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RESEARCH ARTICLE

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The effects of thermal stress on the early development of the lizard *Anolis sagrei*

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Abstract

Across the globe terrestrial ectotherms—amphibians and non-avian reptiles—are facing a range of emerging challenges. Increasing global temperatures, in particular, are affecting all aspects of ectotherm biology and life history. Embryonic development is a thermally sensitive period of the organismal lifecycle, yet the impacts of thermal stress on the early development of ectotherms have significantly lagged behind studies of later stages and adult thermal physiology. Morphogenesis, the stage where the major anatomical systems are actively forming, is particularly sensitive to thermal stress, yet is not studied as often as later stages where growth is the primary process happening within the egg. Here, we focus on the effects of thermal stress on the first 12 days of development, the stages of morphogenesis, in the lizard *Anolis sagrei*. We examine the resiliency of the early developmental stages to heat stress by incubating eggs at temperatures that parallel conditions observed today and predicted over the next 50–100 years of projected climate change. Our results suggest that some anole nests are currently at the thermal limits for which the early embryonic stages can properly develop. Our results emphasize the importance of studying early embryonic stages of development and the importance of studying stage-specific effects of thermal stress on squamate development.

KEYWORDS

Anolis, global warming, thermal biology, thermal stress, lizard embryo

1 | INTRODUCTION

Global temperatures are increasing. These changes, which are happening at unprecedented rates, is driving profound changes across biological scales, from genes to ecosystems (Nadeau, Urban, & Bridle, 2017; Urban et al., 2016). Climate change is expected to have dramatic effects on many aspects of species' physiology (Gunderson, Armstrong, & Stillman, 2016; Pörtner & Farrell, 2008), behavior (Kearney, Shine, & Porter, 2009), and geographic distribution (Parmesan, 2006). Understanding the ways that life on Earth will be challenged by increasing temperatures is one of the priorities of organismal biology, ecology, and evolutionary biology in the 21st century (Nadeau et al., 2017, Urban et al., 2016).

Among animals, terrestrial ectotherms—amphibians and non-avian reptiles, those species that lack the ability to internally control body temperature—are particularly at risk of thermal stress with increasing global temperatures (Janzen, 1994; Mitchell, Kearney, Nelson, & Porter, 2008; Noble, Stenhouse, & Schwanz, 2018). Many ectothermic species persist at or near their thermal maximum (Araújo et al., 2013). It is predicted that within the next 100 years between 40% and 80% of lizard species will be threatened with environmental temperatures surpassing this limit (Böhm et al., 2016; Sinervo et al., 2010). However, most predictive models developed to estimate the effect of climate change on reptiles primarily rely on adult physiological data or only a fraction of the developmental thermal reaction norm (Noble et al., 2018). Predictive models become more accurate with the incorporation of multiple life stages. For example, both Levy et al. (2015) and Carlo, Riddell, Levy, and Sears (2018) have demonstrated a dramatic increase in the threatened populations of the fence lizard, *Sceloporus undulatus*, when considering fluctuations in egg incubation temperature. If biologists hope to accurately predict the impacts of environmental change on ecotherms, it is important to more thoroughly understand the effects of thermal stress on embryonic development, which has been largely overlooked.

Embryonic life is particularly sensitive to heat stress. For example, a relatively narrow window of morphogenesis during mammalian embryonic development, correlating with the first trimester in humans, has been shown to be sensitive to only a 2–4°C increase (Edwards,

