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

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Abstract

Aspects of global change result in warming temperatures that threaten biodiversity across the planet. Eggs of non-avian, oviparous reptiles (henceforth "reptiles") are particularly vulnerable to warming due to a lack of parental care during incubation and limited ability to behaviorally thermoregulate. Because warming temperatures will cause increases in both mean and variance of nest temperatures, it is crucial to consider embryo responses to both chronic and acute heat stress. Although many studies have considered embryo survival across constant incubation temperatures (i.e., chronic stress) and in response to brief exposure to extreme temperatures (i.e., acute stress), there are no standard metrics or terminology for determining heat stress of embryos. This impedes comparisons across studies and species and hinders our ability to predict how species will respond to global change. In this review, we compare various methods that have been used to assess embryonic heat tolerance in reptiles and provide new terminology and metrics for quantifying embryo responses to both chronic and acute heat stress. We apply these recommendations to data from the literature to assess chronic heat tolerance in 16 squamates, 16 turtles, five crocodilians, and the tuatara and acute heat tolerance for nine squamates and one turtle. Our results indicate that there is relatively large variation in chronic and acute heat tolerance across species, and we outline directions for future research, calling for more studies that assess embryo responses to acute thermal stress, integrate embryo responses to chronic and acute temperatures in predictive models, and identify mechanisms that determine heat tolerance.

KEYWORDS
critical thermal maximum, heat stress, lethal temperature, thermal developmental plasticity, thermal optimum, thermal performance

1 | INTRODUCTION

Warming temperatures due to global change threaten biodiversity across the planet. Eggs of non-avian, reptiles (henceforth "reptiles") are particularly vulnerable to heat stress due to a lack of parental care during incubation and limited ability to behaviorally thermo-regulate (Cordero, Telemeco, & Gangloff, 2018; Telemeco, Elphick, & Shine, 2009; Telemeco et al., 2016; but see Du, Shine, Ma, & Sun, 2019; Li, Zhao, Zhou, Hu, & Du, 2014; Shine & Du, 2018; Teng et al., 2014). Consequently, the biotic impacts of global change have motivated a surge in research devoted to understanding the effects of warming nest temperatures on reptile development. Two aspects of global change have been center stage: global climate change (Carlo, Riddell, Levy, & Sears, 2018; Levy et al., 2015) and habitat alteration (Kolbe & Janzen, 2002; Tiatragul, Kurniawan, Kolbe, & Warner, 2017). Both can increase nest temperatures in detrimental ways (Dayananda & Webb, 2017; Hall & Warner, 2018; Tiatragul, Hall, & Warner, 2020). Historically, reptiles have served as a primary

model in studies of thermal developmental plasticity (Warner, Du, & Georges, 2018; While et al., 2018), which has resulted in a large body of literature (reviewed by González et al., 2019; Howard, Bell, & Pike, 2014; Noble, Stenhouse, & Schwanz, 2018; Pezaro, Doody, & Thompson, 2017; Refsnider, Clifton, & Vazquez, 2019; Warner et al., 2018; While et al., 2018) upon which researchers can draw to predict species responses to rising temperatures; however, there are currently no standard assays for measuring heat stress of reptile embryos (unlike post-hatching stages; Angilletta, Zelic, Adrian, Hurliman, & Smith, 2013). Such methods are critical to understand the evolution and ecology of embryo heat tolerance and predict responses to global change. Given the threat of rising temperatures, the available data, and this recent surge in interest, now is an ideal time to (a) define basic terminology that will enable efficient communication among researchers and (b) consider the pros and cons of various protocols for measuring embryo heat tolerance.

Although standard assays exist for determining heat tolerance of post-hatching stages (reviewed by Taylor et al., 2020), these may not be applicable to embryos for biological and methodological reasons. For example, a common measure of thermal tolerance is the critical thermal maximum (CT_{max}; see Table 1 for terms and abbreviations), which is the upper temperature at which an individual loses motor control and is measured by heating individuals until they are immobile (Huey & Kingsolver, 1989). Since eggs do not move, the breakdown of the cardiovascular system has been used to determine embryonic CT_{max} (i.e., CT_{max} of cardiac performance; Angilletta et al., 2013). These endpoints, however, are not comparable because one results in a breakdown of performance and the other results in death. Additionally, adults and juveniles often navigate a thermally heterogenous landscape and are able, even in extreme or novel thermal environments, to maintain preferred body temperatures via behavior (Battles & Kolbe, 2019; Bogert, 1949). Indeed, behavioral thermoregulation is the primary way that ectotherms maintain

functionality despite having relatively narrow thermal safety margins (Sunday et al., 2014). Embryos of oviparous reptiles, however, are generally left to develop in prevailing conditions with limited opportunities to thermoregulate (but see Li et al., 2014; Shine & Du, 2018). Therefore, they are subjected to large changes in mean and variance of body temperature and both chronic and acute thermal stress must be considered to describe heat tolerance. Thus, embryo thermal ecology requires a set of definitions, methods, and interpretations that differ from post-hatching stages.

In this review, we consider several questions. How should we measure and express the upper thermal limits of reptile embryos? How do these upper limits differ across species? Furthermore, how should we interpret measurements of upper thermal limits with respect to ecology? Finally, how can such data be used to make predictions about the future? First, we compare existing methods for measuring the upper thermal limits of reptile embryos and propose metrics that can be used to categorize existing studies and make comparisons across species. Second, we use data for the brown anole lizard (*Anolis sagrei*) as a case study to demonstrate the importance and ecological relevance of different measures of embryo heat tolerance. Third, we present data from the literature to summarize what is currently known about the upper thermal limits of reptile embryos. Finally, we outline directions for future research, calling for more complete thermal reaction norms in studies of developmental plasticity, more studies of embryo responses to acute thermal stress, integration of embryo responses to chronic and acute temperatures in predictive models, and studies that identify mechanisms that determine heat tolerance. Although our focus is on reptiles, the methods we discuss can be applied to studies of embryonic thermal tolerance in other oviparous ectotherms, including insects and amphibians. Indeed, oviparous ectotherms, generally, are predicted to be particularly vulnerable to climate warming (Huey et al., 2012).

TABLE 1 List of terms and abbreviations

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

2 | MEASURING HEAT TOLERANCE OF REPTILE EMBRYOS

2.1 | Chronic versus acute heat stress

Andrews and Schwarzkopf (2012) were first to broadly assess the thermal physiology of reptile embryos with metrics that could be compared across a wide array of species. For 40 squamate species, they calculated a slope of developmental rate across constant temperatures within the optimal temperature range (OTR). The OTR is the range of constant temperatures across which hatching success is relatively high (e.g., 80%). Of course, "high hatching success" is a relative term; however, egg survival across temperature tends to have an "upside down U" or "staple-shaped" curve (van der Have, 2002), such that survival declines quickly at critical temperatures. Thus, the OTR is usually relatively obvious to determine in a qualitative way (see fig. 1A in Andrews & Schwarzkopf, 2012). With this regression (see fig. 1B in Andrews & Schwarzkopf, 2012), they estimated a developmental rate index (DRI) as the slope of developmental rate versus temperature, the lowest temperature for development (i.e., T_0) as the x intercept, and the optimal temperature for development (T_{opt}) as the highest temperature within the OTR. One problem with their method is that it cannot estimate the upper thermal limits of development. Although T_0, T_{opt}, and DRI are important traits to understand how historical factors (e.g., phylogeny, climate) have shaped embryo thermal ecology, the upper thermal limit is necessary to understand how species will respond to warming temperatures in the future. For example, to calculate a thermal safety margin, one needs to know the typical operative temperatures (i.e., pervasive nest temperatures) and an estimate of the upper critical temperature (e.g., CT_{max}; Sunday et al., 2014). Given the limits on embryo thermoregulatory behavior, multiple parameters may be required to describe embryo thermal tolerance. For example, when nest temperatures fluctuate widely throughout the day, embryos may become damaged or die due to a single, brief exposure to a high temperature (Hall & Warner, 2019). Conversely, chronic exposure to sublethal temperatures may also result in damage and death (Carlo et al., 2018; Sanger, Kyrkos, Lachance, Czesny, & Stroud, 2018). Thus, we need standardized methods and terminology to assess the upper thermal limits of reptile embryos, and these should reflect vulnerability of embryos to both chronic and acute heat stress.

