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Received: 20 January 2020 | Revised: 27 March 2020 | Accepted: 30 March 2020

DOI: 10.1002/jez.2359

RESEARCH PAPER

JEZ-A ECOLOGICAL AND INTEGRATIVE PHYSIOLOGY WILEY

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

Joshua M. Hall | Daniel A. Warner

Department of Biological Sciences, Auburn University, Auburn, Alabama

Correspondence
Joshua M. Hall, Department of Biological Sciences, Auburn University, 101 Rouse Life Sciences Building, Auburn, AL 36849.
Email: jmh0131@auburn.edu

Funding information
National Science Foundation, Division of Environmental Biology, Grant/Award Number: 1564563; Alabama EPSCoR, Grant/Award Number: Graduate Research Scholars Program

Abstract

Aspects of global change create stressful thermal environments that threaten biodiversity. Oviparous, non-avian reptiles have received considerable attention because eggs are left to develop under prevailing conditions, leaving developing embryos vulnerable to increases in temperature. Though many studies assess embryo responses to long-term (i.e., chronic), constant incubation temperatures, few assess responses to acute exposures which are more relevant for many species. We 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 sublethal but stressful temperatures on development and hatchling phenotypes and survival. Most embryos died at heat shocks of 45°C or 46°C, which is ~12°C warmer than the highest constant temperatures suitable for successful development. 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 our study highlights the role of both immediate and cumulative effects of high temperatures on egg survival. Such effects must be considered to predict how populations will respond to global change.

KEYWORDS
climate change, critical thermal maximum, heart rate, heat shock, metabolic rate, oxygen-limited thermal tolerance

1 | INTRODUCTION

Multiple aspects of global change (e.g., urbanization and climate change) create novel, stressful thermal environments that threaten biodiversity across the planet (McDonald, Kareiva, & Forman, 2008; Sinervo et al., 2010). Much research on this topic quantifies how adult organisms respond to high temperatures (e.g., Battles & Kolbe, 2019; Sinervo et al., 2010); however, the effect of global change on developing offspring (e.g., embryos) is less studied, but also important (Burggren, 2018; Ma et al., 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

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capacity to behaviorally thermoregulate (Cordero, Telemeco, & Gangloff, 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 (Kaiser, Merckx, & Van Dyck, 2016; Shine, Langkilde, Wall, & Mason, 2005). Finally, egg mortality can drive population cycles (Chalcraft & Andrews, 1999); therefore, the thermal sensitivity of embryos can influence species distributions and population persistence in the face of global change (Carlo, Riddell, Levy, & Sears, 2018; Ma 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).

Non-avian reptiles (henceforth, "reptiles") have contributed greatly to our understanding of embryo thermal ecology (Noble, Stenhouse, & Schwanz, 2018; Refsnider, Clifton, & Vazquez, 2019), and many species are threatened by global change (Santidrián Tomillo, Genovart, Paladino, Spotila, & Oro, 2015; Sinervo et al., 2010). A recent flurry of work has synthesized existing egg incubation studies (Booth, 2018; Noble et al., 2018; Warner, Du, & Georges, 2018; While et al., 2018), demonstrating that most researchers use a series of constant incubation temperatures to define the upper thermal limits for development (Andrews & 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, Carter, & Paitz, 2014; Noble et al., 2018; Warner & Shine, 2011); 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, Zelic, Adrian, Hurliman, & Smith, 2013). This knowledge gap should be filled for two reasons. First, it 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 & Schwarzkopf, 2012). Second, in some contexts, maximum nest temperatures can drive the evolution of life-history traits more so than mean temperatures (Shine, Elphick, & Barrott, 2003). Given that nest temperatures often fluctuate above the critical thermal maximum for some species (e.g. Angilletta et al., 2013; Sanger, Kyrkos, Lachance, Czesny, & Stroud, 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. Protocols for their captive husbandry are established (Sanger, Hime, Johnson, Diani, & Losos, 2008), they are relatively fecund in captivity allowing for robust sample sizes (Hall, Buckelew, Lovern, Secor, & Warner, 2018), and their developmental staging series has been described (Sanger, Losos, & Gibson-Brown, 2008). Females construct shallow nests across a diversity of habitats; thus, in the wild, embryos experience relatively large thermal variation during incubation (Gunderson, Fargevieille, & Warner, 2020; Sanger et al., 2018) and temperature has important effects on embryo development, egg survival, and hatchling phenotypes (Pearson & Warner, 2018). Moreover, daily fluctuations in nest temperature often reach stressfully warm temperatures (i.e., >40°C; Sanger et al., 2018), indicating that embryos might have physiological mechanisms for ameliorating the adverse effects of acute exposure to high temperatures.

