments create novel thermal conditions that differ from adjacent non-urban areas in both mean and extreme temperatures. Because early life stages (i.e. embryos, hatchlings) of reptiles are very sensitive to abiotic conditions, extreme temperatures in urban habitats may have consequences for survival, physiology, development, and growth. Although embryos can adapt or acclimate to local conditions, virtually nothing is known about how they respond when briefly exposed to extreme temperatures, and even less is known about the influence of urban incubation regimes on development. Our experiment does not support the hypothesis that anole embryos have adapted to local urban incubation regimes, nor does it show evidence that embryos acclimate to urban thermal conditions. The extreme ground temperatures of urban environments, however, do have the potential to increase egg mortality and alter patterns of development.

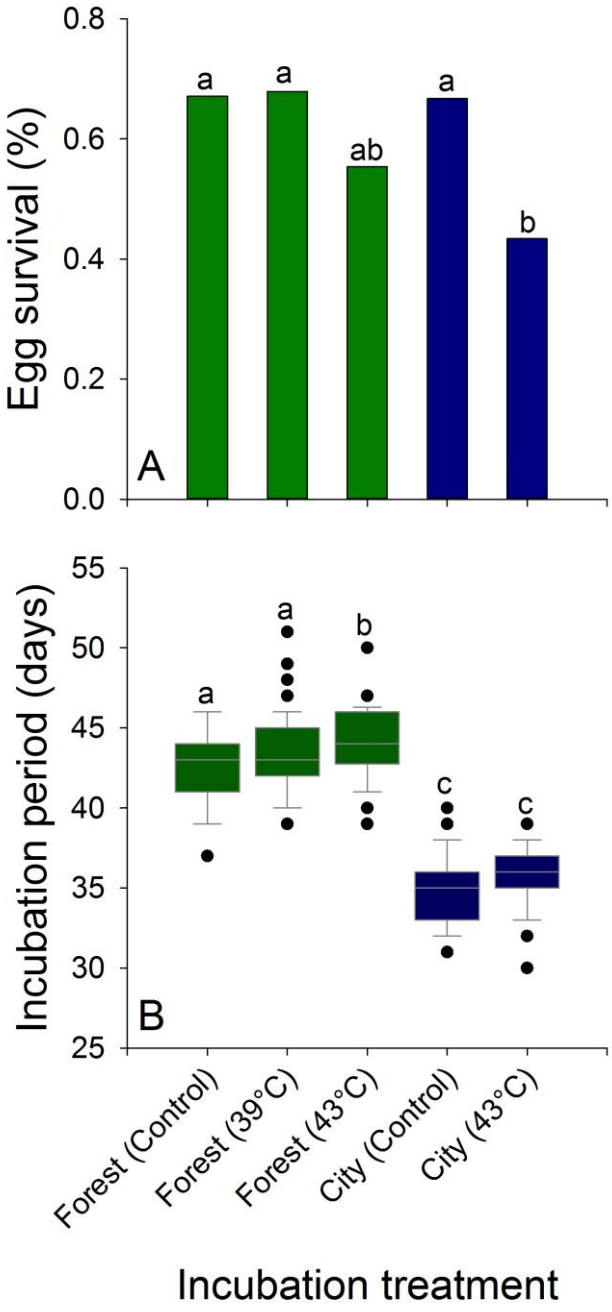

Figure 4. Egg survival (A) and incubation period (B) for each treatment. Green and blue bars denote treatments with the forest and city incubation profile, respectively. Boxes and whiskers show quartiles, solid horizontal lines show the median, and closed circles show raw data points that are above, below the upper, lower quartiles. Letters show groups that were statistically different from one another after false discovery rate correction. Because we found no interaction between population (city vs forest) and incubation treatment, we combined data from both populations for these graphs. See Table 1 and Table 2 for results of statistical tests, estimates, and sample sizes.

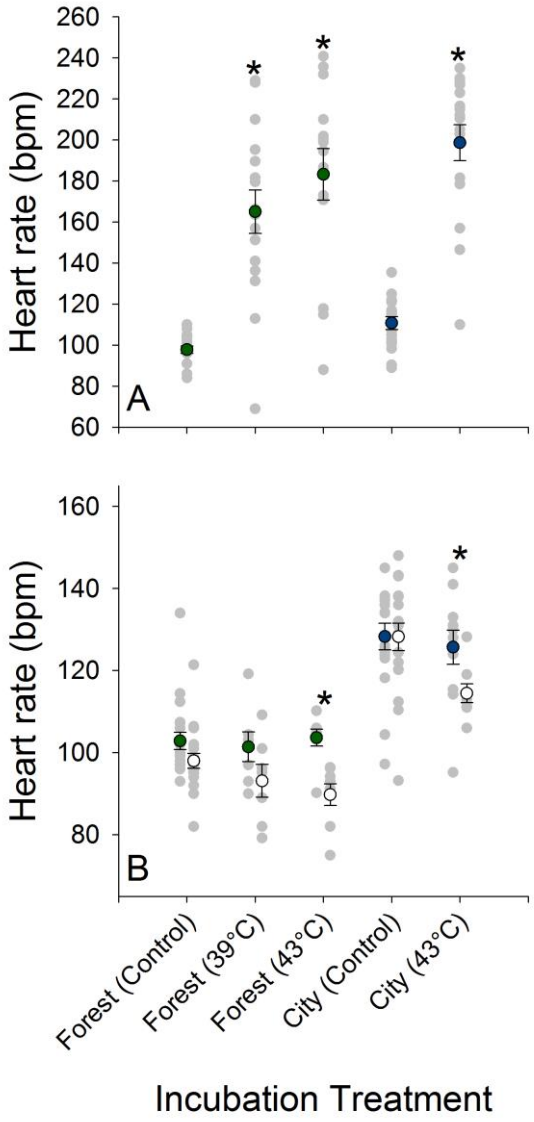

Figure 5. Embryo heart rates before, during, and after a brief thermal spike. Gray circles show raw data and bars represent standard error. Green and blue circles denote treatments with the forest and city incubation profile, respectively. Panel (A) shows mean stressed heart rates (closed green or blue circles) at the peak of each thermal spike. Controls received no thermal spike; thus, heart rates were recorded at the peak of a typical diel cycle. The temperatures at which heart rates were measured were 26, 30, 39, 43, and 43°C, which are the peak temperatures of each treatment listed from left to right. Panel (B) shows mean resting heart rates recorded 24 hours before (closed green or blue circles) and 24 hours after (white circles) a thermal spike. Heart rates were recorded at the peak of a typical diel cycle, 26 and 30°C for forest and city regimes, respectively. Because we found no interaction between population (city vs forest) and incubation treatment, we combined data from both populations for these graphs. Asterisks signify statistical difference between (A) an experimental group and its associated control or (B) heart rate before and after a thermal spike. P values were adjusted for false discovery rate detection. See Table 1 and Table 2 for results of statistical tests, estimates, and sample sizes.

Population-specific effects

Embryos from two different populations may differ in their response to the same developmental conditions if local embryo adaptation has occurred or if maternal effects (i.e., nesting behavior, resource allocation to eggs) influence responses to local developmental environments (these are not mutually exclusive). For example, embryo physiology and maternal nesting behavior of the eastern fence lizard (*Sceloporus undulatus*) are adapted to the cool temperatures and short reproductive season of high-latitude environments (Angiletta et al., 2004). Females in these populations nest in warm, open canopy microhabitats, and their embryos develop more quickly at cool temperatures than those from low-latitude populations.

Relatively few studies have explored local adaptation of reptile embryos (Angilletta et al., 2004; Du et al., 2010b; Angilletta et al., 2013), and only one has explored potential adaptation to urban environments. Although Tiatragul et al. (2017) found no evidence that lizard embryos are adapted to urban thermal conditions, they did not expose embryos to the extremely high temperatures that often occur in urban areas. Reptile embryos can employ a diversity of strategies to physiologically respond to variable nest temperatures (Du and Shine, 2015); however, adaptation of reptile embryos to extremely high temperatures has not yet been observed (Angilletta et al., 2013). This may be because maximum temperatures vary less than minimum temperatures as latitude increases, and thus, critical thermal maximums of adult ectotherms are often uniform throughout their range (Sunday et al., 2011). Extreme temperatures in cities, however, may be above the range of naturally occurring maximum temperatures and induce novel selection on embryo phenotypes.

Maternal effects also have the potential to shield embryos from adverse environmental conditions. For example, females can influence offspring phenotypes through differential

allocation of resources to eggs (i.e., yolk or hormones), and these allocations can be environmentally dependent (e.g., maternal diet; Rutstein et al., 2004; Lovern and Adams, 2008; Warner and Lovern, 2014). Chejanovski et al. (2017) found that, urban anoles can be less responsive when offered food and have greater body size and body condition when compared to non-urban congeners, indicating that food is more abundant in urban areas. Indeed, the city females used in our study had greater body size and condition than those from the forest (Hall and Warner, 2017) and may have started the breeding season with greater fat reserves to fuel reproduction. Therefore, population-specific maternal effects could potentially influence how embryos respond to brief, thermal stress.

Despite the potential for adaptation or maternal effects to influence development, embryos from both populations responded similarly to brief, thermal spikes, and so our data do not support our prediction that embryos from the city are better able to mitigate the adverse effects associated with urban thermal incubation environments than those from the forest. We suggest four, non-mutually exclusive explanations. First, the two study populations are only ~ 1.5 km apart. Although separated by a canal, plenty of roads and walkways cross the water; hence, gene flow between our city and forest populations could stifle local adaptation. Second, our study populations may not have had sufficient time to genetically diverge from one another. Indeed, *A. cristatellus* was first detected in South Florida in 1976, and because these lizards mature in about 1 year, approximately 40 generations have passed. This time is sufficient for local adaptation of adult phenotypes (e.g., thermal tolerances of *A. cristatellus* have adapted to the climate of south Florida; Kolbe et al., 2012; Leal and Gunderson, 2012). We do not know, however, how much time is necessary for embryonic phenotypes to adapt to local conditions.

Embryo phenotypes may take longer to adapt to local conditions than adults if maternal nesting behavior is highly plastic (Doody et al., 2006).

Third, nesting behavior can potentially shield embryos from adverse environmental conditions (Doody et al., 2006; Reedy et al., 2013). Indeed, in urbanized areas, females prefer to nest in relatively cool microhabitats compared to what is generally available (Tiatragul et al., 2020). This may protect embryos from selection pressures that would induce thermal adaptation. If, however, nests are exposed to thermal regimes like those in our study, extreme temperatures may reduce egg survival in the city by as much as 20%. This could be a conservative estimate since we only delivered a thermal spike on a single day during development. Eggs in cities may experience multiple thermal spikes that vary in magnitude (Figure 1), and the compounding effects of such repetitive, acute thermal stresses are certainly detrimental (see Chapter 2). Conversely, thermal spikes may be rare enough through space and time that they induce little selective pressure. Out of 615 daily thermal cycles measured in the city (123 collection days x 5 collection locations), ground temperatures met or exceeded 39°C only 20 times (3.3%).

Although urban-adaptation of embryos has not yet occurred, accounting for variance due to maternal ID improved model fit for egg survival and some aspects of hatchling morphology; thus, there is at least the potential for embryo adaptation to occur since some of this maternal variance is likely genetic. We conclude, however, that city environments impose selection on embryos to adapt to extreme temperatures, on female nesting behavior, or both. Since we do not see evidence of embryo adaptation to urban incubation conditions, nest-site choice may be how lizards cope with these extreme temperatures (Tiatragul et al., 2020).

Effects on embryo and hatchling survival and phenotypes

Tiatragul et al., (2017) suggest that *A. cristatellus* embryos are physiologically robust to variation in nest temperature, and, therefore, urban habitats may enhance fitness due to increased rates of embryonic development. They further speculate this may facilitate the establishment of *Anolis* lizards outside their native range since their spread can be associated with urban sprawl. Like Tiatragul et al. (2017), we found that urban incubation temperatures enhanced developmental rate at no apparent cost to hatchling morphology or survival, and we observed no population-specific effects on embryo development (i.e. no adaptation). However, we show that embryos are not robust to the urban thermal landscape when thermal spikes are considered, and, thus, the positive effects of increased developmental rate in urban nests may come at a cost to egg survival due to thermal spikes. The conflicting conclusions of their work and ours demonstrate that assessing the impact of urban conditions on wildlife is a complex task and will likely require a synthesis of various studies. Additionally, multiple aspects of life history must be considered across all life stages. For example, past work shows that fecundity of lizards may be enhanced in urban areas (Lucas and French, 2012; Hall and Warner, 2017) relative to adjacent rural or natural areas; however, if egg mortality is greater in urban habitats, the combined effect of the urban landscape on reproduction and development may be zero sum with respect to fitness.

Exposure to extreme nest temperatures may be uncommon in the field, but theoretical models demonstrate that even rare exposure to extreme temperatures can substantially alter thermal performance and sensitivity (Buckley and Huey, 2016). This may be particularly important for embryos which have little opportunity to behaviorally compensate for extreme temperatures (Du and Shine, 2015). Although studies of incubation temperature on reptile development abound, most studies have used constant temperatures or fluctuating temperatures

that do not mimic natural conditions (Noble et al., 2018a). The consequences of fluctuating temperatures on development differ from those of constant temperatures, even when mean temperature is the same (Les et al., 2009; Bowden et al., 2014). Given that two aspects of global change, urbanization and climate change, both ensure that extreme nest temperatures will become more common, we need a better understanding of the effects that brief, thermal spikes have on development and survival.

Effects on embryo physiology

For complex organisms like animals, the processes of ventilation and circulation likely set the limits of thermal tolerance: at high temperatures, the body's demand for oxygen exceeds supply (Pörtner, 2002; the oxygen- and capacity-limited thermal tolerance concept, see Pörtner et al., 2017). Reptile eggs obtain oxygen via diffusion through the shell, thus increasing heart rate is necessary to supply oxygen to tissues during heat stress (i.e., no ventilation apparatus). Reptile embryo heart rates generally increase by a factor of 1.5-2.6 for a 10°C rise in temperature (Angilletta et al., 2013), and our measurements of anole heart rates during thermal spikes are similar (Figure 5A).

Few studies have attempted to measure the thermal tolerance of reptile embryos (reviewed in Chapter 1). These studies either increased incubation temperature until embryo mortality reached 100% or quantified the influence of various concentrations of atmospheric oxygen on embryo survival. Studies that use mortality as a metric for thermal tolerance or that only explore the effect of ambient oxygen supply on mortality cannot identify the underlying mechanisms that determine the thermal tolerance of embryos (e.g. Pörtner et al., 2017). Thus, studies that put less emphasis on mortality and more on sublethal thermal constraints of

respiration or metabolism will provide novel insights (e.g., heart rate; Pörtner et al., 2017). To better understand what factors are associated with survival at ecologically-relevant levels of thermal stress, embryos should be pushed to their tolerance limits and given the opportunity to recover. In doing this, we discovered two subtle effects of thermal spikes on reptile embryo physiology: developmental rate and heart rate were depressed in embryos that survived a thermal spike.

