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RESEARCH ARTICLE

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The effects of incubation temperature and experimental design on heart rates of lizard embryos

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Abstract

Many studies of phenotypic plasticity alter environmental conditions during embryonic development, yet only measure phenotypes at the neonatal stage (after embryonic development). However, measuring aspects of embryo physiology enhances our understanding of how environmental factors immediately affect embryos, which aids our understanding of developmental plasticity. While current research on reptile developmental plasticity has demonstrated that fluctuating incubation temperatures affect development differently than constant temperatures, most research on embryo physiology is still performed with constant temperature experiments. In this study, we noninvasively measured embryonic heart rates of the brown anole (*Anolis sagrei*), across ecologically relevant fluctuating temperatures. We incubated eggs under temperatures measured from potential nests in the field and examined how heart rates change through a diel cycle and throughout embryonic development. We also evaluated how experimental design (e.g., repeated vs. single measures designs, constant vs. fluctuating temperatures) and different protocols (e.g., removing eggs from incubators) might influence heart rate. We found that heart rates were correlated with daily temperature and increased through development. Our findings suggest that experimenters have reasonable flexibility in choosing an experimental design to address their questions; however, some aspects of design and protocol can potentially influence estimations of heart rates. Overall, we present the first ecologically relevant measures of anole embryonic heart rates and provide recommendations for experimental designs for future experiments.

KEYWORDS

anolis, embryo, buddy®, diel cycle, developmental plasticity, experimental design

1 | INTRODUCTION

Variation in the developmental environment can have lasting impacts on adult phenotypes in ways that affect fitness (Monaghan, 2008). Many studies of phenotypic plasticity examine the effects of embryo environments on neonate phenotypes (Booth, 2006), yet do not examine the immediate consequences of the environment on the embryos themselves (Goodman, 2008; Pearson & Warner, 2016; Tiatragul, Kurniawan, Kolbe, & Warner, 2017). Understanding the immediate responses of embryos to altered environmental conditions can provide important insights into our understanding of phenotypic plasticity. Eggs of non-avian reptiles are particularly useful for understanding phenotypic plasticity because they are directly affected by the environment, whereas viviparous or brooding animals have more control over their embryo's environment (Warner, 2014). Reptile eggs experience a broad range of environmental conditions, and many researchers have documented the effect of environmental conditions on hatchling phenotypes (Bodensteiner, Mitchell, Strickland, & Janzen, 2015; Booth, 2006; Shine, Elphick, & Harlow, 1997). The structure of the amniotic egg has previously limited our ability to measure certain physiological characteristics of embryos, such as heart rate, which is an important physiological trait because it strongly correlates with metabolic and developmental rate (Du, Radder, Sun, & Shine, 2009; Du, Ye, Zhao, Warner, & Shine, 2010). However, the recent utilization of the Buddy® egg monitor, a device created to measure heart rates in chicken embryos (Avian Biotech), has enabled researchers to noninvasively measure heart rates of embryos that develop in shelled eggs (Lierz, Gooss, & Hafez, 2006; Sartori, Taylor, Abe, & Crossley, 2015). This technology allows us to consider potential physiological adaptations of embryos in response to the environment, and more broadly enables researchers to understand the immediate effects of environmental variation on embryo physiology (Cordero et al., 2017; McGlashan, Loudon, Thompson, & Spencer, 2015; Radder & Shine, 2006).

Reptile embryos respond differently to realistic fluctuating temperatures than to constant temperatures (Du & Shine, 2010; Georges, Beggs, Young, & Doody, 2005; Warner & Shine, 2011). Yet most studies on embryonic physiology in reptiles have used constant temperatures (Aubret, 2013; Birchard & Reiber, 1996; Du et al., 2009; Sartori et al., 2015) or modified incubation temperature for a short time to address specific research questions (e.g., heart rate patterns during acute heat spikes; Angilletta et al., 2013). Accordingly, we lack critical background information on patterns of embryonic metabolism across natural diel thermal cycles and through the duration of development. As researchers increasingly recognize the importance of maximizing ecological relevance in their work, such information will be an important reference for future research. In particular, an increasing number of studies examine developmental responses to conditions that are likely encountered in the field (i.e., urbanization or climate change; Dayananda and Web, 2017; Mitchell et al., 2016; Tiatragul et al., 2017).

Researchers that use the Buddy® to measure embryonic heart rates have employed a variety of experimental designs and protocols, but we lack a critical examination of the biological consequences of this variation. For example, some researchers frequently remove (and unintentionally jostle) eggs from the incubator to measure heart rates and such interference may influence heart rates or have other unintended consequences (Aubret, Blanvillain, & Kok, 2015). When eggs are removed from an incubator and placed on the Buddy® for embryonic heart rate measurements, egg temperatures may rapidly drop, resulting in reduced embryonic heart rates. Moreover, the Buddy® can significantly raise egg temperature and heart rates, if eggs remain on the monitor for long periods of time (Sartori et al., 2015). Though most experimenters obtain measures of heart rate in short periods of time (<90 sec), how much warming can occur due to the Buddy® over short durations is not well established. Addressing these issues will be important to ensure researchers choose proper experimental designs and include appropriate covariates in their analyses.

We have two primary goals in this study. First, we seek to add valuable biological information to understanding embryonic heart rates in reptiles. We describe the nature of changes in embryonic heart rates over a natural thermal diel cycle and through the course of development. To do this, we exposed lizard embryos to natural temperature fluctuations and measured their heart rates at the coolest and warmest parts of the day, and measured heart rates at intermediate temperatures during warming and cooling periods. We made these measurements four times over the course of incubation to determine if heart rate changes through different stages of development. Our second goal is to provide valuable logistical advice to researchers designing their own studies. Specifically, we address the impact of repeatedly measuring embryos over a short timespan (as is required for repeated measures studies), the influence of removing eggs from the incubator to measure embryonic heart rate, and the potential warming effect of the Buddy® on small eggs.

