

Thermoregulatory Behavior Is Widespread in the Embryos of Reptiles and Birds

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ABSTRACT: Recent studies have demonstrated that thermoregulatory behavior occurs not only in posthatching turtles but also in turtles prior to hatching. Does thermoregulatory behavior also occur in the embryos of other reptile and bird species? Our experiments show that such behavior is widespread but not universal in reptile and bird embryos. We recorded repositioning within the egg, in response to thermal gradients, in the embryos of three species of snakes (*Xenochrophis piscator*, *Elaphe bimaculata*, and *Zaocys dhumnades*), two turtles (*Chelydra serpentina* and *Ocadia sinensis*), one crocodile (*Alligator sinensis*), and four birds (*Coturnix coturnix*, *Gallus gallus domesticus*, *Columba livia domestica*, and *Anas platyrhynchos domestica*). However, we detected no significant thermoregulation by the embryos of two lizard species (*Takydromus septentrionalis* and *Phrynocephalus frontalis*). Overall, embryonic thermoregulatory behavior is widespread in reptile as well as bird species but may be unimportant in the small eggs laid by most lizards.

Keywords: amniote, behavioral thermoregulation, egg size, embryogenesis.

Introduction

Thermoregulatory behavior (e.g., sun basking) plays an important role in achieving optimal body temperatures for functional performance (in ectotherms: May 1979; Huey 1982) or in reducing the energetic costs of thermoregulation when ambient temperatures fluctuate (in endotherms: Liwanag 2010). Consequently, both ectothermic and endothermic animals may use thermoregulatory behavior to enhance their fitness (survival and reproductive output) in thermally heterogeneous environments (Madsen and Shine 1999; Du et al. 2000; Gilbert et al. 2009). Although widespread in the postem-

bryonic stages of animals from insects to mammals, thermoregulatory behavior traditionally has been assumed not to occur in the embryonic phase of the life cycle (Hafez 1964; May 1979; Huey 1982; Lustick 1983). That assumption was falsified by recent studies that documented thermoregulatory behavior in turtle embryos. Embryos of the Chinese soft-shelled turtle (*Pelodiscus sinensis*) moved within the egg to find a warmer area (Du et al. 2011), and embryos of an emydid turtle (*Chinemys reevesii*) moved toward warmer areas and away from dangerously high temperatures (Zhao et al. 2013).

Thermoregulatory behavior of embryos thus appears to be genuinely analogous to that of posthatching ectotherms (Zhao et al. 2013), but the generality of this behavior remains unknown. Although many posthatching ectotherms regulate their body temperatures by exploiting thermal heterogeneity in the environment, this behavior is not universal: some species conform to ambient temperatures (Huey 1982; Hertz et al. 1993). An embryo's opportunities for behavioral thermoregulation are limited by the thermal gradient within its egg, which in turn is largely determined by the egg size (i.e., the larger the egg, the wider the thermal gradient). Hence, embryos may be able to show heat-seeking behavior only in large eggs, because the thermal differential in a small egg may not be wide enough for behavioral thermoregulation. Accordingly, we might expect to see both thermoconformity and active thermoregulation in embryos, as we do in posthatching stages of the life history. We conducted experiments on the thermal response of embryos in 12 species from diverse lineages of reptiles and birds (with different egg sizes) to ask whether embryonic thermoregulatory behavior is widespread in oviparous amniotes and to test the prediction that thermoregulatory behavior occurs in embryos within large but not small eggs.