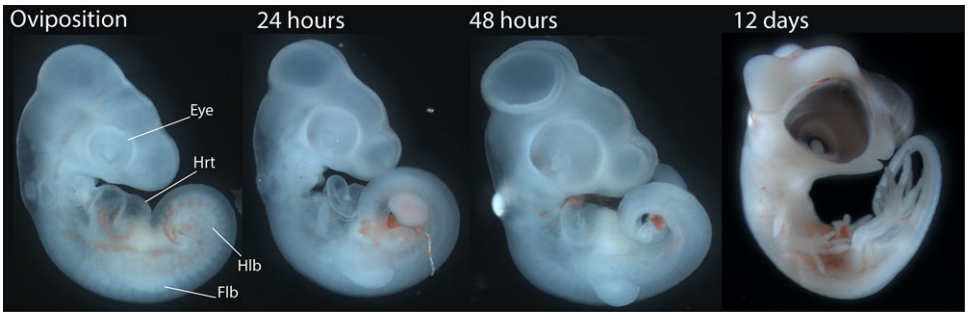

FIGURE 1 The first 12 days of *A. sagrei* development. *Anolis* embryos are laid at the early stages of morphogenesis. At this stage the early eye, brain segments, heart (hrt), forelimb buds (Flb), and hindlimb buds (Hlb) are visible, but not well developed. These structures become more developed over the next 12 days (at 27°C), until the time when the embryo can be readily recognized as a lizard. *A. sagrei* eggs are laid at Sanger stage 4. Stages 5 and 6 emerge sequentially over the next 48 hr. After 12 days of incubation at 27°C an average brown anole embryo reaches stage 11. Further details of *A sagrei* embryology are described in Sanger et al. (2008a) [Color figure can be viewed at wileyonlinelibrary.com]

1998; Walsh, Klein, Hightower, & Edwards, 1987). During these stages of morphogenesis, the major structural and organ systems are rapidly developing and are particularly sensitive to environmental insult (Sadler, 2014; Figure 1). Later stages, where embryonic growth is the primary process taking place, tend to be more robust to environmental perturbation. Strikingly, the homologous and critically important stages of morphogenesis occur when many lizard and snake eggs are laid and parental care ceases (Andrews & Mathies, 2000; Noro, Uejima, Abe, Manabe, & Tamura, 2009; Sanger, Losos, & Gibson-Brown, 2008a; Whiteley et al., 2017). This raises new concerns as to whether reptilian morphogenesis is as sensitive to thermal stress as observed in other vertebrate species. There are many opportunities for embryos at this stage to be exposed to thermal stress. For example, while gravid females search for nest sites, at the time of laying when eggs are briefly exposed to the external environment or from poorly chosen nest sites by females.

Previous studies examining thermal stress on reptilian development have primarily focused on hatching success, posthatching performance, or posthatching learning abilities (Gao et al., 2014; Pearson & Warner, 2016; Warner, Moody, Telemeco, & Kolbe, 2012; Dayananda and Webb 2017). For example, it is has been repeatedly shown that lizard eggs reared at higher temperatures have lower hatching success and tend to give rise to smaller hatchlings (e.g., Andrews, Mathies, & Warner, 2000; Angilletta, Sears, & Winters, ; Goodman, 2008, Warner et al., 2012; Tiatragul, Kurniawan, Kolbe, Warner, 2017). Studies of reptile "embryos" have often relied on coarse and indirect assessments of health status, such as monitoring changes in heart rate using noninvasive methods (Radder & Shine, 2006; Du, Warner, Langkilde, Robbins, & Shine, 2010a, 2010b; Hulbert et al., 2017), rather than direct observation of dissected embryos. Furthermore, heart rate monitoring typically begins at least a week after oviposition to as much as 50% of the total incubation duration (Du et al., 2010a), well after the stages of morphogenesis. To understand how the early stages of reptilian development are affected by thermal stress we must make the effort to dissect and directly examine the embryonic processes unfolding within the egg during morphogenesis.

Anolis lizards, or anoles, have been a model of thermal ecology, adaptation, and evolution for decades (e.g., Gorman & Hillman, 1977; Hertz, Arce-Hernandez, Ramirez-Vazquez, Tirado-Rivera, & Vazquez-Vives, 1979; Huey and Bennett 1987; Huey et al., 2009; Gunderson & Leal 2012; Muñoz et al., 2014; Campbell-Staton et al., 2017). Like previously discussed, however, the vast majority of this research has been conducted on adult stages or without direct observation of embryos. In novel environments, where the climatic environment is warmer than in natural areas, the brown anole, *A. sagrei*, has been observed to have eggs hatch sooner, have lower survival, and result in hatchlings that are phenotypically different to those in natural thermal environments (Schlaepfer, 2003; Pearson & Warner, 2016; Tiatragul, Kurniawan, Kolbe, & Warner, 2017). Herein, we use the embryos of *A. sagrei*, a widespread thermal generalist and model lizard species in thermal biology, to evaluate the effects of thermal stress on the early stages of embryonic development.