Additionally, we provide baseline heart rate information for an important reptilian model, the brown anole (*Anolis sagrei*). *Anolis* lizards have served as a model system in ecology, physiology, development, and evolution (Losos, 2009). The brown anole (*A. sagrei)* is oviparous, highly fecund, and easy to breed in the lab which makes it an excellent species for studies of developmental plasticity and embryonic physiology (Sanger, Hime, Johnson, Diani, & Losos, 2008; Sanger, Losos, & Gibson-Brown, 2008). We selected the brown anole for our experiments because of the ease of obtaining eggs and because, despite being extremely well studied, the literature on embryonic heart rate during development in anoles is scant (Du et al., 2011). The questions addressed in this study will provide essential background information for others hoping to expand our understanding of embryonic physiology in this model system.

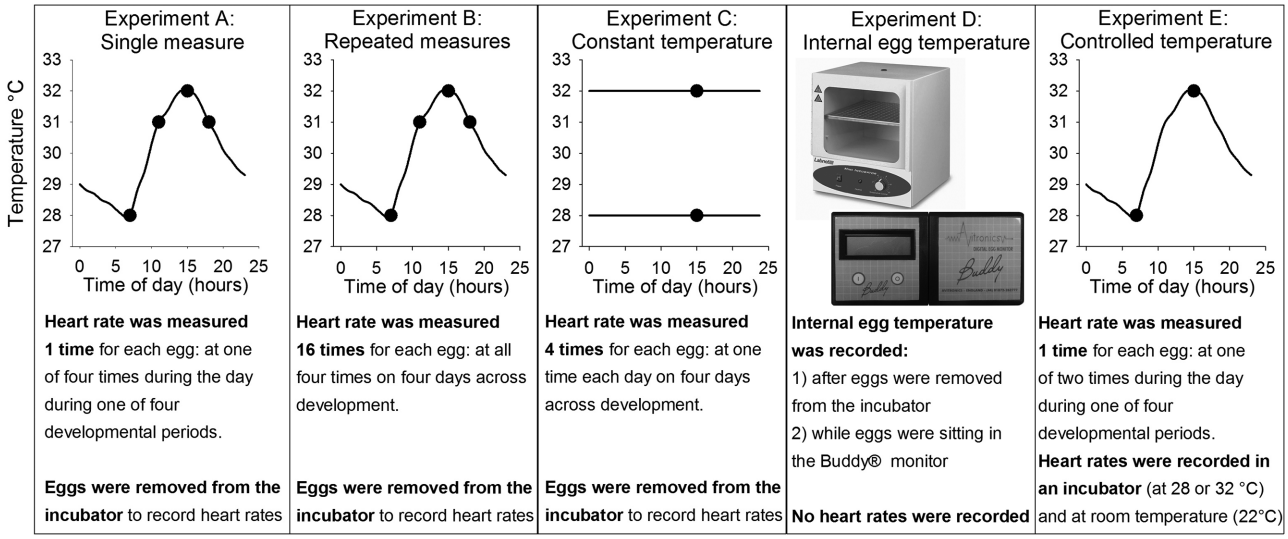
2 | METHODS

2.1 | Egg collection and incubation

We captured adult *A. sagrei* from two spoil islands in Palm Coast, Florida on April 2–5, 2016 and housed them at Auburn University in ReptiBreeze screen cages (46 × 46 × 91 cm). Lizards were housed in groups (male:female) of either 2:2 or 3:3 for a behavior study not described here. We provided nesting boxes filled with a mixture of moist potting soil and peat moss and checked for eggs twice each week. Thus, each egg's oviposition date is accurate to within 4 days. Lizards were fed three crickets each (dusted with vitamins and calcium) twice weekly and cages were misted with water daily.

We measured the mass of each egg on the day it was collected and immediately following each time its heart rate was measured. Each egg was given a unique identification number and incubated individually in moistened vermiculite (−150 kPa) inside 60 × 15 mm (diameter × height) petri dishes sealed with parafilm. Most eggs ($n = 292$) were incubated in a Memmert brand IPP 55 Plus incubator, which we programmed to fluctuate daily (Figure 1). The fluctuations of these incubators approximate realistic conditions measured at nest sites nearby. This approximation was based on iButton temperature logger data collected from August to October 2013 near four *A. sagrei* eggs that were discovered in the field (Supp. Figure S1). At these temperatures our hatch rate was 90.3%. The remaining eggs ($n = 24$) were incubated in VWR INCU-line incubators and held at either constant 28°C or 32°C: the lowest and highest temperatures from the daily, fluctuating regime (Figure 1).

A subset of eggs ($n = 47$) were collected from August 3 to September 5, 2017 from a different group of adult lizards that were also captured from Palm Coast, Florida (April 15–16, 2017). These lizards were housed identically to that previously described with two exceptions: the cages were larger (ReptiBreeze screen cages 60 × 60 × 122 cm) and lizards were kept in groups of (male:female) 2:4, 3:3, or 4:2 for an experiment not described here. Each egg was incubated as previously described in a Memmert brand IPP 55 Plus incubator programmed to fluctuate daily. These eggs were used to measure heart rates at 28°C and 32°C in the controlled temperature design (see Experiment E below). All experiments were approved by Auburn University Institutional Animal Care and Use Committee (Protocol #2016-2812).



Questions addressed in our study	Type of question	Experiments that answer each question
How do embryo heart rates fluctuate across a natural diel cycle?	Biological	A and B
Can embryos physiologically thermoregulate during warming and cooling (hysteresis)?	Biological	A and B
Does embryo heart rate change through development?	Biological	A, B, C, and E
Are anole embryo heart rates repeatable?	Biological	B
Does repeated jostling of eggs influence estimation of heart rate?	Methodological	Contrasting A with B
Do constant temperature experiments give comparable measures of heart rates to fluctuating temperature experiments?	Methodological	Contrasting A and B with C
How rapidly does temperature change when eggs are removed from incubators?	Methodological	D
Does the Buddy monitor appreciably warm eggs over short durations?	Methodological	D
Does using duration as a covariate appropriately account for changes in egg temperature?	Methodological	Contrasting A, B, and C with E

FIGURE 1 Summary of questions and experimental designs. See text for further descriptions of experiments A–E. Experiments A, B, and E show the daily thermal cycle programmed into our fluctuating incubators, and experiment C shows the constant temperatures we used. Closed circles show times of day when heart rates were measured during each experiment

2.2. | Measuring heart rate

To measure the heart rate of each embryo, we removed a petri dish from the incubator, removed the egg from the petri dish, and quickly placed the egg within the Buddy®, which was held at room temperature. We anticipated that eggs would begin to cool upon being removed from the incubator and that this cooling would affect heart rate; therefore, we measured the time to obtain a heart rate (henceforth referred to as "duration") so it could be used as a covariate in our analyses. We measured duration by starting a stopwatch the moment the petri dish was removed from the incubator and stopping it when we had successfully measured the heart rate. We excluded all heart rates from our data that were not obtained within a 60-sec duration. We did not record duration for one experimental design (controlled temperature; see below) because, for this design only, the Buddy® was housed inside an incubator set at a constant temperature while heart rates were recorded.

