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RESEARCH ARTICLE

Thermal tolerance in the urban heat island: thermal sensitivity varies ontogenetically and differs between embryos of two sympatric ectotherms

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ABSTRACT

Most studies of thermal tolerance use adults, but early-life stages (e.g. embryos) are often more sensitive to thermal agitation. Studies that examine effects on embryos rarely assess the potential for thermal tolerance to change with ontogeny or how effects differ among sympatric species, and often utilize unrealistic temperature treatments. We used thermal fluctuations from nests within the urban-heat island to determine how thermal tolerance of embryos changes across development and differs among two sympatric lizard species (*Anolis sagrei* and *Anolis cristatellus*). We applied fluctuations that varied in frequency and magnitude at different times during development and measured effects on embryo physiology and 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. Inter-specific responses and the timing of stochastic thermal events with respect to development have important effects on embryo mortality. Thus, research that integrates ecologically meaningful thermal treatments, considers multiple life-history stages and examines interspecific responses will be critical to make robust predictions of the impacts of global change on wildlife.

KEY WORDS: *Anolis*, Critical thermal maximum, Early-life stage, Global change, Oxygen-limited thermal tolerance, Urbanization

INTRODUCTION

Urbanization dramatically transforms natural landscapes with respect to structural and thermal environments (Battles et al., 2018; Tiatragul et al., 2019). As a result of reduced greenspace and an abundance of heat-absorbing substrates, urban habitats have higher mean temperatures and increased levels of thermal variation compared with 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 (Kaiser et al., 2016; Tiatragul et al., 2017; Hall and Warner, 2018). Embryos, for example, are highly sensitive to thermal stress because of a relatively narrow thermal tolerance (Pörtner et al., 2017) and a limited ability to thermoregulate (but see Du and Shine, 2015). Importantly, successful embryo development is a prerequisite for survival in every environment and must be considered when assessing the impacts of 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 (but see 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. Because of 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., 2018).

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., 2018), 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

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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; Hall and Warner, 2018). 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 (modeled 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*; Fig. 1A) and the Puerto Rican crested anole (*Anolis cristatellus*; Fig. 1B), 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, given 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 and aid predictions of how wildlife will respond to global change.

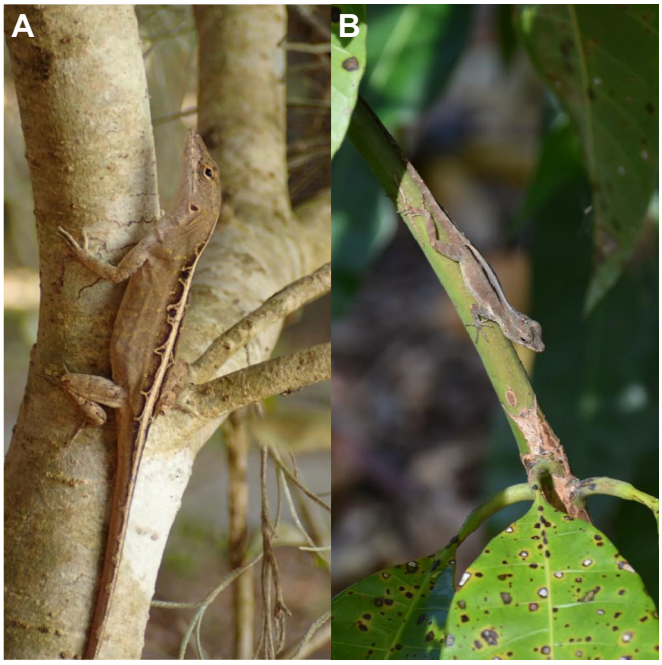

Fig. 1. Anole lizards used in this study. (A) *Anolis sagrei* female (photo credit: A. F. Kahrl, orcid.org/0000-0002-1650-1227) and (B) *Anolis cristatellus* female (photo credit: C. J. Thawley, orcid.org/0000-0002-6040-2613).

MATERIALS AND METHODS

Study species

Anolis sagrei and *A. cristatellus*, both Duméril and Bibron 1837, are relatively small (2–5 g and 3–8 g, respectively), subtropical lizards native to Cuba and Puerto Rico, respectively; however, the two 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., 2016; Winchell et al., 2016; Hall and Warner, 2017, 2018; Tiatragul et al., 2017, 2019; Battles et al., 2018; Lapiedra, 2018; Battles and Kolbe, 2019; Thawley et al., 2019).