2 | METHODS

2.1 | Animal collection and husbandry

All protocols described herein were approved by the Loyola University Chicago IACUC committee. We collected and transported approximately 200 gravid *A. sagrei* females from Miami, FL, to Loyola University Chicago, IL, in May 2016. These were housed in groups of 4–6 females per cage. Over the next 2 months, cages were checked daily for eggs, which resulted in collections of 3–22 eggs per day (average 12.4 eggs per day from the colony). *Anolis* husbandry, egg collection, and egg incubation protocols are described in detail in Sanger, Hime, Johnson, Diani, and Losos (2008b). Briefly, we maintained cages within a walk-in growth chamber maintained at 28.5°C with misting every 2 hr to maintain humidity and provide drinking water. Each cage had a small pot with moist soil for the females to lay their eggs, which we checked daily between the hours of 8:30 and 10:00 AM. Eggs were incubated with moist vermiculite (mixed in equal ratios by weight with water) in sealed culture dishes within a humidified incubator. To test the resiliency of brown anole eggs to thermal stress, we randomly distributed eggs across five incubation temperatures: 27°C, 30°C, 33°C, 36°C, and 39°C. At the time of egg harvest, we also treated a subset

of eggs to a short-term heat shock at a temperature found to be lethal in the long-term incubation experiment (1 hr at 39°C within a day of oviposition; see below). To avoid the potentially confounding effects of embryos stressed during transport, we did not include eggs laid the first week following relocation.

Anole embryogenesis—including fertilization, gastrulation, and neurulation—occurs within the gravid female (Sanger et al., 2008a). To test whether these stages are sensitive to thermal stress, we collected a second series of approximately 50 gravid *A. sagrei* females from Miami in late April 2017. Lizards were transported to Loyola University Chicago and followed identical husbandry as described above, however, were maintained at a constant elevated ambient air temperature (36.5°C), which is known to dramatically lower egg survivorship after oviposition (see below). All eggs from this treatment were incubated at standard incubation temperature (27°C).

2.2 | Embryonic dissection, staging, and body length measurements

We dissected all eggs after 12 days of incubation, marking the completion of morphogenesis (Sanger et al., 2008a). Embryos at this age are, on average, at a stage where the limbs, head, and tail are readily recognizable as those of a lizard (Figure 1). Embryos were staged based on the criteria outlined in Sanger et al. (2008a), which is based on the progression of craniofacial, limb, and digit morphogenesis at the relevant stages. In our analysis, development of the adhesive toes pads is further subdivided into three stages (12a, 12b, and 12c) based on more recent observations (Sanger pers. obs.). Embryonic survivorship was determined by the presence of a heartbeat. Even slow or weakly beating hearts were considered as living.

To assess whether thermal stress during morphogenesis can induce a change in body size, we measured body size following Sanger, Revell, Gibson-Brown, and Losos (2012). Body length of each embryo was measured from scaled digital photographs from a Zeiss V16 dissecting microscope, recorded using *Image J* (Schneider, Rasband, & Eliceiri, 2012). Because animals are growing throughout development we only compared body lengths of embryos at a common stage, stages 10 and 11. The only observable morphological difference between these stages is the small amount of webbing that is regressing from between the digits. Because of the inherent differences in sample sizes, we used a Krustal–Wallis, nonparametric test to assess statistical differences in body size among these embryos. No embryos that were malformed or physically damaged were used in this analysis.