2.3 | Study designs

2.3.1 | Experiment A: Single measure

We anticipated an incubation period of ~29 days for anole eggs incubating at our thermal regime (Mitchell, unpublished data). To ensure our sample included eggs that spanned a broad range of developmental stages, we divided this time into four arbitrary developmental periods: 1–6, 7–13, 14–19, 20–29 days. We selected 232 eggs to be measured once each, and randomly allocated them into four groups representing these arbitrary periods of development. We further subdivided each group four ways so that we could measure embryo heart rates at four different times of day (0700, 1100, 1500, and 1800) within each period. These times correspond to the temperature minimum and maximum of our daily fluctuation, as well as the midpoints between the minimum and maximum (Figure 1). This design ensured that we measured heart rates for an equal number of embryos at each of four temperatures in our daily regime and across the full length of development (days 1–31 from egg collection).

2.3.2 | Experiment B: Repeated measures

We selected 20 additional eggs and measured their heart rates four times in a single day on four different days across development. Therefore, each embryo's heart rate was measured a total of 16 times: on one day during each developmental stage (described above), each embryo was measured at 0700, 1100, 1500, and 1800 hr. Eggs in this experiment were also exposed to the same thermal fluctuation described for experiment A (Figure 1).

2.3.3 | Experiment C: Constant temperature

We selected 24 additional eggs and placed 12 into a constant 28°C incubator and 12 into a constant 32°C incubator. We measured each embryo's heart rate at 1500 hr within 2 days after collection and once per week thereafter at the same time of day (Figure 1). This ensured that each embryo was measured during each of our four developmental stages as in our single and repeated measures experiments.

2.3.4 | Experiment D: Internal egg temperature

Because *Anolis* eggs are very small, we anticipated they would quickly cool once we removed them from the incubator. Moreover, Sartori et al. (2015) showed that the infrared beam of the Buddy® monitor can increase the temperature of eggs if they remain in the monitor for several minutes. We performed two experiments to determine how anole eggs change temperature due to these manipulations. To quantify cooling, we incubated three additional eggs at a constant temperature of 32°C and measured their rate of temperature loss upon removal from the incubator. We punctured each egg with a thermocouple and used superglue to hold it in place. Each egg then was placed into a petri dish filled with moist vermiculite (−150 kPa) and put back into a constant 32°C incubator. We recorded each egg's internal temperature once it stabilized. We then removed each egg from the incubator and placed it on the Buddy®, which was at room temperature (~22°C) and recorded internal egg temperature every 30 sec for 3 min. At the end of each 3-min trial, we returned the egg to the incubator and repeated the trial once the egg reached a steady internal temperature. We performed four trials on each egg.

To quantify warming due to the Buddy® monitor, we used four eggs and inserted a thermocouple into each as described previously. Each egg was placed in the monitor, which was at room temperature, and we recorded both the egg temperature and the air temperature inside the monitor at 10-sec intervals for 60 sec (our longest duration). We did this four times for each egg: twice with the monitor turned on and twice with the monitor off. To quantify longer-term warming, we also performed a separate trial with each egg, recording both the egg temperature and air temperature in the monitor at 30-sec intervals for 5 min. The Buddy® was turned on for each of the 5-min trials. Because of the small size of these eggs, we anticipated that puncturing them with a thermocouple would damage vital embryo structures, rendering heart rates uninformative. Thus, we did not record heart rates for any embryos used to measure internal egg temperature.

2.3.5 | Experiment E: Controlled temperature

We wanted to define the relationship between heart rate and temperature and ensure that using duration as a covariate would accurately correct for any decrease in heart rate due to egg-cooling. Thus, we performed a final experiment where heart rates were measured at three known temperatures (22, 28, and 32°C). We selected 87 eggs that varied in age from 2 to 31 days since oviposition. Forty eggs were cooled to room temperature (22°C), and their heart rates were measured. Twenty-four eggs were removed from the fluctuating incubator at 0700 hr and placed in a VWR INCU-line incubator set at 28°C. Each egg was removed from this incubator, one at a time, and placed inside the Buddy®, which was housed in a larger incubator (VWR Gr Con 2.6CF). We anticipated that the larger incubator would cool slightly as we opened and closed the glass door to move eggs into and out of the Buddy®, so we set this incubator at 28.5°C. We allowed the egg to sit in the Buddy® for 45 sec and then we recorded a heart rate. For each heart rate, we recorded the exact air temperature inside the Buddy® to the nearest 0.1°C using an Omega HH801A thermocouple. With the other 23 eggs, we repeated this procedure at 1500 hr except the incubator was set to 32°C and the incubator with the Buddy® was set to 32.5°C. Mean temperatures inside the Buddy® when heart rates were recorded were 28.2 and 32.07°C at 0700 and 1500 hr, respectively. Measurements at 28 and 32°C were performed in a warm room (mean temperature = 27.1°C) to reduce the chances that eggs would substantially cool when moved between incubators.