Captive husbandry and egg collection

This research was approved by IACUC # 2015-2785. 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 on 2–3 June 2017 from a suburban area in Pinecrest, FL, USA (coordinates 25.678125, −80.287655). Fewer females of *A. sagrei* were required because of their higher fecundity compared with *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 (Zoo Med Inc.) and plant grow bulbs (model F40; General Electric Co.), with a 12 h:12 h light:dark cycle and maintained at a mean ambient room temperature of 25.6°C (range 24.5–27°C). Cage temperatures were measured via 16 temperature loggers (Thermochron iButtons) distributed across four cages. Within each of these cages, iButtons were placed in various locations (e.g. perches, cage floor) to capture the range of temperatures. Because of the light sources, ambient cage temperature was ∼28°C and, during the day, maximum daily temperature was 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) 2 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 2–3 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*).

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We collected eggs 3 times each week and recorded their mass, date of oviposition and maternal identity. Eggs were individually placed in a Petri dish (60×15 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'; Fig. 2). 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 three 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 (Fig. 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 resembled 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 the thermal tolerance of embryos, we raised or lowered the peak of the thermal spike by 1°C (Fig. 2). We started with a peak of 43°C because previous work indicated that survival declines for anole embryos at this temperature (Hall and Warner, 2018); however, this resulted in high survival for *A. sagrei* and near-total mortality for *A. cristatellus* (see Results). Thus, 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 (Fig. 3; Fig. 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 develop quicker than *A. cristatellus* embryos; 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 s.d.) 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 08:00 h, 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 moved to the 28°C incubator for 1 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 1 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-

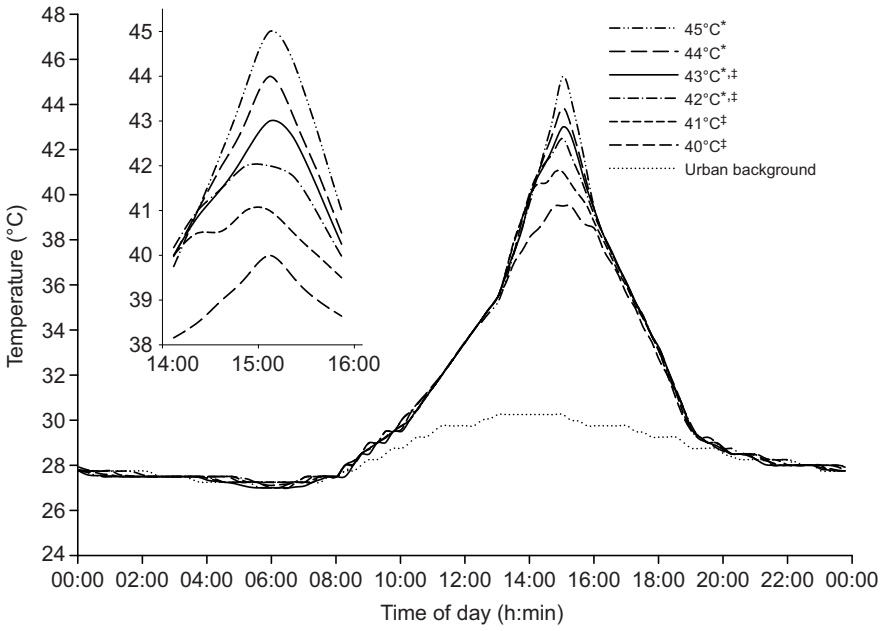

Fig. 2. Thermal fluctuations used to determine the thermal tolerance of *A. sagrei* and *A. cristatellus* embryos. The key shows the peak temperature of each fluctuation. Temperature was recorded by a thermocouple that was placed in the same conditions as the incubating eggs (i.e. in a Petri dish with moist vermiculite, sealed with Parafilm). *Temperatures used for *A. sagrei*; ‡temperatures used for *A. cristatellus*. The inset shows the peaks on a finer scale. The urban background refers to the daily fluctuation used to incubate eggs before and after exposure to thermal spikes.