Compared to respective controls, incubation period was slightly longer for all three experimental groups, though only one was statistically significant (Figure 4B; Table 2). This could be due to differential survival of embryos based on metabolic rate. Embryos with relatively high metabolic rates, and thus higher developmental rates, may exhibit greater oxygen consumption and be more likely to die during a thermal spike due to hypoxia (Smith et al., 2015). Conversely, this slight increase in incubation period may be due to metabolic depression induced by thermal stress since thermal spikes reduced heart rate by ~ 10% (Figure 5B). This heart rate depression was not due to differential mortality because we utilized a repeated measures design and any embryos that were killed during the thermal spike were not included in our analysis. If thermal spikes result in reduced heart rates for one or two days, this would correspond with a subsequent increase in incubation period since incubation period negatively covaries with heart rate (Du et al., 2010b; Du et al., 2009).

The biological significance of these physiological effects is difficult to assess. An increase in incubation period of 1 day is likely not ecologically meaningful, especially in comparison to the 8-day decrease in incubation period caused by the city temperatures, generally (Table 2). Furthermore, because we observed no adverse effects of thermal spikes on post-

hatching growth or development, we have no reason to assume that the observed changes in physiology were permanent or detrimental.

Though the consequences reported here might seem insignificant, the effect could accumulate if embryos are repeatedly exposed to thermal spikes during development (Chapter 2). Indeed, in reptiles with temperature-dependent sex determination (e.g. turtles), repeated, extreme thermal fluctuations at high temperatures can increase the incubation period and reverse sex ratios because developmental rates are depressed at extremely high temperatures (Neuwald and Valenzuela, 2011). Our data, however, compels us to consider that development may not only slow during exposure to such extreme fluctuations but also for some time after exposure. Further studies are needed to determine how long the heart rate depression we observe may last and the extent to which it corresponds with changes in metabolic rate or survival. In other animals, metabolic depression can mitigate the adverse effects of thermal stress (Guppy and Withers, 1999; Pörtner et al., 2017); however, this has never been considered for reptile embryos.

Potential acclimation to urban incubation temperatures

Reptile embryos can acclimate to local nest conditions (Du and Shine, 2015). Du et al., (2010b) found that embryos have relatively high metabolic rates when acclimated to cool temperatures, allowing them to partially compensate for the reduction in developmental rate caused by cooler incubation temperatures. We do not yet know, however, if such acclimation can ameliorate the adverse effects of extreme fluctuations in nest temperature. Since hypoxia is a likely cause of death at extremely high temperatures (Chapter 2), we predicted that embryos incubated at cooler, forest temperatures would have relatively higher metabolic rates due to acclimation than those incubated at warmer, city temperatures and would, thus, be more

susceptible to death during a thermal spike because of their relatively greater oxygen consumption. Embryos incubated at city temperatures would, therefore, better mitigate the adverse effects of urban, thermal spikes. We observed the opposite trend: embryos incubated at city temperatures were least likely to survive a thermal spike (Figure 4A). Likewise, recent studies show that higher incubation temperatures can actually lower the thermal tolerance of early life stages and result in poorer performance and increased mortality under thermal stress (Dayananda et al., 2017). Much of this research is intended to assess the vulnerability of ectotherms to climate change; however, changes in temperature due to climate warming may be comparable to those caused by urbanization (Youngsteadt et al., 2015). We think that further study of this phenomenon is warranted. The adverse effects of high incubation temperatures in urban areas are likely to be exacerbated by future climate-change, and the ability for females to ameliorate these effects through nesting behavior is limited (Telemeco et al., 2009).

CONCLUSIONS

Although we show that thermal spikes caused by the urban heat island effect have the potential to reduce egg survival, we did not detect any evidence that embryos are adapted to urban incubation conditions. We also found no evidence that embryos can acclimate to urban incubation temperatures in ways that mitigate the risks associated with extremely high temperatures. However, we show that brief thermal spikes have the potential to reduce egg survival and alter embryo physiology both during and at least 24 hours following exposure. Though past work demonstrates that urban conditions have positive impacts on body size, reproduction, and embryo development, extreme fluctuations in incubation temperatures may reduce egg survival and, thus, fitness. Most studies of urban adaptation have been conducted on

adults, but we emphasize that studies focused on multiple aspects of life history across all life stages will better assess the impact of urbanization on wildlife. A synthesis of such work would create a more accurate picture of the various ways that global change influences evolutionary processes.

Acknowledgements

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CHAPTER 3 SUPPLEMENTAL MATERIAL

Table S1. Statistics for covariates. Initial morphology refers to the initial body size metric that corresponds with the final metric: initial mass, initial SVL, and initial body condition were covariates for final mass, final SVL, and final body condition, respectively. For hatchling survival, we used initial hatchling mass as the initial morphology covariate. An asterisk (*) denotes estimates of transformed data. Bold text denotes statistical significance. See Figure S2 for graphical representation of each significant covariate.

Dependent Variable	Egg mass		Trials (0, 1, 2)		Initial Morphology		Relative age	
	β±SE	Test stat; *P*	β±SE	Test stat; *P*	β±SE	Test stat; *P*	β±SE	Test stat; *P*
Egg Survival	4.56 ± 4.87	X^2_1=0.88; *P*=0.35	-	-	-	-	-	-
Incubation Period (days)	**-16.38 ± 4.98**	**$F_{1,176}$=10.82; *P*=0.0012**	-	-	-	-	-	-
*Stressed Heart Rate (bpm)	-	-	**-0.003 ± 0.001**	**$F_{1,40}$=6.3; *P*=0.016**	-	-	-	-
Resting Heart Rate (bpm)	-	-	**-9.54 ± 1.33**	**$F_{1,56}$=51.14; *P*<0.0001**	-	-	-	-
Hatchling Survival	-	-	-	-	-0.002 ± 0.005	χ^2_1=0.09; *P*=0.76	0.003 ± 0.003	χ^2_1=1.16; *P*=0.29
Initial Mass (g)	**0.61 ± 0.066**	**$F_{1,176}$=83.43; *P*<0.0001**	-	-	-	-	-	-
Initial SVL (mm)	**12.05 ± 2.21**	**$F_{1,176}$=29.85; *P*<0.0001**	-	-	-	-	-	-
Initial Body Condition	**1.61 ± 0.25**	**$F_{1,176}$=40.52; *P*<0.0001**	-	-	-	-	-	-
Initial Tail Length (mm)	**18.1 ± 4.3**	**$F_{1,176}$=17.7; *P*<0.0001**	-	-	-	-	-	-
Final Mass (g)	-	-	-	-	**2.24 ± 0.82**	**$F_{1,58}$=7.37; *P*=0.0088**	0.005 ± 0.003	$F_{1,55}$=2.3; *P*=0.13
Final SVL (mm)	-	-	-	-	**0.77 ± 0.25**	**$F_{1,60}$=9.22; *P*=0.004**	**0.1 ± 0.041**	**$F_{1,56}$=6.2; *P*=0.016**
Final Body Condition	-	-	-	-	0.35 ± 0.18	$F_{1,60}$=3.7; *P*=0.06	-0.0041 ± 0.003	$F_{1,59}$=2.65; *P*=0.11

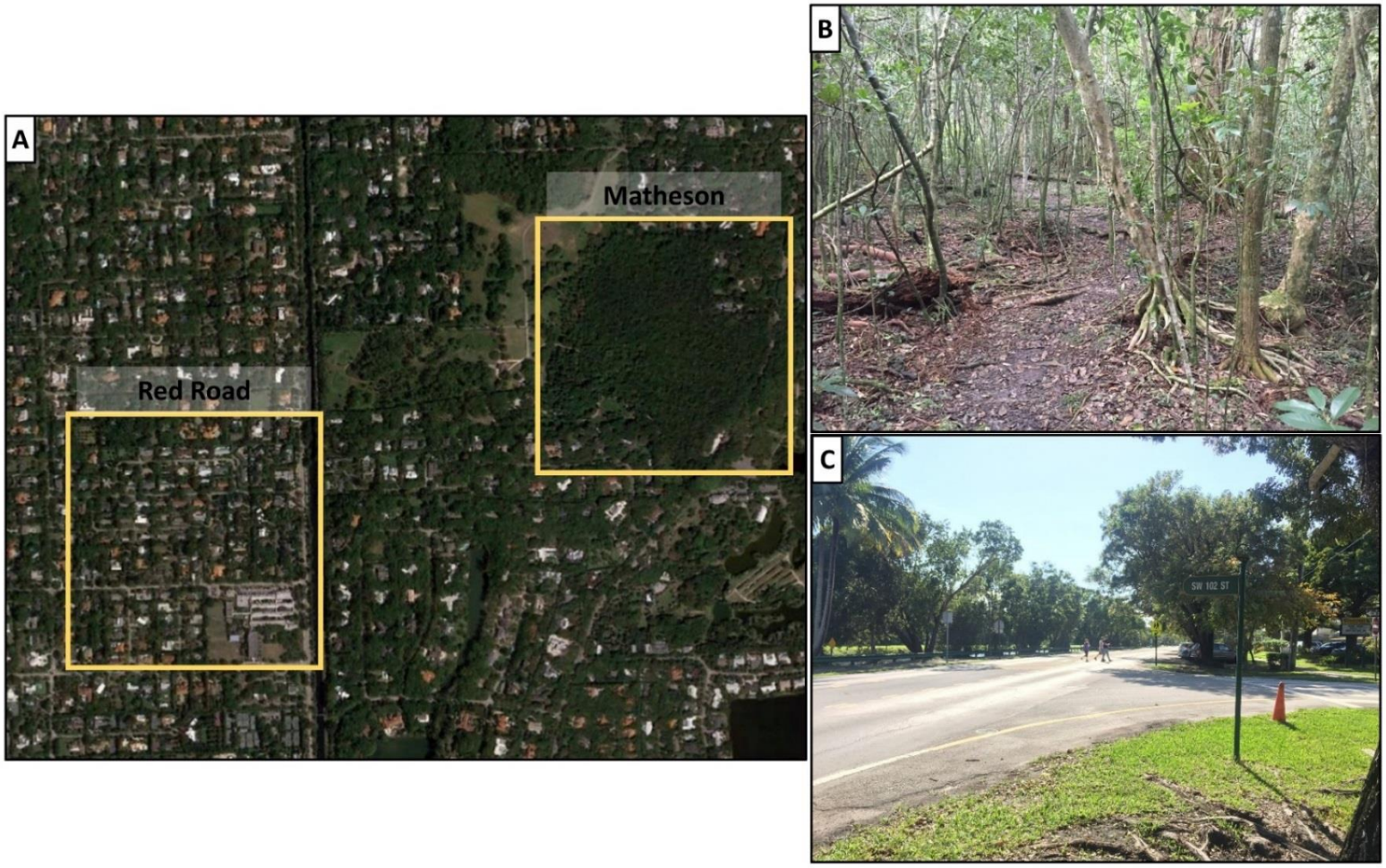

Figure S1. Collection sites for our breeding colonies and thermal data. Panel A shows an aerial view of our study locations (Google Earth). Panels B and C show views from the ground for Matheson and Red Road, respectively, and demonstrate the structural variation between sites. We call Red Road 'urban' (vs suburban or rural) for simplicity and provide important landscape metrics according to MacGregor-Fors (2011): lat and long of center: 25.678125, -80.287655; population density: 1000 people/km^2; yearly population growth rate: 0.04%; study area size: 0.43 km^2; primary use: residential. Population data for Pinecrest, FL is for 2016 and was taken from www.opendata.network.com on 3/1/20

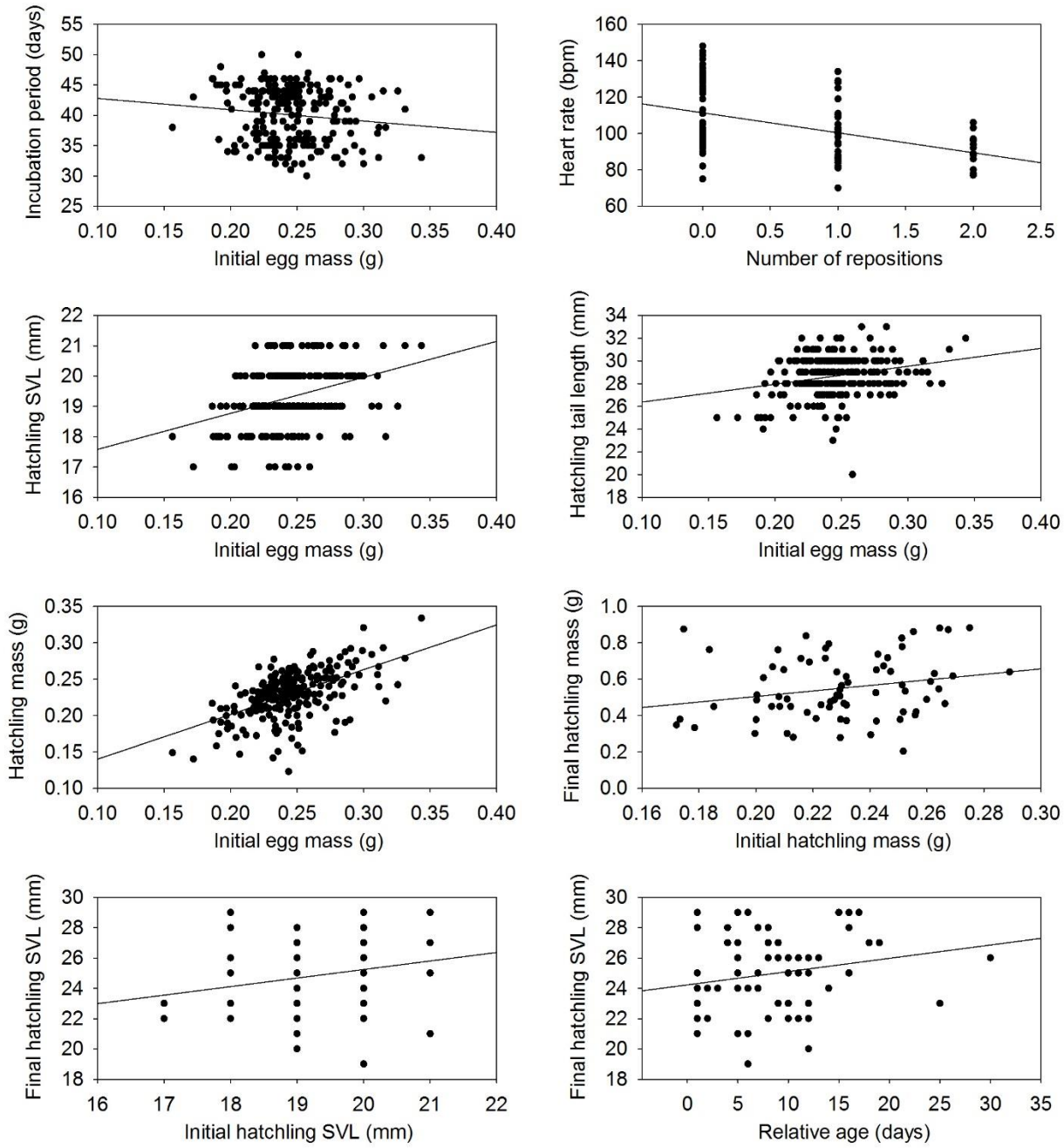

Figure S2. Statistically significant relationships between dependent variables and covariates from Table S1. Two relationships warrant explanation. The negative relationship between incubation period and initial egg mass (top left) is likely not biologically meaningful. Rather, because we checked for eggs every 2-3 days, eggs laid on the day of collection had less time to absorb water from nest pots than eggs laid on the day before collection. Thus, eggs laid on the same day as collection were likely both younger and smaller. The relationship between heart rate and number of repositions (top right) is most likely because eggs whose heart rates were measured after 2 repositions probably cooled some compared to eggs whose heart rates were measured with no repositions (see Hulbert et al., 2017).