Most estimates of embryo heat tolerance have been in response to constant temperatures, even though nest temperatures typically fluctuate in the wild (Booth, 2018). However, Angilletta et al. (2013) were first to measure CT_{max} of embryos by heating eggs of *Sceloporus undulatus* at a constant rate (3°C/hr) until embryos underwent cardiac arrest. This measure of heat tolerance (~46.5°C) was much greater than the warmest constant temperature that results in viable hatchlings (~35°C; Angilletta, Winters, & Dunham, 2000). Since the publication of this groundbreaking study, several researchers have measured CT_{max} of reptile embryos (e.g., Gao et al., 2014; Hall & Warner, 2019; Smith, Telemeco, Angilletta, & VandenBrooks, 2015). However, we propose that when researchers measure CT_{max} using

acute exposures to temperature, they are measuring a different phenotype than assessing heat tolerance with constant temperatures. Although each assay assesses embryo survival, the mechanisms resulting in death may differ: Acute heat tolerance may result from cardiac arrest or oxygen limitation (Angilletta et al., 2013; Hall & Warner, 2020; Smith et al., 2015), while chronic heat stress results in morphological abnormalities (Sanger et al., 2018), or depletion of energy stores (e.g., Sun, Li, Gao, Ma, & Du, 2015; Yang, Niu, & Sun, 2002).

We propose that researchers should use the term *embryo chronic heat tolerance* (ECHT) when referring to constant temperatures that induce high mortality. Thus, the ECHT is the constant temperature at which typical rates of hatching success (i.e., rates within the OTR) are reduced by 50% (i.e., lethal temperature 50; LT_{50}). Moreover, we suggest the term CT_{max} invites confusion given the differences between embryos and post-hatching stages discussed above. We recommend the term *embryo acute heat tolerance* (EAHT) when measuring responses to acute temperatures within relatively short time periods (e.g., heat shocks, extreme thermal fluctuations, or thermal ramps as in Angilletta et al., 2013; Hall & Warner, 2020; Smith et al., 2015, respectively). By using an ecologically relevant heating rate or heat shock (see below) that mimics extreme heat events in natural nests, the EAHT is the mean acute temperature that causes embryo mortality. Finally, it is important to note that repeated measures of ECHT and EAHT are not possible due to egg mortality, which further differentiates these measures from common protocols for assessing the thermal physiology of post-hatching stages (e.g., CT_{max}, individual thermal performance curves).

EAHT should be considered a measure of the total amount of heat stress that an embryo can withstand at a given moment, while ECHT represents thermal damage that is accumulated across time. These two measures potentially have different uses and importance depending on the ecological context. For example, some species nest relatively deep in the ground and temperatures are mostly constant through embryonic development (e.g., *Chelonia*, Booth & Astill, 2001; *Varanus*, Doody, James, Colyvas, Mchenry, & Clulow, 2015; *Chameleo*, Andrews, 2018). For such species with relatively constant developmental temperatures, we expect brief, extreme heat events to be few, and EAHT to be relatively low, accordingly. Moreover, if embryos develop at constant temperatures, EAHT may have little relevance compared with ECHT when assessing potential thermal stress. For other species (e.g., *Sceloporus*, Angilletta et al., 2013; *Emydura*, Booth, 2018; *Anolis*, Hall & Warner, 2020), embryos develop in shallow nests and temperatures fluctuate widely. Accordingly, EAHT may serve as the more important phenotype when considering thermal stress, and it may be relatively higher than embryos developing at constant temperatures. We propose that researchers begin using the terms EAHT and ECHT. This will (a) encourage researchers to consider which phenotype is most appropriate for their system and research question, (b) facilitate comparisons across the literature, and (c) enable a more accurate use of thermal tolerance phenotypes when generating predictive models.

