

7-2016

Reptile Embryos Lack the Opportunity to Thermoregulate by Moving within the Egg

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Abstract

Historically, egg-bound reptile embryos were thought to passively thermoconform to the nest environment. However, recent observations of thermal taxis by embryos of multiple reptile species have led to the widely discussed hypothesis that embryos behaviorally thermoregulate. Because temperature affects development, such thermoregulation could allow embryos to control their fate far more than historically assumed. We assessed the opportunity for embryos to behaviorally thermoregulate in nature by examining thermal gradients within natural nests and eggs of the common snapping turtle (*Chelydra serpentina*; which displays embryonic thermal taxis) and by simulating thermal gradients within nests across a range of nest depths, egg sizes, and soil types. We observed little spatial thermal variation within nests, and thermal gradients were poorly transferred to eggs. Furthermore, thermal gradients sufficiently large and constant for behavioral thermoregulation were not predicted to occur in our simulations. Gradients of biologically relevant magnitude have limited global occurrence and reverse direction twice daily when they do exist, which is substantially faster than embryos can shift position within the egg. Our results imply that reptile embryos will rarely, if ever, have the opportunity to behaviorally thermoregulate by moving within the egg. We suggest that embryonic thermal taxis instead represents a play behavior, which may be adaptive or selectively neutral, and results from the mechanisms for behavioral thermoregulation in free-living stages coming online prior to hatching.

Keywords

Chelydra serpentina, microclim, nest, play, soil, snapping turtle, temperature

Disciplines

Ecology and Evolutionary Biology | Evolution | Population Biology

Comments

This article is from *The American Naturalist* 188 (2016): E13, doi: [10.1086/686628](https://doi.org/10.1086/686628). Posted with permission.

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Submitted June 30, 2015; Accepted February 4, 2016; Electronically published May 2, 2016

Online enhancements: appendixes, supplementary PDF. Dryad data: <http://dx.doi.org/10.5061/dryad.mj8m0>.

ABSTRACT: Historically, egg-bound reptile embryos were thought to passively thermoconform to the nest environment. However, recent observations of thermal taxis by embryos of multiple reptile species have led to the widely discussed hypothesis that embryos behaviorally thermoregulate. Because temperature affects development, such thermoregulation could allow embryos to control their fate far more than historically assumed. We assessed the opportunity for embryos to behaviorally thermoregulate in nature by examining thermal gradients within natural nests and eggs of the common snapping turtle (*Chelydra serpentina*; which displays embryonic thermal taxis) and by simulating thermal gradients within nests across a range of nest depths, egg sizes, and soil types. We observed little spatial thermal variation within nests, and thermal gradients were poorly transferred to eggs. Furthermore, thermal gradients sufficiently large and constant for behavioral thermoregulation were not predicted to occur in our simulations. Gradients of biologically relevant magnitude have limited global occurrence and reverse direction twice daily when they do exist, which is substantially faster than embryos can shift position within the egg. Our results imply that reptile embryos will rarely, if ever, have the opportunity to behaviorally thermoregulate by moving within the egg. We suggest that embryonic thermal taxis instead represents a play behavior, which may be adaptive or selectively neutral, and results from the mechanisms for behavioral thermoregulation in free-living stages coming online prior to hatching.

Keywords: *Chelydra serpentina*, microclim, nest, play, soil, snapping turtle, temperature.

Introduction

Environmental temperature profoundly affects reptiles and other ectotherms, influencing most of their physiolog-

ical processes (Huey and Stevenson 1979; Huey 1982; Chown and Terblanche 2007; Angilletta 2009). Researchers have long recognized that free-living reptiles (i.e., those in the juvenile and adult stages) combat variation in the thermal environment behaviorally (Cowles and Bogert 1944; Huey 1982; Huey et al. 2003). By selecting suitable thermal microenvironments, free-living individuals can maintain stable body temperatures near their physiological optimum during activity (Avery 1982; Huey 1982; Angilletta et al. 2002; Christian et al. 2006). In contrast, the egg-bound embryos of oviparous species (most reptiles) were long assumed to be at the mercy of the nest environment (e.g., Shine et al. 1997; Ackerman and Lott 2004; Refsnider and Janzen 2010). Recent work, however, has demonstrated that the embryos of many amniotes—including species of turtle, crocodile, snake, and bird—adjust their positions within the egg in response to thermal gradients, actively moving toward or away from external heat sources (Du et al. 2011; Zhao et al. 2013; Li et al. 2014). Such observations of embryonic thermal taxis raise the possibility that vertebrate ectotherm embryos behaviorally thermoregulate similar to post-hatching individuals (Du et al. 2011; Du and Shine 2015). Minor changes in temperature (e.g., of 1°C–2°C) can have dramatic impacts on reptile development, affecting everything from embryonic survivorship to incubation duration to offspring phenotype (e.g., body size and shape, performance, and sex in some species; reviewed in Janzen and Paukstis 1991; Birchard 2004; Vitt and Caldwell 2009; Andrews and Schwarzkopf 2012). Embryonic thermoregulation could thus dramatically affect fitness (Du et al. 2011; Du and Shine 2015).

However, before assigning embryonic thermal taxis an adaptive value and labeling it behavioral thermoregulation, we must first demonstrate that this behavior allows embryos to adaptively adjust their body temperature in

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nature. We suggest two simple criteria for assessing the plausibility of adaptive embryonic thermoregulation. First, embryos must be exposed to thermal gradients within natural nests. These gradients must be both steep enough for embryos to alter their body temperature by moving within the egg and stable enough that the embryo can effectively track the thermal environment. Prior work has demonstrated that embryos move slowly in response to thermal gradients. For example, embryos of the Chinese soft-shell turtle (*Pelodiscus sinensis*; the only species for which embryonic thermal taxis speed has been quantified) require 3 days to substantially alter their position within the egg in response to a thermal gradient (Du et al. 2011). All species for which embryonic thermal taxis has been demonstrated appear to require 2–4 days to adjust their position (Zhao et al. 2013; Li et al. 2014). Given the relatively slow speed of embryonic movement, thermal taxis could allow adaptive thermoregulation only if gradients within nests remain relatively stable over time, changing over the course of days or weeks rather than hours.

Second, temperatures within the nest must produce thermal gradients within the egg. Prior studies of embryonic thermal behavior assessed thermal gradients by measuring temperature on the surface of the eggshell (difference between the warm and cool sides of the egg), finding gradients of 1°C–2°C (Du et al. 2011; Zhao et al. 2013; Li et al. 2014). However, because amniote eggs are fluid-filled structures with active circulatory systems (Thompson and Speake 2004), heat convection and conduction within the egg could largely erase external thermal gradients. The extent of this thermal damping will depend on the size of the egg and amount of circulation within the egg. The degree to which external thermal gradients are actually translated to the embryo is currently uncertain.

Using these criteria, we assessed the potential for embryonic thermal taxis to allow adaptive embryonic thermoregulation in reptiles. First, we tested both criteria in the common snapping turtle (*Chelydra serpentina*), a species that displays thermal taxis in the laboratory by moving toward a moderate heat source (Li et al. 2014). Our results suggest that *C. serpentina* embryos in natural nests do not have the opportunity to behaviorally thermoregulate by moving within the egg. To examine the generality of this result, we extended a soil physics model to simulate the thermal gradients available to embryos within eggs of various sizes, across a range of soil depths, and across a range of soil types. Additionally, we examined the global availability of conditions that could lead to embryos experiencing biologically relevant thermal gradients within the egg. Our simulations confirm that reptile embryos in subterranean nests will rarely, if ever, be exposed to thermal gradients within the egg that are large enough or constant enough to allow embryonic behavioral thermoregulation.