CHAPTER 4

Thermal sensitivity varies ontogenetically and differs between embryos of two sympatric ectotherms

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INTRODUCTION

Urbanization dramatically transforms natural landscapes with respect to structural and thermal environments (Battles et al., 2018; Tiatragul et al., 2019). Due to reduced greenspace and an abundance of heat-absorbing substrates, urban habitats have higher mean temperatures and increased levels of thermal variation compared to adjacent rural or natural sites (i.e. the urban heat island effect; Battles and Kolbe, 2019). Despite these novel thermal regimes, many species thrive in urban landscapes via a diversity of mechanisms (Kaiser et al., 2016; Diamond et al., 2017; Tiatragul et al., 2019). Although there has been a recent proliferation of research in urban ecology and evolution, several knowledge gaps remain. First, most studies quantify how adult phenotypes respond to urban temperatures, but few have focused on earlier life stages (see Chapter 3). Embryos, for example, are highly sensitive to thermal stress due to a relatively narrow thermal tolerance (Pörtner et al., 2017; but see Chapter 1) and a limited ability to thermoregulate (Du and Shine, 2015). Importantly, successful embryo development is a prerequisite for survival in every environment and must be considered when assessing the impacts urbanization and other aspects of global change (Burggren, 2018).

Second, because urban-dwelling species vary considerably in many facets of their biology (e.g., microhabitat preference, thermal physiology), the impact of urbanization can be highly species-specific (Niemelä et al., 2002; Diamond et al., 2014; Thawley et al., 2019). This

makes it difficult to draw general conclusions about the effects of this widespread aspect of global change. Though several studies have assessed species-specific responses of adult phenotypes to urbanization, little is known about inter-specific effects on embryos (Kaiser et al., 2016; Tiatragul et al., 2017). A fuller understanding of species differences in embryo thermal tolerance and how these might contribute to population abundance and distribution will provide insight into effects of novel thermal conditions (Ma et al., 2018).

Finally, most studies of urbanization are conducted on a few groups of organisms (e.g. birds, mammals, arthropods) which limits our understanding of its impact on biodiversity (Magle et al., 2012; French et al., 2018). Oviparous, non-avian reptiles (henceforth "reptiles") are an understudied group but make excellent models for addressing these knowledge gaps. Due to a lack of parental care, eggs experience a wide range of incubation conditions during development, resulting in species-specific adaptations to local environments (Ma et al., 2018). Additionally, reptiles vary considerably in their responses to urbanization (French et al., 2018). Moreover, the physiological and ecological responses of reptile embryos to temperature are well understood (reviewed by Noble et al., 2018a).

There are, however, two important knowledge gaps in our understanding of the thermal ecology of reptile development. First, though much work describes embryo survival across incubation temperatures (Noble et al., 2018a), few studies have explored the factors that contribute to thermal tolerance (e.g. Smith et al., 2015; Bentley et al., 2017). Embryos change dramatically through development with respect to shape and size (e.g. single cell to fully-formed organism), gene expression, energetic demands, and thermal sensitivity (Kobayashi et al., 2017). Late-stage embryos may be susceptible to death at high temperatures because of their high oxygen demand (Thompson, 1989); however, early stage embryos may also be susceptible

because they are still completing organogenesis (Sanger et al., 2018). Second, most incubation studies utilize a range of constant incubation temperatures or periodic exposure to extreme temperatures (i.e. heat shocks) to assess the effects of thermal stress on development (Howard et al., 2014; While et al., 2018; Sanger et al., 2018). Such studies provide valuable information about the physiological responses of embryos to thermal stress; however, in the wild, extreme temperatures occur through the daily fluctuation of nest temperatures (Bowden et al., 2014; Chapters 2, 3). Thus, for a more complete understanding of the relationship between extreme temperatures and development in wild habitats (e.g. urban environments), we must utilize incubation treatments that mimic the thermal fluctuations of real nests.

The goal of this study was to address these knowledge gaps by applying ecologically-relevant thermal stress (modelled after urban nest temperatures) to determine how the thermal tolerance of embryos changes across development and varies between two urban-exploiting species. We used temperature data from nest sites in the field and subjected eggs of two lizard species, the brown anole (*Anolis sagrei*) and the Puerto Rican crested anole (*A. cristatellus*), to extreme thermal fluctuations that varied in peak temperature. These fluctuations were delivered to embryos of various ages from oviposition to near hatching. Although these species often co-occur across the urban landscape, they have different microhabitat preferences (Battles et al., 2018; Battles and Kolbe, 2019): *A. sagrei* prefers warmer, open canopy microhabitats and *A. cristatellus* prefers cooler, closed canopy microhabitats. As a result, *A. sagrei* nests are warmer and less thermostable than those of *A. cristatellus* (Sanger et al., 2018; Tiatragul et al., 2019). Thus, we predicted that *A. sagrei* embryos would be more robust to thermal stress than those of *A. cristatellus.* Moreover, due to stage-specific patterns of development and oxygen demands, we predicted that embryos of both species would be more sensitive to thermal stress early and late in

development than mid-development. This work expands our understanding of factors that contribute to thermal tolerance (i.e. EAHT) and aid predictions of how wildlife will respond to global change.

METHODS

Study species

Anolis sagrei and *A. cristatellus* (Duméril and Bibron 1837) are relatively small (2-5 grams, 3-8 grams, respectively), subtropical lizards native to Cuba and Puerto Rico, respectively; however, both species inhabit the same urban areas in Florida, USA, where they are naturalized. They lay a single egg about every one (*A. sagrei*) or two (*A. cristatellus*) weeks and have several features that make them ideal for studying embryo responses to urban thermal stress. First, they lay eggs in shallow nests across diverse habitats and over a broad reproductive season (Mitchell et al., 2018; Tiatragul et al., 2019). Thus, embryos develop under a wide range of mean temperatures and thermal fluctuations. Second, they vary in ecology and thermal physiology, and in their responses to urban conditions (Battles and Kolbe, 2019). Third, methods for their captive husbandry and egg incubation are well established (Sanger et al., 2008a), and patterns of embryo development are described (Sanger et al., 2008b), making them logistically-feasible models for incubation studies. Lastly, they have contributed substantially to our general understanding of urban ecology and evolution (Kolbe et al., 2016a; Winchell et al., 2016; Hall and Warner, 2017; Tiatragul et al., 2017; Battles et al., 2018; Chapter 3; Lapiedra, 2018; Tiatragul et al., 2019; Battles and Kolbe, 2019; Thawley et al., 2019).

Captive husbandry and egg collection

Adult *A. sagrei* (n=30 females; n=15 males) were captured on 4 March 2017 and *A. cristatellus* (n=64 females; n=34 males) were captured 2-3 June 2017 from a suburban area in Pinecrest, FL (coordinates are 25.678125, -80.287655). Fewer females of *A. sagrei* were required because of their higher fecundity compared to *A. cristatellus*. Once lizards were moved to Auburn University, we housed females individually per standard conditions (Sanger et al., 2008a) in single cages (29 × 26 × 39 cm; height × width × depth) illuminated with Reptisun 5.0 UVB bulbs (ZooMedInc.) and plant grow bulbs (model F40; General Electric Co.) with a 12:12 hour light:dark cycle and maintained a mean ambient room temperature of 25.6 (range 24.5 – 27) °C. Cage temperatures were measured via 16 temperature loggers (Thermochron iButtons) distributed across 4 cages. Within each of these cages, iButtons were placed in various locations (e.g. perches, cage floor) to capture the range of temperatures. Due to the light sources, ambient cage temperatures were ~28 °C and, during the day, maximum daily temperatures were 31-33 °C in the warmest part of the cage. Thus, cage temperatures were within the range of field body temperatures measured for both species in our study population (Battles and Kolbe, 2019). Cages included two bamboo perches, an artificial plant, a nesting pot (plant pot filled with a mixture of soil and peat moss) and reptile cage carpet (Zoo Med Inc.) as a floor substrate. Each lizard was fed three crickets (dusted with vitamins and calcium) two times per week, and we misted cages with water daily. Because we had half as many males as females, each male was rotated between the same two females once every two to three weeks to prevent sperm limitation (females can store sperm for many weeks).

We used eggs from both species that were laid from 7 June to 25 October 2017 (n=352 *A. sagrei*; n=388 *A. cristatellus*). We collected eggs three times each week and recorded their mass, date of oviposition, and maternal identity. Eggs were individually placed in a petri dish (60 x 15

mm) half-filled with moist vermiculite (-150 kPa) and wrapped with parafilm to prevent desiccation. Eggs were randomly assigned to one of three incubation treatments (described below) using the "RAND" function in Microsoft Excel and placed in an incubator that repeated a daily thermal fluctuation that was suitable for development of both species (Tiatragul et al., 2017; henceforth "urban background"; Figure 1). This regime fluctuated daily from 27.5 to 30.5 °C and was based on temperatures collected from nests at our field site (Tiatragul et al., 2019). We randomly assigned each female's first egg to one treatment and each successive egg was allocated to a remaining treatment. Once females laid more than 3 eggs, we repeated the sequence. Thus, each female's eggs were randomly and equally distributed among treatments.

Thermal tolerance of embryos

We used nest temperatures from the field (Figure S1) to create an extreme diurnal fluctuation in incubation temperature (henceforth, 'thermal spike'). We made sure that the rate of change in temperature and time spent at the peak temperature were like that of real nest fluctuations because both these factors can influence responses to thermal stress (Chown et al., 2009; Rezende et al., 2011). Our three incubation treatments differed in the number of exposures to this thermal spike (0, 1, or 2 exposures). Thermal spikes were programmed into Memmert brand IPP110 plus incubators.

To assess thermal tolerance of embryos, we raised or lowered the peak of the thermal spike by 1 °C (Figure 1). We started with a peak of 43 °C because previous work indicates that survival declines for anole embryos at this temperature (Chapter 3); however, this resulted in high survival for *A. sagrei* and near total mortality for *A. cristatellus* (see results). Thus,

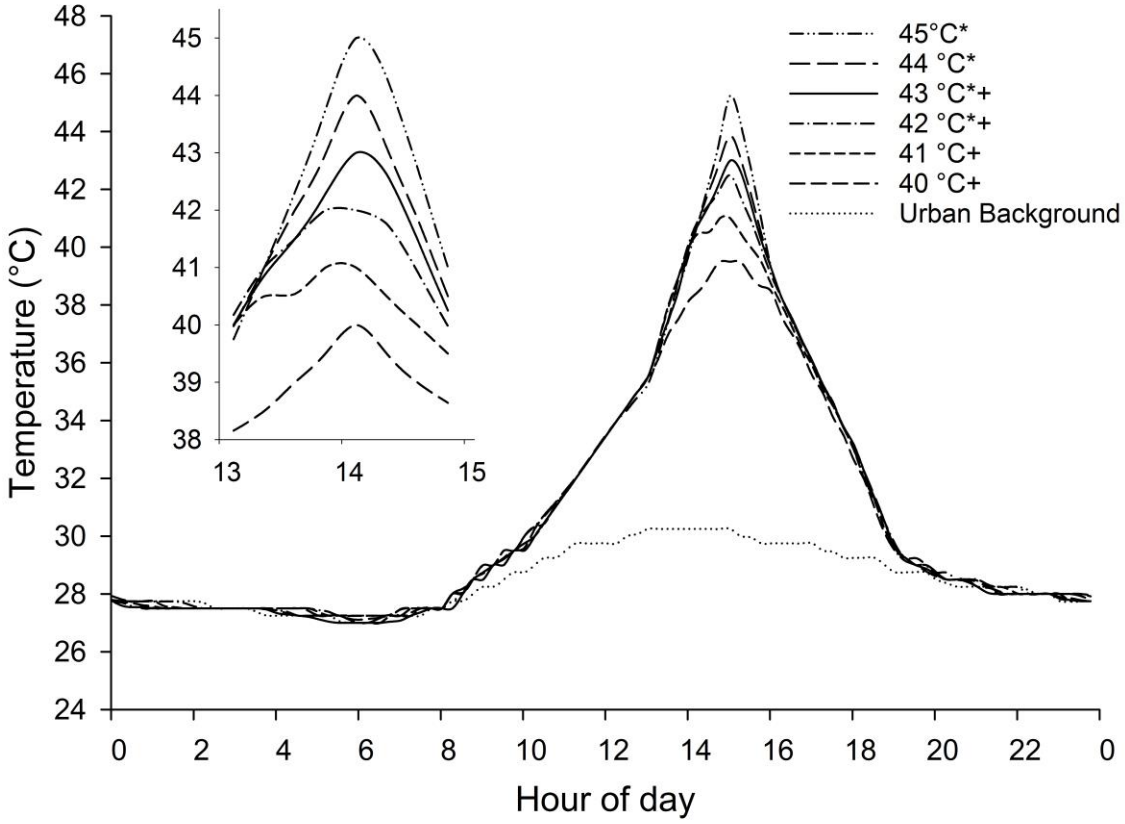

Figure 1. Thermal fluctuations used to determine the thermal tolerance of *A. sagrei* and *A. cristatellus* embryos**.** The legend shows the peak temperature of each fluctuation. Temperatures shown were recorded by a thermocouple that was placed in the same conditions as incubating eggs (i.e. in a petri dish with moist vermiculite, sealed with parafilm). Asterisks denote temperatures used for *A. sagrei* and plus signs denote those used for *A. cristatellus*. The inset figure in top left shows the peaks at a finer scale. The urban background refers to the daily fluctuation used to incubate eggs before and after exposure to thermal spikes.

subsequent treatments increased the peak temperature for *A. sagrei*, and decreased the peak temperature for *A. cristatellus*; but two peak temperatures (42 and 43 °C) were used for both species.