2.3 | Putative nest temperatures

We investigated whether the incubation temperatures and elevated body temperatures of gravid females used in our thermal stress experiments could be important for embryos developing in the wild through field validation. We collected putative nest site temperature data using Thermochron iButton temperature loggers (programmed to record temperatures at hourly intervals) from August 31, 2017–September 6, 2017 at Fairchild Tropical Botanic Gardens, Coral Gables, FL (25.677046, −80.271073). This corresponds with the peak of the nesting season in *A. sagrei*. iButtons were coated in waterproof Plasti-dip sealant and placed individually within sealed ziplock bags and placed in nest sites where *A. sagrei* eggs were discovered. We placed iButtons at a typical nest depth, ca. 3–5cm below surface, and covered them with soil substrate to replicate conditions eggs were discovered in. We assume our iButton sites were valid nest sites because two out of the three iButtons were displaced by a nesting female(s) part way through the data collection period. Prior to this time, data from all three iButtons were averaged to obtain an hourly approximation of nest temperature. After this time, data from the displaced iButons were not included in the analysis. We compared these nest temperatures to air temperature using hourly temperature data maintained by The Weather Company.

To examine whether gravid females reach the thermal limits observed in our incubation experiment, we also collected data on their field body temperatures at the same locality in May 2017. We collected 29 gravid females between 14:00 and 16:30h using a small noose on the end of the fishing pole and immediately collected their cloacal temperatures using an Omega digital thermocouple thermometer (HH501DK, Type K) accurate to within 0.1°C.

TABLE 1 Embryonic survival data

	Number of embryos	Survivors after 12 days	Percent survivorship
27°C	259	245	95%
30°C	51	47	92%
33°C	87	78	90%
36°C	106	41	39%
39°C	94	4	4%
39°C heat shock	60	51	85%
Maternal heat stress	88	53	60%

3 | RESULTS

3.1 | Thermal stress and embryonic mortality

We assigned 657 eggs to six incubation temperatures (27–39°C) for 12 days from May to July of 2016. Embryonic mortality was low (<10% for every treatment) from 27°C through 33°C (Table 1, Figure 2). However, mortality increased significantly at 36°C (61% mortality) and continued such that incubation at 39°C was functionally lethal (96% mortality). A 1-hr heat shock of 39°C on the day or ovipositon resulted in 15% embryo mortality.

In April and May of 2017, we collected an additional 88 eggs from females housed at an elevated ambient temperature (36.5°C). The rate of female egg deposition steadily decreased over the course of this experiment until no further eggs were laid after 4 weeks. Of the 88 eggs laid, 53 survived to incubation day 12 (60%; Table 1).

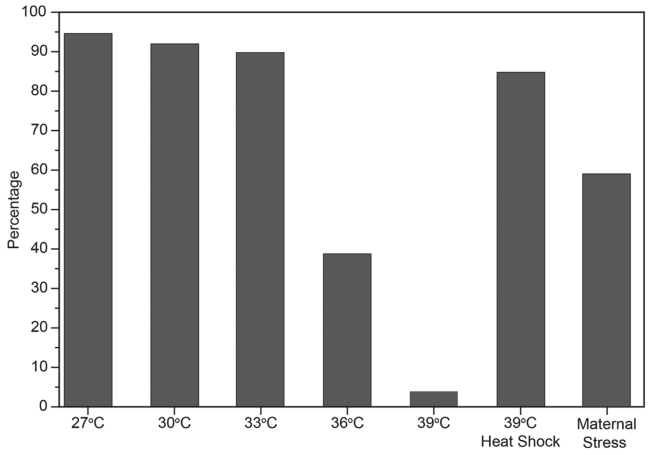

FIGURE 2 Decline in embryonic survival is associated with increasing incubation temperature. From 27°C to 33°C, *A. sagrei* embryos show only a mild drop in survival. However, from 33°C to 36°C, *A. sagrei* embryos show a dramatic decline in survival until only a small fraction of embryos (4%) survive 12 days of incubation at 39°C. A 1-hr heat shock at this otherwise lethal temperature also lowers embryo survival below the standard incubation temperature of 27°C. Maintaining gravid females at 36°C has a similar effect, lowering embryonic survival at day 12 to only 60%, even the eggs were incubated at a more favorable temperature following oviposition