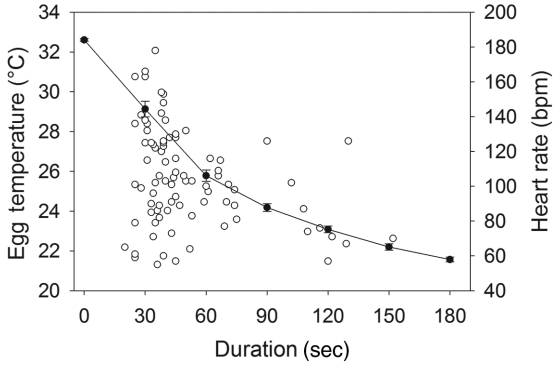


FIGURE 2 Relationships of duration with egg temperature and heart rate. The solid line and closed circles show mean internal *Anolis sagrei* egg temperatures (±SE) after removal from 32°C constant temperature incubator and placement on the Buddy® monitor at room temperature (~22°C). Temperature was recorded at 30 sec intervals. Open circles show the relationship between duration to obtain a heart rate measurement after removal from 32°C constant temperature incubator and embryonic heart rate measurement at room temperature (~22°C). Duration was the total time to measure a heart rate after removing an egg from the incubator

2.4 | Statistical analyses

To assess the effects of developmental age and diel cycle on heart rate, we used an ANCOVA for single measure data (experiment A) and mixed model ANCOVAs for repeated measures (experiment B) and constant temperature trials (experiment C) with individual egg as a random effect. We considered each egg's developmental age to be the number of days since oviposition. Thus, age was modeled as a continuous variable and ages ranged from 1 to 31 days (Supp. Figure S2a). We excluded all heart rates with a duration (time between removal from the incubator and detection of heart rate) greater than 60 sec because of how rapidly eggs cooled once removed from the incubator (Figure 2). Models for repeated measures, single measure, and constant temperature designs included duration as a covariate. Preliminary analyses indicated that heart rate was not associated with egg mass (all P values > 0.09) and that there were no significant interactions between developmental age and diel cycle (all P values > 0.31) or between duration and diel cycle (all P values > 0.25); therefore, we excluded egg mass

and these interactions from all analyses. Mean mass for eggs used in our study was 0.2907 ± 0.0871 g standard deviation (range 0.1285–0.5318 g) (Supp. Figure S2); these values represent eggs from all developmental ages used in our study.

To analyze warming due to the Buddy® monitor (experiment D), we performed a repeated measures ANCOVA with egg temperature as an independent variable and our fixed effects were time (0–60 sec), the status of the Buddy® (on or off) and their interaction. The air temperature in the Buddy® was a covariate and egg identity was a random effect. We used mixed model regressions of egg temperature versus time to analyze egg cooling and the longer-term warming due to the heart rate monitor: egg identity was a random effect.

To correct for slight fluctuations in temperature in the controlled temperature design (experiment E), we performed two linear regressions of heart rate versus exact temperature (measured inside the Buddy®): one for each nominal temperature (28 or 32°C). Separate regressions were necessary because the relationship between heart rate and temperature is nonlinear (Du and Shine, 2010). We used the slope estimates from these regressions to calculate a heart rate for each egg at exactly 28 or 32°C. These corrected heart rates, along with heart rates measured at 22°C were our independent variable (treated as factors) in an ANCOVA that included nominal temperature (22, 28, or 32°C) and developmental age as fixed effects. Preliminary analysis demonstrated that the relationship between heart rate and age was nonlinear, so we built two models that assumed either a linear or quadratic fit for developmental age and compared them with an *F* test to determine the better fit.

To calculate adjusted repeatability of heart rates and estimate 95% CIs, we used parametric bootstrapping in R package "rptR" on our repeated measures data (Nakagawa & Schielzeth, 2010). Repeatability was adjusted for fixed effects (duration, age, diel cycle). We performed all analyses in R (vers 3.3.1; R Core Team, 2015) and utilized package "nlme" for mixed models and made pairwise comparisons using the "lsmeans" package. To assess variation among our study designs, we calculated 95% confidence intervals for estimates of heart rate at 28 and 32°C. We compared these confidence intervals across experiments to determine if different designs yielded similar estimates of heart rate.

3 | RESULTS

3.1 | Experiment A: Single measure

Heart rates of embryos changed throughout the day with corresponding increases and decreases in incubation temperature ($F_{3,99} = 4.39$, $P = 0.0061$) (Figure 3A) and increased through development by 0.24 beats per minute (bpm) per day (±0.32 SE) but this was not significant ($F_{1,99} = 0.57$, $P = 0.45$). We also tested if duration, the time required to measure an egg's heart rate after removing it from the incubator, influenced heart rate. We observed an 18.46 bpm (±7.98 SE) decrease in heart rate for every 30-sec increase in duration ($F_{1,99} = 5.35$, $P = 0.023$).

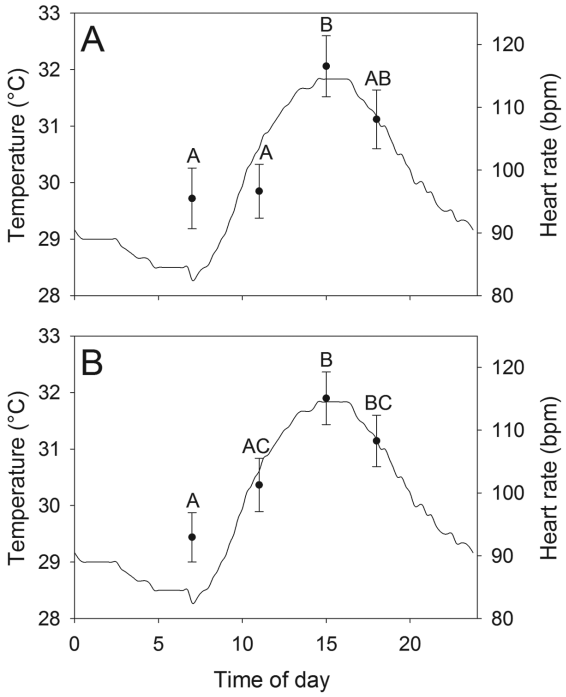


FIGURE 3 Incubation temperature (solid line) and mean embryonic heart rates (circles ± SE) of *Anolis sagrei* eggs throughout incubation at a diel temperature regime for single measure (A) and repeated measure (B) designs. Heart rates were measured at 700, 1,100, 1,500, and 1,800 hr. Letters denote significant differences ($P > 0.05$) between groups. Temperatures were recorded inside petri dishes assembled identically to those used to incubate eggs. Thus, temperatures differ slightly from the ambient temperatures programmed into the incubator (shown in Figure 1A, B, and E)

3.2 | Experiment B: Repeated measures

Our repeated measures experiment gave us similar results to the single egg measurements. Heart rate increased with temperature ($F_{3,134} = 7.23$, $P = 0.0002$) (Figure 3B) but change in heart rate through development (increase of 0.056 bmp per day ±0.24 SE) was not significant ($F_{1,134} = 0.053$, $P = 0.82$). Heart rate decreased by 10.2 bpm (±6.16 SE) for every 30 -sec increase in duration, but this was not statistically significant ($F_{1,134} = 2.75$, $P = 0.09$).