Material and Methods

Study Species

Chelydra serpentina are large freshwater turtles native to much of North America (Conant and Collins 1998). Females produce large nests averaging 26–55 spherical eggs, with clutches of >100 eggs observed (Congdon et al. 2008). Nest cavities are flask shaped and average 45 mm from top to bottom (range: 20–80 mm), with the top of the nest ~100 mm below the soil surface (Congdon et al. 2008). At oviposition, eggs are 26–28 mm in diameter, weigh 9–12 g, and typically increase in diameter and mass during development as a result of water uptake (Ackerman et al. 2008; Congdon et al. 2008). When compared to the vertical axis of other species (most reptiles produce oblong eggs, but the shorter, vertical axis will be most important for maintenance of thermal gradients within subterranean nests), *C. serpentina* eggs are among the largest of nonavian reptile eggs (*Chelonoidis nigra* and *Varanus komodoensis* have the largest, at 5.5 cm; Iverson and Ewert 1991). Because *C. serpentina* produce large nests with large eggs, sufficient thermal gradients plausibly occur within eggs in natural nests to allow embryos to thermoregulate by altering their position. Additionally, *C. serpentina* display temperature-dependent sex determination (type II, female-male-female): females are produced at constant incubation temperatures <21.4°C and >27.8°C, whereas males are produced at intermediate constant temperatures (Janzen and Paukstis 1991; Janzen 2008). The range of temperatures that produce mixed-sex ratios is narrow (<1.0°C for both the lower and upper transitional ranges; Janzen 2008); thus, behavioral thermoregulation might allow *C. serpentina* embryos incubating near either pivotal temperature to control their sex.

Our study site was located at the Upper Mississippi River National Wildlife and Fish Refuge in Carroll County, Illinois. The site is adjacent to the Mississippi River and is composed primarily of relict sand prairie habitat with a riparian zone near the river (Kolbe and Janzen 2002) and a recreational campground (Schwanz et al. 2010). Female *C. serpentina* produce one clutch per season (in late May or early June at this site) and generally oviposit at night.

Egg Collection and Nest Temperature Monitoring

From May 27 to June 2, 2014, we located fresh nests ($N = 10$; discovered within 12 h of construction) by walking the site daily before dawn. For the first three nests, we carefully excavated the eggs on discovery, packed them in sand, and transported them to the laboratory at Iowa State University (see below). For the remaining nests, we carefully placed four iButton thermochron data loggers (DS1921G, Maxim Integrated, San Jose, CA) preprogrammed to record temperature hourly within each nest. Nests were average

size for *C. serpentina* (mean eggs per nest \pm 1.0 SD = 46 ± 10), with eggs arranged in three layers within the nest cavity (fig. 1, inset). Spaces between eggs were generally air filled. To maximize our ability to detect thermal gradients relevant to the embryos, we placed data loggers above and below each egg layer within the nests (fig. 1, inset). We also measured the minimum and maximum depths of each nest (depth above the top egg and depth below the bottom egg, respectively). We then carefully covered each nest with soil and used a digital camera fitted with a 180° fish-eye lens to photograph the sky directly above each nest. Using Gap Light Analyzer software (ver. 2.0; Frazer et al. 1999), we estimated canopy cover (% shade) and incident solar radiation ($\text{MJ m}^{-2} \text{day}^{-1}$) over the course of development for each nest from these photographs (Doody et al. 2006; Mitchell et al. 2013). Nest predation is high at our site, so we placed a metal screen over each nest (a 1×1 -cm mesh aluminum hardware cloth, staked to the ground) to guard nests from predators. Even with these safeguards, two nests were depredated, leaving five intact nests for analyses.

We allowed the data loggers to remain within the nests for the majority of incubation, retrieving them on August 9, 2014 (incubation period averages 75–100 days depending on temperature; Congdon et al. 2008). For four nests, we examined temperatures across the full period where we recorded data for all nests (62 days). The final nest flooded on day 25 of incubation (nest 3 in fig. 2), so we analyzed only

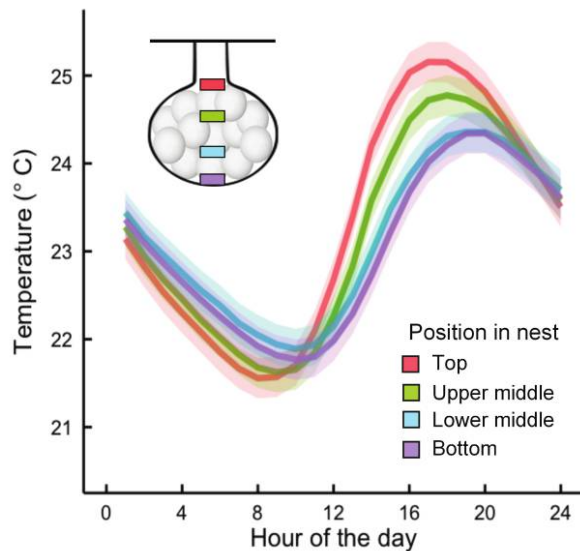


Figure 1: Average temperatures at four depths within natural snapping turtle (*Chelydra serpentina*) nests in Carroll County, Illinois, over the course of a day. Lines are derived from hourly least squares means for each depth, and shaded regions represent ± 1.0 SE. Inset, diagram of a typical flask-shaped *C. serpentina* nest showing the relative placement of thermal data loggers among the eggs.

temperatures recorded prior to the flood for that nest. We used a generalized linear mixed-effects model to examine the effect of nest depth on nest temperature. Time of day, relative nest depth (i.e., top, top-middle, bottom-middle, and bottom; fig. 1, inset), and their interaction were included as fixed effects in the model, and nest identity was included as a random effect.

Assessing Thermal Gradients within Eggs

We examined whether an external thermal gradient could be maintained internally within six live eggs in the laboratory (additional eggs from the three nests returned to the laboratory were used to confirm gradient treatments, assess alternate methods for internal temperature measurement, and for unrelated experiments). Throughout excavation and transport, we carefully avoided rotating or jostling the eggs. On arrival in the laboratory, we immediately placed the eggs into sealed 0.5-L mason jars with flat sides (Ball, model 1440061180) one-third filled with moist vermiculite (-150 kPa; Packard et al. 1987). We buried the eggs two-thirds into the vermiculite and incubated them at a constant 27°C (Packard et al. 1987). At the onset of the experiment, embryos were at the advanced gastrula stage (Ewert 2008).

We created two thermal gradient treatments (large gradient and small gradient) by adjusting the distance of the egg-incubation jars from a supplemental heat source within an environmental chamber set at 22°C . We used Flex Watt heat tape (7.6 cm wide) mounted to a vertical board along the back of a metal shelving unit as a supplemental heat source. We placed the side of the incubation jars directly against the heat tape to create the large-gradient treatment or 10 mm away from the heat tape to create the small-gradient treatment. To ensure that all eggs within treatments experienced the same gradient, we placed the eggs against the side of their incubation jar nearest the heat tape. Because ambient temperature was uniformly maintained at 22°C , increasing the temperature of the warm side of the eggs by placing them closer to the heat source created an increased thermal differential across the eggs (see below).

Prior to measuring the internal gradients within eggs exposed to each treatment, we assessed the external thermal gradients created by the treatments. After allowing the eggs to incubate for 2 weeks at constant temperature, we placed multiple eggs ($N = 20$ for each treatment) on the gradients. After 2 h of exposure, we measured the temperature on the surface of each egg closest to the heat source (mean \pm 1.0 SE = $33.8^\circ\text{C} \pm 0.4^\circ\text{C}$ and $25.1^\circ\text{C} \pm 0.1^\circ\text{C}$ on the large and small gradients, respectively), on the top of the egg (mean \pm 1.0 SE = $27.0^\circ\text{C} \pm 0.2^\circ\text{C}$ and $24.3^\circ\text{C} \pm 0.1^\circ\text{C}$, respectively), and at the point farthest from the heat