We exposed embryos to thermal spikes at different times during development to assess how effects may change with ontogeny (Figures 2, S2). For each peak temperature (n=4 peak temperatures per species), we performed the following procedure: we collected *A. sagrei* eggs for a period of 25 days and those of *A. cristatellus* for a period of 30 days (*A. sagrei* embryos

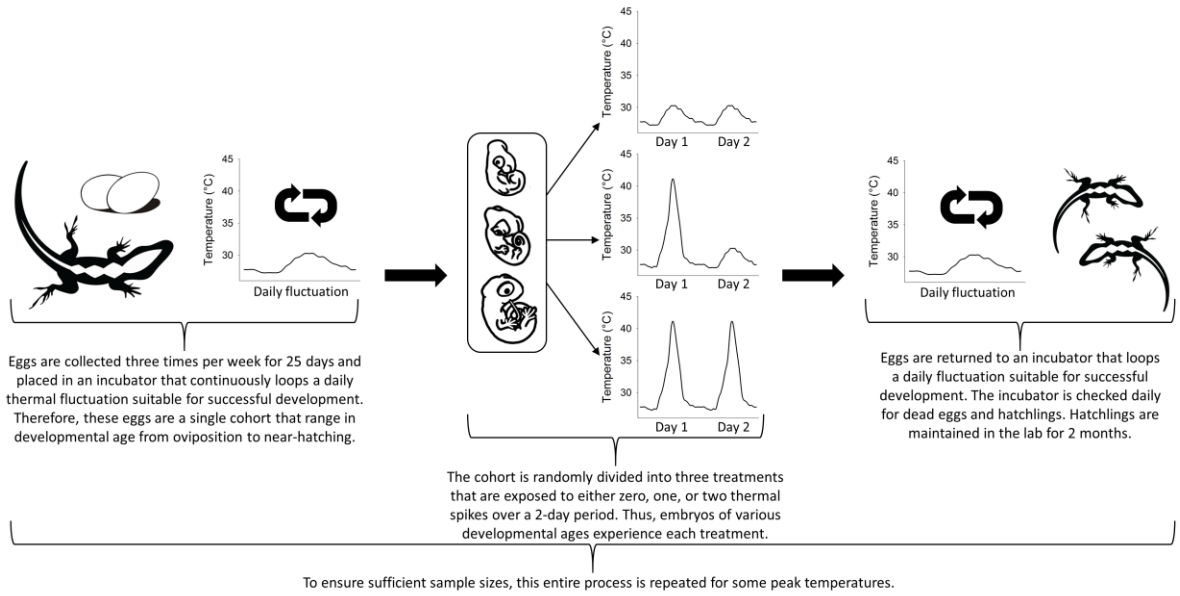

Figure 2. Experimental design for determining thermal tolerance of embryos for *A. sagrei*. The process was identical for *A. cristatellus* except eggs were collected over a 30-day period. For a complete list of sample sizes for each cohort see Table 1.

develop quicker than *A. cristatellus*, Tiatragul et al., 2017) and randomly assigned each egg to our three treatments. During this time, all eggs were incubated at urban background temperatures. Thus, these represent cohorts of eggs that range from oviposition to near hatching. To apply treatments, all eggs were removed from the urban background and placed in a constant 28 °C (± 0.5 °C) incubator (VWR INCU-line). This ensured that control (0 spikes) and treatment (1 or 2 spikes) eggs were treated equally (with respect to being moved among incubators) and that all eggs began the thermal treatment with an internal temperature equal to the starting temperature of the thermal spike (28 °C). The following morning at 0800 hours, control eggs were moved back to the urban background incubator and treatment eggs were moved to an incubator programmed for the thermal spike. The next day, eggs from treatments 0 and 1 were returned to the 28 °C incubator for one day and were then returned to the urban background incubator the following day to complete development. Eggs exposed to two thermal spikes remained in the spiking incubator for one additional day and were then moved to the 28 °C

incubator for one day and returned to the original incubator. These movements were necessary for logistical reasons (e.g. limited number of programmable incubators). Eggs remained in their petri dishes while being moved, which minimized potential disturbance of embryos (e.g. turning of eggs). Moreover, moving and turning eggs has minimal effects on anole embryo development (see Hulbert et al., 2017).

The extreme temperatures of thermal spikes caused small tears in the parafilm sealing the petri dish; therefore, after treatment, we provided fresh vermiculite to every petri dish (including controls) and rewrapped them with new parafilm. This controlled for any moisture that may have been lost during treatment. Thereafter, we checked incubators daily for dead eggs and for hatchlings. Dead eggs were discarded and were not assessed for deformities. See Sanger et al. (2018) for discussion of thermally-induced embryo abnormalities. This design ensured that eggs of various developmental ages, from oviposition to near-hatching, experienced each treatment (0, 1, or 2 spikes) at each peak temperature (full factorial design). However, because we collected eggs 3 times per week, embryo age is only accurate to 3 days. Moreover, like most squamates, *Anolis* embryos are already at the early limb-bud stage at the time of oviposition (Sanger et al., 2008b); thus, our treatments were not applied to the earliest developmental stages (e.g. gastrulation). To ensure sufficient sample sizes, we repeated the procedure shown in Figure 2 as needed. We allocated more eggs to treatments that resulted in higher variation in survival and fewer eggs to treatments resulting in little variation (i.e. total mortality or near 100% survival). See Table 1 for a complete list of sample sizes per peak temperature and per treatment.

Table 1. Sample sizes (n) and survival frequencies of *A. sagrei* and *A. cristatellus* eggs and hatchling. "Age range" is the range of egg ages since oviposition for each group. Beneath each treatment (i.e. control, one spike, two spikes), the sample size for that treatment is given with the survival frequency for that sample in parenthesis. "Peak temp" refers to the peak temperature of the thermal fluctuation (i.e. thermal spike).

			Egg survival				**Hatchling survival**		
	Peak temp (°C)	n	age range (days)	control	one spike	two spikes	n	control	one spike	two spikes
A. sagrei	42	65	1 to 25	19 (0.95)	25 (0.96)	21 (1.00)	62	18 (0.72)	24 (0.63)	20 (0.75)
	43	115	4 to 22	40 (0.93)	39 (0.82)	36 (0.78)	82	28(0.76)	27(0.48)	27(0.26)
	44	83	1 to 25	21 (0.90)	30 (0.90)	32 (0.63)	65	19 (0.47)	27 (0.59)	19 (0.74)
	45	89	4 to 25	27 (0.85)	32 (0.00)	30 (0.00)	23	23 (0.78)	-	-
A. cristatellus	40	87	4 to 32	31 (0.84)	29 (0.90)	27 (0.67)	70	26 (0.35)	26 (0.69)	18 (0.72)
	41	162	2 to 30	58 (0.76)	48 (0.60)	56 (0.45)	97	43 (0.53)	29 (0.59)	25 (0.64)
	42	93	5 to 32	33 (0.94)	31 (0.10)	29 (0.00)	34	31 (0.42)	3 (0.33)	-
	43	46	4 to 22	12 (0.75)	23 (0.00)	11 (0.00)	3	3 (0.33)	-	-

Embryo physiology

We noninvasively measured embryo heart rates using the Buddy® egg monitoring system. This device uses infrared light to detect the heart rate of embryos and measurements have no effect on developmental rate or survival of anole eggs (Hulbert et al., 2017). For 66 *A. sagrei* and 81 *A. cristatellus* embryos randomly selected across treatments, we measured embryo heart rates on the day before and the day after exposure to a thermal spike(s) to confirm that embryos died during thermal spikes and not sometime later in development. To determine any lasting effects of thermal spikes on embryo physiology, for a different subset of *A. sagrei* eggs (n=37) that received 0, 1, or 2 thermal spikes at 43°C, we measured embryo heart rates at 28 °C on the day before and on each of two days immediately after exposure. This was also done for *A. cristatellus*; however, mortality was 100%; thus, we only report data for *A. sagrei*. To measure heart rates before and after thermal spikes, we placed eggs in an incubator set at a constant temperature of 28 °C (± 0.5 °C) for 24 hours. On the day prior to treatment application, we removed eggs from this incubator (one at a time) and quickly placed them inside the heart rate monitor which was housed in another incubator also set to 28 °C. Eggs remained in the monitor for 45 to 60s before we recorded a heart rate. A thermocouple was run into the housing chamber of the heart rate monitor and the air temperature inside the monitor was recorded along with each measure of heart rate (mean temperature = 28.3 °C). Eggs were returned to the 28 °C incubator, and the following morning we put them in their corresponding treatment incubator (control vs thermal spike). At the end of the day (i.e. after treatment) eggs were returned to the 28 °C incubator. Heart rates were measured again on each of two days following the thermal spike(s) (as previously described). Eggs were then returned to the urban background incubator to complete development.

Hatchling phenotypes and survival

For all hatchlings (n=248, *A. sagrei*; n=211, *A. cristatellus*), we measured snout-vent length (SVL) and tail length (nearest 0.01 mm), and hatchling mass (nearest 0.0001 g). Hatchlings were placed in cages identical to those of adults except more perches and leaves were provided. Hatchlings were marked with toeclips and housed communally: we aimed to keep 6 hatchlings per cage (2 from each treatment: 0, 1, or 2 spikes), but due to differential egg mortality among treatments, some cages had more of one treatment than another and some cages had fewer than 6 lizards (n=1 cage with 3 lizards; n= 3 cages with 4; n= 11 cages with 5; n= 51 cages with 6). All cages had at least one lizard from each treatment. Hatchlings were segregated by species and the peak temperature of the thermal spike (e.g. hatchlings from peak temperature of 43°C were not mixed with those from other peak temperature treatments). To minimize large age discrepancies among cage-mates, we filled cages in the order that lizards hatched; however, we calculated a relative age for each hatchling to use as a covariate in analyses. The last hatchling to be put in a cage had a relative age of zero. All other hatchlings were assigned an age that reflected the number of days they were in a cage before the final hatchling was added. Hatchlings were fed *ad lib* with fruit flies dusted with vitamins/calcium and misted with water daily. We measured the body mass of all survivors at 2 months of age. See Table 1 for sample sizes.

Statistical analyses

Because effects of thermal spikes might change across development, for each response variable, we used AICc to compare models that considered the relationship with embryo age (i.e., day of treatment minus day egg was collected) to be either linear or curvilinear (i.e. linear +

quadratic term). Adding the quadratic term improved model fit for egg survival for both species and developmental rate for *A. cristatellus* (Table S1); so, we modeled the relationship with age linearly for all other analyses. Each model initially included the following fixed effects: initial mass (either egg mass at oviposition or body mass at hatching), embryo age, treatment (number of thermal spikes), peak temperature, a treatment by peak temperature interaction, an age by treatment by peak temperature interaction, and an initial mass by treatment by temperature interaction. We dropped three-way interaction terms if they were not statistically significant (alpha = 0.05). The mass by treatment by temperature interaction was never significant (all p values > 0.1), so it was omitted from all final models. We split datasets by either treatment or peak temperature to explore significant interactions. Oviposition date was a covariate to analyse egg survival, developmental rate, and hatchling morphology and relative age was a covariate for models of hatchling survival and growth rate; however, we dropped them from models if they were not significant (see Results).

To determine how each fixed effect influenced embryo survival, we performed generalized linear mixed models (GLMMs) with maternal ID as a random effect and a binomial distribution. To compare thermal tolerance between the species, we calculated embryo acute heat tolerance (EAHT) for the one and two spike treatments for each species using a logistic regression of embryo survival on temperature (see Chapter 1). EAHT is the peak temperature at which half the embryos are predicted to die. We compared 95% confidence intervals of these estimates to assess statistical significance.

We performed 4 separate LMMs with maternal ID as a random effect to assess the influence of fixed effects on developmental rate, hatchling SVL, body mass, and tail length. We calculated a developmental rate for each egg by dividing the number of embryonic stages that

anole embryos traverse from oviposition to hatching (15 stages, Sanger et al., 2008b) by incubation period (days). Peak temperatures where only control eggs survived were not included in analyses of developmental rate or hatchling morphology. To assess longer-term effects on hatchlings, we analyzed hatchling survival with GLMMs using a binomial distribution, and we assessed hatchling growth with LMMs; hatchling cage was a random effect. Hatching growth rate was the final mass of each hatchling minus its body mass at hatching divided by the number of days between measurements. For analysis of heart rate, we performed a general linear mixed effects model. Fixed effects included embryo age, day of measurement (day before, 1 day after, or 2 days after thermal spike(s)), treatment (number of spikes), and a treatment-by-day interaction. Egg ID was a random effect. The temperature inside the heart rate monitor was a covariate. We performed separate analyses for each species. Data analyses were performed in R ver. 3.5.3 (R Core Team 2018).

RESULTS

For experimental eggs that died, 97% had no heart rate one day following treatment, demonstrating that embryos died during the thermal spike and not later in development. Embryo age and treatment interactively affected egg survival for *A. cristatellus* but not *A. sagrei* (Table 2). For *A. cristatellus*, the oldest and youngest embryos were less robust to thermal stress than embryos of intermediate age. We observed the opposite pattern for *A. sagrei*; however, this was only true for eggs exposed to two thermal spikes and the pattern was not statistically clear (Figure 3A, B; Table 2). We also observed treatment by peak temperature effects for both species (Table 2): embryo survival declined with increasingly higher peak temperatures, particularly for eggs exposed to two thermal spikes (Figure 3C, D). EAHTs were greater for

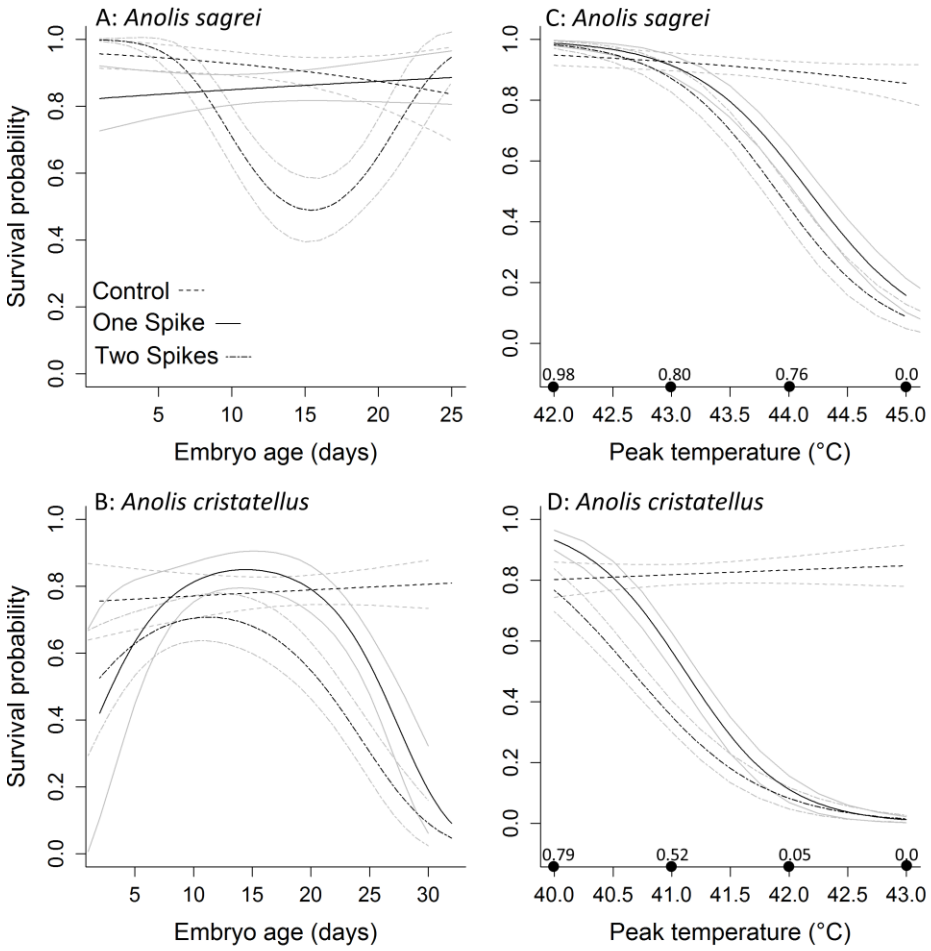

Figure 3. Survival probability of *A. sagrei* (A, C) and *A. cristatellus* (B, D) embryos exposed to thermal spikes. Panels A and B show survival across embryo age at time of treatment. Panels C and D show survival across peak temperatures of thermal spikes. Black lines show survival probabilities; gray lines show standard errors. For panels A and B, data shown exclude peak temperatures with little variation in survival (*A. sagrei*: 42 and 45 °C were excluded; *A. cristatellus*: 43 and 44 °C were excluded). In panels C and D, circles on the x-axis denote the peak temperatures of each thermal spike, and the values above each circle are survival rates for one and two spike treatments combined for each peak temperature. See Table 1 for sample sizes and raw mean survival frequencies and Table 2 for test statistics.