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Material and Methods

Embryonic Movement in Response to Heating Sources

To explore whether thermoregulatory behavior occurs widely in reptiles and birds, we studied embryonic movements in two species of lizards (*Takydromus septentrionalis* and *Phrynocephalus frontalis*), three snakes (*Xenochrophis piscator*, *Elaphe bimaculata*, and *Zaocys dhumnades*), two turtles (*Chelydra serpentina* and *Ocadia sinensis*), one crocodile (*Alligator sinensis*), and four birds (*Coturnix coturnix*, *Gallus gallus domesticus*, *Columba livia domestica*, and *Anas platyrhynchos domestica*). *Takydromus septentrionalis*, a northern grass lizard, is a small oviparous lacertid lizard (snout-vent length [SVL]: up to 70 mm) that is widely distributed in mainland China and lays multiple clutches with 2–5 eggs in each clutch (Du et al. 2005). *Phrynocephalus frontalis*, the steppe toad-headed agama, is a small oviparous lizard (SVL: up to 60 mm) from northern China, with females laying a single clutch of 2–6 eggs each year (Qu et al. 2011a). *Elaphe bimaculata*, the twin-spotted rat snake (SVL: up to 800 mm), *X. piscator*, the checkered keelback snake (SVL: up to 940 mm), and *Z. dhumnades*, the black-striped snake (SVL: up to 2,000 mm) are differently sized colubrid species that are widespread in southern China (Zhao and Adler 1993). *Chelydra serpentina*, the common snapping turtle, is a large freshwater turtle occurring throughout much of North America; females produce multiple clutches in spring and summer (Steyermark et al. 2008). *Ocadia sinensis*, the Chinese stripe-necked turtle, is an aquatic species from the Geomydidae family that is mainly distributed in southern China and Vietnam (Zhang et al. 1998). The four species of birds come from different families and produce eggs of different sizes, ranging from about 10 to 100 g. *Coturnix coturnix*, a quail, and *G. gallus domesticus*, a fowl, are two common and widespread domestic birds from Phasianidae. *Columba livia domestica* is the world's oldest domesticated bird derived from the rock pigeon (Columbidae). *Anas platyrhynchos domestica*, the Pekin duck, is a large species from Anatidae that weighs up to 5 kg in captivity.

In May 2013, we collected 35 *T. septentrionalis* eggs (0.56 ± 0.31 g [mean mass \pm SE]) and 24 *P. frontalis* eggs (0.80 ± 0.37 g) laid in captivity by recently field-collected females. In July 2013, we obtained 30 freshly laid *X. piscator* eggs (4.41 ± 0.33 g), 24 *E. bimaculata* eggs (9.77 ± 0.37 g), and 51 *Z. dhumnades* eggs (12.85 ± 0.25 g) from a commercial snake ranch and 46 freshly laid *C. serpentina* eggs (10.05 ± 0.27 g) and 59 *O. sinensis* eggs (11.33 ± 0.24 g) from a turtle farm in Zhejiang, China. We also obtained 30 *A. sinensis* eggs (35.30 ± 0.33 g) from the Chinese Alligator National Nature Reserve in Anhui, China. In August 2013, we obtained 63 fertilized quail eggs (11.16 ± 0.53 g), 54 fowl eggs (53.16 ± 0.57

g), 50 pigeon eggs (21.94 ± 0.59 g), and 47 duck eggs (95.74 ± 0.61 g) from a commercial bird ranch in Beijing. Lizard, snake, turtle, and small bird (quail and pigeon) eggs were incubated individually in 100-ml jars (with a diameter of 7 mm) containing moist vermiculite (-220 kPa). Alligator and large bird (fowl and duck) eggs were incubated individually in 500-ml jars (with a diameter of 12 mm) containing moist vermiculite. We initiated the thermoregulatory behavior trials when the embryos were approximately 30% through the total development. Given that the embryos of lizard and snake eggs have already completed about one-third of total development by the time of oviposition (Andrews 2004), the trials of these eggs began immediately after they were placed in 28°C incubators for a 2-day acclimation period. The turtle, crocodile, and bird eggs were placed in incubators and incubated at constant temperatures of 28°, 30°, or 38°C for ~25% of the total incubation period prior to thermoregulatory behavior trials. We designed the thermoregulatory behavior experiments for individual species on the basis of the optimal incubation temperatures for each lineage of reptiles and birds. The optimal and overly high temperatures for embryonic development are ~28°C and >32°C in the two lizards (Du and Ji 2006; Qu et al. 2011b), three snakes (Ji et al. 2001; Lin et al. 2010), and two turtles (Steyermark et al. 2008; Du et al. 2010). The optimal temperature for embryonic development is 31°C for the Chinese alligator (Liang and Pan 1990) and ~38°C for the four birds (Deeming and Ferguson 1991).

