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A new approach for measuring temperature inside turtle eggs

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ABSTRACT

For turtles, the thermal environment experienced during development plays critical roles in many biological processes. While the temperature inside an egg is assumed to match the substrate temperature, many factors such as evaporative cooling, metabolic heating and the insulating properties of extra-embryonic components can lead to thermal differences. However, no method developed to date has allowed for measurement of the embryonic temperature in live chelonian eggs. We designed a thermocouple-based technique to measure embryonic temperature, achieving 94% survival in *Trachemys scripta*. This methodology may be applicable to other reptile species. We found that, while the temperature in the substrate adjacent to the eggshell accurately reflects the internal egg temperature, it differs from air temperature (~2°C) in a moisture-dependent manner. Our results demonstrate that external egg temperature, but not air temperature, is suitable for assessing the effects of temperature on biological processes, which could be critical when considering that processes such as temperature-dependent sex determination in turtles occurs within a 4°C window.

KEY WORDS: Embryonic temperature, Thermocouple, Reptile egg manipulation, Egg breaching, Thermal biology, Substrate moisture

Introduction

As in many other non-archosaurian reptile species, turtle eggs are buried in soil or sand by the mother and left to incubate, for a variable period, until hatching. During incubation, there is a complex relationship between the developing egg and the environmental features of the incubation site. The egg–environment relationship has a direct effect on the development of the embryo and can therefore influence the phenotype of the resulting hatchling (Ackerman, 1997; Packard, 1999).

Temperature is one of the most important abiotic factors inside turtle nests that affects the developing embryos. It is well-documented that nest temperature affects the embryo's metabolic rate, hatching success and incubation duration, as well as some hatchling phenotypes, such as body size, locomotor performance and behavior (Ackerman, 1997; Elphick and Shine, 1998; Booth et al., 2004; Tang et al., 2012; Siviter et al., 2017). Additionally, for most turtles, the thermal environment that the embryo experiences during a critical period of incubation directs the sex of the individual through a process known as temperature-dependent sex determination (TSD).

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In addition to temperature, moisture also influences embryonic development, particularly in turtle species with flexible-shelled eggs (Packard, 1999). Previous experiments show that wetter conditions during incubation produce larger and heavier embryos compared with those produced from eggs incubated in drier substrates (Packard et al., 1989; Packard, 1991; Sifuentes-Romero et al., 2018). Wetter conditions also increased the degree of water uptake by the eggs and the length of incubation (Packard et al., 1989; Sifuentes-Romero et al., 2018). Furthermore, Sifuentes-Romero et al. (2018) showed that moisture influenced the sex ratio through its interaction with temperature: cooler and wetter conditions produce more males than drier and warmer conditions. The same relationship was found for *in situ* loggerhead turtles (*Caretta caretta*) by Wyneken and Lolavar (2015).

The intrinsic relationship between the nest environment and developing embryos sparked a variety of efforts by the scientific community to design predictive models for several developmental processes (e.g. growth, developmental rate, sex ratios, etc.) using environmental variables (particularly temperature) (Hulin et al., 2009; Girondot et al., 2018). Such efforts are becoming increasingly relevant in the face of a rapidly changing global climate. However, air temperatures or even nest temperatures are simply not equal to embryo temperature and all can vary independently (Meijerhof and van Beek, 1993). Therefore, in order to design reliable models, it is critical that we understand how the environmental conditions of the nest translate into the environment that the embryo itself experiences inside the egg. This area remains mostly unexplored because techniques to measure the internal temperature of a developing turtle egg over an extended time period were lethal. Historically, manipulations of pliable shelled turtle eggs have been mostly unsuccessful, often reporting embryo survival below 30% across a variety of species (Bull et al., 1988; Crews et al., 1991; Bowden et al., 2001). Here, we describe a new egg breaching technique to measure the temperature inside red-eared slider turtle (*Trachemys scripta elegans*) eggs. This technique was used to answer the following questions: (1) how does the temperature inside a turtle egg relate to the temperature outside the egg (air and sand temperature), and (2) how does nest moisture affect the relationship between the temperature inside and outside the egg?