3.2 | Rates of embryonic development

Embryonic developmental rates steadily accelerated as incubation temperatures rose from 27°C to 33°C (Figure 3). After 12 days of incubation at a standard incubation temperature (27°C) most embryos (70%) were between Sanger stage 10 and 12. At stage 10, embryos have a fully formed body axis, tail, snout, and limbs with a small amount of interdigital webbing remaining (Figure 1). At 33°C, stage 14 was the most commonly observed embryonic stage (range, stage 9–15; Figure 3c). At this stage, embryos have fully formed adhesive pads and the early stages of scale formation elsewhere on their bodies had commenced. There was a wide variance in the Sanger stage of embryos incubated at 36°C. Surviving embryos ranged from stage 4—remaining within the limb bud stages of morphogenesis—to stage 14. Embryos that experienced a 39°C heat shock were, on average, less developed than the embryos incubated only at 27°C for 12 days. The distribution of embryos from the maternal thermal stress experiment is similar to that observed in the standard 27°C treatment.

3.3 | Body size variation

We evaluated differences in body size among stage 10/11 embryos at 27°C, 36°C, and 39°C degrees. Not enough embryos of these stages were collected at the other temperatures to include in this analysis.

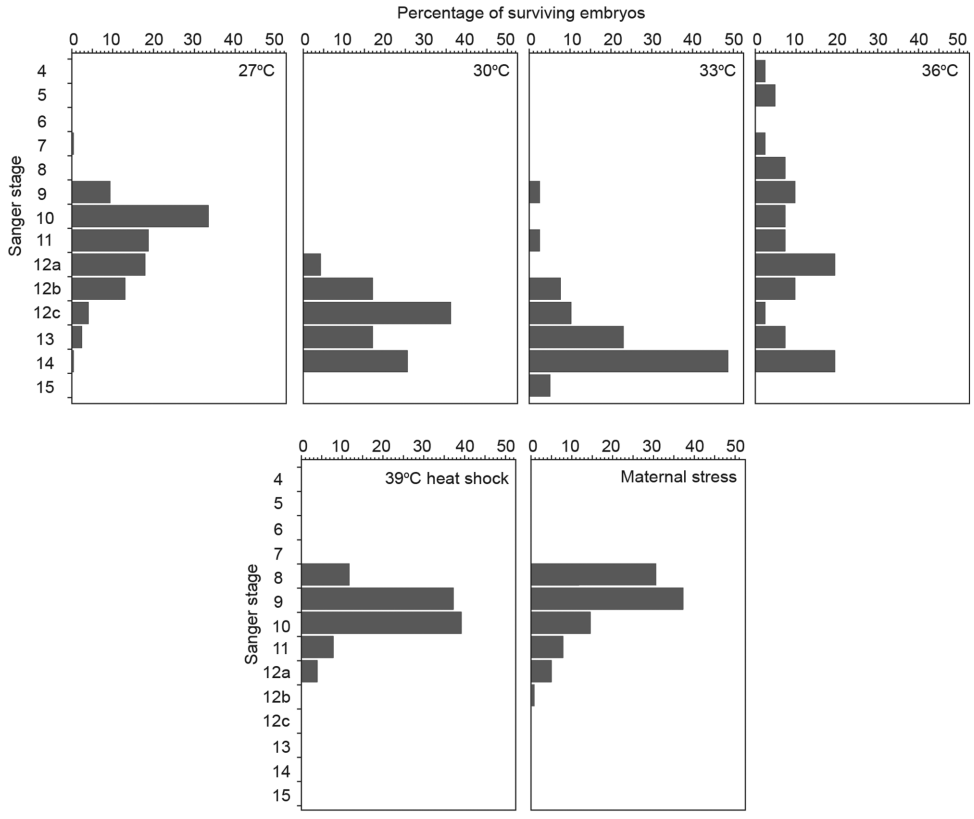

FIGURE 3 Changes in developmental staging associated with incubation temperature. At the standard incubation temperature, 27°C, most embryos are between Sanger stages 10 and 11. The range of stages reached over 12 days of incubation gradually increases with increasing temperature. However, incubation at 36°C generates embryos throughout the typical 12-day spectrum of development. A 1-hr incubation at a super-lethal incubation temperature does little to affect the staging of surviving embryos. Staging is based on the Sanger et al. (2008a). Only surviving embryos are included in the staging data

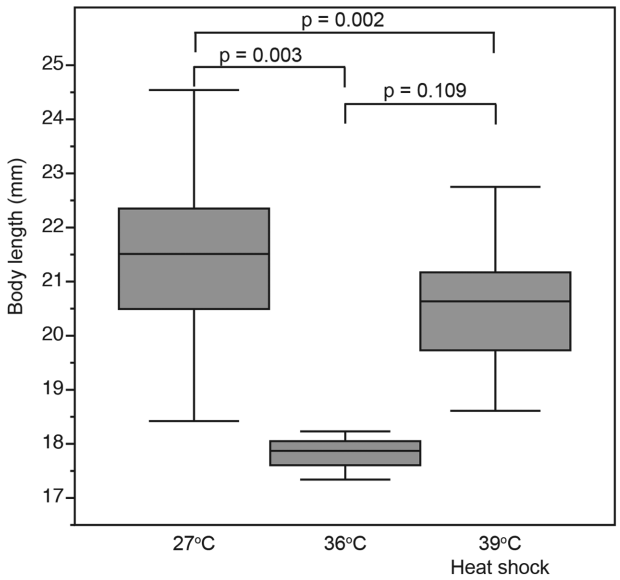

FIGURE 4 Variation in body length among day 12 embryos across incubation temperatures. Increasing incubation temperature during morphogenesis leads to decreased body size. Embryos used in this analysis are stage matched, between Sanger stage 10 and 11, to prevent the confounding effects of developmental progression (older embryos are larger simply because they are older, not because of the treatment)

