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An appraisal of the use of an infrared digital monitoring system for long-term measurement of heart rate in reptilian embryos

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Abstract

Measurement of heart rate (f_H) in embryonic reptiles has previously imposed some degree of invasive treatment on the developing embryo. Recently a non-invasive technique of f_H detection from intact eggs was developed for commercial avian breeders and has since been used in biological research. This device uses infrared light, enabling it to detect heartbeats in very early embryos. However, infrared light is a source of heat and extended enclosure of an egg in the device is likely to affect temperature with consequent effects on physiological processes, including f_H. We studied the effect of use of the monitor on the temperature of eggs and on f_H in two species of reptiles, the snapping turtle (*Chelydra serpentina*) and the green iguana (*Iguana iguana*). Egg temperature increased from a room temperature of 27-28^{o}C, by 26% in turtles and 14% in iguanas over one hour of enclosure, resulting in an increase in f_H of 76-81% in turtles and 35-50% iguanas. These effects on f_H can either be avoided by brief enclosure of each egg in the monitor or measured and accounted for during the design of long-term experiments.

Key words: reptiles; embryonic development; heart rate; Buddy®; infrared radiation; temperature

1. Introduction

Heart rate (f_H) during embryonic development has been the most commonly reported cardiovascular variable taken from a wide range of species of reptile, providing basic data regarding the maturation of cardiovascular function (Crossley et al., 2003; Crossley and Burggren, 2009; Eme et al., 2011; Sartori et al., 2015).

Methods to acquire these data include direct measurements of arterial pressure (Crossley et al., 2003; Crossley and Altimiras, 2005; Eme et al., 2011; Alvine et al., 2013; Eme et al., 2013; Eme and Crossley, 2015), visual counting via a dissecting microscope (Nechaeva et al., 2007; Sartori et al., 2015) or impedance measurements (Bichard and Reiber, 1996, Crossley and Burggren, 2009). While these methods are useful for gathering information regarding maturation of the cardiovascular system they require some degree of invasive instrumentation, possibly disturbing and most often terminating embryogenesis for the individual embryo. Recent longitudinal studies of f_H prior to hatching in embryos of several species of lizards and turtles have utilized a noninvasive method for monitoring f_H using the transmittance or reflectance of infrared light from a digital egg monitoring system (Buddy®, Avitronics, Truro, UK). Publications using this system include: Lierz et al., 2006; Radder and Shine, 2006; Du and Shine, 2008; Du et al., 2009; Du and Shine, 2010; Du et al., 2010a; Du et al., 2010b; Du et al., 2010c; Du et al., 2010d; Du et al., 2011; McGlashan et al., 2012; Spencer, 2012; Angilletta et al., 2013; Aubret, 2013; Loudon et al., 2013; Zhao et al., 2013; Sartori et al, 2015. Infrared radiation (IRR) is an important source of heat (Herschel, 1801: Seigel et al., 2001) and devices emitting IRR are commonly used as a deliberate heating source. If the IRR emitted by the Buddy® system significantly alters the thermal environment of the egg it is likely to affect physiological processes, including f_H. Thus, there is clearly the potential for reporting unreliable data on progressive changes in f_H using this system. However, the potential heating effect of infrared light on the thermal status of reptilian eggs was not overtly considered in previous studies and has yet to be determined.

This investigation set out to characterize the changes in heart rate in the embryonic snapping turtle (*Chelydra serpentina*) and green iguana (*Iguana iguana*),

when exposed to IRR. The snapping turtle represents one of the most extensively studied reptiles during embryonic development, allowing cross study and method comparisons within a species. We hypothesized that the infrared detection method would heat the turtle egg resulting in an elevation in heart rate. To test this hypothesis we studied embryonic snapping turtles at 70% and 90% of incubation and green iguanas from 30% of incubation until close to hatching. The eggs of green iguanas increase in mass during development (Sartori et al, 2015), possibly affecting their response to any heating effect from the infrared monitor.

2. Material and Methods

2.1 Experimental animals

Snapping turtle: On June 2013 eggs from snapping turtles, *Chelydra serpentina* were collected in northwestern Minnesota (Minnesota Department of Natural Resources Permit No. 18337 to DAC) and transported to the Biology Department at the University of North Texas, Denton, USA, where the experiments were performed. Upon arrival, eggs were numbered, weighed and placed in plastic boxes (volume approximately 3 litres) containing vermiculite mixed with water in a 1:1 ratio by mass. Water content of vermiculite was maintained by weighing boxes twice weekly and adding water as needed. The boxes were set in plastic Ziploc bags supplied with normoxic air (21% O_2) bubbled through water to maintain both oxygen and water saturation at adequate levels. The bags were maintained in incubators set to 30°C. Six eggs from different clutches were taken from incubators at each 70% and 90% of incubation time and weighed before assigned to the experiments.