2.2 | Measuring ECHT

Measuring ECHT is logistically simple. Eggs should be incubated at various constant temperatures under normoxic conditions (Figure 1a). Care should be taken to ensure that eggs remain aerated. This can be accomplished by opening incubation containers once weekly to refresh the air. Alternatively, a small hole can be placed in the lid of the incubation container and water can be returned to the incubation medium weekly to prevent desiccation. The water potential of the incubation medium should remain constant through the duration of the experiment and across treatments and support high hatching success. Ideally, at least five temperatures should be used with the two warmest temperatures reducing survival within the OTR by at least 50%. Eggs should be distributed across temperatures in a split-clutch design. Each egg should be categorized as 1 (*hatched*) or 0 (*did not hatch*) and a dose–response model can be used to estimate ECHT. Figure 2 demonstrates a hypothetical example, where 100 eggs were divided among five temperatures (*n* = 20 per temperature). Multiple dose–response curves were fit to the data using the "drc" package in R (Ritz, Baty, Streibig, & Gerhard, 2015) and the best model was used to estimate ECHT. A bootstrap was then applied to generate a 95% confidence interval for ECHT (see Supporting
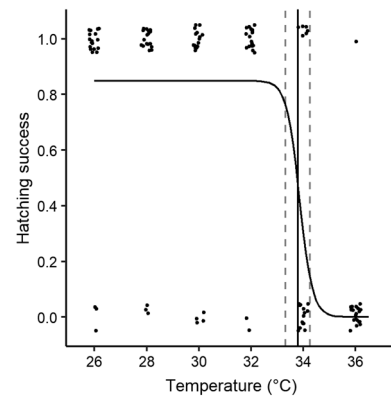
FIGURE 2 Dose–response model applied to hypothetical data. In this example, 100 eggs were incubated at five constant temperatures (*n* = 20 per temperature) and hatching success was recorded for each egg (1 = *hatched*, 0 = *did not hatch*). Closed circles denote the raw data (jittered around 0 and 1 to reduce overplotting). Several dose–response curves were applied to the data and assessed via Akaike's information criterion using the "drc" package in R (best model was a log-logistic model: "fct = LL.3()"). The solid gray vertical line denotes the embryo chronic heat tolerance (ECHT; 33.8°C) and vertical dashed lines denote the 95% confidence interval obtained via applying a bootstrap with 1,000 replicates
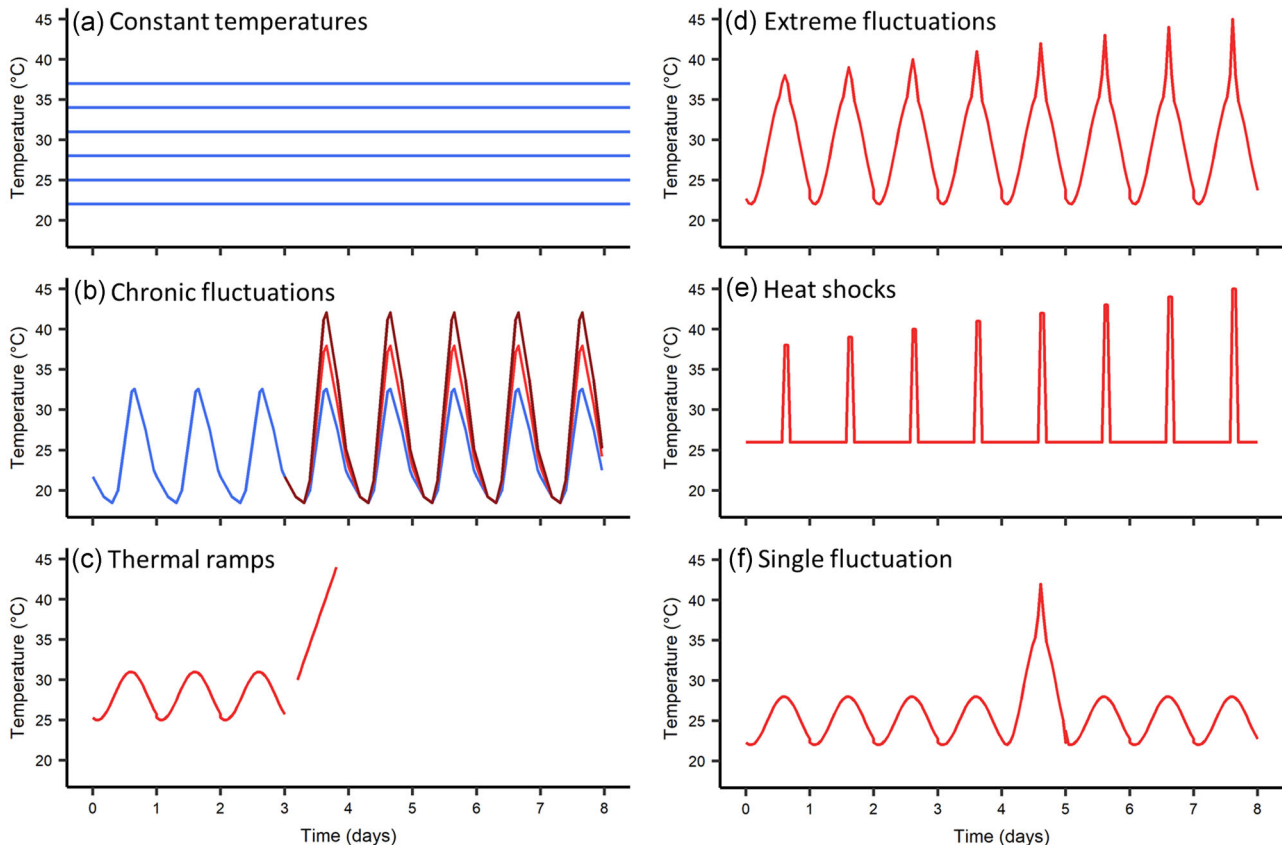
FIGURE 1 Methods for measuring heat tolerance of reptile embryos. Blue and red colors denote methods for measuring embryo chronic heat tolerance (ECHT) and acute heat tolerance (EAHT), respectively. The constant temperature approach (a) is used for measuring ECHT, and the other approaches (b–f) can be used to measure EAHT. The chronic fluctuations method (b) combines exposure of both chronic and acute thermal stress (see text). See text and Table 2 for explanations of each method

Information Material for R code and data). Although many studies report hatching success across a wide range of constant temperatures, a standard metric (i.e., ECHT) is required to make comparisons across studies and species.

We recommend that hatching success be the proper measure for determining ECHT. Studies that dissect eggs before hatching may overestimate ECHT because late-stage embryos may be particularly vulnerable to heat stress due to their relatively high oxygen demand (Hall & Warner, 2019; Kobayashi et al., 2017). Moreover, death at high constant temperatures is likely due to factors that compound across development (e.g., morphological abnormalities, damage from reactive oxygen species, arrest of cell division, cell death; Sanger et al., 2018; van der Have, 2002). The advantage of measuring ECHT is that it is logistically simple, only requiring several relatively inexpensive constant temperature incubators. Moreover, results are easily compared across studies since the methods are simple to repeat and there is a wealth of literature describing embryo responses to constant temperatures (Noble, Stenhouse, & Schwanz, 2018; While et al., 2018). However, because many, perhaps most, eggs do not incubate at constant temperatures in the wild and because responses to constant temperatures differ from responses to fluctuating temperatures (reviewed by Booth, 2018); the ecological relevance of ECHT is questionable. This is the primary limitation to measuring this phenotype. This assay could be conducted in a more ecologically relevant way by using repeated daily thermal fluctuations across a broad range of temperatures (e.g., repeated sine waves with amplitude of 2°C); however, this would make comparisons across species more difficult as both the mean and variance of each treatment must be standardized.

2.3 | Measuring EAHT

Measuring EAHT is more challenging compared with ECHT. Most methods require the use of programmable incubators which may be prohibitively costly (but see Greenspan et al., 2016). To our knowledge, there are five ways that researchers have measured EAHT (Figure 1b–f), and each method has strengths and limitations. Importantly, unlike ECHT, EAHT is measured at a single point during development. Because embryos change dramatically across development with respect to size and physiology, EAHT likely varies with ontogeny (Hall & Warner, 2019). Thus, it is important to control for and report the embryo stage at which EAHT is measured. Additionally, unlike ECHT, most methods of EAHT result in a heat tolerance estimate for each egg, allowing researchers to consider among-individual variation in heat tolerance. Finally, because thermal effects are time-sensitive and depend on warming/cooling rates, the length of exposure to an extreme temperature and rates of warming during EAHT assays will likely influence results. Thus, these factors should always be controlled, reported, and reflect real nest temperatures when possible.

Levy et al. (2015) were first to incorporate acute measures of heat tolerance into species distribution models (SDMs) in reptiles.

They incubated eggs of the eastern fence lizard (*S. undulatus*) at fluctuating temperatures that were suitable for successful development (i.e., blue line, Days 1–3; Figure 1b) and then allocated eggs to incubation treatments that varied in the peak temperature of the daily thermal cycle (i.e., red lines, Days 4–8; Figure 1b). Some eggs remained at the standard fluctuation to serve as controls (i.e., blue line, Days 4–8; Figure 1b). At the end of each day, eggs were placed in a heart rate monitor (Buddy; Avitronics Inc.) to determine survival based on the presence or absence of a heart rate. Eggs remained in these treatments until hatching. The primary advantage of this method is that it is highly ecologically relevant, as extreme temperatures are experienced as they would be in natural nests, which exhibit wide daily thermal fluctuations. There are, however, a few disadvantages. First, this method requires as many programmable incubators as treatments. Second, it confounds the effects of chronic and acute thermal stress since treatments differ in both maximum and mean temperature. Therefore, we cannot determine if this method produces an estimate of ECHT or EAHT. Finally, Levy et al. (2015) subjected eggs to chronic fluctuations at a relatively late developmental stage (from 70% to 95% development completed). Because heat tolerance can change with ontogeny, results may vary depending on the timing of treatment allocation with respect to development.