MATERIALS AND METHODS

Egg incubation and set-up

A total of 227 freshly laid *Trachemys scripta elegans* (Wied-Neuwied 1839) eggs from 21 clutches were obtained from Concordia Turtle Farms (Hammond, Louisiana, USA) and transported to Florida Atlantic University, Boca Raton, Florida, USA. Clutches were divided evenly among four treatments (Tmt 1–Tmt 4). Briefly, the four treatments were: Tmt 1, 30.5°C, with high moisture (50% water saturation or 1.0 m^3 m^{-3}); Tmt 2, 30.5°C with moderate moisture (25% water saturation or 0.06 m^3 m^{-3}); Tmt 3, 32.5°C with high moisture and Tmt 4, 32.5°C with moderate moisture (Fig. 1A). These temperatures are biologically relevant, as incubation temperatures for *T. scripta* eggs in Louisiana normally

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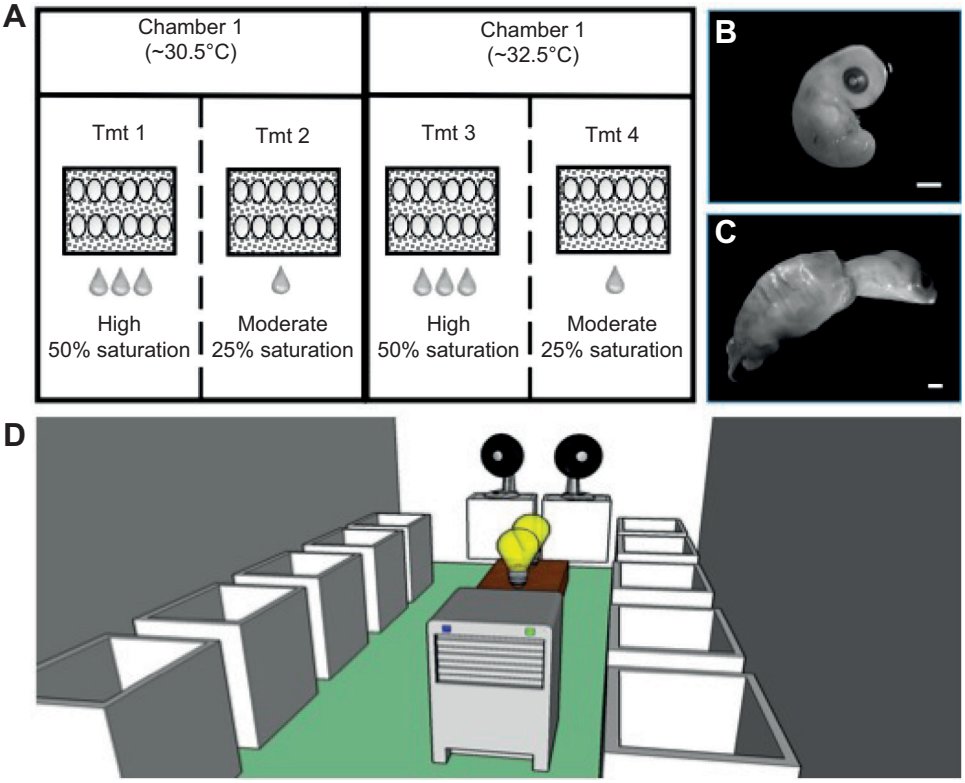

Fig. 1. Experimental set-up for measurement of egg temperature in *T. scripta*. (A) Schematic representation of incubation settings. Eggs were incubated in Styrofoam™ boxes and divided in four different treatments (Tmt 1–Tmt 4): two temperatures (30.5°C, chamber 1 and 32.5°C, chamber 2) and two moisture regimes ('high' and 'moderate' moisture). (B,C) *T. scripta* embryos at the targeted developmental stages. Lateral views of a stage 16 (B) and a stage 23 (C) embryo. Scale bars: 2 mm. (D) Schematic representation of the incubation chamber. Each chamber has five incubator boxes on each side, two fans to keep air circulating, two light bulbs to serve as a heat source, and a humidifier to keep chamber humidity high (80–90%).

range from 26.0 to 32.5°C (Crews et al., 1994; Rödder et al., 2009). Two eggs from each incubation chamber were harvested every 2–3 days to monitor developmental stage. Once the target developmental stages were reached (stages 16 and 23; Greenbaum, 2002), one egg from each treatment and stage was selected at random and fitted with a thermocouple probe (described below). After instrumentation, eggs were returned to their respective treatment condition. Two sets of 24 h thermal monitoring trials were performed per target developmental stage (Fig. 1B,C).