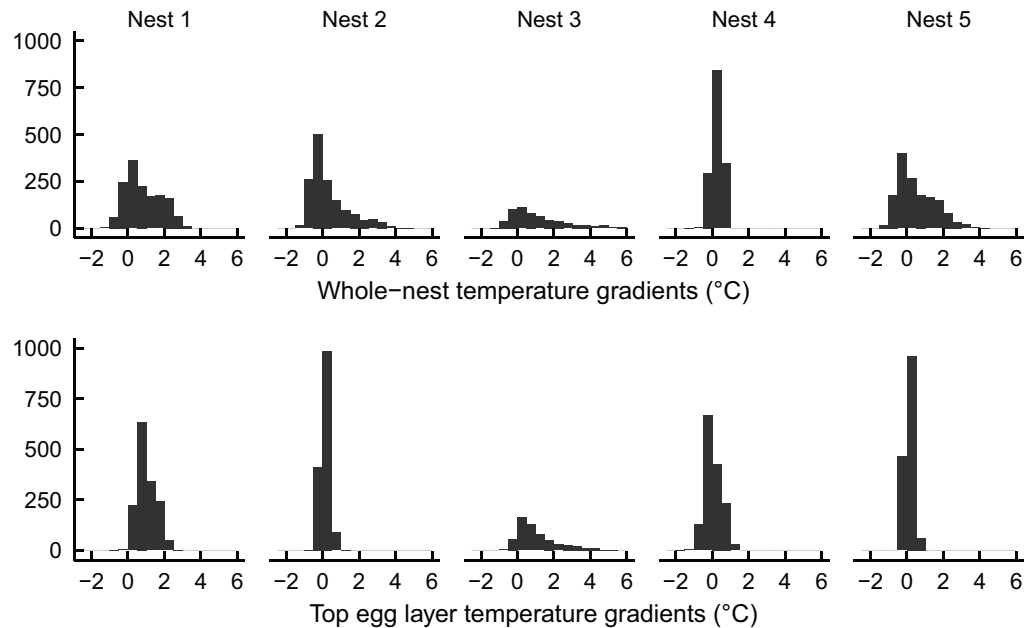


Figure 2: Frequency histograms of gradients observed within five natural *Chelydra serpentina* nests in Carroll County, Illinois, throughout the incubation period. The top row depicts whole-nest thermal gradients observed hourly (temperature at the top of the nest – temperature at the bottom of the nest), whereas the bottom row depicts the thermal gradients observed across a single egg layer within the nests (temperature above the top egg layer – temperature below the top egg layer). Fewer observations are available for nest 3 because we included only temperatures prior to the nest flooding 25 days into incubation.

source (mean \pm 1.0 SE = $25.2^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$ and $24.1^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$, respectively). Thus, the large-gradient treatment exposed eggs to an 8.6°C external gradient, while the small-gradient treatment exposed eggs to a 1.0°C external gradient. We measured temperature by placing a thermocouple (30-gauge, Omega SC-GG-K-30-66) on the surface of each egg, allowing the temperature to stabilize, and recording the temperature with a calibrated handheld digital thermometer (Omega CL3512A). Repeated measurements confirmed that temperatures were stable across the eggs for multiple days.

After confirming that the treatments induced stable gradients, we recorded the internal temperatures of six additional eggs (three each exposed to the large- or small-gradient treatments). Prior to being exposed to the gradients, these eggs completed approximately two-thirds of development at constant temperature (assessed by the number of days since oviposition and by candling). After allowing the eggs to equilibrate to the thermal gradient treatments for 1 day, we inserted fine thermocouple probes (40-gauge, Omega 5SC-TT-T-40-56) into the ends of each egg, with one probe inserted in the side adjacent to the heat source and the other in the side farthest from the heat source. We used sterile 27.5-gauge needles to make incisions on the eggs and inserted the probes within the extraembryonic fluids to a depth of 5 mm. We measured temperature using

the same digital thermometer used to measure external egg temperatures. We recorded the temperature from each probe at six time points approximately evenly spaced over a 24-h period. We confirmed that the embryos were alive prior to inserting the probes by candling the eggs. Embryos continued to display signs of life (blood circulation and motor reflexes) for the duration of the 24-h period that we recorded temperatures. However, embryos from our pilot studies died when exposed to the probes for longer periods, generally as a result of egg dehydration followed by infection. We used a generalized linear mixed-effects model to compare the internal temperatures at either end of the eggs. Initially, we included egg side and treatment (small or large gradient) as fixed effects in the model, with egg identity included as a random effect to account for repeated measurements. However, there was a significant interaction between egg side and gradient treatment on internal temperature (Wald's $\chi^2 = 107.19$, $df = 1$, $P < .000001$), so for the final models, we examined internal egg temperatures for eggs on each gradient separately. To confirm that the gradient treatment affected the size of the internal gradient, we used a generalized linear mixed-effects model with internal gradient size as the dependent variable (temperature adjacent to the heat source – temperature opposite the heat source), gradient treatment as a fixed effect, and

egg identity as a random effect. All analyses were performed using the R programming language (ver. 3.1.1; R Core Team 2015). We constructed the mixed-effects models using the lme function in the nlme package (Pinheiro et al. 2013) and assessed statistical significance using type-III sum of squares with the Anova function in the car package (Fox and Weisberg 2011).

Egg Thermal Gradient Simulation

Using a published soil-temperature model (eq. [8.6] in Campbell and Norman 1998), we estimated temperatures within soils under the full range of conditions that eggs within natural subterranean nests might experience. (See appendix A [apps. A–C available online] and Campbell and Norman 1998 for details of the model.) This model was originally designed to describe heat transfer through the soil and provide a qualitative understanding of soil-temperature patterns. It assumes that the diel pattern of thermal variation is sinusoidal and that soil properties remain uniform with depth. Briefly, the model predicts soil temperature (T) for a given time of day (t), average surface temperature (T_{AVE}), thermal amplitude ($A(0)$), depth within the soil column (z), and damping rate (D). $A(0)$ is half of the diel thermal range at the soil surface, and D is a unitless, soil-type-specific parameter that describes the rate at which the thermal amplitude is reduced with increasing soil depth. We calculated temperature for factorial combinations of the natural range of each parameter: t , for the entire day at 2-h intervals; z , ranging from 0 cm (i.e., soil surface) to 150 cm at 0.5-cm increments; T_{AVE} , ranging from 10°C to 30°C at 2°C increments; $A(0)$, ranging from 2°C to 30°C at 1°C increments; and D , ranging from 0.03 to 0.15 at 0.02 increments. Globally, $A(0)$ can range from 2.5°C to 30°C (Kearney et al. 2014a; fig. 3), and D ranges from 0.062 in organic soils to 0.139 in wet sand (Campbell and Norman 1998). The simulation required 8,065,596 calculations and was performed on a personal computer using the foreach function within the foreach package paired with the doParallel package in R (Revolution Analytics and Weston 2014a, 2014b; R Core Team 2015).

We used the simulated soil temperatures to calculate the external thermal gradient predicted to occur across eggs ranging in size from 0.5 to 10 cm in vertical diameter (0.5-cm increments) given each combination of t , T_{AVE} , $A(0)$, z , and D . This range of egg sizes encompasses all extant amniotes: the largest nonavian reptile eggs are ≈ 5.5 cm in vertical diameter (e.g., Galapagos tortoise [*Chelonoidis nigra*] and Komodo dragon [*Varanis komodoensis*]), whereas the largest avian eggs (ostrich [*Struthio camelus*]) are ≈ 10 cm in vertical diameter (Iverson and Ewert 1991). We calculated thermal gradients by subtracting the predicted soil temperature at the top of the egg from the predicted soil tempera-

ture at the bottom of the egg. Using these data, we constructed heat maps depicting how external egg gradients throughout the soil profile are affected by each model parameter. We performed egg gradient calculations using the foreach function in the foreach package and the rollapply function in the zoo package within R (Zeileis and Grothendieck 2005; Revolution Analytics and Weston 2014b; R Core Team 2015) and constructed heat maps using the ggplot2 package within R (Wickham 2009; R Core Team 2015).

To assess the power of the model to predict temperatures within nests, we compared predicted temperatures from the model to the temperatures that we observed within *C. serpentina* nests. Using the model, we calculated temperatures throughout the day at 10, 15, 20, and 25 cm below the soil surface, which approximates our placement of thermal data loggers within nests. We assumed $T_{\text{AVE}} = 23^\circ\text{C}$, $A(0) = 5^\circ\text{C}$, and $D = 0.11$ because these conditions approximate those at our field site (F. J. Janzen, personal observation; Campbell and Norman 1998). We used a Mantel test and root mean square error (RMSE) to compare observed and predicted nest temperatures. The Mantel test was performed using the function mantel from the vegan package in R (significance assessed with 10,000 permutations; Oksanen et al. 2013; R Core Team 2015), and RMSE was calculated using the rmse function from the hydroGOF package in R (Zambrano-Bigiarini 2014; R Core Team 2015).