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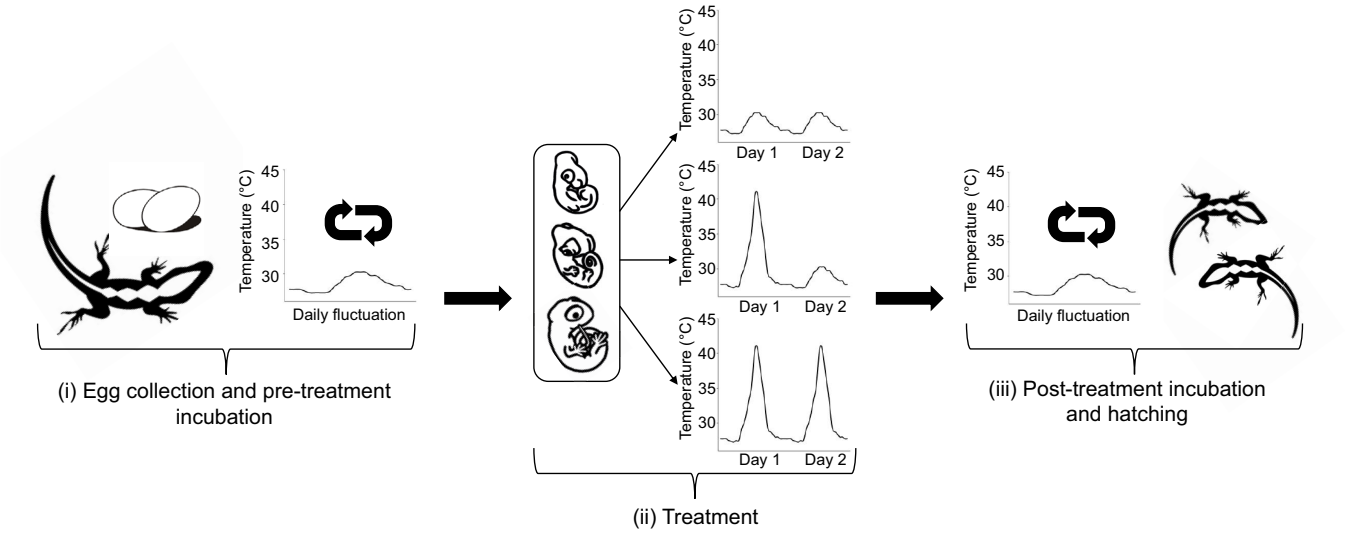

Fig. 3. Experimental design for determining thermal tolerance of *A. sagrei* embryos. (i) Egg collection and pre-treatment incubation: eggs were collected 3 times per week for 25 days and placed in an incubator that continuously looped a daily thermal fluctuation suitable for successful development. Therefore, these eggs were a single cohort that ranged in developmental age from oviposition to near hatching. (ii) Treatment: the cohort was randomly divided into three treatments that were exposed to 0, 1 or 2 thermal spikes over a 2 day period. Thus, embryos of various developmental ages experienced each treatment. (iii) Post-treatment incubation and hatching: eggs were returned to an incubator that looped a daily fluctuation suitable for successful development. The incubator was checked daily for dead eggs and hatchlings. Hatchlings were maintained in the lab for 2 months. To ensure sufficient sample sizes, this entire process (i–iii) was repeated for some peak temperatures. The procedure 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.

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 Fig. 3 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.

Embryo physiology

We non-invasively 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 rate 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 rate at 28°C on the day before and on each of 2 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 rate before and after thermal spikes, we placed eggs in an incubator set at a constant temperature of 28°C (±0.5°C s.d.) for 24 h. 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 45–60 s 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

Table 1. Sample size (*n*) and survival frequency of *Anolis sagrei* and *Anolis cristatellus* eggs and hatchlings

		Egg survival					Hatchling survival			
	Peak temperature (°C)	*n*	Age range (days)	Control	1 spike	2 spikes	*n*	Control	1 spike	2 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)	–	–

Age range is the range of egg ages since oviposition for each group. Beneath each treatment (i.e. control, 1 spike, 2 spikes), the sample size for that treatment is given with the survival frequency for that sample in parentheses. Peak temperature refers to the peak temperature of the thermal fluctuation (i.e. thermal spike).