A. sagrei than *A. cristatellus* and were greater for one spike vs two spike treatments. EAHTs of *A. sagrei* were 44.17 (43.92 – 44.43, 95% CI) and 43.90 (43.66 – 44.14, 95% CI) °C for one and two spike treatments, respectively. EAHTs of *A. cristatellus* were 41.11 (40.91 – 41.32, 95% CI) and 40.66 (40.40 – 40.92, 95% CI) °C for one and two spikes, respectively.

Table 2. Results for final models testing the effects of treatment ("treat", 0, 1, or 2 thermal spikes), age (embryo age at time of treatment), peak temperature ("temp") and interactions and covariates on *A. sagrei* and *A. cristatellus* embryo and hatchling phenotypes and survival. Peak temperatures are 42, 43, 44, 45 and 40, 41, 42, 43 °C for *A. sagrei* and *A. cristatellus*, respectively. Asterisks denote terms that were omitted from the final model due to lack of statistical significance in preliminary analyses. If no asterisk or statistics are provided, that variable was not considered in the analysis. Initial mass refers to egg mass at oviposition for embryo variables, and body mass at hatching for hatchling variables. Bold text denotes statistical significance (alpha=0.05). See Tables 1, S2, and S3 for sample sizes, raw means, and standard deviations.

	Response variable	Oviposition date	Initial mass	Age	Treat	Temp	Treat x temp	Age x treat x temp	Relative age
A. sagrei	Egg survival	*	**χ^2_1=4.1; p=0.04**	**χ^2_2=9.6; p=0.008**	**χ^2_1=10.4; p=0.001**	**χ^2_1=4.0; p=0.047**	**χ^2_1=5.5; p=0.02**	χ^2_2=5.4; p=0.067	
	Developmental rate	**$F_{1,194}$=31.0; p<0.0001**	**$F_{1,194}$=32.6; p<0.0001**	$F_{1,194}$=3.8; p=0.05	**$F_{1,194}$=8.7; p=0.004**	$F_{1,194}$=1.8; p=0.17	$F_{1,194}$=0.6; p=0.45	*	
	Hatchling SVL	*	**$F_{1,195}$=55.4; p<0.0001**	$F_{1,195}$=0.6; p=0.45	$F_{1,195}$=0.1; p=0.82	$F_{1,195}$=1.6; p=0.20	$F_{1,195}$=0.1; p=0.83	*	
	Hatchling mass	*	**$F_{1,195}$=73.7; p<0.0001**	$F_{1,195}$=0.1; p=0.77	$F_{1,195}$=0.4; p=0.51	$F_{1,195}$=0.19; p=0.66	$F_{1,195}$=0.1; p=0.78	*	
	Hatchling tail length	*	**$F_{1,194}$=18.0; p<0.0001**	$F_{1,194}$=0.1; p=0.79	**$F_{1,194}$=8.7; p=0.004**	$F_{1,194}$=0.01; p=0.91	$F_{1,194}$=1.0; p=0.31	*	
	Hatchling survival		**χ^2_1=9.6; p=0.002**	χ^2_1=3.5; p=0.06	χ^2_1=2.1; p=0.15	χ^2_1=2.1; p=0.15	χ^2_1=1.3; p=0.26	*	*
	Hatchling growth rate		**$F_{1,79}$=4.5; p=0.04**	$F_{1,79}$=1.2; p=0.27	**$F_{1,79}$=7.7; p=0.007**	$F_{1,79}$=0.3; p=0.58	$F_{1,79}$=1.3; p=0.27	*	**$F_{1,79}$=7.1; p=0.009**

A. cristatellus	Egg survival	*	*	**χ^2_2=11.3; p=0.004**	**χ^2_1=54.8; p<0.0001**	χ^2_1=1.1; p=0.29	**χ^2_1=24.1; p<0.0001**	**χ^2_2=10.9; p=0.004**	
	Developmental rate	**$F_{1,113}$=5.4; p=0.02**	**$F_{1,113}$=4.1; p=0.046**	**$F_{2,113}$=6.1; p=0.003**	**$F_{1,113}$=19.3; p<0.0001**	$F_{1,113}$=3.0; p=0.09	$F_{1,113}$=3.9; p=0.05	*	
	Hatchling SVL	*	**$F_{1,115}$=73.2; p<0.0001**	$F_{1,115}$=1.2; p=0.28	$F_{1,115}$=0.2; p=0.69	$F_{1,115}$=0.4; p=0.53	$F_{1,115}$=0.2; p=0.68	*	
	Hatchling mass	*	**$F_{1,115}$=111.9; p<0.0001**	$F_{1,115}$=0.01; p=0.97	$F_{1,115}$=1.8; p=0.18	$F_{1,115}$=3.2; p=0.08	$F_{1,115}$=0.2; p=0.68	*	
	Hatchling tail length	*	**$F_{1,114}$=25.6; p<0.0001**	$F_{1,114}$=2.4; p=0.12	$F_{1,114}$=1.5; p=0.23	$F_{1,114}$=0.75; p=0.39	$F_{1,114}$=1.2; p=0.28	*	
	Hatchling survival		**χ^2_1=9.6; p=0.002**	χ^2_1=1.6; p=0.20	**χ^2_1=4.5; p=0.03**	χ^2_1=0.0; p=0.98	χ^2_1=0.9; p=0.36	**χ^2_1=4.8; p=0.03**	*
	Hatchling growth rate		**$F_{1,61}$=4.5; p=0.04**	$F_{1,61}$=2.3; p=0.13	$F_{1,61}$=0.0; p=0.92	$F_{1,61}$=1.6; p=0.20	$F_{1,61}$=0.4; p=0.55	*	**$F_{1,61}$=8.7; p=0.005**

For both species, we observed significant treatment effects on developmental rate (Table 2): each exposure to a thermal spike decreased developmental rates by 0.0051 (± 0.0017 SE) and 0.0089 (± 0.0020 SE) stages per day for *A. sagrei* and *A. cristatellus*, respectively (Figure 4). Heart rates of *A. sagrei* embryos were similar among treatment groups on the day before treatments were applied ($F_{2,32}$= 0.19; p=0.83); however, a single thermal spike decreased embryo heart rate by 13.3 bpm (± 4.3 SE; p=0.004) on the day after the thermal spike compared to controls (i.e. a 13.3% reduction) (Figure 5). Two days after the thermal spike, heart rates were still 8.8 bpm (± 3.5 SE) lower than controls (p=0.02) (i.e. an 8.7% reduction). For embryos exposed to two thermal spikes, heart rates were 17.1 bpm (± 4.4 SE) and 13.4 bpm (± 3.7 SE) lower than controls one day (p=0.0005) and two days (p=0.001) after experiencing the thermal spikes, respectively (i.e. 17.0% and 13.4% reductions).

Treatments had no clear effects on *A. cristatellus* hatchling morphology and only minimal effects on morphology of *A. sagrei* hatchlings (Table 2). For each additional thermal spike, *A. sagrei* tail length decreased by 0.39 mm (± 0.13 SE), but there were no statistically clear effects on *A. sagrei* SVL or body mass (Table 2). Treatments did not affect hatchling survival for *A. sagrei*; however, for each thermal spike, *A. sagrei* growth rates decreased by 0.00064 g/day (± 0.00023 SE) (Table 2). Finally, we observed significant effects of treatment and treatment by peak temperature by age for survival of hatchling *A. cristatellus* (Table 2). At warmer peak temperatures, hatchlings were more likely to die if they were exposed to thermal spikes early or late in development, but this pattern was evident only for those exposed to a single thermal spike (Figure 6). We found no effects of our treatments on hatchling growth for *A. cristatellus* (Table 2). See Tables S2 and S3 for raw means and standard errors of egg and hatchling phenotypes.

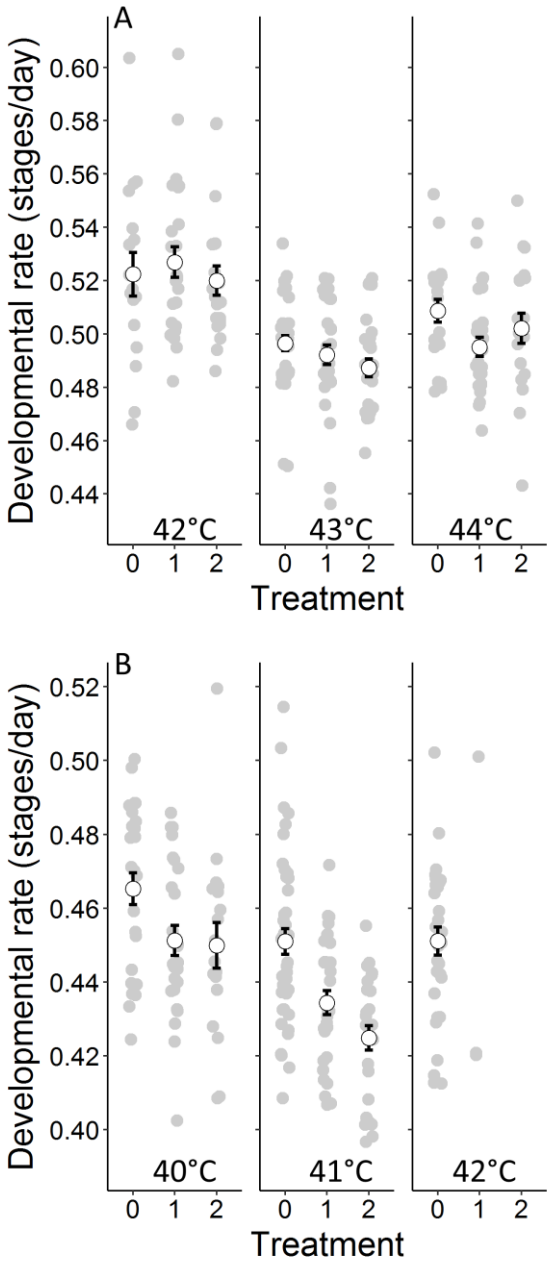

Figure 4. Developmental rates of *A. sagrei* (A) and *A. cristatellus* (B) embryos exposed to 0, 1, or 2 thermal spikes that vary in peak temperature. Open circles show raw means for each group, bars show standard error, and gray circles show raw data. The temperature at the bottom of each panel is the peak temperature of the thermal spike. Data from the 42 °C peak for *A. cristatellus* (B) is shown for consistency but high mortality precluded statistical analysis. See Table S2 for sample sizes, raw means, and standard deviations and Table 2 for test statistics.

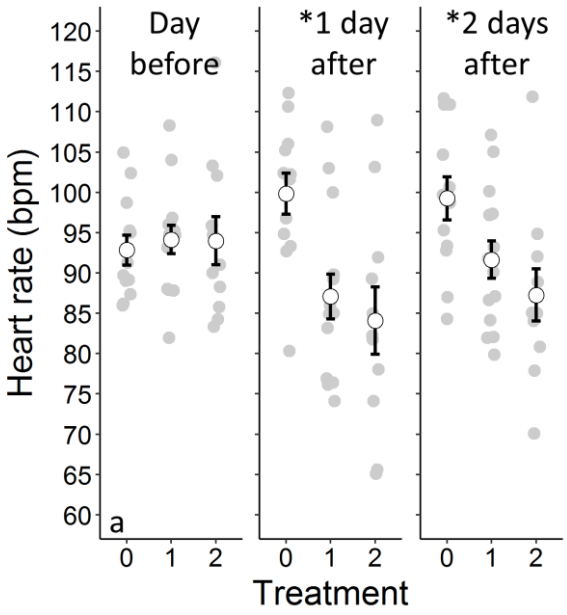

Figure 5. Heart rates of *A. sagrei* embryos exposed to 0, 1, or 2 thermal spikes with a peak temperature of 43 °C. Heart rates were measured at 28 °C on the day before and on one and two days after exposure. Open circles show the raw means for each group, bars show standard errors, and gray circles show the raw data. Asterisks signify a significant decline in heart rate with an increase in the number of spikes. See Table S3 for sample sizes, raw means, and standard deviations.

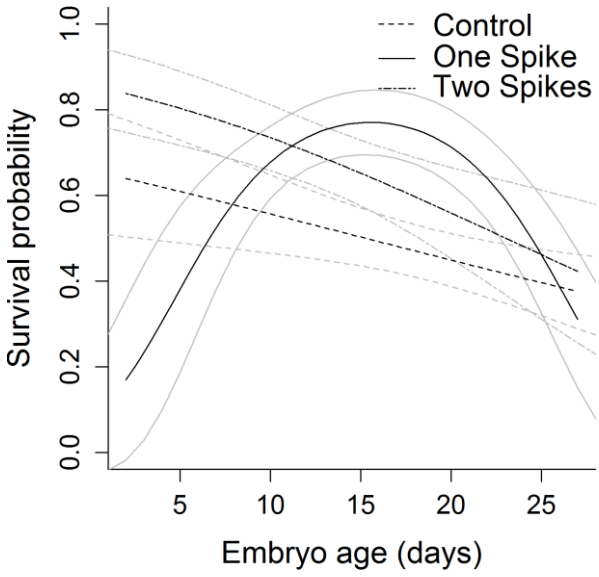

Figure 6. Survival probability of *A. cristatellus* hatchlings that were exposed to thermal spikes on different days during embryo development. Black lines show survival probabilities for each treatment. Gray lines show standard errors. See Table 1 for sample sizes and raw survival frequencies and Table 2 for test statistics

DISCUSSION

We utilized thermal fluctuations from nests in an urbanized landscape and found that thermal tolerance of embryos changed through development: for *A. cristatellus*, early- and late-stage embryos were less robust to thermal stress than those mid-stage; however, this relationship was unclear for *A. sagrei* (discussed below). The greatest effects of acute thermal stress were on embryo physiology and survival, but the nature of these effects differed between the species. EAHT of *A. sagrei*, a lizard that prefers warmer, open-canopy microhabitats, was greater than EAHT of *A. cristatellus*, a lizard that prefers cooler, closed-canopy microhabitats.