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 (a) determine the acute lethal temperature for *A. sagrei* embryos, (b) quantify the thermal sensitivity of embryo physiology across nest temperatures and near-lethal temperatures, (c) and assess the effects of repeated exposure to sublethal, but stressful temperatures on embryo development and hatchling phenotypes and survival. To determine the acute lethal temperature (T_{LETHAL}) for embryos, we exposed eggs to 1-hr heat shocks of increasingly high temperatures. To quantify embryo physiology, we measured embryo heart rates (f_H) and oxygen consumption (VO_2) across temperatures from 22°C to 47°C, which encompasses the range of nest temperatures commonly reported for this species (Gunderson et al., 2020; Pruett, Fargevieille, & Warner, In press; Sanger et al., 2018). 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 are predicted to increase due to climate change, these thermal treatments will provide an ecologically relevant evaluation of the relationship between thermal stress and embryo development under current and future environments. In turn, this study will improve predictions of how reptile populations respond to global change.

2 | METHODS

2.1 | Determining T_{LETHAL} 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 × 45 × 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 three 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 & Warner, 2018).

We subjected all eggs ($n = 72$) to a series of 1-hr heat shocks starting at 44°C because eggs are robust to brief exposures to 43°C (Hall & Warner, 2019). 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., lethal temperature; T_{LETHAL}). To apply heat shocks, we placed eggs in glass jars (59 ml FLINT s/s) that were 3/4 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 hr before 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 min 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, this method was effective.

Due to variation in egg-laying date among females, eggs ranged in age from 4 to 17 days postoviposition (i.e., 11–47% of the incubation period completed). To estimate T_{LETHAL} while controlling for variation in egg size and age, we performed a linear regression with T_{LETHAL} as the response variable and egg age (i.e., days since oviposition) and egg mass (which was measured before treatment) as fixed effects. Egg mass and age were centred at zero before analysis.

2.2 | 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 to 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 before measurements. A constant temperature was used to avoid potential circadian rhythms in f_H. Thus, embryos varied in age from 7 to 14 days postoviposition (i.e., 23–46% of the incubation period 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 hr 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 1/2 hr at various target temperatures (22°C, 26°C, 29°C, 31°C, 34°C, 37°C, 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 45–60 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°C to 47°C. Heart rates were measured at 40°C (after bringing them from 28°C 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). Brief exposure to 42°C has no detectable effect on *A. sagrei* embryo development or survival (Hall & Warner, 2019); 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 ($p = .32$).

A different subset of eggs was used to determine the thermal sensitivity of embryo VO_2 ($n = 45$ eggs; that is, all eggs collected from 23 to 30 July 2018). These eggs were incubated in petri dishes (as previously described) at a constant 28°C for 1 week before measurements. A constant temperature was used to avoid potential circadian rhythms in metabolic rate. Thus, embryos were between 7 and 14 days since oviposition at time of measurement (i.e., 23–46% of the incubation period completed). These eggs were randomly allocated to 1 of 9 temperature treatments (21°C, 25°C, 29°C, 33°C, 35°C, 39°C, 41°C, 44°C, 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 min 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 min. 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 a likelihood ratio test. We excluded eggs incubated at 47°C because they died during treatment which prevents 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.

2.3 | 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 1 or 2 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., 10–57% of the incubation period 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 Hall & Warner, 2018). The peak temperature of this thermal spike was 43°C. A previous study showed that one or two exposures to this fluctuation result in moderate reductions in egg survival and hatchling body size, but most effects were not statistically clear (Hall & Warner, 2019). For example, hatching success was 82% and 78% for 1 and 2 spikes, respectively, compared to 93% for controls. Here, we use four 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