The thermal ramp (Figure 1c) is the most used method to estimate EAHT and was first used by Angilletta et al. (2013). Eggs incubate at temperatures suitable for development (e.g., repeated sine wave shown on Days 1–3; Figure 1c) and then, on a particular day, each egg is placed in a heart rate monitor (Buddy) and heated at a fixed rate that reflects nest temperatures (e.g., 3°C/hr in Angilletta et al., 2013) until cardiac arrest (i.e., no heart rate; shown on Day 3 of Figure 1c). A thermocouple can be attached to the egg or, for large eggs, can be inserted inside the egg, to monitor egg temperature during the assay (see also Tezak, Sifuentes-Romero, & Wyneken, 2018). The average egg temperature at which the heart stops beating is the EAHT (e.g., Angilletta et al., 2013; Gao et al., 2014). One major benefit of this method is its high degree of ecological relevance: Eggs can be heated at a rate similar to diurnal temperature increase of natural nests (e.g., 3°C/hr; Angilletta et al., 2013). Conversely, this method is logistically challenging to perform as researchers must have specific equipment (e.g., heart rate monitor, programmable incubators or water baths), and, unless a lab is equipped with multiple heart rate monitors and programmable incubators (and multiple personnel to run the assays), only one egg can be measured at a time. Moreover, controlling the warming rate of eggs makes the assay strictly time-sensitive. Thus, this method is complicated and may not be feasible for some study questions, particularly those that require measuring EAHT on many individuals of multiple species or populations (e.g., Hall & Warner, 2019).

An alternative method was first used by Smith et al. (2015). Eggs incubate at temperatures suitable for development. On a chosen day during development, eggs are subjected to thermal fluctuations that increase in peak temperature each day (Figure 1d). At the end of each day, eggs are placed on the heart rate monitor to assess survival via

the presence/absence of a heart rate. The peak temperature that kills the embryo is recorded as the EAHT. This method has high ecological relevance—the assay exposes eggs to daily nest fluctuations as in the wild. Moreover, all eggs are treated simultaneously, which is logistically favorable. However, there are three major drawbacks. First, it requires specific equipment (at least one programmable incubator and a heart rate monitor). Second, previous exposure to extreme, nonlethal temperatures can reduce measures of EAHT (Hall & Warner, 2019); thus, damage may accumulate during the first days of the experiment, resulting in lower estimates of EAHT than would be measured by other methods (e.g., thermal ramps). Finally, EAHT likely changes with ontogeny (Hall & Warner, 2019), and the peak temperature is confounded with embryo age.

A modified version of the Smith et al. (2015) method can be used if programmable incubators are unavailable. Heat shocks of 1/2 or 1 hr can be applied. This involves incubating eggs at a constant temperature within the OTR and then placing eggs in an incubator set to an extreme temperature for a short time (e.g., 1/2 or 1 hr), removing the eggs and then assessing survival using a heart rate monitor (i.e., presence vs. absence of heart rate). Survivors can then be heat shocked at a higher temperature the next day (Figure 1e). The lethal temperature is recorded as the EAHT. Logistically, this is the simplest of all methods to measure EAHT as only constant temperature incubators are required. Moreover, all eggs can be tested simultaneously. However, this assay lacks ecological relevance as eggs would not experience such abrupt temperature changes in the wild. Additionally, previous exposure to extreme temperatures may influence the final measure of EAHT and temperature is confounded with embryo age (as in Smith et al., 2015).

A modification of the Smith et al. (2015) protocol has been used by Hall and Warner (2019). In this assay, eggs are randomly allocated to be exposed to a single extreme temperature fluctuation with a pre-determined peak temperature (Figure 1f). These peak temperatures range from below to above an estimated EAHT (based on preliminary data). After exposure, each egg incubates until hatching. A logistic binomial regression (1 = hatch, 0 = did not hatch) is used to estimate EAHT. If programmable incubators are unavailable, this method could be modified to use heat shocks rather than extreme fluctuations. The benefits of this assay are that it is ecologically relevant and relatively simple to perform (e.g., does not require measuring heart rates). Moreover, it eliminates the potential for previous exposures to extreme temperatures to influence EAHT, and peak temperature and embryo age are not confounded. However, the disadvantages are that it requires large sample sizes and an estimate of EAHT is not made for each egg. This limits interpretations, particularly about how EAHT might vary across individuals.

Finally, reproducibility is of vital importance. Constant temperatures, thermal ramps, and heat shocks are easy to reproduce, allowing for comparisons across studies and species. Fluctuating treatments (Figure 1b,d,f) are more challenging to reproduce because the breadth of the thermal fluctuation in addition to the peak temperature will influence embryo survival. Therefore, temperatures across the entire thermal fluctuation, not just the peak temperature,

are required to reproduce the assay. Due to the strengths and limitations of each method to measure EAHT (summarized in Table 2), we do not recommend a preferred method but encourage researchers to consider their model species, available equipment, and study question when selecting a method. Moreover, researchers should consider these limitations and confounding variables when comparing estimates of EAHT across studies. Finally, assessing multiple methods simultaneously may be useful to select a preferred method for a given study system (e.g., Hall & Warner, 2020). Importantly, most methods determine embryo survival/death via the presence/absence of a heart rate. Detecting heart rates is sometimes difficult for very early- and very late-stage embryos (J. M. H., personal observation). Additionally, extreme bradycardia may occur just before death (Angilletta et al., 2013; Hall & Warner, 2020), potentially resulting in no heartbeat detected for a live embryo. Therefore, we recommend that eggs without a heartbeat be placed in an incubator at a temperature suitable for successful development and subsequently monitored to confirm mortality.

2.4 | A case study of embryo heat tolerance: The brown anole (A. sagrei)

The brown anole (A. sagrei) is becoming an important model for developmental ecophysiology because it is hardy in captivity, has relatively high fecundity, protocols are established for egg and embryo collection, and its developmental staging series is described (Hall, Buckelew, Lovern, Secor, & Warner, 2018; Hall, Mitchell, Thawley, Stroud, & Warner, 2020; Sanger et al., 2018; Sanger, Hime, Johnson, Diani, & Losos, 2008; Sanger, Losos, & Gibson-Brown, 2008). To our knowledge, A. sagrei is the only species for which EAHT has been measured using three of the methods described above and data exist to estimate ECHT. Because females construct shallow nests (<5-cm depth), nest temperatures fluctuate substantially across the day (Hall & Warner, 2020; Pruett, Fargevieille, & Warner, 2020); thus, this species is an excellent model to consider the importance of ECHT and EAHT with respect to egg survival and physiology.