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 versus thermal spike). At the end of the day (i.e. after treatment), eggs were returned to the 28°C incubator. Heart rate was measured again on each of 2 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 (both to 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 because of differential egg mortality among treatments, some cages had more of one treatment than another and some cages had fewer than 6 lizards (one cage with n=3 lizards; three cages with n=4 lizards; 11 cages with n=5 lizards; 51 cages with n=6 lizards). 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 a 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 libitum* 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 the corrected Akaike information criterion (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 the 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 (α=0.05). The mass by treatment by temperature interaction was never significant (all P>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 analyze 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 median lethal temperatures (LT_{50}) for the 1 and 2 spike treatments for each species using a logistic regression of embryo survival on temperature. LT_{50} is the peak temperature at which half the embryos are predicted to die. We compared 95% confidence intervals (CI) of these estimates to assess statistical significance.

We performed four separate linear mixed models (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 the 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 v.3.5.3 (http://www.R-project.org/).

RESULTS

For experimental eggs that died, 97% had no heart rate 1 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 (Fig. 4A,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 (Fig. 4C,D). LT_{50} values were greater for *A. sagrei* than for *A. cristatellus* and were greater for 1 spike versus 2 spike treatments. The LT_{50} values of *A. sagrei* were 44.17°C (43.92–44.43°C, 95% CI) and 43.90°C (43.66–44.14°C, 95% CI) for 1 and 2 spike treatments, respectively. LT_{50} values of *A. cristatellus* were 41.11°C (40.91–41.32°C, 95% CI) and 40.66°C (40.40–40.92°C, 95% CI) for 1 and 2 spikes, respectively.

For both species, we observed significant treatment effects on developmental rate (Table 2): each exposure to a thermal spike decreased developmental rate by 0.0051±0.0017 and 0.0089±0.0020 stages per day (mean±s.e.m.) for *A. sagrei* and *A. cristatellus*, respectively (Fig. 5). 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±4.3 beats min^{-1} (P=0.004) on the day after the thermal spike compared with controls (i.e. a 13.3% reduction) (Fig. 6). Two days after the thermal spike, heart rate was still 8.8±3.5 beats min^{-1} lower than in controls (P=0.02) (i.e. an 8.7% reduction). For embryos exposed to two thermal spikes, heart rate was 17.1±4.4 and 13.4±3.7 beats min^{-1} lower than in controls 1 day (P=0.0005) and 2 days (P=0.001) after experiencing the thermal spikes, respectively (i.e. 17.0% and 13.4% reduction).

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Table 2. Results for final models testing the effects of treatment, age, peak temperature and interactions and covariates on *A. sagrei* and *A. cristatellus* embryo and hatchling phenotypes and survival

	Response variable	Oviposition date	Initial mass	Age	Treatment	Peak temperature	Treatment× temperature	Age×treatment× temperature	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**

Age is embryo age at the time of treatment. Treatment corresponds to 0, 1 or 2 thermal spikes. Peak temperature was 42, 43, 44 or 45°C for *A. sagrei* and 40, 41, 42 or 43°C for *A. cristatellus*. Asterisks denote terms that were omitted from the final model because of a 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 (α=0.05). See Tables S2 and S3 for sample sizes, raw means and standard deviations. SVL, snout–vent length.

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±0.13 mm (mean±s.e.m.), 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±0.00023 g day^{-1} (Table 2). Finally, we observed significant effects of treatment and the treatment by peak temperature by age interaction for survival of *A. cristatellus* hatchlings (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 (Fig. 7). 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 deviations of egg and hatchling phenotypes.

DISCUSSION

We utilized thermal fluctuations from nests in an urbanized landscape and found that the thermal tolerance of lizard 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. Embryos of *A. sagrei*, a lizard that prefers warmer, open-canopy microhabitats, were more robust to thermal stress than embryos of *A. cristatellus*, a lizard that prefers cooler, closed-canopy microhabitats.