3.3 | Experiment C: Constant temperatures

Incubating eggs at two constant temperatures yielded similar results to incubating eggs in a diel cycle. Heart rate was 33.06 bpm (±6.12 SE) higher for embryos incubated at 32°C than those at 28°C ($F_{1,20} = 29.22$, $P < 0.0001$). Heart rate increased by 0.69 bpm per day (±0.39 SE) through development, but this was not significant ($F_{1,41} = 3.16$, $P = 0.083$). For every 30-sec increase in duration heart rate decreased by 5.55 bpm (±11.19 SE), but this was not statistically significant ($F_{1,41} = 0.25$, $P = 0.62$).

3.4 | Experiment D: Internal egg temperature

Internal temperature rapidly dropped once eggs were removed from the incubator ($F_{1,32} = 661.07$, $P < 0.0001$) (Figure 2). Over the

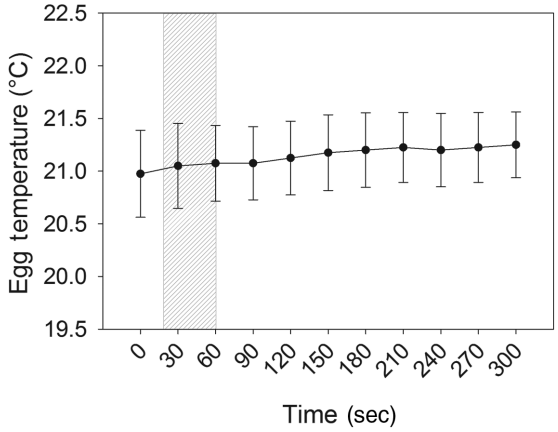


FIGURE 4 Internal temperature of eggs that were in the Buddy® monitor for a 5-min duration. Closed circles show the mean egg temperature at each 30 sec interval and bars show standard error. All heart rates were recorded within a duration of 18–60 sec (shaded region)

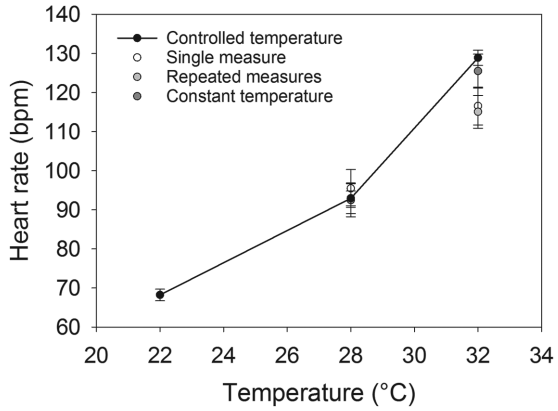


FIGURE 5 Relationship between heart rate and temperature (Experiment E). Estimates of heart rate from experiments A, B, and C are shown to demonstrate that using duration as a covariate will underestimate heart rate at warmer temperatures. Bars show standard error

first 60 sec, temperature dropped 0.114°C each second (±0.0044 SE). The Buddy® monitor, however, did not significantly increase internal egg temperature within a 60-sec duration: egg temperature was not warmer when the Buddy® was on versus when it was off ($F_{1,88}$ = 0.22, P = 0.64), nor was there an interaction between the Buddy®'s status (on vs. off) and time ($F_{1,88}$ = 0.016, P = 0.90). We detected a slight increase in egg temperature over a 5-min period ($F_{1,38}$ = 38.81, P < 0.001). Egg temperature increased 0.0486°C per minute (±0.00078 SE) (Figure 4).

3.5 | Experiment E: Controlled temperatures

Heart rate increased with temperature ($F_{2,82}$ = 295.15, P < 0.0001; Figure 5). At 32°C, mean heart rate was 35.96 bpm (±2.68 SE) and 60.67 bpm (±2.50 SE) higher than at 28 or 22°C, respectively. Unlike other experimental designs, we observed a significant increase in heart rate through development, and this relationship was better explained by a quadratic model than a linear one ($F_{1,82}$ = 15.90, P = 0.0001, R^2 = 0.31; Figure 6).

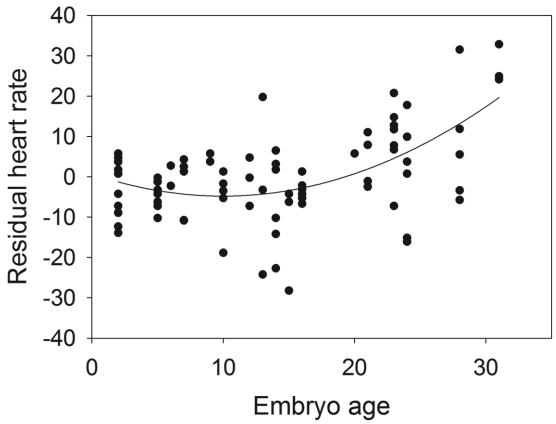


FIGURE 6 The relationship between embryo heart rate and embryo age. Heart rates were measured at 22, 28, and 32°C, so plotted values (closed circles) are residuals from a model that included only these temperatures as fixed effects. The solid line is a nonlinear regression of residual heart rate and embryo age that included a quadratic term

3.6 | Comparisons between experiments and heart rate repeatability

Our estimates of heart rate did not differ among experimental designs at 28°C; however, our controlled temperature experiment yielded a higher estimate of heart rate at 32°C than the repeated measures experiment (Table 1). Additionally, we found that heart rate within individual was repeatable (R_{adj} = 0.138; 0.071 SE; P = 0051) and, thus, some of the wide variation in heart rate at any given duration (Figure 2) is attributable to interindividual differences (Figure 7).