We collected estimates of EAHT from published studies (Hall & Warner, 2019, 2020) and used unpublished data (Pruett and Warner) of hatching success at eight constant temperatures (21°C, 23°C, 25°C, 27°C, 29°C, 31°C, 33°C, and 35°C) to estimate T_{opt} (as in Andrews & Schwarzkopf, 2012) and ECHT. To estimate ECHT, we analyzed mean survival at each temperature with multiple dose–response models (two-, three-, four-, and five-parameter log-logistic models; Weibull I & II; log-normal; gaussian; quadratic) using the drc package in R (Ritz et al., 2015). A three-parameter logistic regression (i.e., lower bound fixed at zero with a symmetrical inflection) was the best model according to Akaike's information criterion. We used mean survival at each temperature (rather than raw data as in Figure 2) to illustrate that ECHT can be calculated when raw data are not available (as when making estimates from past studies). Estimates were made according to the following equation:

TABLE 2 Comparison of methods used to measure reptile embryo heat tolerance

Method; figure	Description	Data recorded	Estimates	Example studies	Advantages	Disadvantages
Constant temperatures; Figure 1a	Eggs are incubated at multiple constant temperatures in a split-clutch design. Warmest temperature should induce at least 50% mortality compared with OTR	Hatching success for each egg expressed as a binary (0,1)	DRI, ECHT, OTR, T_{opt}, T_O	Mueller et al. (2019), Telemeco (2015), Ligon and Lovern (2009)	Logistically simple. Need only constant temperature incubators. Assesses embryo responses to chronic thermal stress. Easy to reproduce	Constant temperatures are not ecologically relevant for many species. Does not consider embryo responses to acute thermal stress. Does not provide estimate of heat tolerance for each egg
Chronic fluctuations; Figure 1b	Eggs are first incubated at fluctuating temperatures suitable for development. Later, eggs are allocated to one of multiple repeated fluctuations which differ in peak temperature	Hatching success for each egg expressed as a binary (0,1)	See text	Levy et al. (2015)	High degree of ecological relevance. Exposes embryos to increasing chronic and acute temperatures as would be experienced due to global change	Requires many programmable incubators. Confounds the mean and peak temperature of thermal treatments. Does not provide estimate of heat tolerance for each egg
Thermal ramp; Figure 1c	Eggs are placed (individually) in a heart rate monitor, temperature is increased at a steady, ecologically relevant rate, heart rate is monitored across temperature until cardiac arrest	Temperature at which heart rate is zero (i.e., cardiac arrest) for each egg	EAHT	Angilletta et al. (2013), Gao et al. (2014), Hall and Warner (2020)	Ecologically relevant measure of EAHT. Allows for estimation of thermal sensitivity of heart rate. Reproducible	Logistically difficult as eggs must be measured one at a time. Requires specific equipment (water baths, programmable incubator, heart rate monitor). May overestimate EAHT (see text)
Extreme fluctuations; Figure 1d	Eggs are exposed to extreme thermal fluctuations that increase in peak temperature each day. Survival is assessed via heart rate at the end of each day	Peak temperature that causes death for each egg	EAHT	Smith et al. (2015)	High level of ecological relevance for species that exhibit wide fluctuations in nest temperature. All eggs can be treated simultaneously	Potentially underestimates EAHT due to compounding effects of sublethal exposures to thermal stress. Confounds embryo age with peak temperature. Difficult to reproduce
Heat shocks; Figure 1e	Eggs are exposed to 1-hr heat shocks that increase in temperature each day. Survival is assessed via heart rate at the end of each day	Heat shock temperature resulting in death for each egg	EAHT	Hall and Warner (2020)	Logistically simplest way to measure EAHT. Does not require programmable incubators. Easy to reproduce	Low ecological relevance. Similar disadvantages as described for extreme fluctuations method
Single fluctuation; Figure 1f	Eggs are exposed to a single extreme fluctuation with a randomly selected peak temperature. Eggs then incubate until hatching	Hatching success for each egg expressed as a binary (0,1)	EAHT	Hall and Warner (2019)	Ecologically relevant measure of EAHT. Decouples embryo age and peak temperature. Avoids the effects of previous, sublethal exposures	Requires multiple programmable incubators and large sample sizes. Does not provide an estimate of EAHT for each egg. Difficult to reproduce

Abbreviations: DRI, developmental rate index; EAHT, embryo acute heat tolerance; ECHT, embryo chronic heat tolerance; OTR, optimal temperature range; T_O, minimum developmental temperature; T_{opt}, optimal temperature for development.

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

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

Figure 3a demonstrates the log-logistic curve and the parameters of interest. Here, T_{opt} and ECHT are 31°C and 32.8°C, respectively. EAHT of *A. sagrei* is 44.17°C (95% confidence interval [CI]: 43.92–44.43°C), 45.3°C (95% CI: 45.15–45.45°C), and 46.15°C (95% CI: 45.87–46.44°C) for extreme fluctuations, heat shocks, and thermal ramps, respectively. Thus, extreme fluctuations provided the lowest estimate of EAHT, thermal ramps provided the highest estimate, and heat shocks resulted in an intermediate estimate (95% confidence intervals do not overlap). Finally, we observed safety margins of 7.7°C and 2.9°C for EAHT and ECHT, respectively (Figure 3b).

There are biological and methodological explanations for the observed differences in EAHT. With respect to biology, measures of EAHT were assessed on two different populations and in different years, and we do not know how EAHT changes across space and time. Ideally, we would compare EAHT estimates taken from a single population during the same study, but such data are not available for any species. Regardless, there are several methodological explanations for why EAHT estimates might vary. First, when exposed to extreme fluctuations (e.g., Figure 1d), additional thermal damage may occur after the embryo has reached the peak temperature and is cooling down. Heat shocks and thermal ramps do not require embryos to "come down" from the test temperature; thus, estimates of EAHT taken from thermal fluctuations may be lower than heat shocks or thermal ramps. Second, thermal fluctuations and heat shocks may generate lower estimates of EAHT compared with thermal ramps because these methods use different metrics to assess survival. Thermal fluctuations and heat shocks assess embryo survival, per se, while thermal ramps require continuous monitoring of cardiac function (and cardiac arrest). There is some evidence that acute exposure to sublethal temperatures damages the cardiovascular system (Hall & Warner, 2018, 2019, 2020); thus, embryos may suffer potentially lethal damage at temperatures below the point of cardiac arrest, resulting in higher estimates of EAHT from thermal ramps compared with other methods.