Accordingly, lizard, snake, and turtle eggs were randomly assigned to three treatments: control (constant temperature of 28°C), warm (lateral heating to 30°C), and hot (lateral heating to 34°C), directed toward the pointed ends of eggs. Alligator and bird eggs were randomly assigned to two treatments: control (a constant temperature of 30°C for alligator eggs or 38°C for bird eggs) and warm (lateral heating to 33.5°C for alligator eggs or 39.5°C for bird eggs). The eggs were heated using 75-W electronic heating mats (500 × 450 mm), with the distance between the jars and the heating mats adjusted to obtain the desired egg-surface temperatures at the end of the egg that was closest to the heat source. For reptile eggs, we quantified the position (midpoint) of the embryo by candling at the beginning of the experiment, normally close to the midline of the egg, and again after 5 days of incubation to determine the distance (± 0.01 mm, using Mitutoyo calipers [Mitutoyo, Kanagawa, Japan]) that the embryos had shifted from their original position along the long axis of the egg. For lizard and snake eggs, we scored the embryo's midpoint as the point where the umbilical cord connects to the embryo because this is an obvious morphological feature in squamate embryos (fig. 1). In addition, we followed the procedure reported by Du et al. (2011) to measure the em-

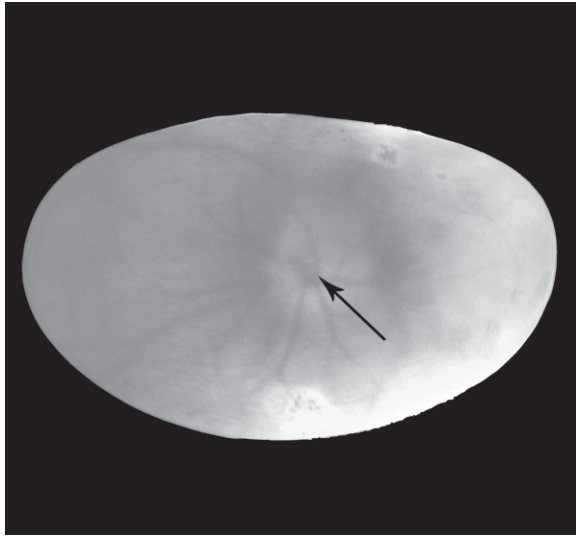


Figure 1: Position of an embryo of the checkered keelback snake *Xenochrophis piscator* inside its egg, as observed by candling. The arrow indicates where the umbilical cord connects to the embryo; this point was used to quantify the embryo's body position in squamates.

bryonic movement in the round turtle eggs of *C. serpentina* and the procedure reported by Zhao et al. (2013) to measure embryonic movement in the oval-shaped eggs of other turtles and alligators. For bird eggs, we sampled the eggs and removed part of the eggshell to locate the embryo beneath at the beginning of the experiment and after 4 days of incubation, because the thick eggshell did not allow us to see the embryos by candling. The distance of deviation (from the midpoint of the embryo to the midpoint of its egg) was used as our measure of the position of an embryo.

Data Analysis

We used Kruskal-Wallis ANOVAs and Mann-Whitney *U*-tests to analyze the effect of heat source on embryonic positions in reptile and bird eggs. The post hoc Nemenyi test was used to identify significant differences in the embryonic movement among treatments. Data are presented as mean \pm SE. Data underlying figures 2 and 3 are deposited in the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.830d7> (Li et al. 2014).