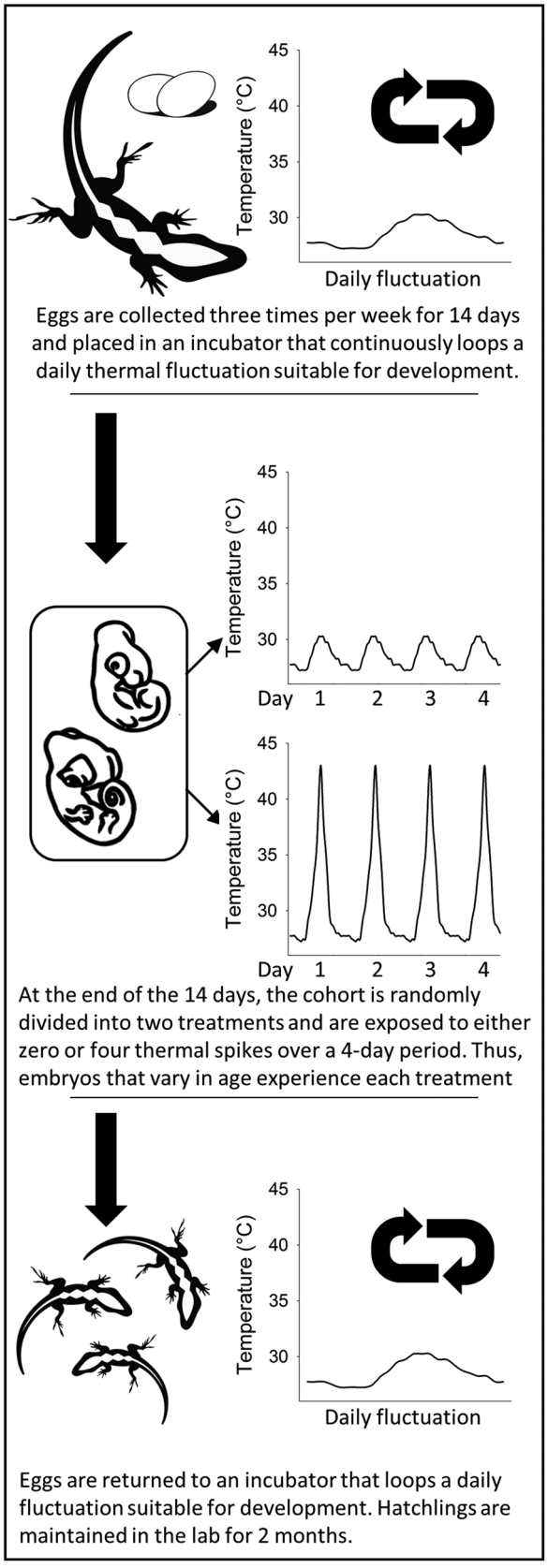

FIGURE 1 Overview of experimental design to assess repeated exposure to sublethal temperatures

development (see results). Thus, four 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 2 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 six hatchlings per cage (three 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.

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 $p > .13$). Embryo age was the first day of treatment minus the day the egg was collected. To convert incubation periods to developmental rates, we divided one by the incubation period (days from oviposition to hatching; Andrews & Schwarzkopf, 2012). Hatchling growth rate was the final mass of each hatchling minus its body mass at hatching and 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, and 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; $\chi^2 = 2.62$; $p = .96$), 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, 1 day posttreatment, or 2 days posttreatment), 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).

2.4 | 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. (In press) ($n = 47$ 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. (In press) 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 hr during May, June, and July of 2013. Pruett et al. (In press) 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., In press). 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 (Wittmann 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 (Wittmann et al., 2008). We selected 40°C based on results from this study (see below). Indeed, brief daily exposure to similar temperatures (33.4°C and 39.7°C) induces high rates of egg mortality (42.9% and 60.3%, respectively; Gunderson et al., 2020).

3 | RESULTS

3.1 | Determining T_{LETHAL} via heat shock

Most embryos died at 45°C or 46°C (Figure 2). There was no statistically clear effect of egg age (0.006 ± 0.02 standard error [*SE*]; $t_{1,69} = 0.23$; $p = .80$) or initial egg mass (0.00004 ± 0.003 *SE*; $t_{1,69} = 0.01$; $p = .99$) on T_{LETHAL}. Thus, we consider the intercept of the linear model, 45.3°C (45.14–45.44; 95% confidence interval [CI]), to be the mean value of T_{LETHAL}.

3.2 | 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*; $t_{1,97} = 2.62$; $p = .01$) and the quadratic term was 0.071 (±0.019 *SE*; $t_{1,97} = 3.71$; $p = .0003$). The temperature at which f_H was no longer detectable (i.e., T_{LETHAL}) was 46.15°C (45.87–46.44; 95%

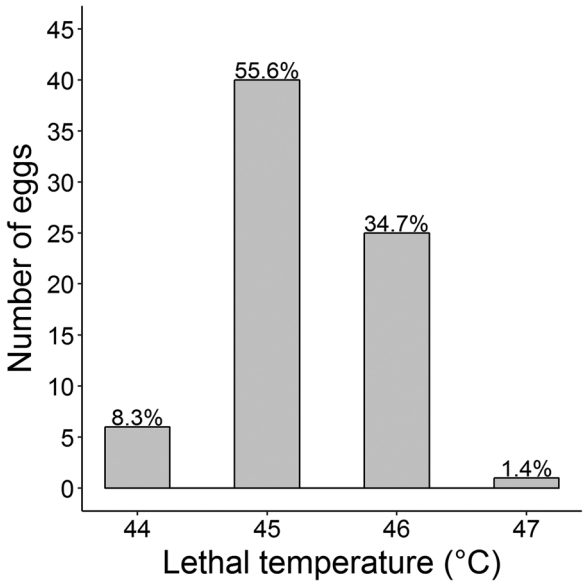

FIGURE 2 Histogram of T_{LETHAL} for *Anolis sagrei* eggs exposed to 1-hr heat shocks. Values above each bar are the percentage of total eggs (n = 72)

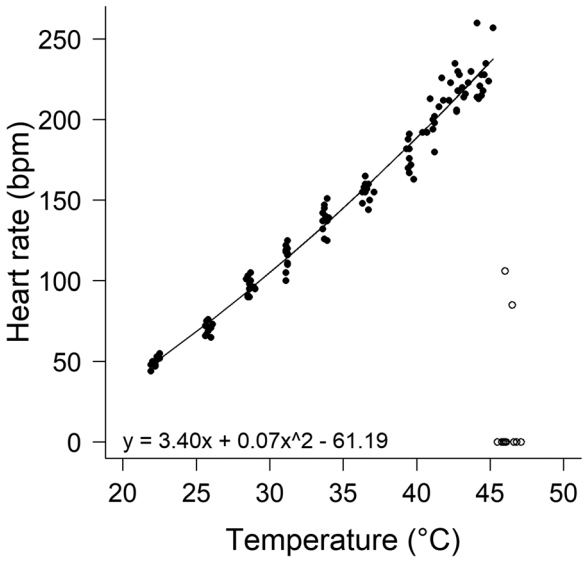

FIGURE 3 Heart rates of *Anolis sagrei* embryos across temperature. Closed and open circles show data that are included or excluded from the regression, respectively. The equation for the regression is given in the panel