Consistent with previous analyses of hatchlings, our analysis found a decrease in body size at elevated incubation temperatures (Figure 4).

3.4 | Field temperatures

We collected field temperatures of gravid females and putative nest sites to address whether field temperatures exceed the 33–36°C threshold. Maximum field body temperatures of gravid females were 34.2°C ($N = 29$, Mean = 31.8°C). The putative nest sites we measured varied extensively over the course of the day (Figure 5) but would periodically reach temperatures up to 44°C during the middle of the afternoon (Six day mean nocturnal temperature [18:00 to 7:00h] = 26.6°C; Six day mean diurnal temperature [8:00 to 17:00] = 36.6°C, Max = 44.5°C).

4 | DISCUSSION

The early stages of *A. sagrei* development, including embryogenesis within gravid females or during morphogenesis after deposition in the nest site, are sensitive to thermal stress. Our incubation experiments demonstrate that *A. sagrei* embryonic survival significantly decreases at temperatures greater than 33°C (Figure 2), above which typical developmental progression is also disrupted (Figure 3). A 1-hr pulse of elevated heat shock at 39°C also lowered embryonic survival and reduced embryonic body size. Embryos also experience decreased survival when the body temperatures of gravid females are raised to 36°C. The range of experimental incubation temperatures used was within the range of our field-collected temperature data of known *A. sagrei* nest sites. On average, putative nest sites are at or above 36°C for the majority of daytime hours before falling below stressful temperatures during the evening. Furthermore, the fraction of embryos that cannot hatch or show phenotypic changes could potentially increase throughout the latter two thirds of development. Although these potentially lethal temperatures represent the maximum range of what these lizards experience today, global climate change may increase the likelihood of embryos encountering these temperatures in the future.