Results

Lizard embryos did not move inside their eggs. All embryos in the control and lateral heating treatments remained near the midpoint of eggs in *Takydromus septentrionalis* ($H_{2,35} =$

2.06, $P = .36$; fig. 2A) and *Phrynocephalus frontalis* ($H_{2,24} = 2.29$, $P = .32$; fig. 2B). In contrast, the embryos of snakes, turtles, and alligators moved inside their eggs to track the heat source. During the 5 days of experiments, embryos in the constant temperature treatment remained near the midpoint of eggs, embryos in the warm lateral heating treatment moved closer to the heat source, and embryos in the hot lateral heating treatment moved away from the heat source in three snake species, *Xenochrophis piscator* ($H_{2,30} = 18.94$, $P = .0001$; fig. 2C), *Elaphe bimaculata* ($H_{2,24} = 13.21$, $P < .01$; fig. 2D), and *Zaocys dhumnades* ($H_{2,51} = 37.69$, $P < .0001$; fig. 2E); two turtle species, *Chelydra serpentina* ($H_{2,46} = 13.86$, $P = .001$; fig. 2F) and *Ocadia sinensis* ($H_{2,59} = 10.79$, $P = .005$; fig. 2G); and one species of crocodilian, *Alligator sinensis* ($Z = 2.97$, $P = .003$, $n = 30$; fig. 2H).

Bird embryos also showed thermoregulatory behavior, seeking warm regions inside the egg. During the 4 days of experiments, embryos exposed to the constant temperature treatment remained near the midpoint of eggs in *Coturnix coturnix* ($Z = 0.89$, $P = .38$, $n = 41$), *Gallus gallus domesticus* ($Z = 0.70$, $P = .48$, $n = 36$), *Columba livia domestica* ($Z = 0.25$, $P = .80$, $n = 33$), *Anas platyrhynchos domestica* ($Z = 0.98$, $P = .33$, $n = 30$), whereas embryos in the lateral heating treatment moved to the heat source in *C. coturnix* ($Z = 3.40$, $P < .001$, $n = 42$), *G. gallus domesticus* ($Z = 2.92$, $P < .01$, $n = 38$), *C. livia domestica* ($Z = 2.46$, $P = .01$, $n = 34$), and *A. platyrhynchos domestica* ($Z = 3.67$, $P < .001$, $n = 34$; fig. 3).

Discussion

The ability of embryos to reposition themselves within the egg to seek warm regions or avoid dangerously high temperatures occurs in the snakes, crocodile, and birds, as well as in the turtle species in which this phenomenon was originally described (Du et al. 2011; Zhao et al. 2013). The wide phylogenetic separation between squamates and chelonians (Chiari et al. 2012; Crawford et al. 2012; Bradley Shaffer et al. 2013) suggests that the ability of reptilian embryos to move about within their eggs may be ancestral in these major clades of amniote vertebrates. This hypothesis is supported by the substantial differences among our study taxa with respect to characteristics such as eggshell and embryo morphology, the degree of embryogenesis at the oviposition, and nest-site environments (Zhao and Adler 1993; Deeming 2004). Further studies on embryonic thermoregulation in other squamate, chelonian, and crocodilian taxa would be of great interest, as would research on the embryos of tuataras. The latter study would not only clarify interspecific variation in embryonic capacity for embryonic thermoregulation but also track the origin of this behavior in tuataras, the most ancient sur-