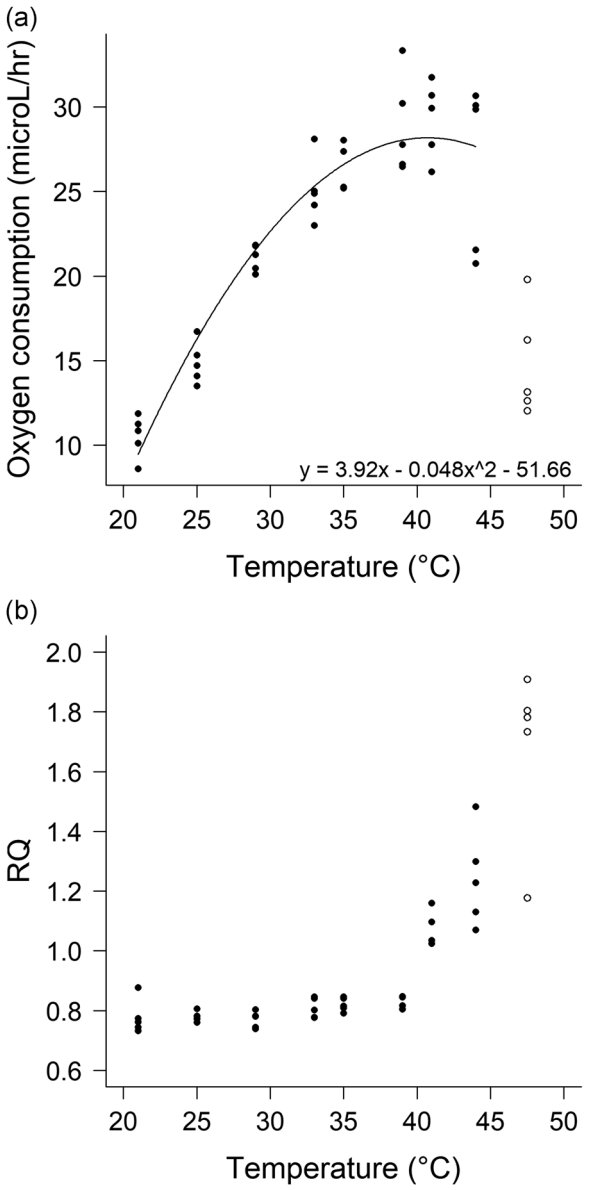

FIGURE 4 Oxygen consumption (a) and respiratory quotient (b) of *Anolis sagrei* embryos across temperature. Closed and open circles show raw data for eggs that did and did not survive measurements, respectively. Only surviving eggs were used to analyze oxygen consumption. The solid line in panel (a)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

CI). This estimate of T_{LETHAL} differs from that of the heat shock experiment (i.e., confidence intervals do not overlap).

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°C 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 < .0001) and the quadratic term was −0.048 (±0.0079 *SE*; $t_{1,36}$ = −6.06; p < .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 = .26). RQ was stable from 22°C 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 overlap with 1 (Figure 5).

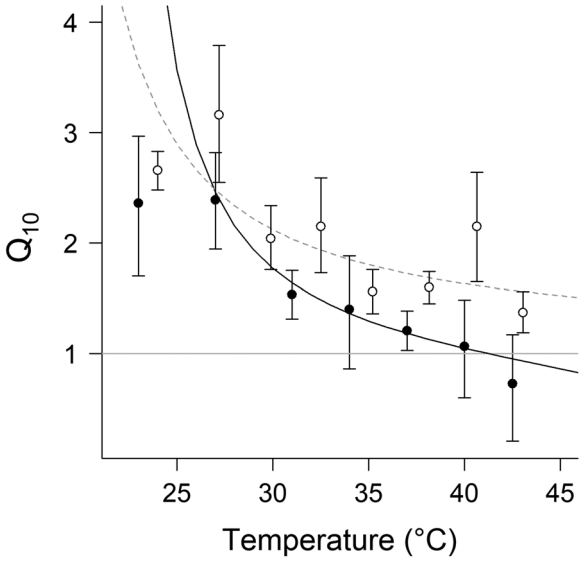

FIGURE 5 Temperature coefficient (i.e., Q_{10}) of heart rate (open circles; dashed gray line) and oxygen consumption (closed circles; solid black line) across temperature. The horizontal gray line shows the point at which reactions are insensitive to temperature (i.e., $Q_{10} = 1$). Regression lines 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°C and 25°C is plotted at 23°C)

3.3 | 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 ($F_{2,123} = 11.73$; $p < .0001$): f_H of experimental embryos were 10.3 bpm (±2.0 *SE*; $p < .0001$) and 9.0 bpm (±1.9 *SE*; $p < .0001$) lower than controls on 1 and 2 days after experiencing the 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 Tables S2, S3, S4, and S5 for raw means, sample sizes, and standard deviations of all variables.