Noninvasive methods of assessing embryonic metabolism, such as heart rate monitoring (Radder & Shine, 2006; Du et al. 2008; 2010a; 2010b), provide a useful technique for assessing relatively late embryonic stages, but cannot be used to assess the effects of thermal stress on morphogenesis. To study the effects of thermal stress on morphogenesis, dissection and direct observation of embryos is critical. Based on our results and comparisons with mammals (Walsh et al., 1987; Edwards, 1998; Hutson et al., 2017), these may be the most thermally sensitive stages of embryonic development for diverse vertebrate species. Within mammals and birds, thermal stress during morphogenesis may cause craniofacial abnormalities, neural tube defects, or mental retardation (Webster & Edwards, 1984; Shiota, 1988; Walsh et al., 1987; Edwards, 1998; Edwards, Saunders, & Shiota, 2003). The severity of these malformations is correlated with the degree and duration of the thermal insult. Our study provides direct evidence that squamate morphogenesis is affected by thermal stress. It remains to be determined whether the phenotypic effects observed at hatching or differences in heart rate at later stages of development are downstream effects of these earlier embryonic changes or whether they arise through dysfunction in stage-specific processes.

Many studies, including ours, show that lizard embryos die due to thermal stress. However, we feel strongly that a greater evaluation of precisely why these embryos die is an area of research that needs greater attention. In addition to obtaining deeper knowledge of stage-specific effects, it is also important to understand whether some developing tissues are more sensitive to thermal stress than others. When armed with this information we can better assess function and performance of hatchlings and more wisely address the mechanism of phenotypic changes. Furthermore, multiple mechanisms can generate similar phenotypic outcomes, again stressing the need to drill into the cellular and molecular level changes associated with embryonic thermal stress.

Our study showed that increasing incubation temperature from 27°C to 33°C led to a predictable increase in developmental rate (Figure 3). But paradoxically, embryos incubated at 36°C and those that experience a brief heat shock experienced developmental retardation and were younger and smaller than embryos reared at typical incubation temperatures (Figures 3 and 4). It is possible that embryonic thermal stress leads to transient arrest of cell division or widespread, but low levels, of cell death. After the stress is alleviated, either cell division may return to normal or cell death ceases allowing the embryos to return to their normal developmental trajectory, just being a little delayed. Either of these distinct mechanisms could generate the similar phenotypic outcomes. Observed embryonic mortality may be the result of either prolonged cell arrest or too much cell death in vital organ systems.