Finally, we utilized published global microclimate data (microclim data set; Kearney et al. 2014a) to explore the global availability of the conditions that our simulations predicted can result in biologically relevant thermal gradients across eggs. The microclim data set is composed of gridded hourly estimates of average monthly microclimate conditions across the globe (Kearney et al. 2014a). These estimates are derived from the microclimate model of the Niche Mapper biophysical model, are based on first principles, and are well verified (Beckman et al. 1973; Porter et al. 1973; Kearney et al. 2014a, 2014b). Generally, the simple soil physics model that we used for our simulations (Campbell and Norman 1998) and the microclim data set (Kearney et al. 2014a) perform similarly, although our simple model better reconstructed temperatures in natural *C. serpentina* nests than did microclim (details in app. B). Even so, the simple soil physics model does not allow us to ascertain the global distribution of microclimatic conditions relevant for eggs, whereas the microclim data set does (Kearney et al. 2014a). Thus, we employed a hybrid approach: we used the simple soil physics model of Campbell and Norman (1998) to assess the conditions that can result in biologically relevant thermal gradients because of its improved fit to our observed data and computational simplicity and then the microclim data set to assess the global availability of such conditions. We first used the microclim data set to estimate global distributions of mean temperature

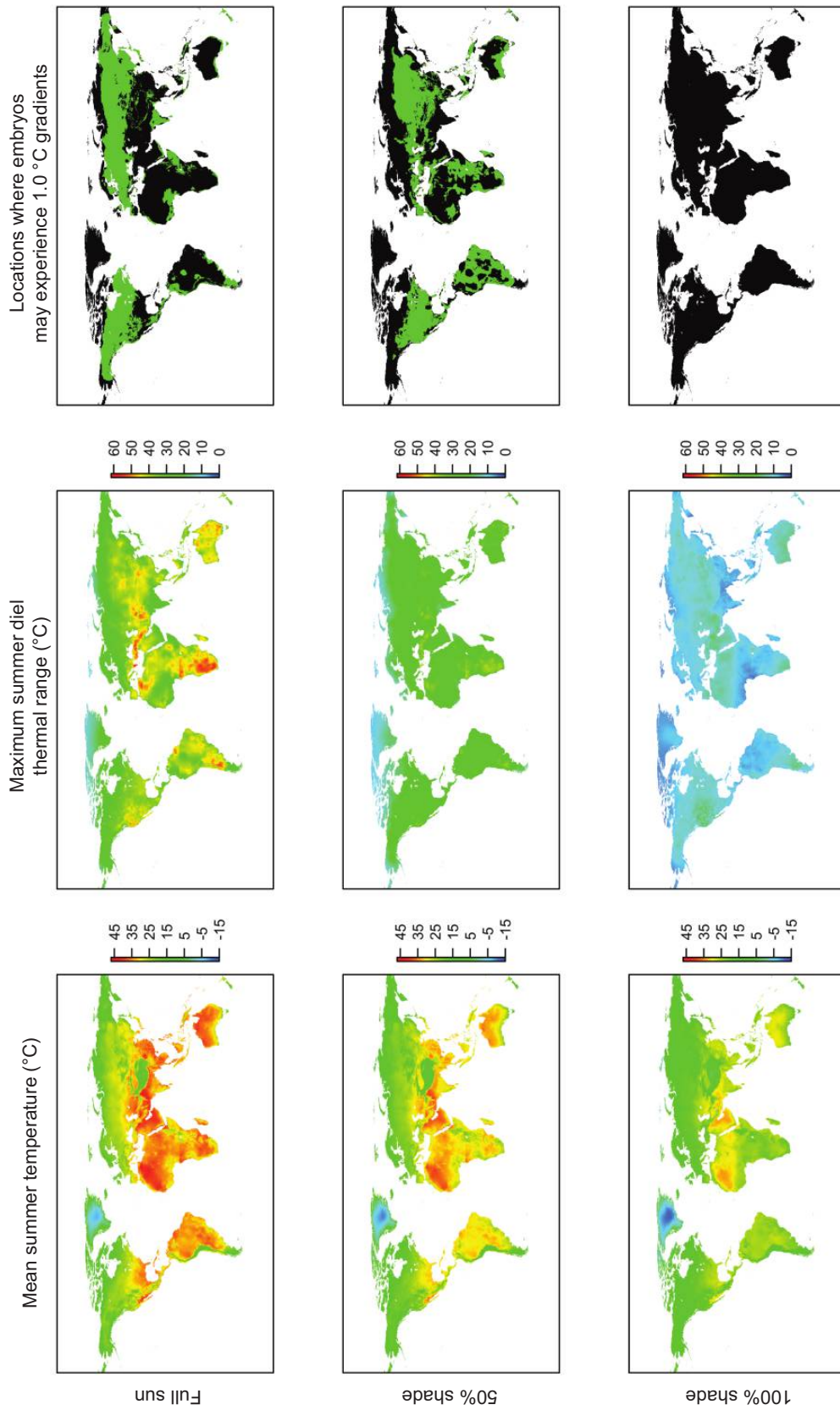


Figure 3: Predicted global distributions of key model parameters (mean temperature [T_{AVE}] and maximum diel thermal range [$2 \times A(0)$] at the soil surface) and embryonic thermal gradients given three shade conditions. All values are derived from the microclim data set (Kearney et al. 2014a), assuming a soil substrate. Values in the Northern Hemisphere are for July, whereas Southern Hemisphere values are for January. For the thermal gradient plots, green indicates areas where biologically relevant thermal gradients (1.5°C external and, thus, 1.0°C internal) are predicted to occur at least once during development assuming a 3-cm-diameter egg buried 10 cm below the surface (approximates a snapping turtle egg at the top of the nest). Importantly, all gradients will be transient, reversing direction every 12 h. Black areas denote either locations where gradients are not predicted to occur or locations that experience conditions where reptiles cannot viably develop (mean egg temperature exceeds 50°C or falls below 0°C).

(T_{AVE}) and diel thermal range ($2 \times A(0)$) at the soil surface for summer (July in the Northern Hemisphere and January in the Southern Hemisphere) assuming three shade regimes (full sun, 50% shade, and 100% shade). To calculate the minimum conditions needed for biologically relevant thermal gradients, we assumed eggs have a 3-cm vertical diameter and are buried 10 cm below the soil surface in moist loam (model *C. serpentina* eggs at the top of the nest). We considered an external gradient of 1.5°C to be biologically relevant, because this could result in an internal thermal gradient of 1.0°C (see “Thermal Gradients within Live Eggs” results). Finally, we conservatively assumed that any location where eggs could maintain median temperatures between 0°C and 50°C is viable for development (i.e., it encompasses the complete range of thermal tolerance for reptile development; Birchard 2004). Using these criteria, we determined locations that are both viable for development and experience biologically relevant thermal gradients at some point during the day, assuming nests are buried under full sun, 50% shade, or 100% shade. This analysis is highly conservative as it assumes large eggs with broad thermal tolerance buried in shallow nests. Moreover, we only considered maximum daily gradients, whereas actual gradients will be transient and reverse direction twice daily. Nest and egg temperature data along with output from the soil-temperature simulations are deposited in the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.mj8m0> (Telemeo et al. 2016).

Results

Thermal Gradients within Natural Nests

On average, nest egg chambers were 8.0 cm in height (top depth – bottom depth; range = 6.3–9.5 cm), with the top egg 9.5 cm below the soil surface (range = 7.9–11.9 cm; table C1, available online). Shade cover over nests ranged from 13.8% to 68.6% (mean = 51.1%), resulting in incident solar radiation ranging from 6.4 to 11.6 MJ m⁻² day⁻¹ (mean = 8.0 MJ m⁻² day⁻¹; table C1; additional nest details are also provided in app. C). Time of day and nest depth interacted to affect temperature within the nests (Wald’s $\chi^2 = 525.48$, $df = 69$, $P < .000001$). This interaction resulted from diel variance in nest temperature decreasing with nest depth (fig. 1). Thus, temperature at the top of the nests could be warmer, cooler, or identical to temperature at the bottom of the nests depending on the time of day (fig. 1). Based on least squares (LS) means, the greatest differences in temperature between the top and bottom of the nests occurred at 14:00, with the top of the nests averaging 1.5°C warmer than the bottom, and at 07:00, with the bottom of the nests averaging 0.4°C warmer than the top (fig. 1). The greatest difference in LS mean temperature

across a single egg layer was 0.6°C, which occurred across the top egg layer at 14:00. Mean (± 1.0 SD) absolute thermal gradients across the incubation period within each nest ($|\text{top temperature} - \text{bottom temperature}|$) were 0.9°C \pm 0.8°C, 0.8°C \pm 0.7°C, 1.2°C \pm 1.3°C, 0.2°C \pm 0.3°C, and 0.8°C \pm 0.7°C (fig. 2). Although nest gradients were generally small ($<1.0^\circ\text{C}$), the distributions were right skewed, with occasional large gradients (fig. 2), the largest of which were two occurrences of 6.0°C gradients within nest 3 (fig. 2). Not surprisingly, nest gradients across single egg layers were generally smaller than gradients observed in whole nests, with gradients across the top egg layer greater than gradients across the other layers (fig. 1). Mean absolute thermal gradients ± 1.0 SD across the top layers of eggs ($|\text{temperature above the egg} - \text{temperature below the egg}|$) were 0.8°C \pm 0.5°C, 0.2°C \pm 0.3°C, 0.9°C \pm 1.0°C, 0.4°C \pm 0.3°C, and 0.2°C \pm 0.2°C (fig. 2). Generally, gradients across the top egg layers were less right skewed than gradients measured across the whole nests (fig. 2).