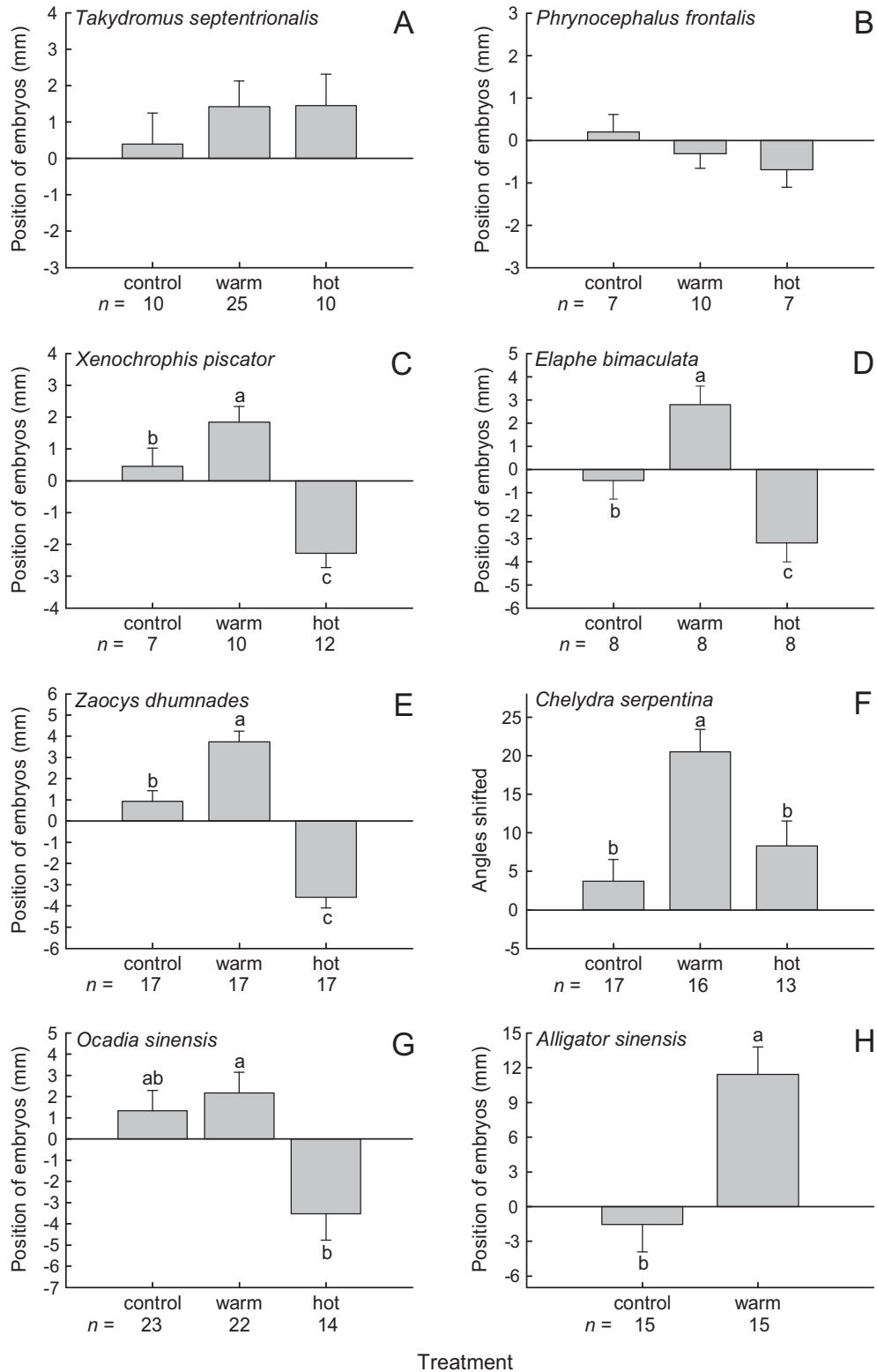


Figure 2: Shifts in the position of reptile embryos inside eggs that were incubated at constant temperatures or heated from the side. Changes in an embryo's location within its oval-shaped egg are shown by the distance shift of the embryo's body from the midpoint of the egg along the long axis. Means with different letters above their error bars were statistically different (post hoc Nemenyi test). Control: constant temperature of 28°C (or 30°C for alligator eggs); warm: lateral heating to 30°C (or 33.5°C for alligator eggs); and hot: lateral heating to 34°C.