Sympatric species may evolve divergent thermal physiologies due to selective pressures exerted by their respective microhabitats (Scheers and Van Damme, 2002). Thus, even when embryos of each species are exposed to similar heat stress (e.g. the urban heat island), they may respond in different ways. Species that prefer cooler microhabitats are often less robust to high temperatures than those that prefer warmer microhabitats, but these findings are usually based on adult phenotypes (e.g. Scheers and Van Damme, 2002; Li et al., 2017). Only a few studies have assessed the thermal physiology of embryos of sympatric reptile species (e.g. Ma et al., 2018). Because *A. sagrei* nests reach warmer daily maximum temperatures than those of *A. cristatellus* (Sanger et al., 2018; Tiatragul et al., 2019), thermally-sensitive phenotypes of each species may have evolved to maximize embryo survival in each respective microhabitat.

Alternatively, the inter-specific differences we observed could be due to phylogenetic history (Scheers and Van Damme, 2002; Garland et al., 2005). Indeed, the common ancestor of our study species lived as long as 72.7 million years ago (Nicholson et al., 2012). Despite this deep divergence time, patterns of embryo development appear highly conserved across anole species (Sanger et al., 2008b). Because our study did not incorporate multiple species from each

clade (i.e. *Norops* and *Ctenonotus* clades; see Nicholson et al., 2012), we cannot distinguish between adaptive and phylogenetic hypotheses. It is worth noting, however, that these mechanisms are not mutually exclusive: physiological adaptation may be a driver of phylogenetic differences during the divergence of clades (Garland et al., 2005).

In addition to adaptation and phylogeny, differences in embryo thermal physiology may result from thermal acclimation of embryos or maternal effects (Du et al., 2010c; Ma et al., 2014). Thermal acclimation is unlikely since all eggs were subjected to the same incubation conditions prior to treatment. Though much development occurs before oviposition, females were housed in the same thermal conditions which decreases the possibility that inter-specific patterns of maternal thermoregulation could result in embryo acclimation. Additionally, maternal effects can influence offspring phenotypes (Warner et al., 2015); however, we tightly controlled the maternal environment throughout the study, decreasing the possibility of species-specific maternal effects. One caveat is that females were occupying species-specific thermal microhabitats prior to capture, which could have contributed to maternal effects on embryos. However, anole reproduction rapidly responds to immediate environmental conditions (Hall et al., 2018); so, thermal conditions prior to capture likely had little influence on our results. We cannot, however, completely rule out this possibility.

Regardless of the cause, *A. sagrei* EAHT was greater than EAHT of *A. cristatellus*. Although *A. cristatellus* is relatively abundant in urbanized habitats both within (Winchell et al., 2016) and outside (Kolbe and Battles, 2018) its native range, this species prefers high canopy cover and is restricted to pockets of relatively dense vegetation (e.g. groves of fig trees, *Ficus citrifolia*, *F. aurea*) throughout the urban matrix (Kolbe et al., 2016b). Though differences in the occupancy and abundance of these species throughout urban landscapes are related to thermally-

sensitive phenotypes of adults (Battles and Kolbe, 2019), nesting behavior may also play an important role. Indeed, urban *A. cristatellus* females prefer nesting in areas that are relatively shaded and cool compared to what is generally available in urban habitats (Tiatragul et al., 2020). This may protect developing embryos from thermal stress but limit dispersal throughout the urban environment. Thus, we suggest that interspecific variation in colonization success of urban habitats is, in part, attributable to differences in thermal sensitivity of embryos. Throughout the urban matrix, suitable nesting habitat may be more readily available for some species compared to others due to variation in embryo responses to the magnitude and frequency of thermal fluctuations within the urban heat island.

Though interspecific variation in the thermal tolerance of embryos has been observed in many species, changes in thermal tolerance across stages of development have rarely been considered (but see Kobayashi et al., 2017). *Anolis cristatellus* embryos were most robust to thermal stress midway through development. For *A. sagrei*, however, no trend was statistically clear, but we observed a weak trend in the opposite direction (lower survival mid-development). The relatively high thermal tolerance of *A. sagrei* embryos may have hindered our ability to detect variation in survival across embryogenesis. Moreover, this may also be due to lower sample sizes for the earliest and latest stage embryos of *A. sagrei* compared to *A. cristatellus* (see Figure S3), which was an unintended consequence of our study design. Indeed, Sanger et al., (2018) exposed freshly laid *A. sagrei* eggs (i.e. within 1 day of oviposition) to a 1-hour heat shock of 39 °C, which resulted in 15% mortality. Thus, *A. sagrei* likely have lower thermal tolerance at early stages like *A. cristatellus*. We suggest that the relationship between embryo age and thermal stress exhibited by *A. cristatellus* is biologically meaningful, while the pattern for *A. sagrei* is an artefact of methodological issues. Moreover, we reiterate that the earliest stages of

development occur within the oviducts of the female and were not considered in our study. Because females can shield these earliest stages from thermal stress via thermoregulatory behaviour (Shine and Harlow, 1993), our study design is sufficient to determine stage-specific responses to thermal stress in nests, where embryos are little able to compensate for adverse conditions (Telemeco et al., 2016). We recommend more studies consider how thermal tolerance changes through development.

Though many studies demonstrate that embryos die at high temperatures, the direct cause of death is not well understood and debated (Gangloff and Telemeco, 2018). The oxygen- and capacity-limited thermal tolerance concept posits that complex organisms experience reduced performance and death at high temperatures because of a mismatch between oxygen supply and demand (Pörtner et al., 2017). Most of this work has been conducted with aquatic animals and some authors speculate it may have little relevance to terrestrial species due to their efficient ventilation systems (e.g. insect spiracles and tracheae) and the comparatively high amount of oxygen in air (vs water) (McCue and De Los Santos, 2013). Reptile embryos, however, depend on diffusion of oxygen through the eggshell and often incubate in subterranean nests where oxygen levels can be lower than ambient conditions (Seymour and Ackerman, 1980). Although the chorioallantoic membrane increases in size through development (Andrews, 1997), the resulting increase in oxygen supply might not be enough to sustain late-stage embryos at extremely high temperatures. Thus, oxygen limitation is likely an important determinant of embryo survival (Smith et al., 2015; Liang et al., 2015; Chapter 2), and this would explain reduced thermal tolerance at latter embryo stages when O_2 demand is high due to tissue growth (Thompson 1989).

Due to the relationship between oxygen demand and survival, our study may have been more ecologically relevant had we incubated eggs in open containers, rather than petri dishes sealed with parafilm. There are multiple reasons, however, we believe that eggs had sufficient access to oxygen during thermal spikes and that differences in the absolute consumption of oxygen between species (i.e. *A. cristatellus* eggs are larger than *A. sagrei*) did not influence our results. Using respiration rates of anole eggs of various ages and at various temperatures (Hall and Warner, 2020b), we estimate that relatively old (~ 75% development) or young (~ 20% development) eggs would have to consume oxygen at their maximum rate (i.e. the rate at 40 °C) for 7.4 and 19.6 hours, respectively, to use just 10% of the oxygen available in our petri dishes. Moreover, small cracks often form in the parafilm during normal incubation, and they always form during thermal spikes. These tears would greatly increase the permeability of oxygen. Additionally, refreshing the vermiculite in the petri dish following treatment (see methods) returned the air to normoxic conditions. Finally, two additional studies further demonstrate the large difference in thermal tolerance between *A. sagrei* and *A. cristatellus* embryos. In one study, *A. cristatellus* eggs were exposed to thermal spikes with a peak temperature of 42 °C, and the vast majority died (70%) (Tiatragul et al., 2020). Moreover, a heat shock experiment conducted with *A. sagrei* eggs revealed that most eggs die between 44 and 46 °C when exposed to a 1-hour heat shock (90.3% die at 45 or 46°C; see Chapter 2). In these studies, eggs were incubated in glass jars which contained ~ 2.5 times more oxygen than our petri dishes, yet these studies produced results like the current study.

For eggs that survived our treatments, we observed important effects on embryo physiology. Increased temperatures should increase developmental rates, but exposure to thermal spikes reduced developmental rates for both species (Figure 4). This could be due to differential

survival of embryos based on metabolic rate: faster-developing individuals may be more likely to die during thermal stress. However, this may also be related to the reduction in heart rate that is caused by thermal spikes and persists for at least 48 hours (Figure 5). Developmental rate positively correlates with embryo heart rate in reptiles (Du et al., 2010b,c). Although mortality precluded heart rate measurements of *A. cristatellus* embryos, they experience a comparable reduction in heart rate after exposure to thermal spikes (Chapter 3). Another possibility for the decrease in developmental rate is that embryos at extreme temperatures (e.g. greater than 34 °C, i.e. pejus temperature – see Chapter 2) may undergo an arrest of cell division or experience moderate levels of cell death (van der Have, 2002; Sanger et al., 2018). In the field, faster developmental rates are advantageous because they reduce the likelihood of exposure to predators or harmful conditions prior to hatching (Doody, 2011). The decrease in developmental rate we observed equates to an increase in the incubation period of 1 to 2 days. Therefore, the ecological effect may be quite small. However, a 2-day increase in incubation period is comparable to reducing mean nest temperature by 1 °C (Tiatragul et al., 2019). Thus, from a physiological perspective, the effect of thermal spikes on developmental rate is quite large. Moreover, this effect appears to compound with increasing exposures to thermal spikes (Chapter 2).

The greatest treatment effects were on egg survival and physiology, and we observed minimal effects on hatchling morphology (Table 2). Likely, individuals that survive heat stress can successfully complete development. This is further evidenced by the relatively few morphological abnormalities we observed among hatchlings (n=5 of 459 hatchlings; Table 3). Moreover, all these abnormalities were from individuals treated prior to day 9 or after day 21,

Table 3. Morphological abnormalities of hatchling lizards. "Age" represents the age of the embryo in days since oviposition at the time it was exposed to a brief thermal spike; however, deformities were assessed at the time of hatching. We did not assess dead eggs for embryo deformities.

Species	Age (days)	Deformity
A. cristatellus	26	deformed tail
A. sagrei	22	deformed tail
A. sagrei	8	extra hind limbs protruding from abdomen
A. sagrei	6	deformed tail
A. sagrei	6	missing digits (#1) on both rear feet; fused digits (1 and 2) on front feet

further demonstrating the sensitivity of early- and late-stage embryos to thermal stress. Thermal spikes decreased growth rates for *A. sagrei* hatchlings; however, this did not translate into any noticeable effects on hatchling survival. Thus, the ecological significance of this depression of growth rate is questionable. Thermal treatments did reduce hatchling survival for *A. cristatellus*, which further demonstrates the relative robustness of *A. sagrei* to thermal stress compared with *A. cristatellus*. Of the *A. cristatellus* hatchlings that died, a greater portion were exposed to thermal spikes early or late in development than mid-development (Figure 6) which indicates that detrimental stage-specific effects of thermal stress may continue after hatching. One caveat is that we only measured hatchling morphology, growth, and survival. Other factors like thermoregulatory behavior may have been influenced by our treatments and future studies should incorporate such measures. Indeed, the effect of incubation conditions on thermal ecology traits of hatchlings and juveniles are an understudied aspect of reptile developmental plasticity (Refsnider et al., 2019).

Finally, the methods used to measure thermal-sensitivity of embryos can influence conclusions and predictions about how populations will respond to aspects of global change (see Chapter 1). Tiatragul et al., (2017) incubated eggs of *A. sagrei* and *A. cristatellus* at relatively warm, urban nest temperatures. They found no differences in egg survival between the species and concluded that both species were robust to urban thermal environments. Our results and conclusions contrast with their study, and the differences are likely methodological. They utilized repeated, diurnal temperature fluctuations created from mean nest temperatures and did not incorporate the unusually high thermal fluctuations that characterize anole nests (Chapter 2). Studies that utilize constant temperatures or repeated fluctuations of the same temperatures fail to capture the true nature of thermal variability in the environment and may lead to inaccurate conclusions about the effects of thermal phenomena (e.g. climate change, urban heat island) and predictions about future responses by wildlife (Bowden et al., 2014; Carter et al., 2018). Indeed, increased thermal variation may be more detrimental to species than increases in mean temperatures, and this has not yet been fully explored for most systems (Vasseur et al., 2014). The frequency and magnitude of extreme thermal events can induce species-specific changes in relative fitness, which can alter the composition of natural communities (Ma et al., 2015).

CONCLUSIONS

Climate projections indicate increases in mean temperatures, thermal variation, and occurrences of extreme temperatures; effects that mirror those of the urban heat island. Understanding how these events influence survival and physiology will be critical to understand their potential impacts and mitigate risks. Our data confirm that the timing of these events, with respect to embryo development, will have important effects on egg mortality and physiology.

This is particularly critical for populations of threatened species that nest within a relatively narrow timeframe each year (e.g. marine turtles; Howard et al., 2014). A heat wave occurring when most embryos are in the earliest or latter stages of development may produce greater mortality than one occurring mid-development. This is vital to consider when modelling the effects of temperature-induced mortality on population viability or when conducting laboratory studies on thermal tolerance. The interactions between thermal stress, developmental age, and species must be considered when examining thermal tolerance of organisms or life stages that have limited capacity for behavioural thermoregulation.

Acknowledgements

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CHAPTER 4 SUPPLEMENTAL MATERIAL

Table S1. Comparisons of models that considered the effect of embryo age at time of treatment to be linear or curvilinear for *A. sagrei* and *A. cristatellus* embryos. We selected the model with the lowest AICc value; however, if adding the quadratic term did not improve AICc by 2, we selected the most parsimonious model (linear). Bold type denotes the model we selected based on AICc values.

	Response	Fixed effects	K	logLik	AICc	Delta AICc
A. sagrei	*Egg Survival*	**Age + Age^2**	**10**	**-121.4**	**263.4**	**0**
		Age	8	-126.1	268.6	5.2
	Developmental Rate	**Age**	**11**	**559.9**	**-1096.5**	**0**
		Age + Age^2	13	561.8	-1095.9	0.6
	Hatchling SVL	**Age**	**10**	**-223.5**	**468.1**	**0**
		Age + Age^2	12	-222.1	469.6	1.5
	Hatchling Mass	Age + Age^2	12	643.8	-1262	0
		Age	**10**	**641.5**	**-1261.9**	**0.1**
	Hatchling Tail Length	**Age**	**10**	**-421.9**	**864.9**	**0**
		Age + Age^2	12	-420.6	866.8	1.9
	Hatchling Survival	**Age**	**9**	**-130.4**	**279.8**	**0**
		Age + Age^2	11	-129.9	283.1	3.4
	Hatchling Growth	**Age**	**11**	**592.8**	**-1161.2**	**0**
		Age + Age^2	13	593.1	-1157	4.3
A. cristatellus	Egg Survival	**Age + Age^2**	**11**	**-163.1**	**348.9**	**0**
		Age	9	-173.5	365.5	16.7
	Developmental Rate	**Age + Age^2**	**13**	**425.3**	**-822.2**	**0**
		Age	11	420.7	-817.6	4.6
	Hatchling SVL	**Age**	**10**	**-155.4**	**332.1**	**0**
		Age + Age^2	12	-154.5	335	2.9
	Hatchling Mass	**Age**	**10**	**427.9**	**-834.4**	**0**
		Age + Age^2	12	429	-832	2.4
	Hatchling Tail Length	**Age**	**10**	**-301.8**	**625**	**0**
		Age + Age^2	12	-299.7	625.4	0.4
	Hatchling Survival	**Age**	**10**	**-97.1**	**215.7**	**0**
		Age + Age^2	12	-95.6	217.2	1.5
	Hatchling Growth	**Age**	**11**	**441.8**	**-858.4**	**0**
		Age + Age^2	13	442	-853.6	4.81

Table S2. Sample size (n), raw mean, and standard deviation (sd) for egg incubation period and hatchling phenotypes of *A. sagrei* and *A. cristatellus*. "Peak temp" refers to the peak temperature of the thermal fluctuation (i.e. thermal spike). Total sample sizes are listed for each peak temperature first and then also for each treatment within each peak temperature.