These data demonstrate the potential ecological importance of various thermal parameters. For example, *A. sagrei* is a highly successful urban colonizer (Hulbert, Hall, Mitchell, & Warner, 2020; Stroud et al., 2019), which may result from relatively warm urban nest temperatures which enhance embryonic development (Tiatragul et al., 2017). In our example nest (Figure 3b), the mean temperature (29.9°C) is close to T_{opt}, but below ECHT, and peak nest temperatures (mean = 36.6°C) do not reach EAHT. Thus, warm urban nests
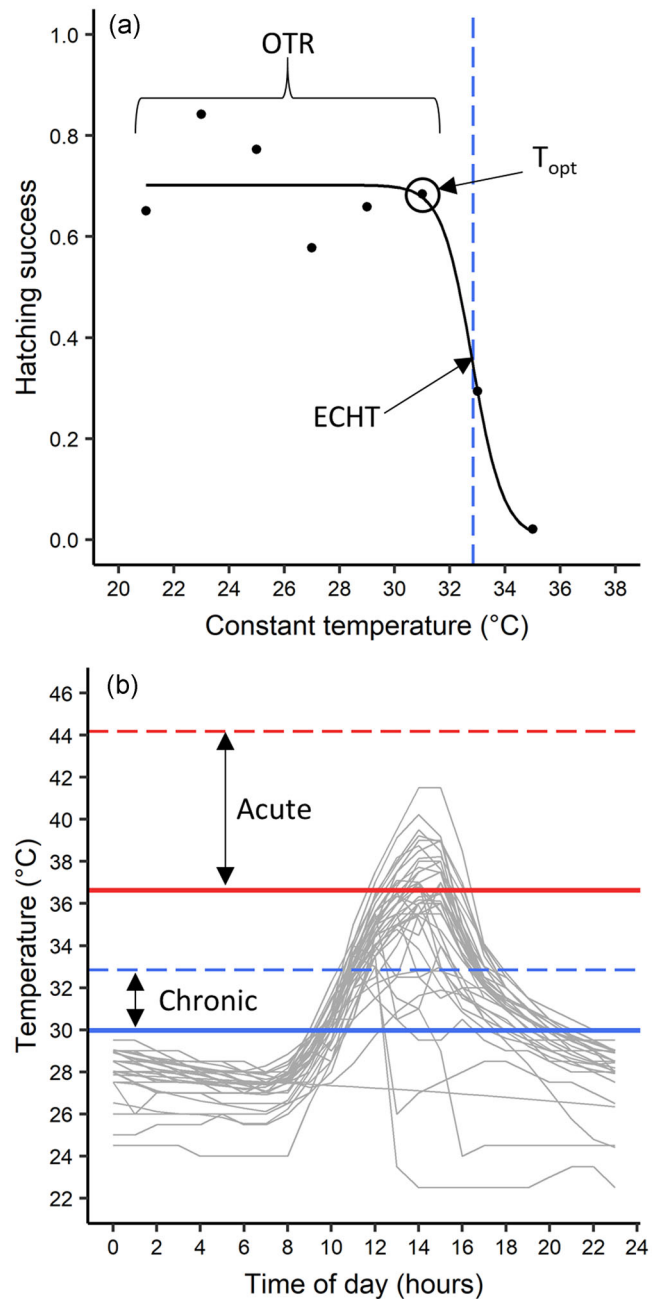
FIGURE 3 *Anolis sagrei* (a) egg survival across constant temperatures and (b) nest temperatures from an urbanized habitat. (a) Closed circles are the mean hatching success at each temperature, the black line is the model fit, and the vertical blue dashed line denotes the ECHT. (b) The dashed blue and red lines show the ECHT and EAHT, respectively. Gray lines show daily temperatures collected from a single nest in Pinecrest, FL (see Tiatragul et al., 2020). The solid blue and red lines show the mean nest temperature and the mean daily maximum temperature, respectively. Arrows denote the acute and chronic thermal safety margins. EAHT, embryo acute heat tolerance; ECHT, embryo chronic heat tolerance; OTR, optimal temperature range

may allow *A. sagrei* eggs to incubate at near-optimal temperatures, but still allow for substantial thermal safety margins with respect to both acute and chronic heat stress. In general, *A. sagrei* occupy relatively warm, open canopy environments (Battles & Kolbe, 2019),

and their embryo thermal physiology may have evolved to maximize fitness in relatively warm nests that exhibit wide thermal fluctuations. Urban environments replicate such conditions, and embryo thermal physiology may explain, in part, their success in urban habitats (Hall & Warner, 2019). Species like *A. sagrei* may exhibit wide divergence in EAHT and ECHT. However, species that nest in relatively cool, thermostable microenvironments may exhibit little difference between ECHT and EAHT. Such a hypothesis has not yet been tested but must be considered when modeling responses to climate change because nest temperatures will likely increase in both mean and variance in the future (Carlo et al., 2018; Dayananda, Gray, Pike, & Webb, 2016).

3 | A QUANTITATIVE REVIEW OF REPTILE EMBRYO HEAT TOLERANCE

In this quantitative review, our goal is to provide a summary of what is known about the chronic and acute heat tolerance of reptile embryos. Our motivation is not to explain heat tolerance according to ecological and evolutionary factors (e.g., nest temperatures, phylogeny). Such analyses are beyond the scope of this review but will be addressed in a future study. Rather, we aim to illustrate the paucity of data available while concomitantly demonstrating the large variation in estimates of heat tolerance. We hope to spark discussion among those interested in the thermal physiology of embryos, motivating researchers to utilize the methods above to estimate EAHT and ECHT in their own study systems so that our collective knowledge of embryo thermal physiology will be enhanced.

Specifically, we aim to obtain estimates of EAHT and ECHT for as many species as possible to (a) assess the number and diversity of species for which these estimates are available, (b) qualitatively assess variation in EAHT and ECHT across species, and (c) assess how EAHT and ECHT differ. We predict that insufficient data will result in relatively few species with an estimate of ECHT and even fewer with an estimate of EAHT. Moreover, these estimates will be highly clustered with respect to phylogeny. Additionally, we predict that there will be large variation among species for both ECHT and EAHT and that estimates of EAHT will be much greater than ECHT. Ultimately, we hope this review will expose gaps in our current understanding and illustrate pathways for future research.

3.1 | Literature review

We combined data from the Reptile Development Database (www.repdevo.com) with literature collected from our own Web of Science search to estimate ECHT for as many species as possible. We used the same search terms reported in Noble, Stenhouse, Riley, et al. (2018) and Noble, Stenhouse, and Schwanz (2018; incubat* and one of the following: reptil*, lizard*, squamat*, snake*, turtle*, chelon*, testudin*, crocodil*, alligator*, tuatara*, sphenodon*) to find studies from 2017 through 2019 to add literature published since the last update of the Reptile Development Database. From this search, we included only papers that reported hatching success at constant incubation temperatures. Studies that manipulated environmental variables other than temperature (e.g., hormones) were excluded. From each study, we extracted estimates of hatching success expressed as percentages or proportions. For some studies, we extracted data from figures using ImageJ (https://imagej.nih.gov/ij/). All percentages were converted to proportions (i.e., the number of eggs that hatched/total eggs).

From our data set, we selected species for which hatching success is reported for at least four different constant temperatures. Of these, we excluded species that did not have at least one warm temperature that reduced hatching success by 50% of hatching success in the OTR. We excluded some studies that exhibited unusually low hatching success within the OTR (compared with other studies of the same species). For each species remaining, we searched using "incubat*" and the species name to try and find additional studies reporting hatching success at extremely warm temperatures, but no additional estimates were found. Because crocodilians were underrepresented in our data set ($n = 0$ species), we used literature reviewed by González et al. (2019) to add additional studies. For two species (*Alligator mississippiensis* and *Caiman crocodilus*), hatching success was not provided for each temperature within the OTR but was described generally (e.g., hatching success was "greater than 90%" at all temperatures). We assigned each temperature a hatching success above this threshold (e.g., 0.91) because we wanted to include as many crocodilian species as possible. Our final data set included 16 squamates, 16 turtles, five crocodilians, and the tuatara (Table S1). See Figure S1 for a flowchart illustrating our review.

To our knowledge, only the studies listed in Table 2 have measured EAHT of reptiles ($n = 5$ squamates and 1 turtle). To increase sample size, we added our own unpublished data ($n = 4$ *Takydromus* species; assessed via thermal ramp; Table S2).