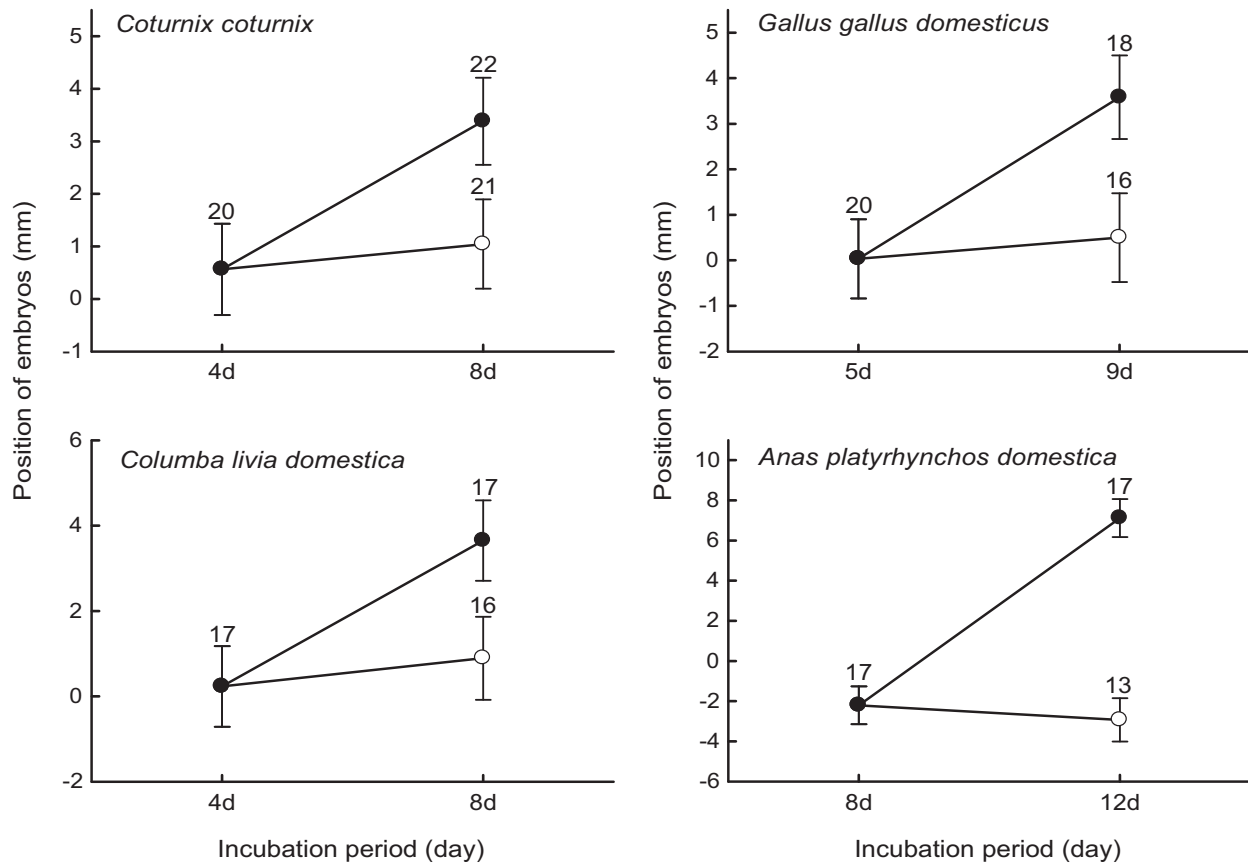


Figure 3: Shifts in the position of bird embryos inside eggs that were incubated at constant temperatures or heated from the side. Changes in an embryo's location within the egg are shown by the distance shift of the embryo's body from the midpoint of the egg along the long axis. The embryos moved to the heat source when heated laterally. Open circle: constant temperature of 38°C; filled circle: lateral heating to 39.5°C. The trials were initiated at 4, 5, or 8 days since incubation, depending on the incubation period of each species (17 days for *Coturnix coturnix*, 21 days for *Gallus gallus domesticus*, 18 days for *Columba livia domestica*, and 28 days for *Anas platyrhynchos domestica*), such that the trials began at ~25% (ranging from 22% to 28%) through the total incubation period in all species.

living group of reptiles. Nonetheless, our documentation of this capacity in a diverse array of reptiles and birds suggests that the embryos of many oviparous amniotes are able to reposition themselves to exploit thermal heterogeneity within the egg. Thus, the organism's ability to regulate its own body temperature may be present, at least to some degree, throughout most of an organism's life rather than arising posthatching in reptiles and birds (Huey 1982).