Temperature settings

Eggs were placed in Styrofoam™ boxes (incubators) containing sterilized sand from a local beach. The incubators were then placed in two different chambers (Fig. 1D). Temperature of the chambers was set at either 30.5°C (chamber 1) or 32.5°C (chamber 2, Fig. 1A) and was controlled by an Omega iSeries Temperature & Process Controller Model CNi3233 (Stamford, Connecticut, USA). The temperature of the chambers was set to be 1.5°C warmer than the intended incubation temperature based on previous observations from Sifuentes-Romero et al. (2018) showing that the chamber air temperature was significantly warmer than the sand temperature. Humidity in the chambers was maintained at high levels (~80–90% relative humidity) using a fan-assisted mist humidity system (Walgreens Cool Moisture Humidifier, Model 890-WGN). Chamber temperature and air humidity were monitored every 30 min using HOBO U12 Temperature & Relative Humidity data loggers (accuracy±0.35°C, resolution=0.03°C; Onset Corp., Cape Cod, Massachusetts, USA).

Moisture levels of sand nesting substrate

The moisture levels selected for this study are within the reported range of what is necessary for successful egg development for *T. scripta* (Tucker et al., 1998). From here on, incubators with 25% moisture content are referred to as 'moderate' moisture incubators while incubators with 50% moisture are referred to as 'high' moisture incubators. The equivalent value of volumetric sand moisture ($m^3\ m^{-3}$) in each incubator was measured with Decagon EC-5 soil moisture probes fitted to HOBO Micro Station Data Loggers [Onset Computer Corp. Model H21-002, resolution 0.0007 m^3 sand m^{-3} water (0.07%), accuracy ±0.031 $m^3\ m^{-3}$ (±3.1%)] where 25% moisture=0.06 $m^3\ m^{-3}$ and 50% moisture=1.0 $m^3\ m^{-3}$. Moisture in the incubator was maintained by spraying the surface sand with distilled H_2O (Di-H_2O) once a day, every day until they reached the values previously established (0.06 and 1.0 $m^3\ m^{-3}$ respectively). Di-H_2O was kept inside the chambers to maintain it at the same temperature as the air.

Instrumentation and measurement of internal temperature of eggs

Once the target developmental stage was reached, the eggs were carefully taken out of the sand and disinfected with 0.015% chlorhexidine gluconate solution and 70% isopropanol swabs to prevent infection. Natural beeswax was melted and molded by hand to the shape of one end of the egg to seal it and prevent leaking after penetration into the albumen by the thermocouple probe. After the beeswax was molded to fit the egg, the thermocouple probe (1 cm) was poked through the wax plug. A cyanoacrylate tissue adhesive (3 mol l^{-1} Vetbond™, 3 mol l^{-1} Corporation, St Paul, MN, USA) was applied to the beeswax to ensure adhesion to the eggshell. The disinfected egg was candled to ensure that it was viable and to identify the location of the embryo. The thermocouple probe was advanced into the egg through the beeswax on the opposite side to where the embryo was located, ensuring that the beeswax sealed the puncture, preventing fluid from leaking out (Fig. 2A,B). After the probe inside the egg was stabilized, the egg was carefully placed back in the sand in its own incubator box and a second thermocouple probe located in the sand directly next to the egg in contact with the eggshell (Fig. 2C). Each egg instrumented with its

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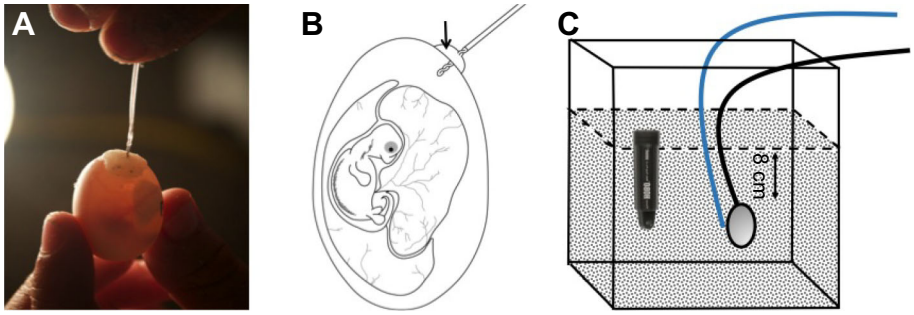