4 | DISCUSSION

Heart rate is a useful and frequently used physiological metric in animal research. In lizards, heart rate has been used as a proxy for metabolism and corresponds with developmental rate (Du et al., 2009; Du & Shine, 2010; Du et al., 2010; but see Sartori et al., 2017). Since we do not know the exact relationship between heart rate and oxygen consumption in *A. sagrei*, we cannot make assumptions about metabolic rate during development in this species. However, heart rate still provides general information about cardiac output and the transport of substances throughout the body (Crossley & Burggren, 2009). The ease of measuring heart rates of reptilian embryos with the Buddy® monitor has allowed a recent flurry of research in reptilian embryonic physiology and plasticity (Du & Shine, 2008; Loudon, Spencer, Strassmeyer, & Harland, 2013; Radder & Shine, 2006). While this research has investigated a host of novel and intriguing questions, several basic biological and methodological questions remain. The first goal of our study was to investigate embryonic heart rates of a small reptile, *A. sagrei*, under natural diel fluctuating temperatures. To date, no other work has documented the responsiveness of heart rates to diel temperature fluctuations, even though fluctuating temperatures are naturally experienced by eggs in the field (Booth, 2006; Georges, 1992; Warner & Shine, 2009). The second goal of our study was to examine whether different

TABLE 1 Heart Rate Estimates for the Four Experimental Designs

	Single Measure*		Repeated Measure*		Constant Temperature*		Controlled Temperature	
Experimental Design	Estimate	Upper, Lower CI	Estimate	Upper, Lower CI	Estimate	Upper, Lower CI	Estimate	Upper, Lower CI
Heart rate at nominal 28°C	95.49	105.09, 85.89	92.95	101.17, 84.73	92.45	101.25, 83.59	92.92	96.68, 89.16
Heart rate at nominal 32°C	116.55	126.16, 106.94	115.08	123.85, 106.31	125.5	134.5, 116.51	128.87	132.73, 125.00

Note: Confidence intervals (upper, lower CI) are 95%.
*Designs that required heart rate to be measured at room temperature and corrected using duration as a covariate.

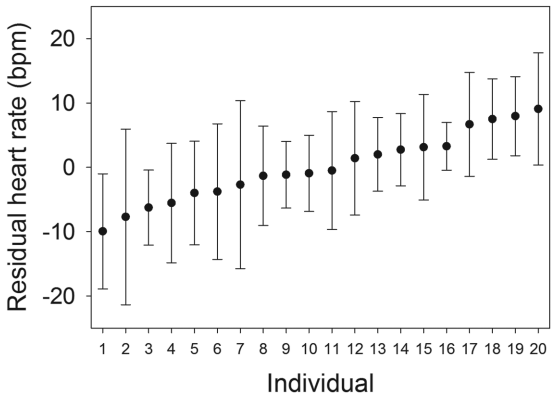


FIGURE 7 Residual heart rates (bpm) of individual embryos from the repeated measures design (experiment B) ranked from low to high. Residual heart rates are corrected for duration, variation in embryo age, and temperature at which they were measured. For each individual, the mean was calculated from all heart rates measured (four times of day across all four developmental periods). Circles show raw residual means and bars are SE. We excluded heart rates measured at a duration greater than 60 sec

experimental designs might contribute to variation in results and affect interpretation. Here we provide several biological and methodological advances for research on physiology of reptile embryos.

4.1 | How do embryo heart rates fluctuate across a natural diel cycle?

As expected, embryonic heart rates tracked the diel fluctuation in incubation temperature in both our single (A) and repeated (B) measure experiments. Heart rates were lowest in the early morning (0700) when temperatures were lowest, and highest in the afternoon (1500) when temperatures were highest (Figure 3). Embryos at the mid-morning (1100) and evening (1800) intermediate temperatures had intermediate heart rates. As the embryo warms, the heart must beat faster to supply enough nutrients and oxygen to keep up with increasing developmental rates (Du et al., 2009, 2010) and this positive relationship between heart rate and temperature has been observed across reptile lineages (Du et al., 2011, 2009; Sartori et al., 2015). The range of temperatures experienced by embryos in our experiment were within the range suitable for development; however, in natural nests, embryos occasionally encounter harsh temperature spikes, which may reduce heart rates and cause cardiac arrest (Angilletta et al., 2013). Thus, the positive relationship between heart rate and temperature observed in our experiment will break down at extremely high temperatures. Although the rise and fall of heart rate through the day was expected, we did not detect statistical difference between some time periods (Figure 3).

4.2 | Can embryos physiologically thermoregulate during warming and cooling (hysteresis)?

Adult lizards can physiologically thermoregulate by increasing heart rate when warming and decreasing heart rate when cooling; these responses have been shown to accelerate heat absorption and slow heat loss, respectively (Bartholomew & Tucker, 1963; Seebacher & Franklin, 2004). To see if a similar phenomenon occurs in embryos, we measured heart rates at 1100 and 1800, which corresponded to the thermal midpoint as temperatures rose and fell. Heart rates were lower when eggs were warming and higher while eggs were cooling (though not significantly different), which is inconsistent with the pattern described in adult lizards. The nonsignificant pattern we observed in our embryos was detected in embryos of two snake species (Du, Tu, Radder, & Shine, 2013), though we do not understand the biological significance of this pattern (but see Du and Shine, 2015). Because anole eggs are so small (~0.15 g), it seems unlikely that physiological thermoregulation via altered heart rates could substantially influence embryo temperature. A second (and not mutually exclusive) explanation for the observed nonsignificant pattern in our experiment is methodological rather than biological. Though we targeted measuring eggs at the exact same temperature during the warming and cooling phase of the fluctuation, our afternoon measurements (during cooling) were taken at slightly higher egg temperatures (~31.2°C) than during the morning (~30.6°C), warming phase (Figure 3). Our eggs were incubated inside sealed petri dishes with moist vermiculite; we placed temperature loggers inside similar dishes and found that the temperature in these dishes rose and fell more slowly than the ambient conditions in the incubator. The observed pattern could therefore simply reflect this discrepancy, and we recommend that this issue is considered in future studies.

4.3 | Does embryo heart rate change through development?

