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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°C, by 26% in turtles and 14% in iguanas over 1 h 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.
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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), and 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

☆ M.R.S is a postgraduate student in the laboratory of A.S.A. and performed the experiments and analyzed the data; D.A.C. initiated the study and supervised the work on turtles; E.W.T. inspected the data and amended the manuscript.

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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

2.1.1. Snapping turtle

On June 2013 eggs from snapping turtles, *C. 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 l) 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.

2.2. Green iguana

Freshly laid eggs of green iguana, *I. 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 × 28.5 × 6.5 cm) containing water saturated vermiculite held at a constant temperature of 30 ± 0.5 °C in incubators (Eletrolab, EL101/3, SP, Brazil). All eggs were examined daily for signs of mortality and the vermiculite was 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.

2.3. 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 manufacturer's 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 2 h. Egg temperature and f_H were collected every 10 min 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:

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

2.4. 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 ± 0.8 g at 70% incubation to 12.9 ± 0.3 g at 90% incubation, an increase of 7.5%. Temperature stabilization occurred 40 min after the egg monitor was turned on at 70% incubation and 50 min at 90%. The mean temperature recorded after a period of 140 min 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 1). 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 ± 1.8 g at 30% incubation to 33.8 ± 0.1 g 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 1). 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

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