Thermal Gradients within Live Eggs

Exposure to either the large or small gradient treatment induced thermal gradients within live eggs, with internal egg temperatures near the heat source warmer than those opposite the heat source (large-gradient treatment: Wald’s $\chi^2 = 248.87$, $df = 1$, $P < .000001$; small-gradient treatment: Wald’s $\chi^2 = 72.52$, $df = 1$, $P < .000001$; fig. 4). Moreover, internal gradients were larger in the eggs exposed to the large-gradient treatment than those exposed to the small-gradient treatment (Wald’s $\chi^2 = 29.09$, $df = 1$, $P < .000001$; fig. 4). Even so, internal egg temperature gradients were relatively small (LS means ± 1.0 SE = 2.45°C \pm 0.24°C and 0.69°C \pm 0.23°C, respectively) and uniformly lower than external gradients measured on the surface of the shell (fig. 4). Given that external gradients were 8.6°C and 1.0°C in the large and small gradient treatments (see “Material and Methods”), internal gradients were reduced by 72% and 31%, respectively. Thus, for a 1.0°C internal gradient to be maintained within a *C. serpentina* egg, external gradients between 1.5°C and 3.6°C would be necessary.

Nest Thermal Gradient Simulation

The model accurately and precisely predicted temperatures within natural *C. serpentina* nests (RMSE for temperature at each depth = 0.52°C, RMSE for gradient sizes = 0.37°C, Mantel $R = 0.942$, $P = .001$; figs. S1, B1; figs. S1–S5, B1–B3 available online), confirming the ability of the soil-temperature model to predict temperatures within reptile nests. Time of day, egg depth, soil type, diel thermal range, and egg size all affected predicted external-egg ther-

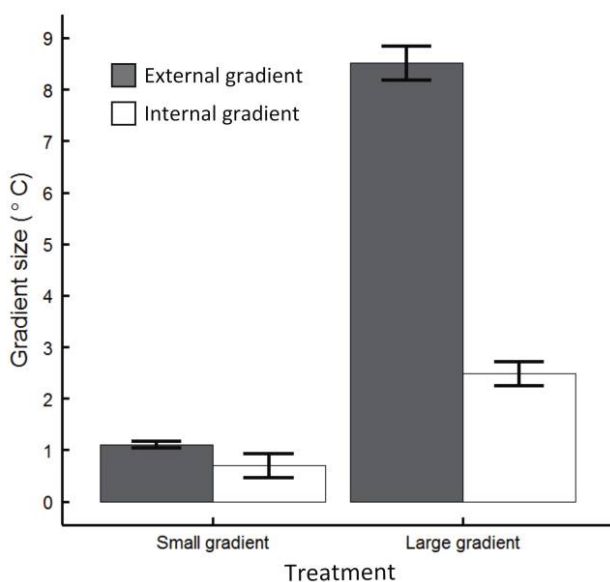


Figure 4: Thermal gradients inside and outside of live common snapping turtle (*Chelydra serpentina*) eggs exposed to small and large thermal gradient treatments. Internal temperatures are least squares means \pm 1.0 SE (to account for repeated measures), whereas external temperatures are absolute means \pm 1.0 SE.

mal gradients (figs. 5–7; see figs. B1, S2–S5 for the additional parameters and details). Predicted thermal gradients were negatively affected by nest depth (exact rate depended on soil type) but positively affected by egg size and diel thermal range. For all parameter combinations, external egg gradients rarely were predicted to occur at depths ≥ 20 cm and were never predicted to occur > 50 cm. When gradients were predicted, they were transient, lasting for short periods and reversing direction twice daily (figs. 5, 6).

Under conditions usually encountered by developing reptiles (i.e., eggs less than 2.5 cm in vertical diameter, buried ≥ 10 cm below the surface, in moderate or shaded environments), predicted thermal gradients were nominal and likely not biologically relevant (figs. 5–7). For example, to produce momentary exposure to a 1.5°C external gradient (i.e., 1.0°C internal gradient) for an egg with a 3-cm vertical diameter buried 10, 15, or 20 cm below the soil surface, diel thermal ranges of 24°C, 32°C, or 56°C are necessary. Even so, large gradients were predicted to occur under some conditions, especially when both eggs and the diel thermal range are large and when eggs are buried close to the soil surface (fig. 7). In the most extreme case, a 10-cm egg buried at the soil surface given a 60°C diel thermal range could briefly experience a 25°C external thermal gradient twice per day. However, this gradient reverses direction every 12 h.

Examination of the microclim data set confirmed that environments with conditions that can produce biologically

relevant thermal gradients are limited (fig. 3). First, environments that produce large thermal gradients are frequently inviable for development, with median egg temperatures predicted to drop below 0°C or rise above 50°C, regardless of egg size. This is because large thermal gradients primarily result from exposure to large diel thermal ranges, which are achieved under full sun in extreme habitats such as desert or high elevation (fig. 3). Because both mean temperature and diel thermal range are extreme in these habitats (fig. 3), eggs experience median egg temperatures outside of viable limits for a portion of the day. In more moderate habitats, transient gradients large enough to be biologically relevant are possible but only in relatively sunny sites (fig. 3). Moreover, this phenomenon will be most common at high latitudes where reptile diversity is low (fig. 3).

Discussion

Our results demonstrate that while thermal gradients can occur within natural reptile nests, these gradients are generally too small, transient, and poorly transferred into the internal egg environment to allow adaptive behavioral thermoregulation by embryos. For example, the maximum average gradient that we observed across an egg layer at the warmest part of the day within natural *Chelydra serpentina* nests was 0.6°C. When exposed to a similar gradient in the laboratory (1.0°C, small-gradient treatment), the internal egg gradient was reduced 31%. Assuming a similar reduction in nests, the average gradient experienced by *C. serpentina* embryos at the warmest part of the day would equal 0.4°C. Embryos might subtly alter their body temperature by adjusting their position within the egg under a constant 0.4°C gradient. However, *C. serpentina* eggs experienced thermal gradients approaching this magnitude for only brief periods of the day, with minimal or nonexistent gradients more common. Furthermore, thermal gradients reversed direction every 12 h. Because reptile embryos require multiple days to adjust their position within the egg (Du et al. 2011; Li et al. 2014), embryos are physically unable to track such diel thermal variation behaviorally.