In three of our experiments (two fluctuating temperature (A and B) experiments and the constant temperature experiment (C)), embryonic heart rates increased slightly from the start to the end of incubation, by less than one beat per minute per day (0.056–0.69 bpm/day); however, these increases were not significant. In these experiments, removing eggs from the incubator to measure their heart rates resulted in greater variation in heart rate than when measurements were made

inside an incubator (e.g., controlled temperature design (D)). This variation might mask subtle, but biologically meaningful, changes in heart rate through development and could explain why our controlled temperature design was the only one that detected such a change. The pattern we detected, heart rates rapidly increasing at the end of development (Figure 6), has not yet been shown for non-avian reptiles but has previously been described for some birds (Pearson & Tazawa, 1999; Tazawa, Watanabe, & Burggren, 1994). Notably, if we expand our single measures, repeated measures, and constant temperature datasets to include heart rates measured at durations greater than 60 sec, the positive relationship between embryo heart rate and embryo age is significant in all three designs (all P values < 0.024). This is reason to suspect that heart rate indeed changes throughout development; however, most prior research in reptiles has not identified a relationship between embryonic heart rate and age (Birchard and Reiber, 1996; Du and Shine, 2008; Radder and Shine, 2006; Sartori et al., 2015; but see Aubret et al., 2016), or has shown a slight decrease in heart rate with age (Angilletta et al., 2013; Zhao, Chen, Wang, Ding, & Du, 2013). If a positive relationship exists, its biological significance is yet to be determined.

4.4 | Are anole embryo heart rates repeatable?

At any given duration, the heart rates of anole embryos exhibit wide variation (Figure 2). Our repeated measures design allowed us to estimate the adjusted repeatability (Nakagawa & Schielzeth, 2010) of anole heart rates and determine how much variation is the result of differences among individuals. We found heart rates to be repeatable and mean heart rate varied among individuals by as much as 20 bpm (Figure 7). The detection of interindividual variation serves as the foundation for important future research. Avian reptiles show substantial interindividual variation in embryonic heart rate (Tazawa, 2005); however, to our knowledge, no study conducted on non-avian reptile embryos has calculated repeatability estimates for individuals nor explored the role that interindividual variation plays in various biological phenomena. For example, we know that developmental rate increases and incubation period decreases with heart rate when we compare groups of individuals that differ in incubation temperature or treatment (Aubret et al., 2016; Du et al., 2009); however, we do not yet know if this trend is detectable when comparing individuals. Individual variation in heart rate may explain some interindividual differences in developmental rate and timing of hatching in lizards (Pearson & Warner, 2016; Tiatragul et al., 2017). Studies that quantify repeatability of heart rates, the range of interindividual variation within species, and the influence of this variation on other phenotypes (i.e., incubation period) could provide important insight into mechanisms underlying phenotypic variation within populations.

4.5 | Does repeated jostling of eggs influence estimation of heart rate?

Numerous studies repeatedly measure heart rates of the same embryos throughout incubation (Aubret, 2013; Du & Shine, 2008; Radder & Shine, 2006; Zhao et al., 2013), but it is possible that the repeated jostling and movement experimenters must exert on eggs during measurement influences estimation of embryonic heart rates. While bird eggs are frequently moved by their parents during brooding (Shaffer et al., 2014), reptile eggs are stationary during incubation, so such movements are unnatural and can reduce post-hatching survival (Aubret et al., 2015). To address this issue, we used alternative experimental designs to answer the same biological questions. The single measure experiment utilized many embryos that were each measured only once. The repeated measure experiment utilized relatively few embryos, but measured each embryo 16 times (four times a day on four different days). Between single and repeated measure experiments, we found remarkably consistent results for heart rates throughout the thermal diel cycle (Figure 3 and Table 1). Heart rates between the fluctuating temperature experiments differed by less than 2.6 bpm at both the low and high daily temperature. Thus, we found no evidence that repeated measurements have adverse effects on embryos, even when measurements are made just a couple hours apart. Moreover, all 20 eggs used in our repeated measures analysis hatched, thus, design did not impact egg survival. We suggest that researchers can select experimental designs based on logistical considerations and based on the type of approaches required to answer their questions. For example, if limited quantities of eggs are available, repeated measures will allow researchers to obtain more measurements from few embryos, and this increased egg movement during measurement will not obscure the interpretation of results.

4.6 | Do constant temperature experiments give comparable measures of heart rates to fluctuating temperature experiments?

Most work on heart rates of reptile embryos has used constant incubation temperatures (Aubret, 2013; Birchard & Reiber, 1996; Du et al., 2009; Sartori et al., 2015), even though eggs naturally experience fluctuating temperatures (Booth, 2006; Georges, 1992; Warner & Shine, 2009). The effects of fluctuating versus constant temperatures on developing embryos are not trivial. For example, because embryonic metabolism increases exponentially with temperature, embryos incubated at fluctuating temperatures develop more quickly than those incubated at an equivalent constant temperature (Du & Shine, 2010). Furthermore, incubating eggs at a constant temperature can result in thermal acclimation: at a given temperature, heart rates are lower for eggs that have been constantly incubated at a higher temperature when compared with those incubated at a lower temperature (Du et al., 2010). For these reasons, we might expect the results of our constant and fluctuating experiments to differ. For example, when heart rates are measured at 32°C, embryos incubated at a constant 32°C might have lower heart rates than those incubated in a diel thermal cycle due to acclimation to constant, higher temperatures.

Thus, we compared heart rates of embryos held at a constant 28 or 32°C to the heart rates of embryos that experienced those same temperatures during a diel fluctuation. At the low temperatures, heart rates incubated at a constant temperature were similar to those at fluctuating temperatures; however, heart rates of embryos in the high constant temperature were ~10 bpm greater than heart rates at the

peak temperature of the fluctuation (Table 1). We expect this discrepancy was caused by methodological issues rather than a biological phenomenon. Though both incubators were set to 32°C, the peak temperature of the diel cycle was 31.8°C (see 1500 hr on Figure 3), whereas the eggs in the constant temperature incubator were at 32.6°C (see time 0 on Figure 2). Regardless, the 95% CI for these estimates overlap (Table 1); therefore, this difference is likely not meaningful. Generally, we expect that heart rates of anole embryos at constant temperatures and equivalent temperatures experienced during a fluctuation are comparable. The primary advantage of using fluctuating temperatures is to enhance ecological relevance.

4.7 | How rapidly does temperature change when eggs are removed from incubators?