3.4 | 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°C 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 1 day. In 2013 and 2018, six (27%) and seven (15%) nest sites, respectively, exhibited peak temperatures of 40°C or higher on at least 1 day. Four (18%) and five (11%) nesting sites exhibited temperatures >40°C on at least 3 days in 2013 and 2018, respectively. Only one (5%) and two (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.

4 | 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 the T_{LETHAL} of embryos, quantify the thermal sensitivity of embryo physiology, and assess the cumulative effects of sublethal but stressful temperatures. Most embryos died at 45°C 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 T_{LETHAL}, 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.

4.1 | Determining T_{LETHAL} 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 & Schwarzkopf, 2012). The OTR has not been defined for *A. sagrei*, but it is probably between 20°C and 33°C. Eggs incubated from 26°C to 30°C have high hatching success (~93%; Warner, Moody, Telemeco, & Kolbe, 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°C and 36°C. In the latter study, eggs were dissected at Day 12 and hatching

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 covariates on *Anolis sagrei* embryo and hatchling phenotypes and survival

	Initial mass		Age		Treatment	
Response variable	**Estimate (*SE*)**		**Estimate (*SE*)**		**Estimate (*SE*)**	
Egg survival	5.87 (29.44)	$\chi^2_1 = 0.04$; $p = .84$	−0.35 (0.13)	**$\chi^2_1 = 10.97$; $p = .001$**	–	–
Developmental rate ($days^{-1}$)	−0.003 (0.009)	$F_{1,34} = 0.13$; $p = .72$	0.00001 (0.00003)	$F_{1,34} = 0.05$; $p = .82$	−0.0017 (0.00028)	**$F_{1,34} = 35.05$; $p < .0001$**
Hatchling initial SVL (mm)	7.51 (5.00)	$F_{1,34} = 2.3$; $p = .14$	0.02 (0.02)	$F_{1,34} = 1.2$; $p = .29$	−0.25 (0.18)	$F_{1,34} = 2.0$; $p = .16$
Hatchling initial mass (mg)	357.5 (107.8)	**$F_{1,34} = 11.0$; $p = .002$**	0.6 (0.4)	$F_{1,34} = 2.5$; $p = .13$	−8.0 (3.2)	**$F_{1,34} = 6.2$; $p = .02$**
Hatchling initial tail length (mm)	22.60 (12.00)	$F_{1,34} = 3.6$; $p = .07$	0.044 (0.039)	$F_{1,34} = 1.3$; $p = .27$	−0.93 (0.34)	**$F_{1,34} = 7.5$; $p = .01$**
Hatchling survival	0.44 (0.34)	$\chi^2_1 = 1.8$; $p = .18$	−0.46 (0.34)	$\chi^2_1 = 2.0$; $p = .16$	−1.31 (0.68)	**$\chi^2_1 = 4.0$; $p = .045$**
Hatchling growth rate (mg/day)	−23.6 (26.8)	$F_{1,20} = 0.78$; $p = .37$	−0.001 (0.08)	$F_{1,20} = 0.001$; $p = .99$	−1.5 (0.7)	$F_{1,20} = 4.2$; $p = .053$

Note: Bold text denotes statistical significance ($p < .05$). See the Supporting Information 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.

success, per se, was not quantified. Given that thermal tolerance declines through development (see Figure 6a), assessing egg survival via dissection, rather than hatching success, may overestimate the OTR. Other data (Pruett and Warner, unpublished) indicate that hatching success begins declining at 29°C and reaches zero at 35°C under constant temperature incubation. 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 versus 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 & 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 T_{LETHAL}, potentially maximizing T_{LETHAL} across the range. Thus, population-specific thermal adaptation differs with respect to chronic versus acute temperatures.

Although there are established protocols for quantifying the critical thermal maximum of adults, no common protocols exist for estimating T_{LETHAL} of reptile embryos (Angilletta et al., 2013). 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 our estimates of T_{LETHAL}. Our f_H study gave an estimate of T_{LETHAL} that was nearly 1°C higher than the heat shock experiment (i.e., 46.2°C vs. 45.3°C). Furthermore, a recent study that subjected eggs to thermal fluctuations of increasingly high temperature estimated T_{LETHAL} to be ~44.5°C for *A. sagrei* embryos (Hall & Warner, 2019). The differences in T_{LETHAL} 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 & 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 (Gao et al., 2014; Nechaeva, 2011). 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 (Sanger et al., 2018), we expect the upper thermal limit of *A. sagrei* embryos to be high compared to species that develop in cooler nests (e.g., *Anolis cristatellus*; Hall & Warner, 2019; Tiatragul, Hall, Pavlik, & Warner, 2019). However, few studies have determined the upper thermal limit of reptile embryos using acute