Fig. 2. Egg instrumentation. (A) Photograph of thermocouple probe being introduced inside the egg. (B) Schematic representation of the egg instrumentation showing the probe, the beeswax plug (black arrow) and the position of the probe on the opposite side to the embryo. (C) Diagram depicting the placement of the instrumented egg in the incubator. Each 'nest' box is equipped with a temperature logger recording sand temperature, and two thermocouples to measure the internal (black) and external (blue) temperature of the egg.

thermocouple was incubated individually in order to minimize the potential for damage and infection. The egg was then covered with ∼8 cm of sand and a moisture probe was placed in the sand to monitor sand moisture. Temperature was measured inside the egg, outside the egg and in the air (chamber air) using a wireless high-accuracy data logger (Omega HH806AU) equipped with a T-type thermocouple wire (Omega TT-T-30-25). A total of 17 eggs were instrumented with a thermocouple probe. We performed four trials with four instrumented eggs each (one egg per treatment) and temperature readings were taken hourly throughout the course of the 24 h trials. Embryos from trial 1 and trial 2 were at developmental stage 16 (Fig. 1B) while embryos from trial 3 and trial 4 were at stage 23 (Fig. 1C). After the first temperature reading, Di-H_2O (at chamber temperature) was added using a 1 liter spray bottle until desired moisture level was reached. Temperatures were then recorded using the thermocouple every hour for 24 h. The location of the probes was checked every 4 h by gently removing the top layer of sand and visually verifying that one probe remained inside the egg while the other was still in direct contact with the eggshell. Following completion of the trial, eggs were opened to verify that the embryo remained alive throughout data collection. The same procedure was followed for at least four different eggs in each of the four treatments.

Statistical analyses

All statistical tests were performed using IBM SPSS Statistics software (2017). Statistical procedures followed those described in Zar (1999). A Levine's Test was performed to verify the assumption of normality of the data was met. A repeated-measures ANOVA was used to determine: (1) if the temperature inside of the egg differed from that of the air and the sand directly surrounding it, and (2) if the moisture treatment had an effect on the temperature inside the egg.

RESULTS AND DISCUSSION

Embryo survival

Techniques that breach the egg of oviparous species (birds and lizards) provide powerful tools to study the physiology of developing embryos (Crews et al., 1991; Sinervo, 1993; Lipar, 2001). However, similar attempts to study embryonic development in turtles have been unsuccessful, mainly because of high embryo mortality (Crews et al., 1991; Sinervo, 1993; Lipar, 2001; Bowden et al., 2009). It remains unclear whether high rates of mortality were due to infections following penetrating the eggshell and associated membranes, or if other fundamental factors inhibited further embryo development once the shell and extra-embryonic membranes were breached (Bowden et al., 2009). In this study, a total of 17 eggs were fitted with a thermocouple probe; 16 embryos remained alive after the course of our trials (94% survival rate). We performed a total of four trials with four instrumented eggs each (one egg per treatment). Embryos from trials 1 and 2 were at developmental stage 16 while embryos from trials 3 and 4 were at stage 23 (Fig. 1B,C, respectively). Trial 1 had an additional egg that was not included in the remaining analysis as it died during the trial. We anticipated the death of this embryo, as the beeswax did not properly adhere to the surface of the egg after the introduction of the probe and the egg lost considerable amounts of fluid. This result suggests that the shortcomings of previous studies are likely to be due to extrinsic factors, such as fluid loss. Furthermore, unlike the Bowden et al. (2009) study, the developmental stage of the embryos at the time of instrumentation did not affect embryo survival in our study. However, there are a few fundamental differences between the two studies. The Bowden et al. (2009) study breached the egg for yolk biopsy and the eggshell puncture site was immediately sealed using tissue adhesive after yolk removal; whereas our study required us to maintain the thermocouple probe inside the egg for the duration of the 24 h trials and we did not puncture the yolk sac. In addition, Bowden et al. (2009) compared the survival rate of non-turgid eggs 24 h and 72 h post oviposition, while our study used turgid eggs ≥16 days post oviposition. Turtle eggs become turgid by water uptake from the substrate as incubation progresses.