Green iguana: Freshly laid eggs of green iguana, *Iguana iguana* were collected during the months of September and October of 2013 from captive gravid females that were part of the breeding program operating at the Jacarezário, Departamento de Zoologia, São Paulo State University (UNESP), Rio Claro, SP, Brazil. Eggs were weighed and immediately placed in trays (38 x 28.5 x 6.5 cm) containing water saturated vermiculite held at a constant temperature of 30±0.5^{o}C in incubators (Eletrolab, EL101/3, SP, Brazil). All eggs were examined daily for signs of mortality and the vermiculite sprayed with dechlorinated tap water to maintain humidity high. Six eggs were selected from different clutches at the developmental times of: 30%, 50%, 70%, 90% and just prior to hatching.

2.2 Instrumentation: Experiments were performed according to approved animal care protocols (UNT IACUC 11- 007 and CEUA-UNESP no. 6597 and no. 3680). The study utilized a digital egg monitor (Buddy® System, Avitronics, Truro, UK) that records f_H non-invasively by detecting movement via infrared sensors, and amplifies the resulting signal, enabling recordings to be obtained from early embryos. The digital egg monitors used in this study were customized by the manufacturers to provide an analog output signal via a BNC connector that was digitally transformed using a data acquisition system (PowerLab; ADInstruments, Bella Vista, NSW, Australia).

For temperature measurement, both snapping turtle and green iguana eggs were weighed and candled to detect a place for insertion of a thermocouple through the eggshell that avoided direct contact with the embryo or yolk. A patch of 1 cm^2 of latex glove was attached to the eggshell using cyanoacrylate glue (Loctite, USA). The

eggshell was then punctured, through this patch, with a 26-gauge needle, and a flexible implantable thermocouple probe (BAT-4, Physitemp Instruments, NJ, USA or T-type, ADInstruments) was inserted approximately 5 mm into the egg. Eggs were then placed in the Buddy® chamber which was housed in a constant temperature chamber (EGC, OH, USA/Caltech EIP-010, PE, Brazil) held at 30±0.5°C and the lid of the instrument was closed following the manufacturers directions for use. Iguana eggs were surrounded by a ring of wet gauze in order to minimize evaporative water loss. The signal outputs from the egg monitors and from the thermocouples in the eggs and in the environmental chambers were relayed to the data acquisition system, (ADInstruments, PowerLab), and recorded simultaneously and continuously via LabChart software (ADInstruments, Bella Vista, NSW, Australia). Recordings were closely monitored and conducted until no major changes in temperature were detected, after a minimum of two hours. Egg temperature and f_H were collected every 10 minutes from the recordings for statistical determination of the time elapsed until stabilization of egg temperature and relationships between temperature and f_H (Table 1). Temperature coefficients (Q_{10}) were calculated according to the following equation:

$$Q10 = \left(\frac{R2}{R1}\right)^{\frac{10}{T2-T1}}$$

2.3 Statistical analysis

Egg mass, initial and final temperatures (T_{min} and T_{max} respectively) and initial and final f_H (If_H and Ff_H, respectively) were tested within turtles with paired T-test and within iguanas with one-way ANOVA. A repeated measures ANOVA with time as the independent factor and temperature as the dependent factor was used to detect the

point of stabilization of egg temperature. A post hoc Student-Newman-Keuls test was used to identify possible significant differences between the incubation groups. Linear regression analysis was performed with changes in egg temperature (independent variable) and f_H (dependent variable) at each point of incubation period. Tests for snapping turtle data were performed with the STATISTICA version 12 software package and for iguana data with SigmaPlot version 10.0. Significance was attributed at a level of 95% confidence. Data are presented as mean ± SEM.

3. Results

Eggs left inside the Buddy® warmed with time until they had reached a stable temperature, which was approximately 34°C in snapping turtle embryos (Fig. 1A) and to 32°C in green iguana (Fig. 1B). A summary of the data for each species and developmental group is detailed on Table 1.