The gradients that embryos experience may be far smaller than the external gradients to which they are exposed. In the *C. serpentina* eggs that we examined, internal gradients did not increase in parallel with their external gradients. Internal thermal gradients were likely smaller than external thermal gradients because eggs are fluid-filled structures with active circulatory systems (Thompson and Speake 2004), and these properties will act to homogenize internal temperatures. As the internal thermal gradient increases, progressively greater external thermal gradients might be necessary to combat the homogenizing tendency of the egg and induce equivalent increases in the internal thermal gradient. Alternatively, the dif-

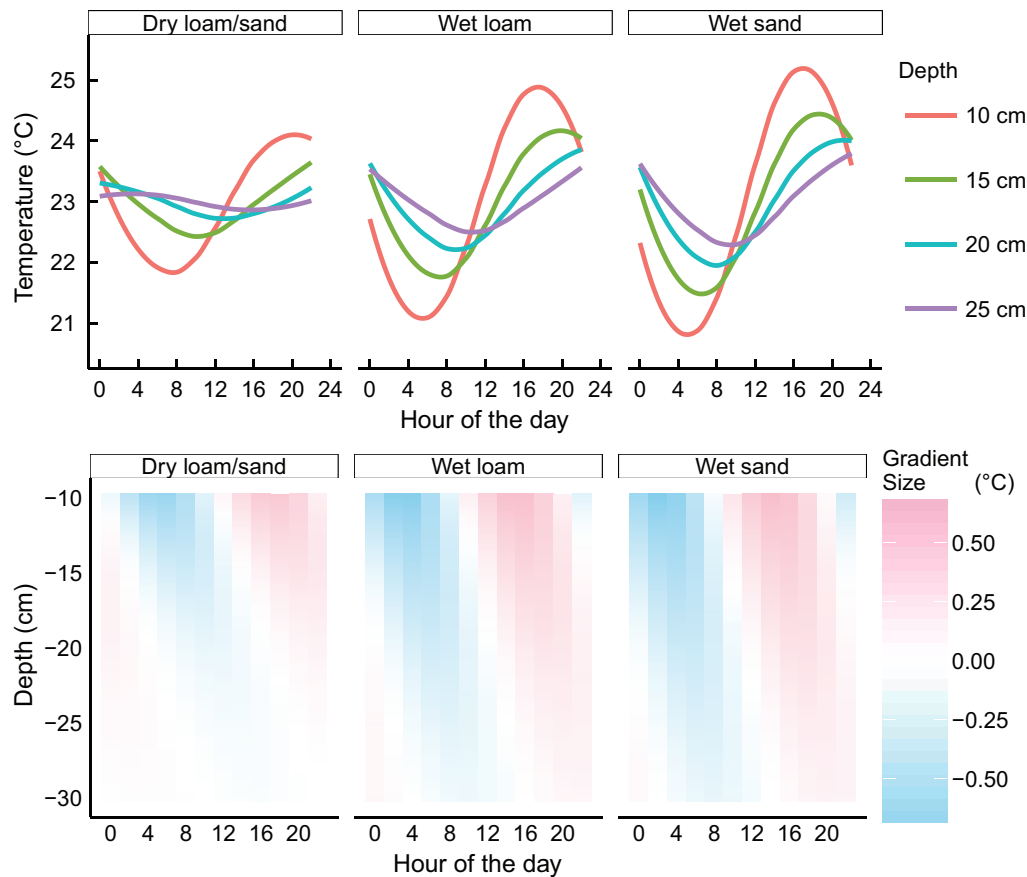


Figure 5: Temperatures predicted to occur within *Chelydra serpentina* nests constructed in three soil types. Data are from simulations using a published soil-temperature model (see text for details). The top row depicts predicted temperatures at 10, 15, 20, and 25 cm below the surface, which approximate the depths at which we placed data loggers in field nests (see fig. 1 for comparison). Most nests that we examined were in wet loam. The bottom row depicts heat maps of predicted external egg gradients across the day and across the range of depths observed in *C. serpentina* nests. Gradient size assumes eggs are 3 cm in diameter, as are *C. serpentina* eggs. Positive (red) gradients indicate that the top of the egg is warmer than the bottom, and negative (blue) gradients indicate that the bottom of the egg is warmer than the top. For these simulations, we assumed an average temperature at the surface of 23°C and a diel thermal range of 10°C. Damping rates were assumed to be 0.07, 0.11, and 0.13 for dry loam/sand, wet loam, and wet sand, respectively.

ference in slope that we observed between the internal and external gradients could result from how we created the treatments. Eggs were partially buried in a moist medium and exposed to a point source of heat on one side. Thus, gradients had to be created in the surrounding medium as well as in the egg. Because of the buffering power of the moist medium, the temperature dropped rapidly with distance from the heat source. This phenomenon was also apparent in our field-nest measurements and model simulations, similarly resulting from the thermal damping properties of the soil. Thus, rather than an anomaly, the mismatch between the size of the external and internal thermal gradients that we observed should be applicable to all eggs in subterranean nests.

Our simulations broadly accord with our *C. serpentina* observations and suggest that thermal gradients suitable

for embryonic behavioral thermoregulation will rarely be accessible to embryos of any reptile species. Only eggs ≥ 2.5 cm in vertical diameter, buried ≤ 5.0 cm from the soil surface, and in environments that experience diel thermal ranges at the soil surface of 18°C or more are predicted to experience external egg gradients $\geq 1.5^\circ\text{C}$ (potential internal gradient of 1.0°C) at any time of day. The vast majority of reptile eggs are substantially smaller than 2.5 cm in vertical diameter, with many < 1.0 cm (Iverson and Ewert 1991; Shine 2005; Vitt and Caldwell 2009). Such small eggs are predicted to experience a detectable external thermal gradient only when placed at the soil surface. However, reptiles generally bury their eggs 5 cm or more below the soil surface, and only limited gradients are ever predicted to occur at such depths. Finally, gradients are expected to be highly

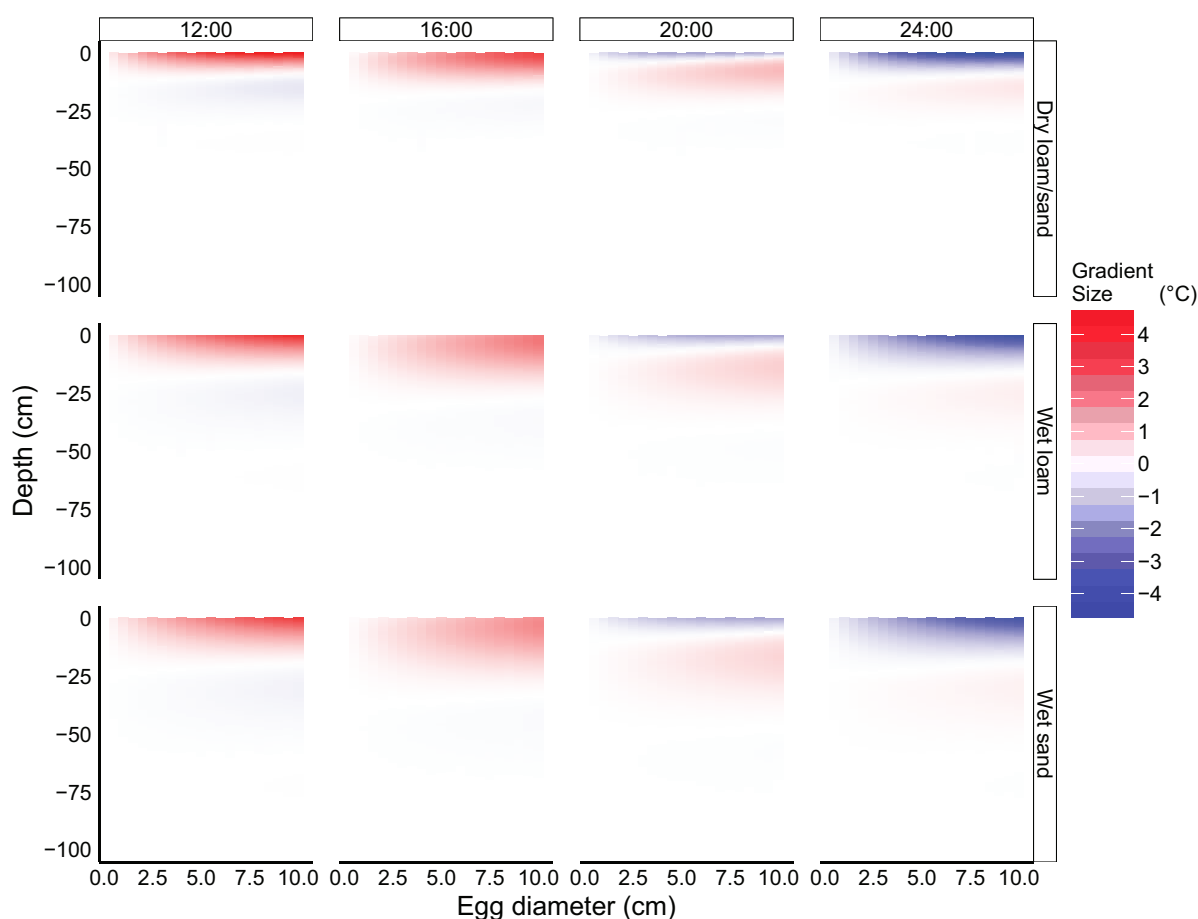


Figure 6: Effect of egg size and depth on external egg thermal gradients predicted using a published soil-temperature model (see text for details). Columns are times of day ranging from the warmest to coolest time at the surface, and rows are soil types. Damping rates were assumed to be 0.07, 0.11, and 0.13 for dry loam/sand, wet loam, and wet sand, respectively. Positive (red) gradients indicate that the top of the egg is warmer than the bottom, and negative (blue) gradients indicate that the bottom of the egg is warmer than the top. For these simulations, we assumed an average temperature at the surface of 23°C and a diel thermal range of 12°C.

transient, occurring for only short periods of the day and reversing direction every 12 h.