Air temperature versus sand and egg temperature

The success of introducing a temperature probe inside the eggs without killing the embryos allowed us to study the relationships among the air, sand and egg temperatures. In all four treatments, the air temperature was significantly warmer than the sand ($F_{24,2}$=127.22, P<0.001) and egg temperatures ($F_{24,2}$=127.22, P<0.001), where the mean air temperature was 3°C warmer than the mean sand and egg temperature. While we did find that the air temperature was consistently higher than the sand temperature in all four treatments, the temperature inside the egg did not significantly differ from the temperature of the surrounding sand ($F_{24,2}$=127.22, P=0.999; Table S1A,B). These findings confirm that measuring the external eggshell temperature is adequate for inferring internal egg temperature. Our results highlight the difficulties associated with using indirect parameters like air temperature to draw conclusions about the environment directly experienced by the eggs and their embryos. It is important to keep in mind that the relationship between the air temperature of an incubation chamber and the temperature inside an incubator box in an experimental setting is likely to be different than under natural conditions, because natural nests have cycling temperatures, vary in size, depth, number of eggs, exposure to sunlight, rainfall, etc. Consequently, we strongly suggest that indirect parameters like air temperature are no longer used to infer conditions inside of a nest.

Moisture treatment versus temperature inside the egg

After identifying that the temperature experienced by the embryo inside the egg did not differ from the temperature in the surrounding sand independently of the air temperature, we tested if the moisture

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treatment had an impact on egg temperature. We found a significant difference in the temperature experienced by the embryo based on the moisture treatment ($F_{24,2}$=22,990.62, P<0.001; Table S2A,B). The mean temperature of the eggs incubated under high moisture conditions was 0.7°C lower than that in eggs incubated under moderate moisture conditions because of evaporative cooling. This is consistent with the findings of Sifuentes-Romero et al. (2018), where moisture had a significant impact on the relationship between air and substrate temperature. In our study, the eggs incubated under high moisture conditions (∼50% saturation) were cooler overall than the eggs incubated at the same air temperature under moderate moisture conditions (∼25% saturation). We observed the same pattern in both incubation chambers (30.5°C and 32.5°C; data not shown). Additionally, after plotting the temperature data for each trial individually, we noticed a trend in the four treatments in which, after 15–18 h into the trials, the difference in temperature between the eggs in moderate and high moisture treatments became more pronounced (Fig. 3). Initially, the eggs in both moisture conditions underwent a similar rate of evaporative cooling (for the first ∼12 h); however, after a period of time, the rate of evaporation decreased in the drier treatment (due to less available water), reducing evaporative cooling and allowing eggs to warm, whereas the wetter treatment continued to cool through evaporation. Although the relationship between substrate moisture levels and rate of evaporative cooling is likely to differ in natural nests with greater substrate volume (thermal mass), our experimental observations provide a mechanistic perspective that may explain why some studies have found an impact of substrate moisture on a variety of developmental processes such as developmental rate and sex ratio (Packard, 1999, Sifuentes-Romero et al., 2018, Wyneken and Lolavar, 2015). However, it is important to note that our experiment was performed under a constant incubation temperature. Future experiments should study the relationship between substrate moisture and nest temperature under natural conditions, as cycling temperatures might influence egg temperature profiles.

Interestingly, it appears that the relationship between sand and egg temperature was not affected by the range of moisture levels of the sand or by the limited range of external air temperature used in our study (Fig. 3; Table S1). Therefore, if the relationship between the temperature inside and outside the egg is not affected by other external factors (e.g. air temperature, substrate water content), our study supports the use of data loggers or other temperature measuring devices placed inside a nest, in contact with eggs, for the purpose of characterizing embryonic temperature.

Egg temperature at different developmental stages

When comparing the temperature of eggs at two developmental stages (Fig. 3), we noticed a trend at stage 16 in which both inside and outside egg temperatures were cooler (average of 0.05°C) in comparison to temperatures at stage 23. Egg temperature in the later stage increased (regardless of surrounding environment) because of an increase in metabolic heat production as the embryo increased in size (Ackerman et al., 1985; Zbinden et al., 2006; Howard et al., 2014). Although metabolic heating is well documented in developing turtles (Standora and Spotila, 1985; Broderick et al., 2001; Zbinden et al., 2006) and alligator embryos (Ewert and Nelson, 2003), the magnitude of difference in temperature in our study was unexpected because the eggs were incubated individually in their own incubator boxes. The metabolic thermal increase in individual *T. scripta* embryos is higher than previously considered