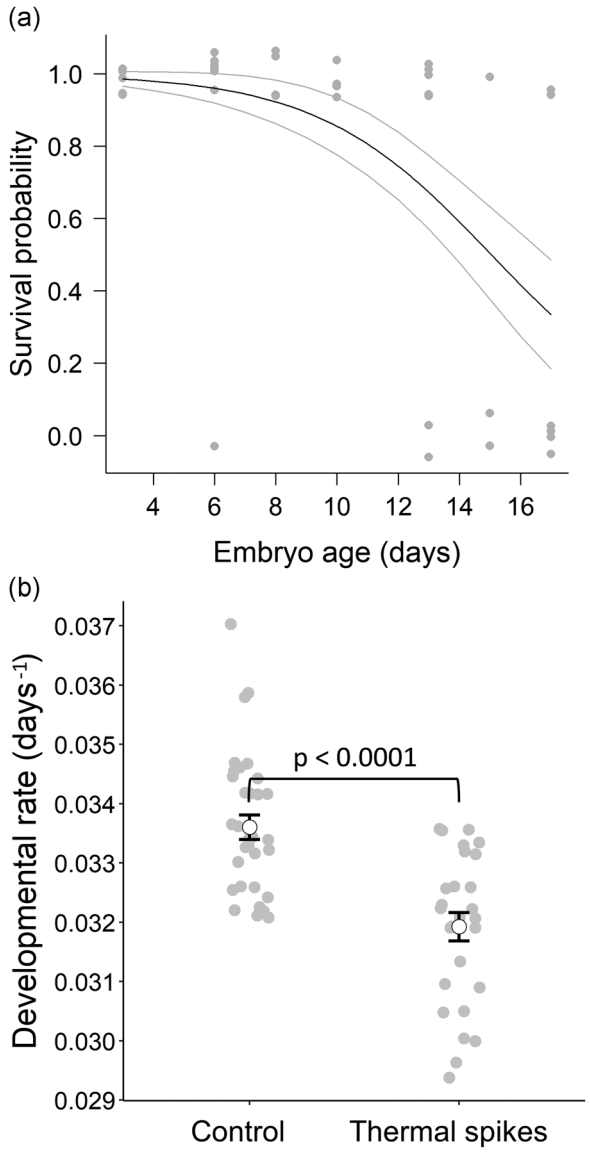

FIGURE 6 Effects of zero ("Control") or four exposures ("Thermal spikes") to thermal spikes on *Anolis sagrei* embryos. (a) How survival probability of embryos in the experimental treatment declines with age. The solid line shows a regression of the raw data and gray circles are the raw data, jittered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

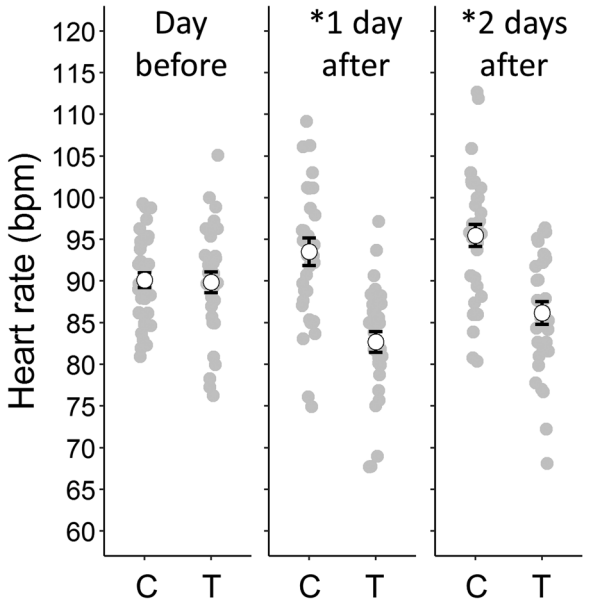

FIGURE 7 Heart rates of *Anolis sagrei* embryos exposed to 0 (C for "Control") or 4 (T for "Thermal spikes") thermal spikes. Heart rates were measured at 28°C on the day before exposure and on 1 and 2 days after exposure. Open circles show the raw means for each group, bars show standard error, and gray circles show the raw data. Asterisks signify a statistically significant difference in heart rate between groups

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 T_{LETHAL} 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°C, 45°C, 47°C, and 41°C, respectively (Angilletta et al., 2013; Gao et al., 2014; Smith, Telemeco, Angilletta, & VandenBrooks, 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.

4.2 | 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 2–3 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 (i.e., 23–30°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 T_{LETHAL}. At high temperatures, VO_2 should plateau (i.e., maximum oxygen capacity, see Gangloff & Telemeco, 2018), causing f_H to decline or plateau due to low oxygen supply to cardiac muscle (Crossley & Altimiras, 2005). Thus, we expected a similar relationship between f_H and VO_2 as temperatures