In the turtle egg mass increased from 12.0±0.8g at 70% incubation to 12.9±0.3g at 90% incubation, an increase of 7.5%. Temperature stabilization occurred 40 minutes after the egg monitor was turned on at 70% incubation and 50 minutes at 90%. The mean temperature recorded after a period of 140 minutes was 34.0±0.1°C (n = 6) at 70% and 34.0±0.2°C (n = 6) at 90%. The increasing temperature had a direct effect upon f_H. At 70% mean f_H increased from 55±1 (beat · min^{-1}) to 96±3 (beat · min^{-1}), which represents a 76% increase. At 90% mean f_H increased from 45±3 (beat · min^{-1}) to 85±1 (beat · min^{-1}), representing 89% increase. Initial and final f_H of turtles were lower at 90% of incubation when compared to values at 70% incubation (Table I). Calculated temperature coefficients (Q_{10}) were 2.4 and 2.5 for 70% and 90%, respectively. A linear relationship between f_H and temperature of turtle eggs was strongly supported by data analysis at both 70% incubation ($R = 0.90$; $R^2 = 0.82$; $P <$

0.001) and at 90% incubation (R = 0.84; R^2 = 0.70; P < 0.001) (Fig. 2A). f_H increased according to the following equations:

$$70\%: \ f_H = 6.4\ T - 123.7$$

$$90\%: \ f_H = 6.5\ T - 131.3$$

In the green iguana egg mass increased from 22.7±1.8g at 30% incubation to 33.8±0.1g immediately prior to hatching at 100% incubation, an increase of almost 50%. The increase in egg size was statistically different from initial values at 70% 90% and 100% of incubation (Table I). Data on egg temperatures and f_H for each of the embryonic periods tested are provided in Table 1. Temperature stabilized after 60 min at 30%, 50% and 90% of incubation, after 70 min at 70% and after 80 min at 100% incubation (Fig. 1B). As the resultant mean values of f_H with time were statistically similar we have reported the combined data. The combined mean temperature after stabilization for all periods of incubation was 31.9±0.1°C. Temperature affected f_H directly. The overall mean combined f_H increased from 73±1 to 105±2(beat • min^{-1}), representing an average increase of 44% (range of 35-50%). The combined temperature coefficient (Q_{10}) was calculated as 2.8. The linear regression of the combined data for all periods of incubation tested indicates a positive linear relationship between f_H and temperature (R = 0.80; R^2 = 0.65; P < 0.001) (Fig. 2B) that follows the equation:

$$f_H = 7.9\ T - 150.0$$

4. Discussion

The Buddy® monitor is a very effective non-invasive system for documenting embryonic viability, apparently delivering everything that the company (Avitronics, Truro, UK) describes on its web site. They state that it is the first digital egg monitor in the world. Using infrared transmitters and sensors it is capable of amplifying the "cardiovascular signal" of an embryo within the egg by as much as 20,000 times, allowing detection of the heartbeat of the embryo as early as 5 days after incubation has started. The monitor gives a digital read out of f_H onto a small screen. As such it gives the bird breeder "warming knowledge that everything is fine" with their valuable embryos. The company cautions that eggs may cool after removal from the nest and in a trial run they recorded f_H within the Buddy® as reducing from 260 to 190 (beat · min^{-1}) within 5 minutes. What they do not mention is the physical warming effect of the Buddy® upon the eggs that we illustrate above. This will, of course, not be a problem for the bird breeder who merely wants to check the vitality of the egg by briefly placing it in the monitor. Neither is it a problem with the bulk of the experimental biologists that have checked embryo f_H from time-to-time by briefly placing them in the monitor. However, long-term measurements intended to establish how f_H changes during development in a given species must account for and document the heating effects of the system. In our studies these effects were observed during the first hour of enclosure within the Buddy® system. Turtle eggs warmed from 27°C to 34°C, causing an increase in f_H of about 80%. This relatively large warming effect may relate to the size of the eggs and the fact that they were placed directly in the monitor, with no protection against desiccation. Iguana eggs warmed from room temperature of 28°C with a f_H of around 73 (beats min^{-1}) up to a stabilized rate of about 105 (beat · min^{-1}) at a temperature of 32°C, taking between 60 and 80

min to stabalize. This reduced warming effect may again relate to the relative size of the eggs and to the fact that they were protected against desiccation by being encircled by a wet gauze. Recorded differences in the stabilization times of iguana egg temperatures indicated an apparent trend for smaller eggs to warm faster. The 70% incubation turtle egg weighing around 12 g took half the time of a 100% incubation iguana egg at around 34 g to stabilize. When we measured some eggs with the lid of the Buddy® held open the temperature of the eggs remained below 30°C, with the heart beating at around 75 (beats min^{-1}), despite being held in an incubator set at 30°C. Clearly, any investigation of long-term changes in f_H using enclosure of eggs within the Buddy® system must account for the warming effect. In most other studies using the Buddy® system (see Introduction) f_H was measured by brief enclosure of the egg in the monitor. For example, Du and Shine (2008) enclosed each egg for 2 min. However, McGlashan et al, (2012) when exploring the phenomenon of synchronous hatching in turtle embryos, subjected batches of eggs to different temperatures for 7 days then combined them at a set temperature or at a complex series of fluctuating temperatures and measuring outcome as hatching times and post-hatching development and growth. Metabolic compensation by embryos was measured as rate of carbon dioxide production and heart rate. To measure heart rate individual eggs were enclosed in a Buddy® egg monitor and a digital camera was used to record heart rate at 5 min intervals over 30 min with f_H taken as the average over this period. If the egg remained in the monitor throughout the 30 min period then it would have been subject to a similar heating effect to that recorded from turtle eggs in the present investigation. For eggs held at 26°C the warming effect may have raised temperature to 31°C. This implies that f_H was not measured under a steady-state