	A. sagrei	control			one spike			two spikes		
Incubation period (days)	Peak temp °C	n	mean	sd	n	mean	sd	n	mean	sd
	42	17	28.82	1.85	24	28.54	1.47	21	28.9	1.34
	43	37	30.24	1.04	32	30.53	1.32	28	30.82	1.12
	44	19	29.53	1.07	27	30.33	1.11	20	29.95	1.54
	45	23	29.48	0.9	-	-	-	-	-	-
SVL (mm)	Peak temp °C	n	mean	sd	n	mean	sd	n	mean	sd
	42	17	17.95	0.67	24	18.2	0.72	21	18.09	0.56
	43	37	17.94	0.78	32	17.86	0.91	28	17.77	0.77
	44	19	17.64	0.85	27	17.77	0.68	20	17.86	0.79
	45	23	18.13	0.5	-	-	-	-	-	-
Body mass (g)	Peak temp °C	n	mean	sd	n	mean	sd	n	mean	sd
	42	17	0.1612	0.0177	24	0.1738	0.0193	21	0.1649	0.0138
	43	37	0.1666	0.0175	32	0.1639	0.0184	28	0.1625	0.0152
	44	19	0.1613	0.0212	27	0.1664	0.0138	20	0.1638	0.02
	45	23	0.1693	0.015	-	-	-	-	-	-
Tail length (mm)	Peak temp °C	n	mean	sd	n	mean	sd	n	mean	sd
	42	17	29.83	1.85	24	30.37	1.56	21	29.78	1.31
	43	37	29.73	4.17	32	29.84	2.18	28	29.21	1.84
	44	19	30.05	2.24	27	29.75	1.44	20	29.31	2.09
	45	23	30.1	1.75	-	-	-	-	-	-
Growth rate (mg/day)	Peak temp °C	n	mean	sd	n	mean	sd	n	mean	sd
	42	13	4.32	1.98	15	3.79	2.39	15	4.31	2.23
	43	21	4.49	2.05	13	3.48	2.47	7	2.05	1.08
	44	9	5.19	2.17	16	3.45	1.88	14	2.97	2.06
	45	18	3.46	1.79	-	-	-	-	-	-

Table S2. Continued

	A. cristatellus	control			one spike			two spikes		
Incubation period (days	Peak temp °C	n	mean	sd	n	mean	sd	n	mean	sd
	40	26	32.3	1.54	26	33.3	1.54	18	33.4	1.92
	41	44	33.34	1.67	29	34.59	1.4	25	35.36	1.41
	42	31	33.32	1.56	3	34	3.46	-	-	-
	43	9	34.11	3.3	-	-	-	-	-	-
SVL (mm)	Peak temp °C	n	mean	sd	n	mean	sd	n	mean	sd
	40	26	19.03	0.84	26	19.03	0.73	18	19.18	0.92
	41	44	19.32	0.69	29	19.18	0.74	25	19.3	0.78
	42	31	18.48	0.86	3	19.77	0.55	-	-	-
	43	9	18.95	0.64	-	-	-	-	-	-
Body mass (g)	Peak temp °C	n	mean	sd	n	mean	sd	n	mean	sd
	40	26	0.207	0.031	26	0.209	0.024	18	0.216	0.028
	41	44	0.227	0.026	29	0.217	0.027	25	0.226	0.025
	42	31	0.2	0.032	3	0.226	0.014	-	-	-
	43	9	0.207	0.031	-	-	-	-	-	-
Tail length (mm)	Peak temp °C	n	mean	sd	n	mean	sd	n	mean	sd
	40	26	27.63	2	26	27.45	1.63	18	27.74	1.29
	41	44	27.88	2.49	29	27.27	1.16	25	27.91	1.84
	42	31	26.66	1.94	3	27.95	2.97	-	-	-
	43	9	26.11	1.36	-	-	-	-	-	-
Growth rate (mg/day)	Peak temp °C	n	mean	sd	n	mean	sd	n	mean	sd
	40	9	5.92	3.79	18	3.85	3.05	13	3.88	2.49
	41	23	3.4	2.55	17	3.27	3.11	16	3.07	1.33
	42	13	3.27	2.46	1	3.5	-	-	-	-
	43	1	2.01	-	-	-	-	-	-	-

Table S3. Mean and standard deviation (sd) for heart rates (Hr) and temperatures (Temp) at which heart rates were measured for *A. sagrei* embryos. Day 1 was the day before treatment (43°C thermal spike), day 2 was the day after treatment, and day 3 was two days after treatment. Sample sizes (n) are listed for each treatment group. This was a repeated measures design and the same individuals were measured across all three days. Eggs that died during the thermal spike were excluded from analysis.

			Day 1				Day 2				Day 3			
			Hr (bpm)		Temp (°C)		Hr (bpm)		Temp (°C)		Hr (bpm)		Temp (°C)	
Treat	n	Age range (days)	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
Control	12	4 to 22	92.83	6.38	28.23	0.16	99.83	8.89	28.12	0.17	99.25	9.31	28.23	0.3
One spike	14	4 to 22	94.14	6.62	28.24	0.17	87.07	10.43	28.26	0.18	91.64	8.63	28.34	0.39
Two spikes	11	4 to 22	94	9.9	28.33	0.11	84.09	13.8	28.25	0.18	87.27	10.7	28.34	0.12

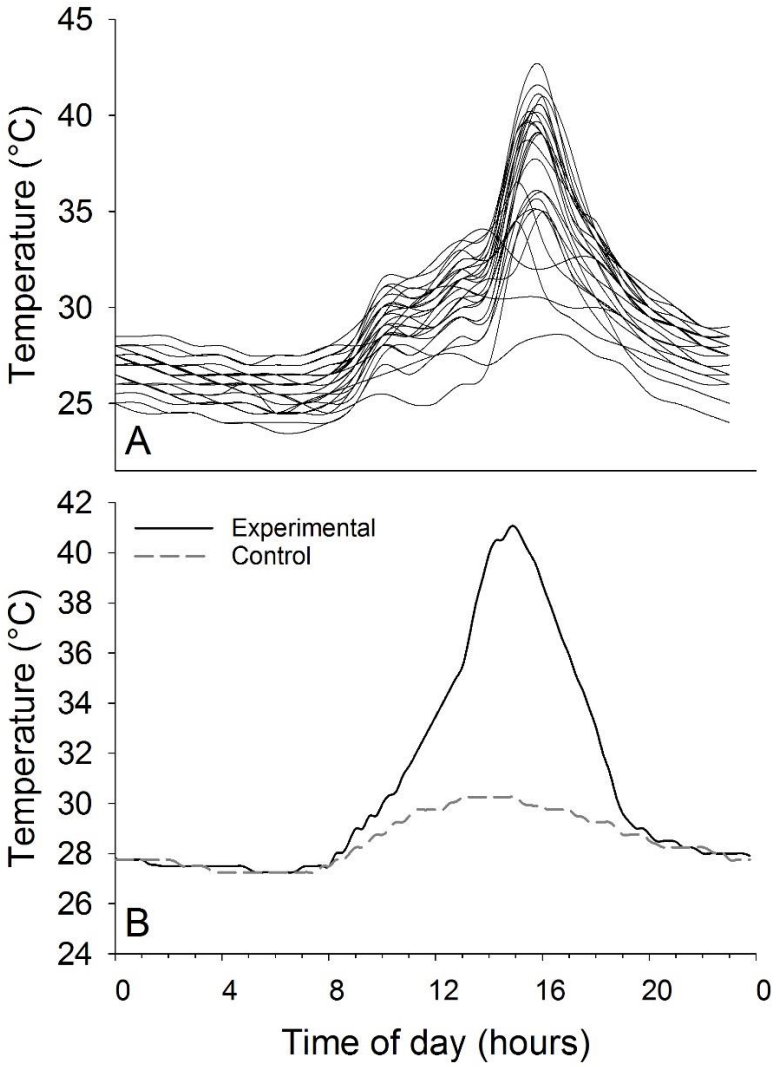

Figure S1. (A) Various thermal spikes measured from nesting areas at our field site in Pinecrest, FL and (B) two fluctuations used in the study (B). The thermal spike shown in panel (B) has a magnitude of 41 °C. Spikes of greater magnitude were also used and are shown in Figure 1 of the main text. In Panel (B), control refers to the "urban background" fluctuation (see main text).

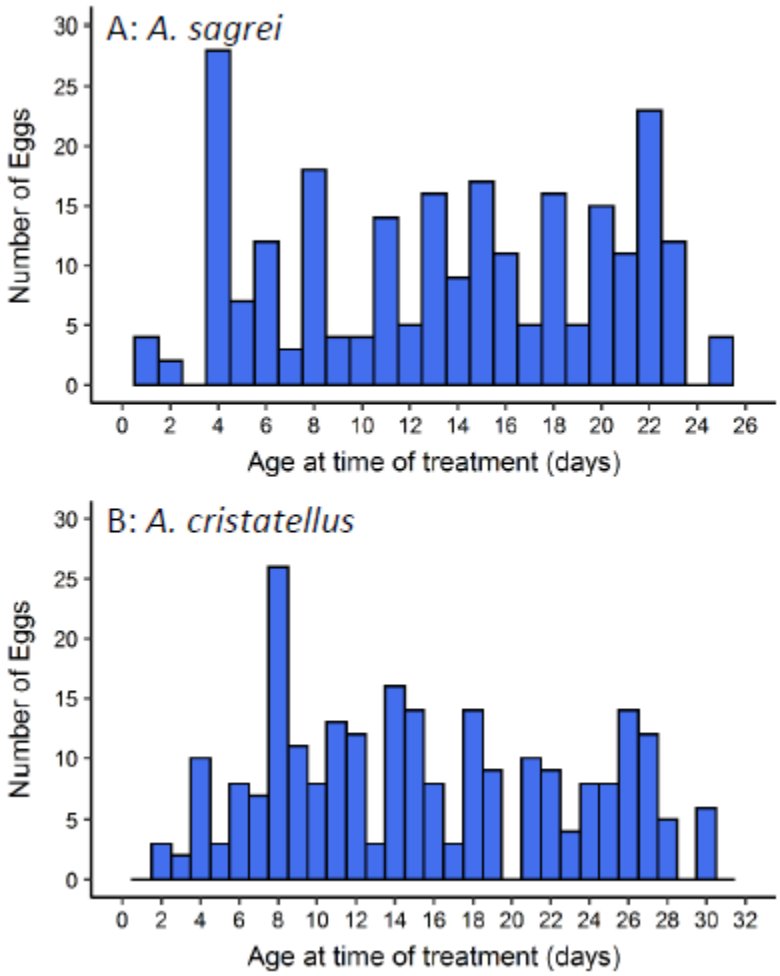

Figure S2. Histograms showing the number of eggs exposed to thermal spikes at each age of development for *A. sagrei* (A) and *A. cristatellus* (B). Age is the number of days since oviposition. One and two spike treatments are combined, and controls are not included in these counts.

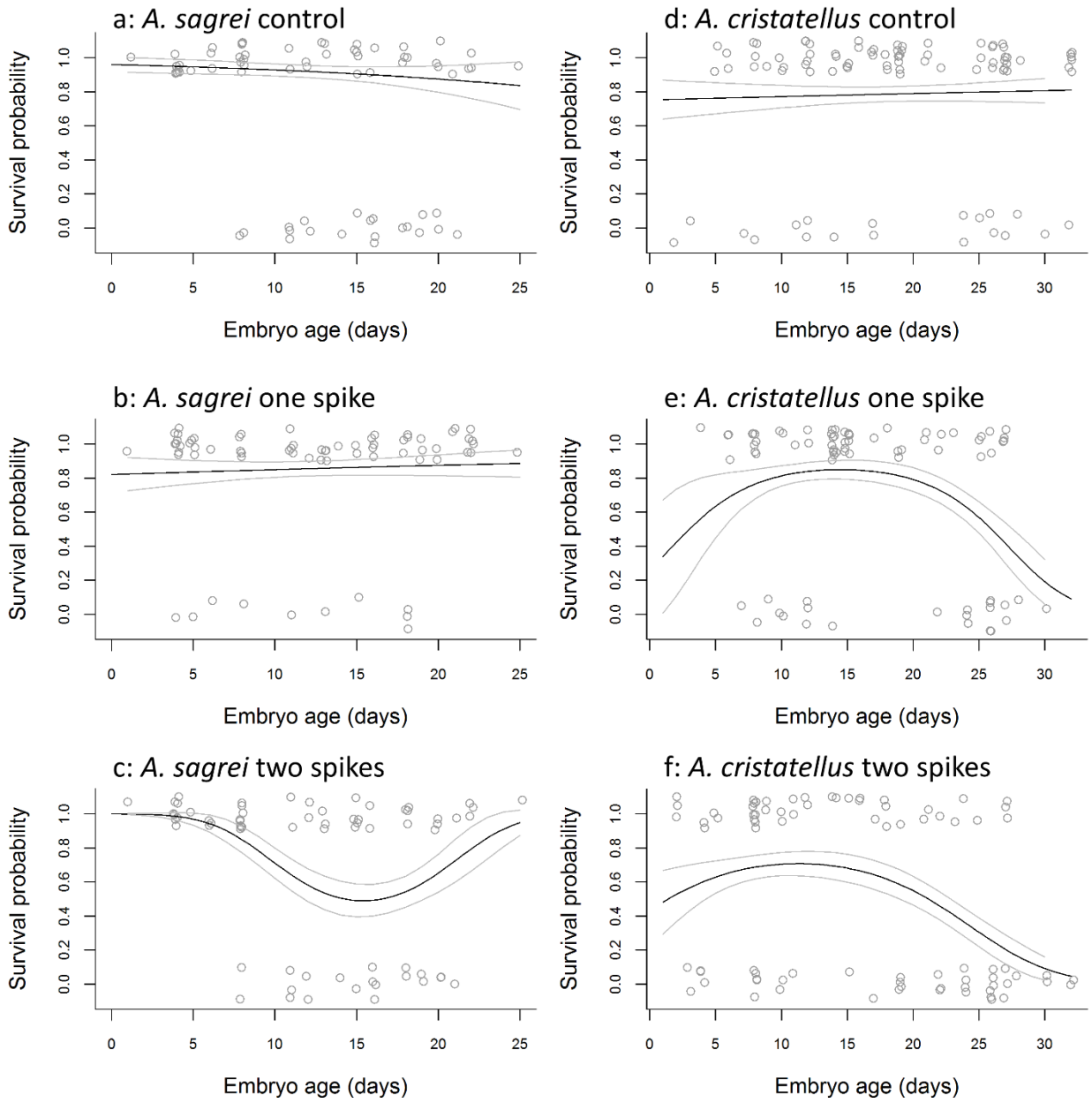

Figure S3. Egg survival according to treatment and embryo age. For each treatment (listed in each panel), solid lines show model predictions of survival and grey lines show standard errors. Open circles show the raw data jittered around 0 and 1 to avoid over plotting. Note that there were fewer relatively young and relatively old embryos for *A. sagrei* than *A. cristatellus*.