Examination of the microclim data set (fig. 3) allows identification of global habitats where embryos of large-egg species might experience biologically relevant thermal gradients at some point during the day. Biologically relevant egg gradients are most likely to occur in relatively mild habitats, such as low-elevation high-latitude sites, when nests are placed within bare soil exposed to large amounts of solar radiation. Even so, these gradients will be moderate in size (1.5°C–3.0°C external gradient and, thus, 1°C–2°C internal gradient), brief, and reverse direction twice daily. While similarly open sites in more extreme habitats could produce large gradients, they also result in extreme conditions that are not suitable for development, regardless of embryo position within the egg. Importantly, whether species actually experience egg gradients in habitats where they

are possible will greatly depend on maternal behavior. If females place their nests under shaded soils (tree or grass cover) or bury their eggs ≥ 20 cm below the soil surface, eggs will not experience gradients of biologically relevant magnitude regardless of other environmental conditions. While nesting behaviors vary widely among reptile species, in general, females of large-egg species (e.g., sea turtles, crocodilians, large squamates) place their eggs within deep, sometimes well-shaded nests, and this behavior will largely erase the potential for exposure to thermal gradients (eggs are within aboveground mounds in many crocodilians but well buffered by organic material; e.g., Ernst et al. 1994; Ackerman and Lott 2004; Vitt and Caldwell 2009).

Even if we suppose that they are occasionally exposed to thermal gradients, embryos face difficulties effectively altering their body temperature via movement. For example, *C. serpentina* embryos are insufficiently developed to alter

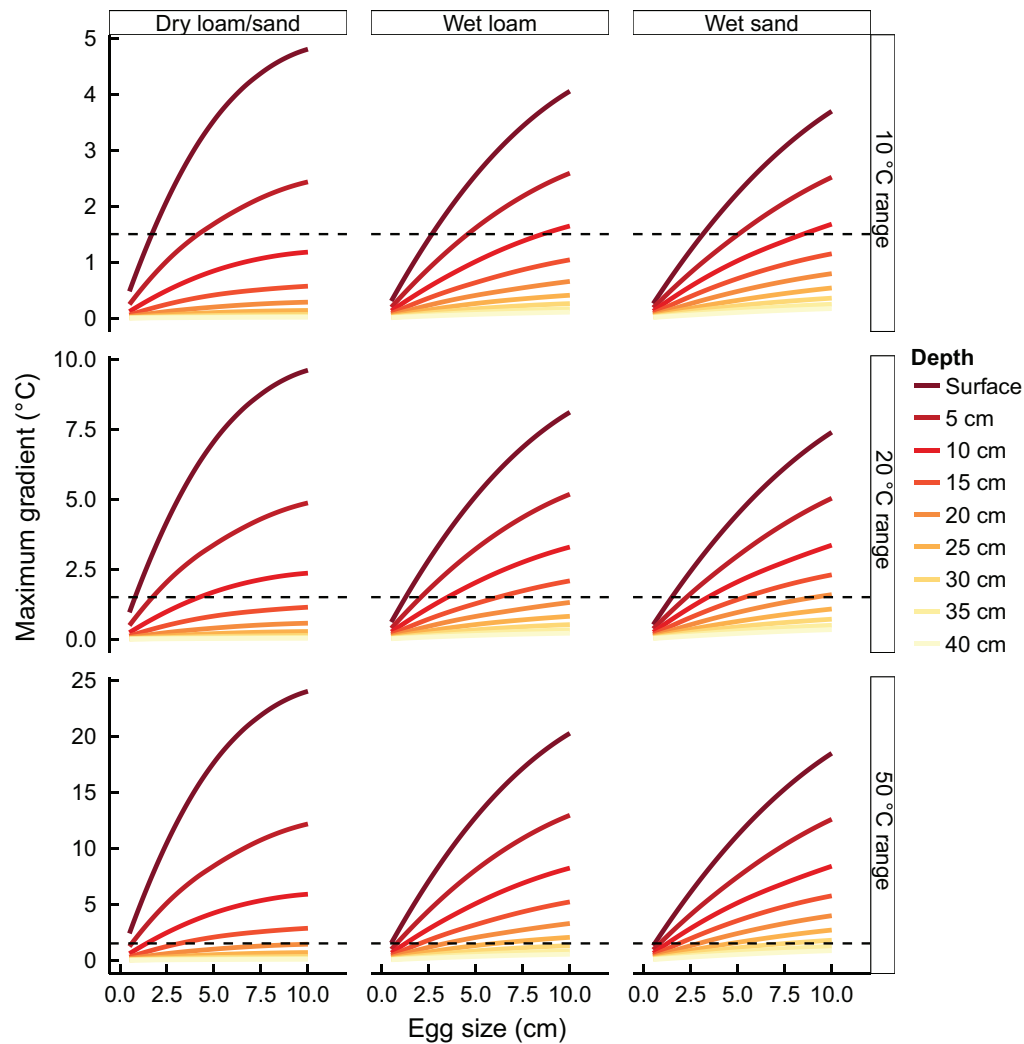


Figure 7: Effects of egg size, egg depth, soil type, and diel thermal range at the soil surface on the maximum external-egg gradient predicted to occur at any time of day. For these simulations, we assumed an average temperature at the surface of 23°C. Damping rates were assumed to be 0.07, 0.11, and 0.13 for dry loam/sand, wet loam, and wet sand, respectively. The horizontal dotted line indicates maximum external gradients of 1.5°C across the egg, which could result in a 1.0°C internal egg gradient.

their position for the initial third of development (Tuge 1931; Decker 1967). Unfortunately, these early stages are when development is most thermally sensitive (Yntema 1968; Shine and Elphick 2001; Birchard 2004), and embryos could most affect their fitness by thermoregulating. Still, thermoregulation in the latter two-thirds of development could provide some benefit, such as increasing the rate of development, allowing heat-stress avoidance, and potentially adjusting offspring sex (Georges et al. 2005; Janzen 2008; Du and Shine 2015). However, as embryos enter later stages of development, they grow rapidly and quickly fill the majority of the egg's space (Andrews 2004). Thus, during the period when embryos are most physically able to al-

ter their position, they are also the most space restricted (Decker 1967). As a result, embryonic movements during later stages when embryos are most mobile are more analogous to adjusting posture than position and will have limited effect on embryonic body temperature.

For those few species with large eggs buried close to the surface, we can still imagine situations where thermal taxis might allow individuals to alter their body temperature in a fitness-relevant way. First, thermal taxis might allow embryos to escape acute exposure to lethal temperatures. However, the situations where this would be possible seem remote (e.g., subtle changes in body temperature would allow survival, lethal temperature exposure occurs only dur-

ing stages when embryos can effectively shift position, and enough warning is available for the embryos to begin moving to the proper egg position days in advance of the lethal event). Also, embryos of some species, such as *C. serpentina*, have only been observed moving toward heat sources (Li et al. 2014), which would increase the probability of lethal exposure to high temperatures but may allow embryos to avoid lethal cold exposure. Alternatively, embryos might move to the position within the egg that maximizes development while minimizing the risk of damage over time and then remain there. Such a strategy would negate the need to track diel thermal variation. Although such an optimum position should exist, it seems unlikely that a developing embryo could detect it. Embryos would need to assess temperature variation across both space (gradient within the egg) and time (diel and seasonal changes in the gradient) and then integrate that information to select the overall optimum position within the egg—all prior to having a fully developed brain. Moreover, observations of embryonic thermal taxis suggest that embryos respond to current thermal stimuli rather than historic thermal patterns (Du et al. 2011; Zhao et al. 2013; Li et al. 2014).