regime and, assuming a Q_{10} of 2, it will have increased up to 37% during the time of measurement.

5. Conclusion

The Buddy® digital egg monitoring system provides a convenient and highly reliable technique for the non-invasive monitoring of f_H in very young bird embryos. For long-term measurements in reptiles it has to be noted that the infrared sensors cause a heating effect, which can have significant effects upon f_H. This effect may be a particular problem when studying relatively small eggs as they may heat more rapidly. The heating effect should always be measured and taken into account in the design of experiments and analysis of data or avoided by placing each egg in the monitor for brief periods, which is the intended primary use of the instrument.

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Table 1 Measured egg temperature and heart rate (f_H) profiles for snapping turtle (Tur) and green iguanas (Ig) within each percentage incubation period studied. T_{min} is the initial egg temperature recorded; T_{max} is the maximum egg temperature recorded; St is the time to temperature stabilization**,** initial and final f_H (I f_H and F f_H respectively) are the heart rates recorded at the T_{min} and T_{max}**,** respectively; Q_{10} is the calculated temperature coefficients; regression equations were provided by linear regression analysis with the respective R^2. Data presented as mean ± SEM. Statistical significance (P<0.001) is represented by lower case letters in turtles and capital letters for iguanas.

Group	Egg mass (g)	T_{min} (°C)	T_{max} (°C)	St (min)	If_H (beat · min^{-1})	Ff_H (beat · min^{-1})	Q_{10}	Regression Equation	R^2
Tur 70	12.0±0.8^{a}	27.6 ± 0.2^{a}	34.0 ± 0.1^{a}	40	54±1^{a}	96±3^{a}	2.4	f_H = 6.4T - 123.7	0.82
Tur 90	12.9±0.3^{a}	27.2 ± 0.4^{a}	34.0 ± 0.2^{a}	50	45±3^{b}	85±1^{b}	2.5	f_H = 6.5T – 131.3	0.70
Ig 30	22.7±1.8^{A}	27.4 ±0.4^{A}	32.0 ± 0.2^{A}	60	70±3^{A}	104±7^{A}	2.4	f_H = 10.1T - 217.7	0.73
Ig 50	25.0±0.9^{A}	27.7 ± 0.3^{A}	31.4 ± 0.2^{A}	60	69±4^{A}	104±3^{A}	3.0	f_H = 10.5T - 228.8	0.96
Ig 70	31.6±1.2^{B}	28.1 ± 0.4^{A}	32.0± 0.2^{A}	70	73±2^{A}	109±3^{A}	2.8	f_H = 8.1T - 155.5	0.78
Ig 90	33.2±0.9^{B}	28.2 ± 0.7^{A}	31.9 ± 0.2^{A}	60	79±2^{A}	107±4^{A}	2.3	f_H = 5.1T - 60.3	0.42
Ig100	33.8±0.1^{B}	29.3 ± 0.2^{A}	32.2 ± 0.2^{A}	80	72±4^{A}	100±3^{A}	3.1	f_H = 8.5T - 174.4	0.77

Figure captions

Figure 1 Profile of temperature change over time for A) snapping turtle egg measured at 70% (closed circle) and 90% (open circle) of incubation and B) green iguana eggs measured at 30% (closed square), 50% (open square), 70% (closed circle) 90% (open circle) and 100% (closed diamond) of incubation. The dashed line represents mean chamber temperature measured in all periods of incubation. Time zero represents the point of the first reliable measurement of heart rate. Snapping turtle egg temperature changed significantly until 40 min at 70% incubation (indicated by a single asterisk) and until 50 min at 90% (indicated by a double asterisk). Egg temperature from green iguanas changed significantly until 60 minutes (indicated by a single asterisk) at 30%, 50% and 90% of incubation. At 70% the temperature changed significantly until 70 minutes (indicated by a double asterisk) and at 100% until 80 minutes (indicated by a triple asterisk). Data are presented as mean ± SEM.