Sympatric species may evolve divergent thermal physiologies as a result of 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

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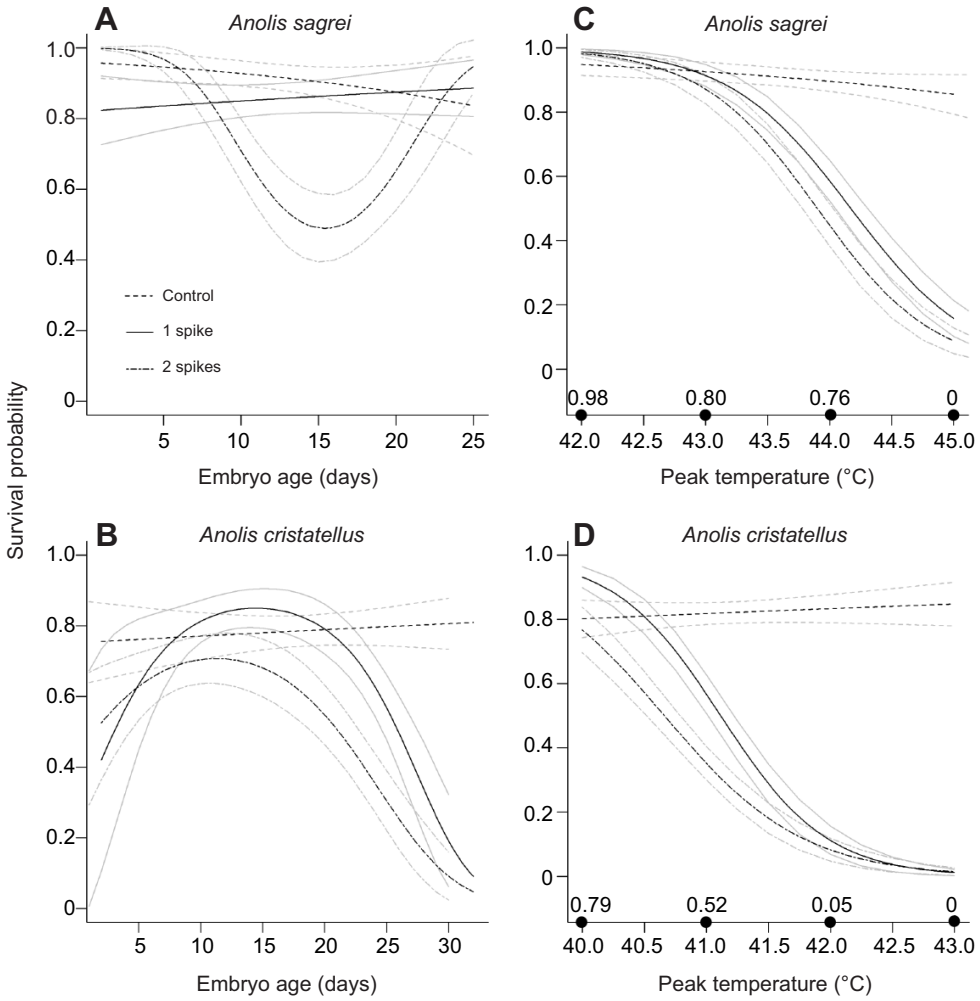

Fig. 4. Survival probability of *A. sagrei* (top) and *A. cristatellus* (bottom) embryos exposed to thermal spikes. (A,B) Survival across embryo age at the time of treatment. (C,D) Survival across peak temperatures of thermal spikes. Black lines show survival probabilities; gray lines show s.e.m. For 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). For 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 1 and 2 spike treatments combined for each peak temperature. See Table 1 for sample sizes and raw mean survival frequencies, and Table 2 for test statistics.

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., 2010a; Ma et al., 2014). Thermal acclimation is unlikely as 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, embryos of *A. sagrei* were more robust to thermal stress than those of *A. cristatellus*. Although *A. cristatellus* is relatively abundant in urbanized habitats both within (Winchell et al., 2016) and outside (Battles and Kolbe, 2019) 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*, *Ficus aurea*) throughout the urban matrix (Kolbe et al., 2016). 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 with what is generally available in urban