While our results demonstrate that eggs primarily heated by the soil will rarely experience the necessary thermal gradients for behavioral thermoregulation, suitable gradients might become available if eggs are heated by additional sources. For example, in species that produce exceptionally large nests (e.g., sea turtles and crocodylians), metabolic heat production by the embryos can produce a thermal gradient from the center to the periphery of the nest (Godfrey et al. 1997; Booth and Astill 2001; Ewert and Nelson 2003; DeGregorio and Williard 2011). However, such gradients occur late in development, when embryos have grown to fill the majority of the egg's space (Ackerman et al. 1985; Godfrey et al. 1997; Ewert and Nelson 2003; Zbinden et al. 2006). Moreover, these thermal gradients are small, with gradients from the center to periphery of the nest generally ranging from 0.5°C to 3°C (Godfrey et al. 1997; Booth and Astill 2001; Broderick 2001; DeGregorio and Williard 2011), and gradients at the egg scale substantially smaller. The largest recorded thermal gradients attributed to metabolic heat production are from green sea turtles (*Chelonia mydas*). While gradients within *C. mydas* nests are generally minimal, increasing from 0.4°C to 1.0°C throughout the majority of development, gradients of 3°C–5°C were recorded just prior to hatching (Booth and Astill 2001). Because metabolically induced thermal gradients only become biologically relevant in magnitude after embryos are too large to migrate within the egg, adaptive embryonic behavioral thermoregulation to take advantage of metabolic heating does not appear possible.

Taken together, the available evidence implies that reptile embryos have extremely limited opportunity for adaptive behavioral thermoregulation and that behavioral thermoregu-

lation might never be possible. While we demonstrate that thermal gradients can be maintained within the largest of reptile eggs, suitable conditions for such gradients are globally limited. Moreover, these gradients are primarily driven by diel thermal variation, which results in gradients being short-lived and reversing twice daily. Thus, natural gradients vary more rapidly than embryos can respond (Du et al. 2011; Zhao et al. 2013; Li et al. 2014). Finally, throughout the majority of development, embryos are unable to respond to any gradient, regardless of its constancy, because they are either too early in development for directed movement or because they are too late in development and have grown to fill the majority of the egg (Tuge 1931; Decker 1967; Andrews 2004).

If most embryos are unable to behaviorally thermoregulate by moving within the egg, selection for behavioral thermoregulation cannot be the mechanism generally responsible for maintaining embryonic thermal taxis in reptiles. Why, then, do embryos of so many species display thermal taxis? We suggest that embryonic thermal taxis may represent play behavior. Play in animals is defined as “seemingly nonfunctional behavior differing from more adaptive versions structurally, contextually, or developmentally, and initiated when an animal is in a relaxed, unstimulated, or low-stress setting” (Burghardt 2014, p. 91, and citations therein). Although seemingly implausible, play behaviors have been demonstrated in free-living life stages of turtles and other reptiles (Burghardt 1998, 2015), and thermal taxis by *C. serpentina* embryos appears to meet the requirements of a play behavior. Developing embryos are largely isolated from the external environment and minimally stimulated, and embryos respond to nondangerous temperatures (Li et al. 2014). Moreover, embryos move around the egg randomly when maintained at constant temperature (Decker 1967; Gottlieb 1973; Li et al. 2014; G. A. Cordero, personal observation), not unlike mammalian fetuses kicking and wiggling in the womb (Gottlieb 1973).

A leading hypothesis for the adaptive value of play is that it allows animals to learn or perfect skills that are necessary later in life (Fagen 1974; Graham and Burghardt 2010; Burghardt 2014). Importantly, neonate reptiles must successfully thermoregulate behaviorally as soon as they hatch, and incubation conditions can affect thermoregulatory decisions by hatchlings (Deeming 2004). Thus, the mechanisms required to sense and respond to the thermal environment develop prior to hatching so that they are in place at hatching (Gottlieb 1973; Deeming 2004). Thermal taxis in the egg might enhance development of behavioral thermoregulation (akin to practice; Gottlieb 1973; Fagen 1974; Graham and Burghardt 2010). If so, we might expect species that tightly thermoregulate as adults to display greater thermal taxis behavior as embryos. Similarly, experimental manipulation of thermal gradients during

incubation would be predicted to positively affect juvenile thermoregulatory precision. Currently, sufficient data are not available to test such predictions, making these ripe avenues for further research.

Alternatively, embryonic thermal taxis might not be directly adaptive but could instead represent spandrels (e.g., Gould and Lewontin 1979) of behavioral thermoregulation by free-living stages: a neutral by-product of the behavioral-thermoregulation mechanism coming online (Gottlieb 1973; Graham and Burghardt 2010). Reptile embryos move both randomly and in response to physical touch (Tuge 1931; Decker 1967). These behaviors are almost certainly neutral by-products of the development of the nervous and muscular systems. Thermal taxis might be similarly neutral. If so, we might expect species that tightly behaviorally thermoregulate as adults to display increased embryonic thermal taxis as above, but the presence of gradients during development should not affect the thermoregulation of later stages.

In conclusion, our results demonstrate that reptile embryos will rarely have the capacity or opportunity to behaviorally thermoregulate by moving within the egg. While some few species may experience the narrow conditions necessary to allow embryonic behavioral thermoregulation, this cannot be the norm. Moreover, even in situations where behavioral thermoregulation by embryos is plausible, the thermal variation to which embryos have access will be much smaller than the variation controlled by female placement of the eggs in the environment. Still, adjusting position within the egg might allow embryos on rare occasion to avoid acute exposure to extreme temperatures, and research into the capacity of embryos to do this is warranted. As an alternative hypothesis, we suggest that embryonic thermal taxis represents a play behavior. As play, thermal taxis might be adaptive if it enhances the development of behavioral thermoregulation mechanisms or might simply be a neutral by-product of the development of such mechanisms.

Acknowledgments

We thank the US Fish and Wildlife Service and the US Army Corps of Engineers for access to the Upper Mississippi River National Wildlife and Fish Refuge. For constructive comments and conversation, we thank C. Adams, M. Angilletta, L. Buckley, L. Hoekstra, and R. Huey, as well as *American Naturalist* editors J. Bronstein and M. Kearney. This research was conducted under an approved animal-care protocol (IACUC 4-14-7784-J) and an Illinois Department of Natural Resources permit (NH14.0073). The research was supported by National Science Foundation (NSF) grant DEB-1242510 to F.J.J. and an Environmental Protection Agency Science to Achieve Results (EPA STAR) Fellowship

to R.S.T. Additionally, R.S.T. was partially supported by an NSF grant to L. Buckley (EF-1065638), E.J.G. was partially supported by a fellowship from the Office of Biotechnology at Iowa State University, and T.S.M. was partially supported by an NSF Postdoctoral Research Fellowship in Biology (DBI-1402202).

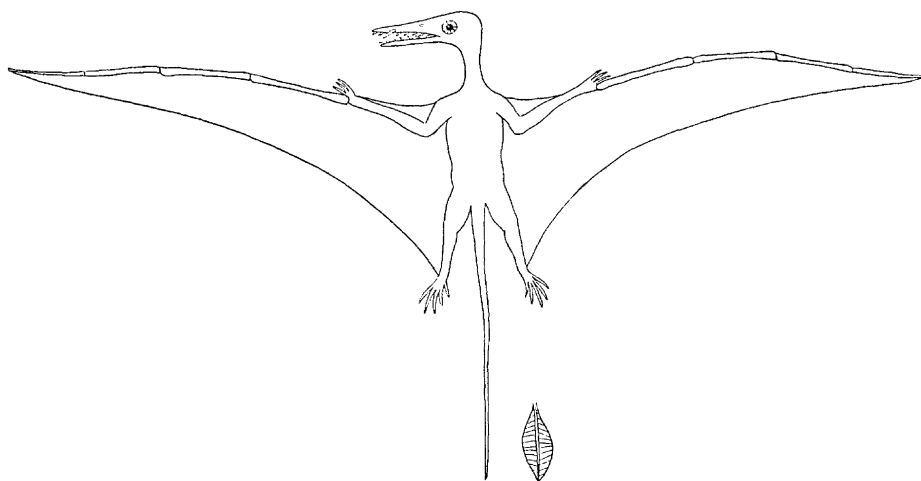
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Associate Editor: Michael Kearney
Editor: Judith L. Bronstein



“The adaptation of the pterodactyls for flight is a very perfect one, implying not only the development of true wings, but a specially modified respiratory apparatus coupled with a highly developed pneumaticity of the bones as in birds.” From “Volant Adaptation in Vertebrates” by Richard S. Lull (*The American Naturalist*, 1906, 40:537–566).