3.2 | Analyses

For each species, we estimated T_{opt} as described previously. Because we did not have the raw data for each species, we analyzed mean survival at each constant temperature (as in Figure 3a). Moreover, sample sizes, with respect to temperature, were necessarily low (i.e., usually one study per temperature), and studies differ with respect to the intervals between incubation temperatures (e.g., every 2°C vs. every 3°C). For these reasons, had we applied a model selection process for each species, among-species differences in final models would probably represent methodological rather than biological variation. Therefore, we applied a three-parameter log-logistic model to each species to estimate ECHT (as above). This function is biologically appropriate because it aligns with theory concerning ectotherm embryo survival (i.e., high survival in the OTR and then a sharp decline; van der Have, 2002). Moreover, it is often used to describe sex ratios in studies of temperature-dependent sex determination (e.g., Carter, Sadd, Tuberville, Paitz, & Bowden, 2018);

therefore, ecologists interested in reptile developmental plasticity are familiar with its application. Based on our goals (outlined above), we did not perform analyses on estimates of ECHT and EAHT but (a) provide a geometric mean and range of ECHT for each major clade and for EAHT estimates combined and (b) discuss data qualitatively.
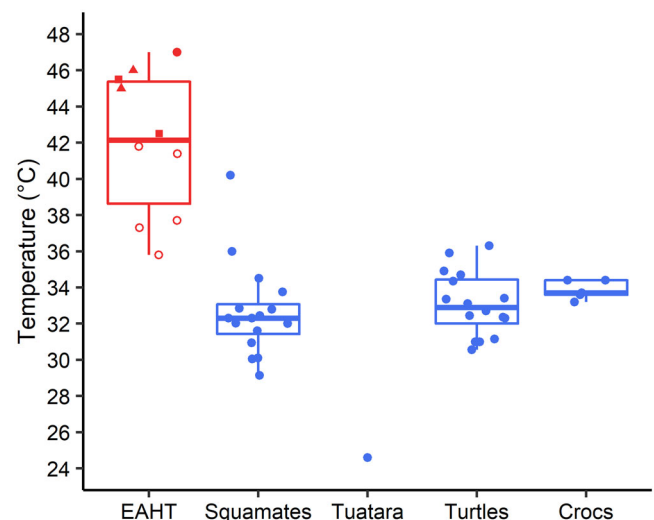
3.3 | Results and discussion

Means and ranges for each clade are provided in Table 3 and plotted in Figure 4. Mean ECHT is similar among crocodilians, squamates, and turtles, but is comparatively low for the tuatara. Our sample, however, vastly underrepresents the diversity within reptiles. Moreover, representation is not equal across major clades. All rhynchocephalian families (1 of 1), 2 of 3 crocodilian families (66.6%), 8 of 14 turtle families (57.1%), and 8 of 55 squamate families (14.5%) have at least one species with an estimate of ECHT (Figures S2, S3, and S4). At the species level, this represents 100%, 20%, 4.5%, and 0.15% of rhynchocephalian, crocodilian, testudines, and squamate species, respectively (total species per group taken from www.reptile-database.org). Thus, squamata is by far the most understudied order when considering the diversity within the clade. Moreover, some of the most speciose groups (e.g., Gekkota), have no estimates.

Despite the low sample size, there is a relatively large variation in ECHT across reptiles. This is best exemplified by the extreme difference in ECHT between the desert iguana (*Dipsosaurus dorsalis*) and the tuatara (*Sphenodon punctatus*; ~15°C), which represent the highest (40.2°C) and lowest (24.6°C) estimates of ECHT, respectively. Differences in ECHT could be due to lineage-specific thermal adaptation (Andrews & Schwarzkopf, 2012; Du et al., 2019). Indeed, nest temperatures of *D. dorsalis* often exceed 40°C (Muth, 1980) while those of *S. punctatus* are between 16°C and 20°C in mean temperature (Thompson, Packard, Packard, & Rose, 1996). These extreme examples of ECHT aside, there is still considerable variation in ECHT, and we plan to explore this variation according to ecological variables in a future study.

FIGURE 4 Estimates of ECHT (blue) of squamates (*n* = 16), tuatara (*n* = 1), turtles (*n* = 16), and crocodilians (*n* = 5) and EAHT (red). EAHT includes nine squamates (*Takydromus* [open circles], *Anolis* [squares], *Sceloporus* [triangles]) and one turtle (*Pelodiscus* [closed circle]). Each point indicates the mean of each species. EAHT, embryo acute heat tolerance; ECHT, embryo chronic heat tolerance

As predicted, estimates of EAHT are few and highly clustered with respect to phylogeny. All nine estimates of squamate EAHT come from three genera (*Anolis*, *Takydromus*, *Sceloporus*) which represent two families (Iguanidae and Lacertidae; Figure S2). Only one estimate is available for turtles (*Pelodiscus sinensis*) and none are available for crocodilians or the tuatara (Figures S2, S3, and S4). Thus, our knowledge of how EAHT varies across species is extremely limited. Overall, estimates of EAHT are much higher than ECHT (~9–10°C), indicating that reptiles can withstand brief exposures to temperatures much greater than ECHT (Figure 4). The highest EAHT is 47°C (Chinese softshell turtle; *P. sinensis*) and the lowest is 35.8°C (southern grass lizard; *Takydromus sexlineatus*). Intriguingly, there appears to be greater variation in EAHT than ECHT (Figure 4). For example, just within the genus *Takydromus*, EAHT ranges across 6°C, which is nearly equal to the total variation in ECHT of turtles and squamates (without considering *D. dorsalis*—an outlier). Moreover, there is some overlap between EAHT and ECHT across reptiles as demonstrated by the relatively high ECHT of the desert iguana (*D. dorsalis*; 40.2°C) and Bibron's agama (*Agama impalearis*; 36.0°C) and the relatively low EAHT of some *Takydromus* lizards (35.8–37.7°C). These anecdotes, collectively, indicate great potential for lineage-specific adaptation of both ECHT and EAHT to abiotic conditions (e.g., climate). Unfortunately, only three species have measures of both ECHT and EAHT, which precludes analyses that explore the relationship between these phenotypes. Moreover, estimates of EAHT are confounded with taxon, which precludes any meaningful analysis to compare the methods outlined above. For example, all *Takydromus* lizards were measured via thermal ramp but *Anolis* lizards were measured with single fluctuation (Table S2).

TABLE 3 Estimates of optimal developmental temperature (T_{opt}), embryo chronic heat tolerance (ECHT), and embryo acute heat tolerance (EAHT) for major reptile clades

	n	Mean (°C)	Range (°C)
Crocodilia, T_{opt}	5	32.9	31.0–33.5
Crocodilia, ECHT	5	33.9	33.2–34.4
Squamata, T_{opt}	16	30.5	28.0–38.0
Squamata, ECHT	16	32.7	29.2–40.2
Sphenodontia, T_{opt}	1	24.0	–
Sphenodontia, ECHT	1	24.6	–
Testudines, T_{opt}	16	31.7	29.0–34.0
Testudines, ECHT	16	33.1	30.6–36.3
EAHT	10	42.0	35.8–47.0

Note: Data are combined for EAHT due to low sample size of most clades.

Both urbanization and climate change can potentially increase nest temperatures by 1–2°C, even after accounting for maternal adjustments in nesting behavior (Telemeco et al., 2009; Tiatragul et al., 2020). T_{opt} for crocodilians, squamates, and turtles are only 1.0°C, 2.2°C, and 1.4°C lower than mean ECHT, respectively. Therefore, if species are currently nesting at temperatures that optimize development, future warming will result in increased mortality in the absence of embryo adaptation or compensatory adjustments of nesting behavior (Carlo et al., 2018; Telemeco et al., 2009, 2017).