CHAPTER 5

General conclusions

In this final chapter, I precipitate out of this dissertation four important considerations for future research. First, even brief exposure to ecologically-relevant thermal stress can have important effects on developmental and hatchling phenotypes; therefore, it will be vitally important for future studies to consider the true thermal heterogeneity of natural environments in studies of thermal developmental plasticity. This applies to all oviparous ectotherms, not just reptiles. Second, despite a recent surge in interest in the mechanisms that determine the thermal limits of complex life, we still have a relatively poor understanding of why embryos die at extreme temperatures. Thus, more work is required to understand the evolution of critical limits with respect to past and future thermal environments. Third, predicting the impacts of future climate change on biodiversity is important, but urbanization is an immediate threat and will continue to be so even if humans alter their behavior in ways that mitigate or reverse temperature changes due to climate warming. Therefore, we need a better understanding of how populations acclimate and/or adapt to the urban heat island. Finally, I present several practical considerations for measuring thermal tolerance of reptile embryos. Due to the pervasive threat of global change to biodiversity coupled with a need to consider embryo responses to both acute and chronic thermal stress, research considering embryo heat tolerance will likely expand in the future and these suggestions will be vital for researchers to consider.

Replicating thermal heterogeneity is critical in studies of developmental plasticity

Over the last three decades, studies of reptile thermal developmental plasticity have made substantial progress in recreating real world temperatures in the lab. One of the most important discoveries is that fluctuating temperatures have different effects on developmental and hatchling phenotypes compared to constant temperature treatments (Georges et al., 2005). This is particularly notable at extreme temperatures (Les et al., 2009). Rather than use constant temperatures, a common contemporary practice is to incubate eggs at daily repeating sine waves with an amplitude similar to daily fluctuations of nest temperatures. However, real thermal environments exhibit daily, weekly, and seasonal variation (Pearson and Warner, 2018); thus, repeating the same fluctuations still does not fully reflect natural nest temperatures (Hall and Warner, 2020b). Moreover, due to global change, extreme thermal environments, like heatwaves, will become more frequent and add additional layers of thermal complexity onto the landscape.

Results from recent studies (e.g. Carter et al., 2018), including this dissertation, indicate that the next revolution in the study of thermal developmental plasticity is to understand how the stochastic nature of real thermal environments influences development. For example, even brief exposure to extreme fluctuations in nest temperature has important effects on morphology, physiology, and survival (Chapters 2, 3, and 4), and such extreme fluctuations occur in both natural (Chapter 2) and human modified habitats (Chapter 3). As we approach this new frontier, it will be vitally important to have standardized metrics and validated methods to make comparisons across studies. The recommendations of Chapter 1 make progress toward this goal by assessing existing methods for measuring heat tolerance and recommending metrics that can be used for comparisons across studies.

The methods used in this dissertation can be extended to all ectotherms that complete development in thermally heterogenous environments. For example, the effects of urbanization

on arthropods have been of interest to researchers for many decades (reviewed by McIntyre, 2000), but very few studies have considered how the urban heat island influences development (e.g. Kaiser et al., 2016; Johnson et al., 2019). These studies have not considered exposure to acute thermal stress nor have they specifically replicated thermal variation in their experimental design. Future studies considering the effects of the urban heat island on arthropod development could benefit from using methods like those in this dissertation. Additionally, my results have implications for species that exhibit parental care, like birds. Bird embryos are essentially ectotherms and depend on environmental temperature, often regulated by parents, to properly develop; however, at this critical stage, individuals are occasionally subject to brief changes in temperature and acute thermal stress when parents are off the nest (Conway and Martin, 2000; Ben-Ezra and Burness, 2017). As in non-avian reptiles, bird incubation studies have mostly been conducted with constant temperatures (Ben-Ezra and Burness, 2017); however, recent work demonstrates the importance of considering acute thermal stress during early-life stages (Hoffman et al., 2018; Rubin, 2019). Many of the physiological mechanisms that govern embryo responses to temperature are comparable in birds and non-avian reptiles (Du and Shine, 2015); therefore, the methods, results, and conclusions of this dissertation are surely applicable to avian systems. Ultimately, studies that ignore the true thermal heterogeneity of the environment will fail to capture how temperature interacts with development to produce phenotypes in the wild. Such a failure will leave us unable to accurately predict population responses to global change.

The mechanisms that determine heat tolerance are still poorly understood

Regardless of the methods used to study the thermal ecology of development, what is clear is that developmental processes have thermal limits. However, the mechanisms that set

these limits are not yet obvious. Proteins and cellular membranes denature at high temperatures; however, these basic biochemical explanations typically fail to explain thermal tolerance of complex organisms (Pörtner et al., 2017). van der Have (2002) proposed a proximate model to explain the thermal limits of ectotherm development, and his model is cited by current textbooks on thermal ecology and adaptation (e.g. Angilletta, 2009). This model proposes that the limits of development are determined by thermal limits on the cell cycle: i.e. embryos die at extreme temperatures because they are unable to complete development due to temperature-induced stalling of cell division. There are two important reasons why van der Have's model could use a rethink, and each can be demonstrated by considering heat tolerance of reptile embryos.

First, van der Have's model was based entirely on chronic heat stress (i.e. constant incubation temperatures), and thus, has essentially nothing to say about embryo survival with respect to acute exposures. It is difficult to imagine how stalling of the cell cycle would cause death during a 1-hour heat shock (as in Chapter 2). Indeed, many reptile embryos (including birds) experience diapause for extended periods as a natural part of development (Du and Shine, 2015); thus, a momentary halt to embryo growth during heat stress should have little effect on survival. Second, van der Have's model was created using insect eggs as a model. Due to the relatively large size of vertebrate embryos, we can dissect eggs and study the morphology resulting from heat stress. One thing is clear from such studies, at lethal temperatures, embryos continue developing, but do so poorly, exhibiting specific morphological abnormalities (Sanger et al., 2018). Were van der Have's model accurate, squamates, like anoles, killed via incubation at a high, constant temperature would be at the limb bud stage when dissected (i.e. their stage at oviposition). Rather, embryos are typically advanced, but with a host of morphological

abnormalities (most often a shortened cranium, deformed limbs and tails; Sanger et al., 2018; personal observation). Thus, van der Have's model fails to explain these empirical data.

Many recent papers indicate that oxygen limitation is the cause of death at high temperatures for both avian and non-avian reptile embryos. These studies have subjected eggs to both chronic (Liang et al., 2015) and acute (Smith et al., 2015; Vimmerstedt et al., 2019) heat stress in the presence of hypoxic, normoxic, and hyperoxic conditions. Additional studies that incubate eggs at different oxygen concentrations and at different temperatures will add little value to our understanding of the relationship between oxygen and heat tolerance. What is now needed are studies that quantify metabolic processes (e.g. heart rate, oxygen consumption, heat shock protein expression, anaerobic respiration) at near-lethal temperatures (Wang et al., 2014; Wang et al., 2017). Chapter 2 of this dissertation is the first study, to my knowledge, to quantify reptile embryo heart and metabolic rates at near-lethal temperatures; thus, defining important thermal breakpoints (i.e. pejus and critical temperatures). This research will hopefully inspire additional research to uncover the relationship between metabolism and heat tolerance.

In addition to defining how oxygen relates to thermal limits, we should expect important interactions between oxygen-limited thermal tolerance and global change. For example, the primary response of montane species to climate warming has been to move upward in elevation (Elsen and Tingley, 2015). Interestingly, higher elevation nests often experience greater maximum temperatures than those at lower elevations (Shine et al., 2003). Nesting females at high elevations must construct relatively shallow nests in locations with reduced canopy cover to ensure that mean nest temperatures are warm enough for egg survival. Shallow nests are less thermostable than deeper nests and experience greater maximum daily temperatures. Concomitantly, oxygen availability declines with elevation, therefore, upward movements in

distribution may further put embryos at risk of heat stress due to the combination of increased maximum temperatures and lower oxygen availability (Li et al., 2020). Understanding the ecological effects of such range shifts will need to consider the relationships between oxygen and acute heat stress. Currently, such studies are lacking. For example, one recent study has considered the relationships between temperature and elevation induced hypoxia in reptile embryos (Li et al., 2020); however, this study used chronic temperature treatments and did not consider responses to acute heat stress. Hopefully, the chapters of this dissertation, particularly Chapter 2, will pave the way for an increase in research that considers acute exposure of embryos to extreme temperatures.

Embryo responses to the urban heat island deserve further investigation

Such intense focus has been placed on the relationship between climate change and thermal developmental plasticity (Janzen, 1994; Telemeco et al., 2009; Mainwaring et al., 2017; Noble et al., 2018a; Valenzuela et al., 2019), but habitat destruction is the most immediate threat to biodiversity across the planet (Tilman et al., 2017). Studies that assess the effects of global climate change on development often use temperature treatments that reflect worse-case warming scenarios that are 30, 50, or even 100 years in the future (e.g. Levy et al., 2015; Dayananda and Webb, 2017; Carlo et al., 2018); however, the effects of habitat alteration (e.g. deforestation, urbanization, agriculture) on ground temperatures are immediate, sometimes extreme, and can potentially influence nest temperatures in ways that alter developmental and hatchling phenotypes (Kolbe and Janzen, 2002; Schlaepfer, 2003; Freedberg et al., 2011; Tiatragul et al., 2019). Urbanization has a homogenizing effect on biodiversity and many reasons and provided for this phenomenon (McKinney, 2008), but effects of landscape changes on egg

survival and embryo development are not often considered (with the exception of birds; Reynolds et al., 2019). As the world becomes more urbanized, it will be vital to consider how we can construct cities in ways that provide appropriate nesting habitat for species of conservation concern. Future work will need to consider the nesting behavior of species in urban areas with respect to embryo development under both chronic and acute heat stress to understand how species are responding to urbanization and what measures will be necessary to ensure population persistence. Currently, city planners weigh the costs and benefits of various strategies that reduce the urban heat island effect for human benefit (Mohajerani et al., 2017); however, some strategies may be more beneficial than others for wildlife. Until we have a fuller understanding of the ways that the urban heat island influences egg survival and nesting behavior in a diversity of species, we will not know the best strategies to preserve urban biodiversity.

Practical considerations for measuring heat tolerance of reptile embryos

Because this research required the use of various methods to assess heat tolerance of reptile embryos, I offer practical advice for researchers attempting to do the same, particularly with respect to acute thermal stress. I have explained this advice more fully in a collaborative paper concerning methods for studying thermal ecology of reptiles and amphibians (Taylor et al., 2020). First, the method for measuring embryo heat tolerance will influence results and, thus, conclusions about the potential for heat stress due to global change. Embryo thermal tolerance has historically been assessed by incubating eggs at various constant temperatures and monitoring egg survival and/or embryo development (e.g., Sanger et al., 2018). Responses to constant temperatures provide important baseline information regarding embryo thermal physiology; however, temperature varies considerably throughout the incubation period in the

wild and constant temperatures are not ecologically relevant for many species. Importantly, the difference in critical temperatures obtained via chronic vs acute methods can be dramatic (Chapter 2). Thus, both mean and maximum nest temperatures may need to be considered to effectively predict egg survival in the wild (Chapter 1).

Additionally, acute thermal tolerance has been measured using many methods, but each method can produce slightly different results (Chapters 1, 2). Thus, when selecting a method, researchers should consult Table 2 in Chapter 1 and, when sample sizes allow, use multiple methods for comparison (as in Chapter 2). When measuring acute thermal tolerance, cooling/heating rates should reflect temperature changes in natural nests, and egg temperature should be monitored throughout the assay (Chapter 2). When using heat shocks or thermal fluctuations, researchers must consider that egg containers (e.g. petri dishes, glass jars, plastic boxes) may not reach the same temperatures as incubators; therefore, temperatures inside these containers must be monitored with temperature loggers and/or thermocouples (e.g. Chapter 4).

A second issue to consider is that chronic heat tolerance is assessed across the entire incubation period, but acute thermal tolerance is measured at a single time point during development. Responses to acute thermal stress may change with ontogeny: younger and older embryos may be more sensitive to thermal stress (Chapter 4). Thus, a basic protocol for any research program should be to determine embryo responses to extreme temperatures at a minimum of three developmental time points (e.g., early, mid, and late developmental stages). This is challenging due to among-species diversity in the timing of oviposition with respect to development. For example, squamate embryos are often oviposited at the limb bud stage, and responses of earlier stage embryos to temperature (e.g. gastrula stage) may be difficult to assess independent of the maternal environment prior to oviposition. Other species (e.g. turtles) deposit

eggs at a much earlier embryonic stage. Therefore, researchers must explicitly describe the timing of assays with respect to both oviposition and developmental stage.

Third, the ecology of the study organism will dictate the relative importance of responses to chronic versus acute thermal stressors. There is vast diversity in nesting behavior across reptiles and, not surprisingly, concomitant diversity in the variability of incubation temperatures in the wild (Booth, 2018). For example, some reptiles construct relatively shallow nests while others construct nests deep underground. Embryo responses to acute thermal stress may be highly ecologically relevant for the former, while responses to chronic thermal conditions may be more relevant for the latter. The best practice will be to determine both the embryo chronic and acute heat tolerance for the study species as described in Chapter 1, and then determine important break points in metabolism (e.g. pejus, critical temperatures) and assess the potential for embryos to experience chronic and acute heat stress as well as thermal break points by using temperatures collected from natural nests (as in Chapter 2).

Closing remarks

Natural thermal environments are complex. Thus, understanding the relationship between temperature and development depends on our willingness and ability to replicate natural complexity in controlled experiments. Past research, however, has been largely focused on mean temperatures and constant conditions. Regardless, I greatly appreciate those who performed this foundational work and the knowledge they created. I never feel qualified to be critical, but in offering criticism, I am reminded of a quote by Steve Jobs, "*Everything around you that you call 'life' was made up by people who are no smarter than you*." Future studies should consider the complexity of natural environments to understand embryo heat tolerance in a changing world.

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