Figure 2 Pooled heart rate (f_H) responses for all embyros to increasing egg temperature (Temp) for A) snapping turtle eggs measured at 70% (closed circle) and 90% (open circle) of incubation and B) green iguana eggs measured at 30% (closed square), 50% (open square), 70% (closed circle) 90% (open circle) and 100% (closed diamond) of incubation. Data points represent f_H for all animals included in the regression for each age group. Linear regression lines for the 70% (dashed line) and the 90% (solid line) are presented for the turtles only. For clarity purposes statistical analysis results are presented in Table I.

Figure 1

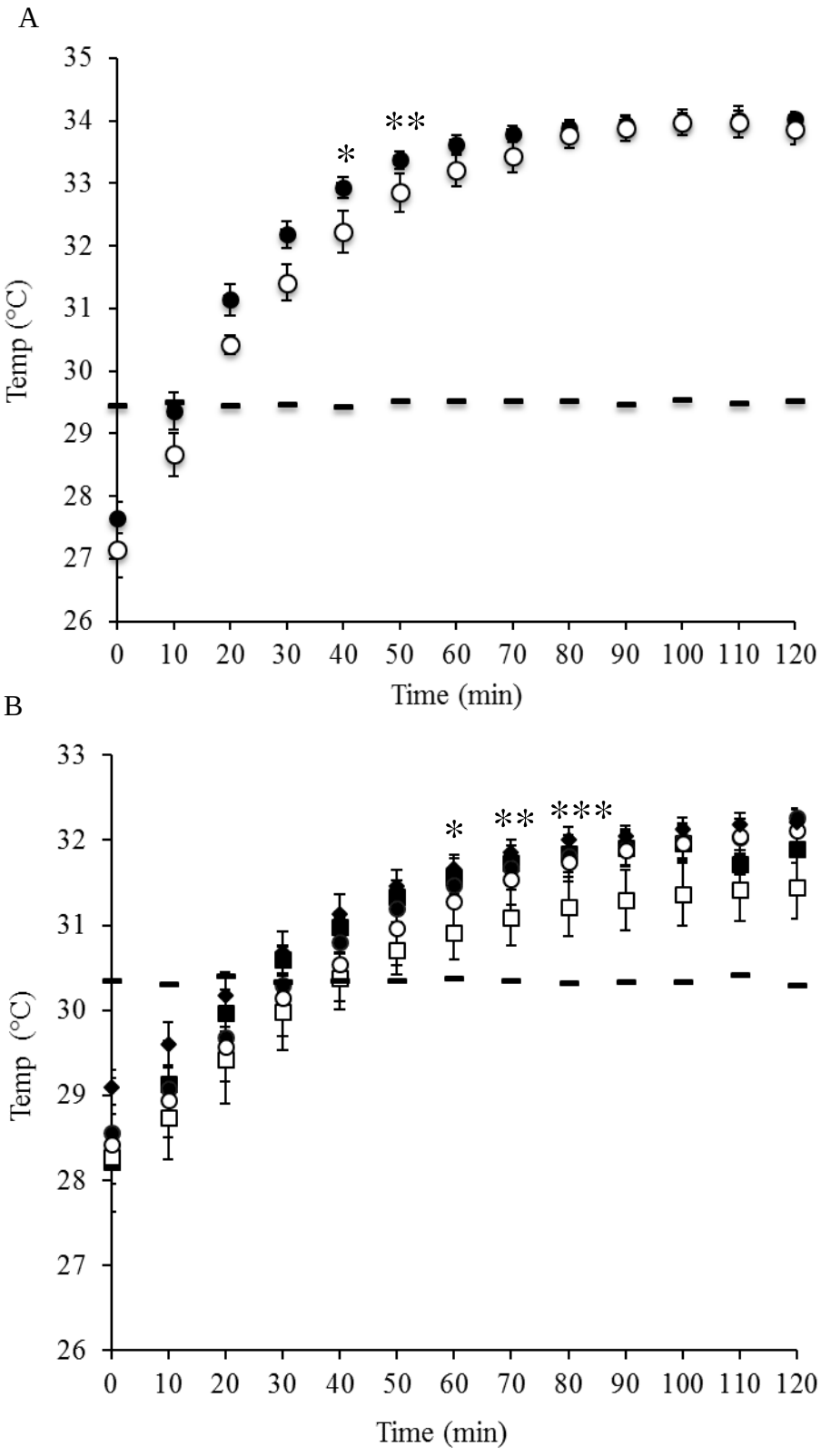

Figure 2