We need more predictive models that consider embryo responses to global change (e.g., Carlo et al., 2018; Levy et al., 2015); however, these models will be hindered by only considering responses of embryos to chronic conditions (i.e., ECHT). Measuring EAHT can increase our understanding of embryo thermal physiology. For example, it is often assumed, based on constant temperature incubation, that embryos have a narrower thermal tolerance breadth than adults. Clusella-Trullas, Blackburn, and Chown (2011) estimated the mean CT_{max} of adult squamates to be ~42°C, which is nearly 10°C greater than squamate ECHT but essentially equal to mean EAHT. EAHT is not perfectly compatible with CT_{max} because the former results in death and the latter results in loss of motor function; however, the large difference between EAHT and ECHT should compel us to abandon comparisons between chronic incubation conditions of embryos and the CT_{max} of post-hatching stages and induce skepticism concerning the assumption that the thermal tolerance breadth of embryos is far less than that of later life stages (van der Have, 2002).

There are some caveats to our review. First, because studies are often limited with respect to sample size, most researchers incubate eggs within the OTR and at widely spaced intervals of temperatures (e.g., 26°C, 30°C, 34°C). Thus, sample sizes were small for estimating ECHT, and there are many "gaps" among treatments, which may reduce the accuracy of our estimates. Second, although hatching success is relatively constant across a broad range of temperatures, many fitness-relevant traits have a thermal optimum (e.g., performance, body size); thus, considering only hatching success with respect to temperature may obscure the true relationship between temperature and fitness (e.g., Mueller, Ruiz-García, García-Gasca, & Abreu-Grobois, 2019). Finally, most studies have used constant incubation temperatures; thus, we used constant temperatures to estimate ECHT. Thermal variation, however, typifies most nests and alters the relationship between temperature and hatchling phenotypes (Les, Paitz, & Bowden, 2007). Future work could incorporate fluctuating temperatures (e.g., repeated sine waves) into calculations of ECHT.

4 | FUTURE DIRECTIONS

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

First, we need more studies that characterize embryo responses across a wide range of temperatures, including extreme temperatures. The relationship between incubation temperature and survival/phenotypes is often curvilinear (Noble, Stenhouse, & Schwarz, 2018); therefore, complete reaction norms may be necessary to understand relationships between temperature, physiology, and fitness. Moreover, most studies have examined the effects of temperatures within the OTR, but we need a better understanding of development at extreme temperatures to predict responses to global change. When possible, researchers should quantify embryo responses to the full range of constant temperatures from the lower to upper lethal limits as a foundational part of their research program. Both the upper and lower limits for development are vital to describe responses to rising temperatures (Levy et al., 2015). For perspective, only eight species in our data set have measures of both the upper and lower limits for development. Moreover, we could only estimate ECHT for 38 species, representing 24.5% of species in the Reptile Development Database ($n = 155$; Noble, Stenhouse, Riley, et al., 2018). Our estimates span a wide range of families across lepidosaurs, testudines, and crocodilians; however, there are many groups for which no estimates are available (Figures S2, S3, and S4). Importantly, studies should calculate and report the ECHT. Current studies report survival rates across temperature, but ECHT will allow for comparisons across studies and species.

Second, we need more studies that quantify EAHT across a range of species. Current estimates are few and highly clustered with respect to phylogeny. Regardless, there is potential for large variation in EAHT among even closely related species. For example, Hall and Warner (2019) found that two species of *Anolis* differ by nearly 3°C in EAHT. Thus, there is likely great variation across reptiles which may relate to lineage-specific ecology and physiology. For example, we predict EAHT will exhibit latitudinal or altitudinal trends and trends associated with the relative thermostability of nest temperatures (e.g., shallow- vs. deep-nesting species). Only two studies have considered geographic variation in EAHT, but they found conflicting results: Geographic variation was detected for *Takydromus septentrionalis* (Sun, unpublished) but not *S. undulatus* (Angilletta et al., 2013). Moreover, there may be interesting relationships between EAHT and ECHT (e.g., coevolution); however, there is some evidence that ECHT and EAHT may not evolve in concert. Eastern fence lizards (*S. undulatus*) exhibit geographic variation in embryo responses to chronic thermal conditions; however, EAHT has not diverged across populations (Angilletta et al., 2013). To our knowledge, population-specific sensitivity to chronic and acute conditions has not been evaluated in any other species. Many more studies are

required to understand the ecology and evolution of EAHT, including methodological studies (e.g., comparing methods, assessing repeatability).

Third, several studies predict species responses to climate change by incorporating embryo physiology into SDMs (Carlo et al., 2018; Levy et al., 2015); however, future studies should consider both EAHT and ECHT. Indeed, warmer average temperatures and extreme high temperatures are both predicted to increase in the future. Such changes are challenging to mimic in laboratory studies. For example, more frequent heat events would not only expose embryos to their EAHT, they would increase the average temperatures at which embryos develop, causing nest temperatures to approach ECHT. Integration of EAHT and SDM or mechanistic models can be used to predict the heat stress frequency for embryos in the context of climate warming (Carlo et al., 2018; Levy et al., 2015). For example, Sun et al. (unpublished) integrated EAHT of different populations of *T. septentrionalis*, and SDM to predict the heat stress frequencies embryos might face within projected distribution areas. In the future, more work is needed to integrate EAHT and heat tolerance of other life history stages to predict the vulnerabilities of species to climate warming (e.g., Wang, Ding, Li, Wang, & Dong, 2017). Future research could integrate hatching success under different chronic warming conditions (i.e., ECHT) with survival possibilities after extreme heat events (i.e., EAHT). Moreover, with EAHT, we can calculate thermal safety margins for embryos and combine the thermal safety margin of hatchlings, juvenile and adults, to calculate the thermal safety margin of the entire life cycle. Furthermore, we can determine the effect of migration on buffering the vulnerabilities of species with consideration of embryonic EAHT. Within predicted migration and new distribution areas, we can quantify potential heat stress of embryos and determine if temperatures are suitable for embryonic development and species survival.

Finally, we need a better understanding of the ecological and physiological factors that determine embryo heat tolerance. Indeed, the mechanisms that determine the thermal limits of complex life are debated and may result from complications at the cellular (van der Have, 2002) or organ-system levels (Pörtner, Bock, & Mark, 2017), or both (Gangloff & Telemeco, 2018). Several studies have demonstrated a strong link between oxygen supply and thermal tolerance in reptile embryos for both ECHT (Liang, Sun, Ma, & Du, 2015; Parker & Dimkovikj, 2019) and EAHT (e.g., Smith et al., 2015); however, we need more studies that assess embryo physiology at near-lethal temperatures (Hall & Warner, 2020). Studies that incubate eggs at hypoxic and normoxic conditions (e.g., Liang et al., 2015; Smith et al., 2015) have identified oxygen availability as an important factor determining heat tolerance, but they cannot identify the mechanisms that mediate the relationship between oxygen, temperature, and survival. We recommend more studies that expose embryos to acute heat stress (i.e., measure EAHT) under normoxic conditions and measure physiology at near-lethal temperatures (e.g., oxygen consumption, expression of heat shock proteins, anaerobic respiration). Indeed, studies that estimate pejus and critical temperatures by measuring aerobic versus anaerobic metabolism at high

temperatures would be most helpful (Wittmann et al., 2008). Such studies will move beyond our current "black box" understanding of the relationships among temperature, oxygen, and survival. Finally, it is currently unknown how temperature might interact with other environmental variables (e.g., moisture) to determine EAHT, although moisture availability certainly influences egg temperature in the nest (Tezak et al., 2018). Future studies should consider such variables to assess how temperature and developmental ecology interact to determine heat tolerance. This is particularly crucial given that climate change will influence a myriad of environmental factors other than temperature (e.g., rainfall).

5 | CONCLUSIONS

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

CONFLICT OF INTERESTS
The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

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