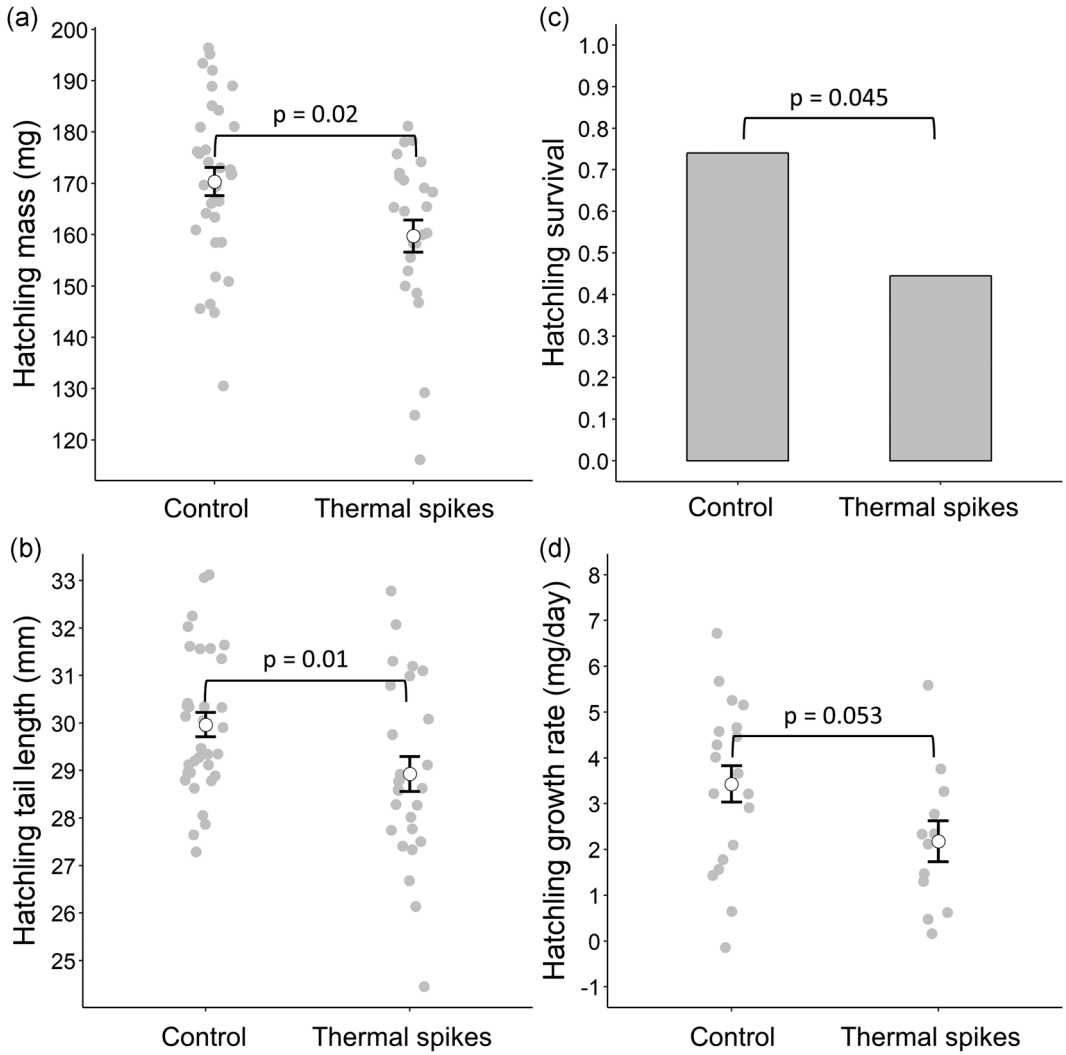

FIGURE 8 Effects of zero ("Control") or four exposures ("Thermal spikes") to extreme thermal fluctuations (43°C peak temperature) during development on hatchling mass (a) and tail length (b) at time of hatching and hatchling survival (c) and growth rate (d) over a 2-month period in the laboratory. In panels (a), (b), and (d), open circles show the raw mean, bars show the standard error, and gray circles show the raw data

approached T_{LETHAL}. Rather, when approaching T_{LETHAL}, f_H continued rising. Moreover, RQ values increased near T_{LETHAL}, indicating the use of anaerobic respiration since lizard embryos derive energy from lipids and protein (RQs between 0.7 and 0.9, Thompson, Speake, Russell, & McCartney, 2001). These data implicate a mismatch between oxygen demand and supply as a cause for death at high temperatures (Gangloff & Telemeco, 2018; Wittmann et al., 2008). Indeed, other studies find that hypoxic and hyperoxic incubation conditions decrease or increase, respectively, the thermal tolerance of reptile embryos (e.g., Liang, Sun, Ma, & Du, 2015; Smith et al., 2015; Vimmerstedt, Padilla Pérez, Angilletta, & VandenBrooks, 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 before 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 before 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.