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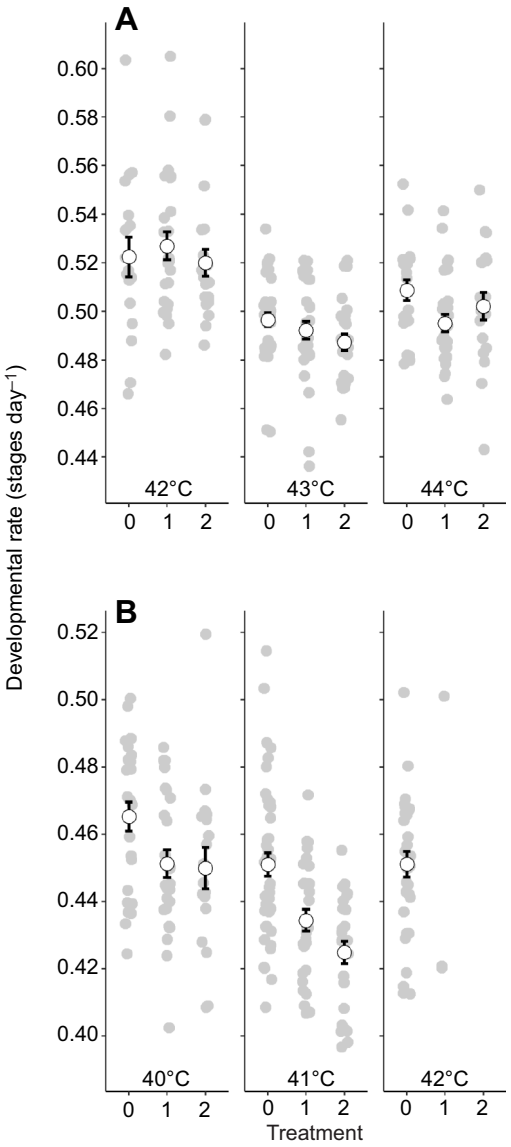

Fig. 5. Developmental rates of *A. sagrei* and *A. cristatellus* embryos exposed to 0, 1 or 2 thermal spikes that varied in peak temperature. (A) *A. sagrei*; (B) *A. cristatellus*. Open circles show raw means for each group, bars show s.e.m. and gray circles show raw data. The temperature at the bottom of each panel is the peak temperature of the thermal spike. Data from the 42°C peak for *A. cristatellus* (B) are 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.

habitats (Tiatragul et al., 2019). 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 the thermal sensitivity of embryos. Throughout the urban matrix, suitable nesting habitat may be more readily available for some species than others as a result of 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

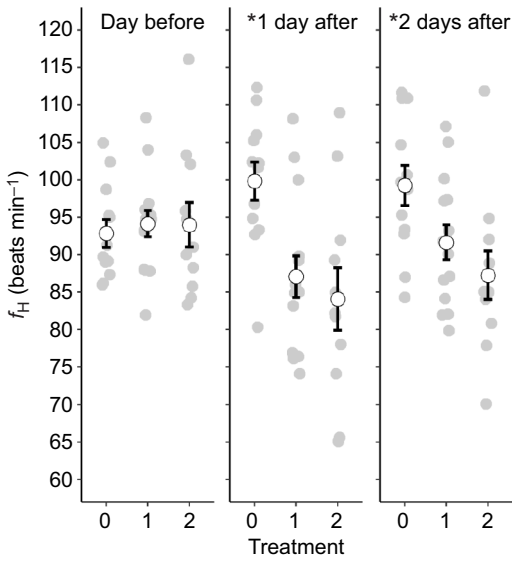

Fig. 6. Heart rates of *A. sagrei* embryos exposed to 0, 1, or 2 thermal spikes with a peak temperature of 43°C. Heart rates (f_H) were measured at 28°C on the day before and 1 and 2 days after exposure. Open circles show the raw means for each group, bars show s.e.m. and gray circles show the raw data. Asterisks signify a significant decline in f_H with an increase in the number of spikes. See Table S3 for sample sizes, raw means and standard deviations.