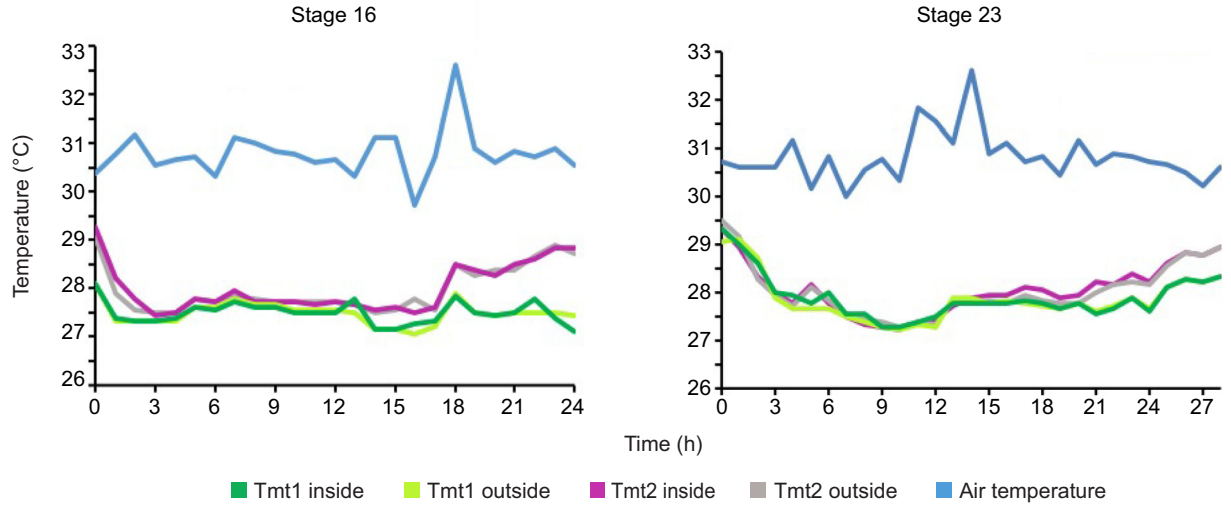

Fig. 3. Comparison of the air temperature with the temperature outside and inside the egg over a 24 h trial. Two different moisture conditions, 'high' (Tmt 1) versus 'moderate' (Tmt 2), and two different developmental stages, stage 16 (left) and stage 23 (right) are plotted. While there was no difference between the temperatures inside and outside the egg, the temperature of the air was higher than the temperature of the egg (both outside and inside) in a moisture-dependent manner. A slight increase (0.05°C) in temperature was recorded between stage 16 and stage 23 embryos due to metabolic heating. Note that these graphs represent the comparison between Tmt 1 and Tmt 2 (air temperature ∼30.5°C with high and moderate moisture, respectively) during trial 1 (stage 16 embryos) and trial 3 (stage 23 embryos) and are representative of the remaining trials. Fluctuations in chamber air temperature are mostly attributed to opening and closing of the chamber door and human body heat when the temperature and position of the probes was checked during the trial.

in other turtle species (Broderick et al., 2001). Because the temperature(s) within the nest can be affected by the metabolic heating of multiple eggs (Kaska and Downie, 1999; Booth and Astill, 2001; Broderick et al., 2001), it remains important to characterize a nest environment by incubating multiple eggs together and measuring the temperatures of eggs at different locations within a nest (Ackerman, 1997). Such studies can provide valuable insight into where temperature probes should be placed in natural nests in order to provide measurements that are representative of the temperatures experienced by all the embryos.

Conclusions

Our study provides a method for measuring temperature inside developing turtle eggs. Additionally, our results highlight the importance of including moisture data in predictive models that attempt to infer developmental processes. Including moisture measures alongside temperature is especially important because the mechanism of TSD in turtles occurs within a narrow 4°C window; a difference of 2°C may account for considerable error in temperature-based sex estimation. The understanding of thermal and hydric effects is relevant when considering nesting phenology in the wild, as temperature and rainfall often vary by nesting season and are predicted to change rapidly in the future.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: B.M.T., I.S., J.W.; Methodology: B.M.T., I.S.; Validation: B.M.T., I.S.; Formal analysis: B.M.T., I.S.; Investigation: B.M.T., I.S.; Writing - original draft: B.M.T., I.S.; Writing - review & editing: B.M.T., I.S., J.W.; Supervision: J.W.; Project administration: J.W.; Funding acquisition: B.M.T., I.S., J.W.

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Supplementary information

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