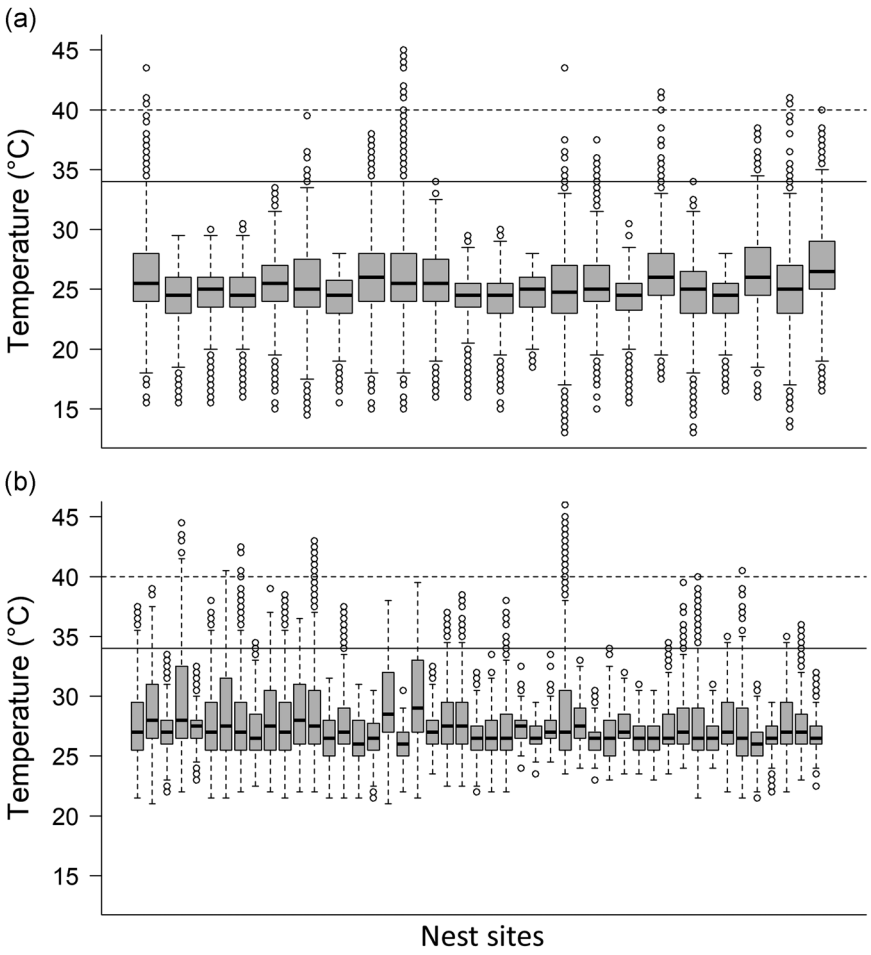

FIGURE 9 Temperatures from potential *Anolis sagrei* nest sites in Palm Coast, Florida collected during May, June, and July of 2013 (a) and from actual nest sites June and July of 2018 (b). Solid and dashed horizontal lines denote the pejus (34°C) and critical temperatures (40°C), respectively

4.3 | Repeated exposure to sublethal temperatures

In a separate study (Hall & Warner, 2019), 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- versus late-stage embryos (Thompson & 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 the incubation period, but those in the heat shock experiment had completed only 47%. This may, in part, explain why survival correlated with embryo age in the thermal spike assay but not the heat shock experiment. Indeed, in squamates, the second half of the incubation period is characterized by rapid growth and a concomitant increase in oxygen demand, while the first half is characterized by organogenesis. Death at high temperatures likely results from multiple factors at different levels of biological complexity (Gangloff & 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, Radder, Sun, & Shine, 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 & Paull, 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, Zamudio, Doughty, & Huey, 1992); however, other factors, like the timing of hatching, may be much more important (Pearson & 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 & 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.

4.4 | Temperatures of nest sites in the field

The mean incubation temperatures for our control and experimental groups were 28.7°C 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 (Pearson & Warner, 2018; Warner et al., 2012). 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 study 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. (In press) 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°C 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 (Pruett et al., In press; Tiatragul, Hall, & Warner, 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. How do embryos survive such extreme temperatures in the field? This is an open question; however, the answer may lie in the interactions between a myriad of abiotic factors that are present in the wild (e.g., moisture, solar radiation, oxygen availability, substrate composition, and shade cover). The effects of such factors and their interactions with nest temperature on development are less often assessed than temperature alone (Warner et al., 2018) but are warranted.

4.5 | 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 & Huey, 2016). Our results indicate that brown anole embryos have an upper lethal temperature 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 T_{LETHAL}, 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.

ACKNOWLEDGMENTS

We thank A. Dees, A. DeSana, J. Miracle, C. Reali, M. Turner, and K. Wilson for help with animal care, A. Appel for logistical advice, C. Guyer for helpful comments on an earlier draft, and J. Pruett for providing nest temperature data. This study was supported by the National Science Foundation (Grant Number DEB-1564563) and approved by the Auburn University IACUC committee (protocols 2017-3027 and 2018-3233). J. Hall acknowledges financial support from the Alabama Graduate Research Scholars Program (GRSP) funded through the Alabama Commission for Higher Education and

administered by the Alabama EPSCoR. This is publication number 907 of the Auburn University Museum of Natural history.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

ORCID

Joshua M. Hall http://orcid.org/0000-0002-5587-3402
Daniel A. Warner http://orcid.org/0000-0001-7231-7785

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Hall JM, Warner DA. Thermal sensitivity of lizard embryos indicates a mismatch between oxygen supply and demand at near-lethal temperatures. *J. Exp. Zool*. 2021;335:72–85. https://doi.org/10.1002/jez.2359