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 with *A. cristatellus* (see Fig. 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 h heat shock of 39°C, which resulted in 15% mortality. Thus, *A. sagrei* likely have lower thermal tolerance at early

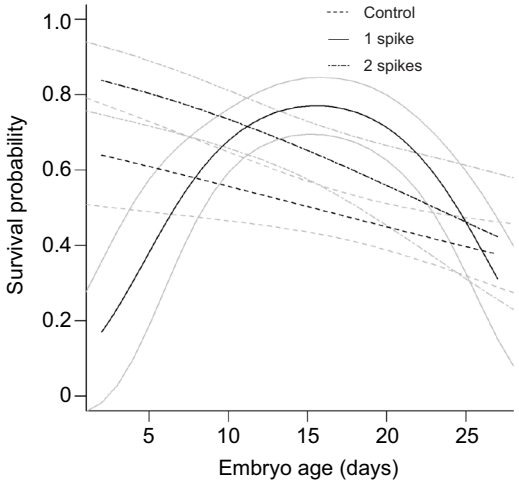

Fig. 7. Survival probability of *A. cristatellus* hatchlings exposed to thermal spikes on different days during embryo development. Black lines show survival probabilities for each treatment. Gray lines show s.e.m. See Table 1 for sample sizes and raw survival frequencies, and Table 2 for test statistics.

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. However, because females can shield these earliest stages from thermal stress via thermoregulatory behavior (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 that future studies consider how thermal tolerance changes through development.

Although many studies demonstrate that embryos die at high temperatures, the direct cause of death is not well understood and is 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 because of their efficient ventilation systems (e.g. insect spiracles and tracheae) and the comparatively high amount of oxygen in air (versus 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), and this would explain reduced thermal tolerance at later embryo stages when O_2 demand is high as a result of tissue growth (Thompson, 1989).

Because of 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. For 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* eggs) did not influence our results. Using respiration rates of anole eggs of various ages and at various temperatures (J.M.H. and D.A.W., unpublished data), 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 h, 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 arise during thermal spikes. These tears would greatly increase the permeability of oxygen. Additionally, refreshing the vermiculite in the Petri dish following treatment (see Materials and Methods) returned the air to normoxic conditions. Finally, two follow-up 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%) (S. Tiatragul, J.M.H. and D.A.W., unpublished data). Moreover, a heat shock experiment conducted by J.M.H. with *A. sagrei* eggs revealed that most eggs die between 44 and 46°C when exposed to a 1 h heat shock (90.3% die at 45 or 46°C). 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 those of the current study.

Table 3. Morphological abnormalities of hatchling lizards

Species	Age (days)	Deformity
A. cristatellus	26	Deformed tail
A. sagrei	22	Deformed tail
	8	Extra hind limbs protruding from abdomen
	6	Deformed tail
	6	Missing digits (no. 1) on both rear feet; fused digits (nos 1 and 2) on front feet

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.

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 (Fig. 5). 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 h (Fig. 6). Developmental rate positively correlates with embryo heart rate in reptiles (Du et al., 2010a,b). Although mortality precluded heart rate measurements of *A. cristatellus* embryos, they experience a comparable reduction in heart rate after exposure to thermal spikes (Hall and Warner, 2018). Another possible reason for the decrease in developmental rate is that embryos at extreme temperatures (e.g. greater than 36°C) may undergo an arrest of cell division or experience moderate levels of cell death (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 1–2 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 be compounded by increased exposures to thermal spikes.

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, 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 (Fig. 7), 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 is an understudied aspect of reptile developmental plasticity (Refsnider et al., 2019).

Finally, the methods used to measure the thermal sensitivity of embryos can influence the conclusions and predictions about how populations will respond to aspects of global change. 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. 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).

Climate projections indicate increases in mean temperature, 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 understanding 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 time frame each year (e.g. marine turtles; Howard et al., 2014). A heat wave occurring when most embryos are in the earliest or later stages of development may produce greater mortality than one occurring mid-development. This is vital to consider when modeling 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 behavioral thermoregulation.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: J.M.H., D.A.W.; Methodology: J.M.H.; Formal analysis: J.M.H.; Resources: D.A.W.; Writing - original draft: J.M.H.; Writing - review & editing: J.M.H., D.A.W.; Visualization: J.M.H.; Supervision: D.A.W.; Funding acquisition: D.A.W.

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Data availability
All relevant data are archived at Auburn University's data repository (AUrora): https://aurora.auburn.edu//handle/11200/49589

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.210708.supplemental

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Summary: Embryos of two lizard species differ in thermal tolerance in ways that correspond with species-specific nest microhabitats and patterns of occupancy throughout the urban matrix.

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