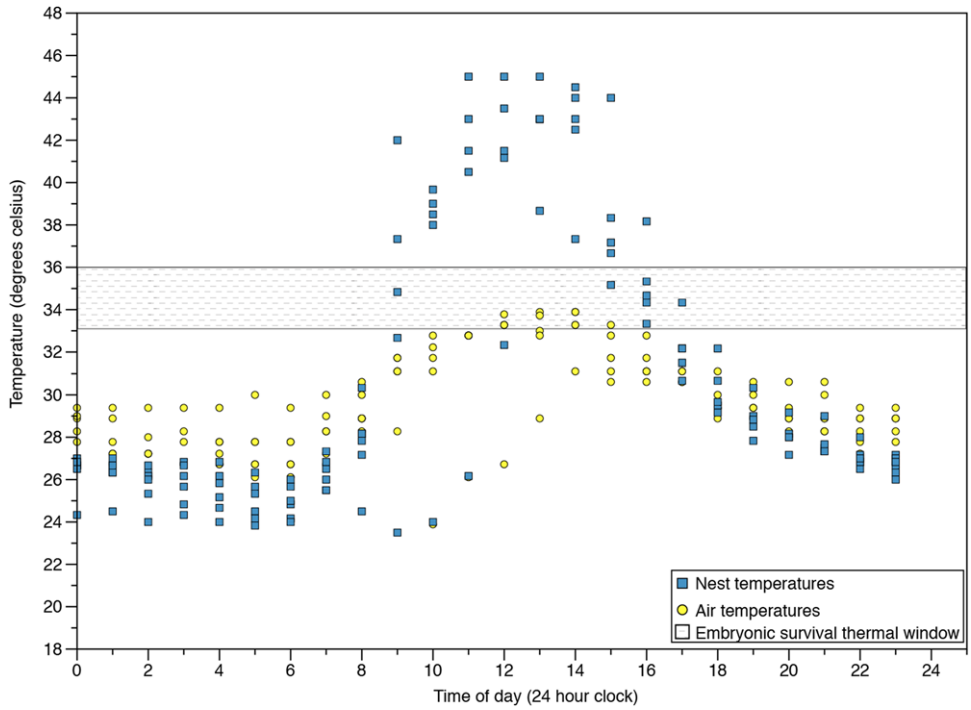

FIGURE 5 Current nest temperatures. Nest temperatures of known *A. sagrei* nesting sites (blue) compared to air temperautres (yellow). The hashed bar represents the thermal window, 33–36°C, where embryonic survivorship dramatically declines [Color figure can be viewed at wileyonlinelibrary.com]

Our results also raise questions about the abundance of *A. sagrei* in the south-eastern United States, where it is commonly the most abundant vertebrate in many ecological communities it inhabits. If nest sites regularly reach temperatures as high as 44°C yet embryo survival drops off precipitously above 33°C one might expect brown anoles to be on the brink of extinction. However, brown anoles are prolific egg layers (Sanger et al., 2008b) and nest sites in nature are diverse, spanning a range of forested, urban, and horticultural environments. For example, Pearson and Warner (2016) did not report brown anole nest temperatures above 30°C on spoil islands in north Florida. Furthermore, the *average* female when active in the field only had a body temperature of 31.4°C, below the critical thermal threshold. Our study demonstrates that some putative nest sites surpass the critical thermal window of anole embryology for at least several hours of the day. Further field study on the range of natural nest temperatures in different environments, the proportion of nest sites that exceed the critical thermal range, and differences in nest temperatures between seasons are needed to thoroughly assess how thermal stress could affect anole embryology are needed. These analyses are critical for the development of accurate predictive models that account for the greater proportion of nest sites that will surpass the critical thermal threshold over the next century as global temperatures continue to rise. Further attention should also be given to species that may have lower thermal limits than *A. sagrei*, or those that have lower reproductive outputs (e.g., Huey et al., 2009).

In this analysis, we used constant incubation temperatures to uncover the thermal limits of brown anole embryos. In contrast, our field data elucidated dramatic fluctuations in ground temperatures over the course of the day and over the 5 days we collected data. Although highly controlled lab experiments are typical for embryologists, they do not reflect the complexities of natural nest sites. Our results demonstrate that greater attention to the early stages of development, whether within the gravid female or shortly after oviposition, are needed. In later experiments, short-term temperature spikes up to 44°C that are focused at distinct stages of morphogenesis are needed to further elucidate the spectrum of effects of temperature stress on anole development. More refined experiments such as these will determine whether some stages of development, perhaps stages where vital organs are forming, are more sensitive to thermal stress than others. This will help address why anole embryos die under thermal stress, not just if they die.

Rising global temperatures and increasing frequency of extreme heat events mean that terrestrial ectotherms will likely experience novel climatic conditions near their upper thermal limits within the next 50–100 years (Sinervo et al., 2010, Böhm et al., 2016). Predicting how these changes to global climate will affect biodiversity is crucial in identifying species and ecosystems most at risk from climate-driven extinction. Incorporating empirical physiological data from all stages of a species' life cycle has so far been widely overlooked in vertebrates. Obtaining a more thorough understanding of both the developmental stage and tissue-specific effects of thermal stress is an important step in this direction. We recommend that in future studies consider the particular stage of the embryo as the operational unit, not more generalized designations, such as the egg. These analyses will require the integrative efforts of both field and laboratory biologists that can bridge fields such as ecology, physiology, evolutionary and

developmental biology, and ecology. With these efforts, we can create more accurate predictive models and better focus conservation efforts over long- and short-term time scales.

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