Since *A. sagrei* eggs are very small, they are more susceptible to heat loss compared with larger eggs (Turner, 1985). Our protocol required briefly removing eggs from the incubator to take embryonic heart rate measurements at room temperature, which caused rapid cooling of eggs (Figure 2). After removal from an incubator set at 32°C, internal egg temperatures lowered by 7°C within the first minute and reached equilibrium with ambient room temperature after three minutes (Figure 2). In the single measure experiment, we showed that duration was a significant covariate: the longer it took to get a measurement after removing the egg from the incubator, the more the heart rate dropped. However, duration did not covary with heart rate in the repeated measures and constant temperature experiments. If we expand our dataset to include heart rates measured at durations longer than 60 sec, duration is a significant covariate in all three experiments (all P values < 0.011). Additionally, the expanded dataset shows a significant interaction between temperature and duration ($F_{3,270} = 4.93$, $P = 0.0024$): the rate of embryonic heart rate drop is faster for warmer (32°C) eggs than cooler (28°C) eggs (Figure 2). This would create a bias by underestimating the heart rates of eggs at warmer temperatures and overestimating heart rates of eggs at cooler temperatures, enhancing the possibility of a type II error, and obscuring our ability to detect true differences between high and low temperature treatments.

4.8 | Does the Buddy® monitor appreciably warm eggs over short durations?

Because anole eggs are relatively small, we anticipated that the Buddy®'s infrared beam would substantially increase egg temperature; however, this was not the case. We observed no warming due to the Buddy® over a 60 sec period and very little warming over 5 min (Figure 4). Sartori et al. (2015) found the Buddy® increased egg temperatures of turtle and iguana eggs over a long duration and suggest that relatively smaller eggs should warm more rapidly. Our data do not corroborate this prediction. Notably, the eggs used by Sartori et al. (2015) were about 100 times larger than anole eggs. Because of the relatively large surface area to volume ratio of anole eggs, heat created by the Buddy® may quickly transfer from the egg to the ambient air inside the monitor, leaving internal egg temperature unchanged over a relatively short duration.

Though Sartori et al. (2015) show that the Buddy® heats eggs over a long period (>20 min), there are two reasons why researchers should be cautious about how the Buddy® might heat eggs over a short duration (<10 min). First, their design resulted in eggs being removed from the incubator, allowing them to cool slightly, and then being reinserted into the incubator and placed on the Buddy®. They began measuring egg temperature immediately upon inserting the egg into the Buddy®, and for this reason, the temperature increase of eggs over the first 10–15 min was mostly due to the eggs heating back up to the incubator's ambient temperature. Thus, one cannot determine how much initial warming was due to the Buddy® versus how much was simply the result of placing relatively cool eggs into a warm incubator. Thus, their data cannot demonstrate how the Buddy® heats eggs over the first 10–15 min and to assume that it can would be an extrapolation rather than an interpolation of data. Second, they argue that if the monitor's chamber was left open, eggs did not warm above the temperature in the incubator. This indicates that the reason eggs warmed was actually a result of the housing chamber accumulating heat over time. Such accumulation could be due to the infrared beam itself, or simply the result of the Buddy® monitor heating up when left on for an extended period since it does not have a cooling fan (or both). Regardless, this heat accumulation is not likely to occur over short durations like those used in our study because the housing chamber was opened once every 1–2 min. In addition, a recent study also found that small lizard eggs were not warmed by the Buddy® over a short duration (~5 min; Cordero et al 2017). Because most studies obtain a heart rate measure within a relatively short duration, a future study should determine how the Buddy® heats eggs of various sizes over short durations.

4.9 | Does using duration as a covariate appropriately account for changes in egg temperature?

The results from our controlled temperature experiment show that using duration as a covariate can accurately correct for decreases in egg temperature: measuring heart rates inside an incubator gave us estimates similar to those obtained when measuring heart rate outside the incubator and using duration as a covariate (Table 1 and Figure 5). However, we note that the controlled temperature experiment generated a significantly higher estimate of heart rate at 32°C than the repeated measures experiment (95% CI did not overlap). Since we observed no difference at 28°C, this is likely due to the interaction between temperature and duration: egg temperature will drop more rapidly when removed from a warmer versus a cooler incubator (Figure 2) causing experimenters to underestimate heart rates at warmer temperatures (Figure 5). Thus, we recommend that experimenters that measure heart rates outside the incubator be cautious when the difference between the target temperature and room temperature is substantial.

Ideally, the Buddy® monitor should be placed inside an incubator for heart rate measurements (Aubret, 2013; Du et al., 2009; Du & Shine, 2008; Sartori et al., 2015; Spencer, 2012), but many studies, including ours, do not employ this protocol (Angilletta et al., 2013; Loudon et al., 2013; McGlashan et al., 2015). We could not place the Buddy® inside our fluctuating incubators because they did not have a

portal for an electrical wire. If it is not possible to place the Buddy® within an incubator, one should record the duration and include it as a covariate. The rate of temperature change will vary according to egg size, and we recommend that experimenters define a cooling curve for their model organism to determine how quickly heart rate measures should be obtained. Moreover, increasing the ambient room temperature will help reduce the rate of cooling.

4.10 | Conclusions

The ability to non-invasively measure heart rates of reptile embryos has advanced our understanding of embryonic physiology. We used the Buddy® monitor to measure heart rates of *A. sagrei* embryos exposed to fluctuating thermal environments that mimic conditions in the field. We show that natural diel fluctuations generate daily changes in embryonic heart rates which suggest developmental and metabolic rates are highest during the warmest parts of the day. Though this finding is not unexpected, it is novel. Research on the effects of developmental environments on offspring phenotypes will continue to be enhanced by studying embryonic physiology which can document immediate responses to the developmental environment. Future work in this area will be well-served when recognizing the logistical freedoms and constraints we outline here. We demonstrated no negative effect of repeatedly measuring the same embryos, which enables researchers to utilize repeated-measures designs if necessitated by their experimental methodology. We also highlighted an important bias that can be induced when embryos are not measured within the temperature controlled incubators, and provided suggestions for how to alleviate or reduce this concern. We provide valuable biological and methodological information that will be useful for future research in the fast-growing area of embryonic adaptation and physiology.

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COMPETING INTERESTS

The authors declare no competing or financial interests.

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SUPPORTING INFORMATION

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