Notably, however, we did not detect thermoregulatory behavior in embryos inside the small eggs of lizards, consistent with our prediction that embryos will show heat-seeking behavior only in large eggs. The absence of thermoregulatory behavior in lizards may be attributable to phylogenetic constraint or simply because small eggs probably provide little opportunities for thermoregulatory behavior. To clarify this issue, we need future studies on

embryonic thermoregulation of lizard taxa that produce large eggs (e.g., varanid lizards) as well as those of turtles, snakes, and birds that produce very small eggs (e.g., *Sternotherus odoratus*, *Leptotyphlops blanfordii*, and *Chlorostilbon canivetii*).

Reptile embryos are often exposed to dramatically fluctuating temperatures during development, which can substantially affect developmental rates and hatchling phenotypes (e.g., body size, locomotion, and sex; Deeming 2004). Thermoregulatory behavior by embryos thus might enhance hatching success and offspring quality (Zhao et al. 2013). However, the potential fitness benefits of thermoregulatory behavior in avian embryos are less obvious, because parental brooding maintains high and relatively constant temperatures for embryonic development in birds. If thermoregulatory behavior is unnecessary, then why do avian embryos move to seek heat within an egg?

The answer lies in the heat source provided by the brooding parents during incubation. Heat for avian egg incubation mainly comes from the brood patch of the parent (Lea and Klandorf 2002), which may deliver heat to only a part of an egg, creating a thermal gradient inside a nest or even within an egg (Caldwell and Cornwell 1975; Turner 2002; Boulton and Cassey 2012). Eggs at the center of a bird nest are likely to be warmer than those at the periphery of the nest (Boulton and Cassey 2012) due to the three-dimensional temperature fields created by differential warming (brood-patch contact) and cooling (environmental contact; Turner 2002). In addition, a brooding parent bird cannot cover all of the peripheral eggs with her brood patch (especially in a large clutch), creating thermal gradients within these eggs (Niizuma et al. 2005). Embryos in peripheral eggs may be able to warm themselves by moving to the end of the egg closest to the brood patch and therefore obtain a fitness benefit by thermoregulatory behavior. Unlike bird eggs, reptile eggs mainly receive heat from the sun-heated ground surface, with warmer eggs in the upper layer rather than the bottom layer of the nest (Thompson 1988).

Together with our previous work (Du et al. 2011; Zhao et al. 2013), the current study clarifies the generality of thermoregulatory behavior in reptile and bird embryos. The inference that this behavior is ecologically important (Zhao et al. 2013) needs to be tested with field experiments. Such studies could measure the thermal environment of reptile and bird eggs within field nests and evaluate the ecological consequences of embryonic thermoregulation. Such studies face significant methodological difficulties but are crucial for understanding the adaptive significance of embryonic thermoregulation. Future work also could usefully address the neuromuscular mechanisms underlying embryonic thermoregulation. For example, the ability of bird embryos to exhibit hypothalamic neuronal thermosensitivity (Tzschentke and Basta 2000; Tzschentke et al. 2004) may play an important role in embryonic thermoregulation.

Our results mesh well with an emerging shift in paradigms about the biology of embryos. Embryos of many species are more active than we thought and are capable of complex behaviors that can affect their own destinies. For example, encapsulated embryos of pond snails display a cilia-driven rotation behavior that is influenced by temperature and oxygen concentration; this behavior may enhance oxygen delivery to embryos within egg capsules (Shartau et al. 2010). In many species from insects to birds (and including turtles), synchronous hatching of embryos occurs, which may facilitate group emergence from the nest, reducing the risk of predation (Colbert et al. 2010). Amphibian and turtle embryos use immediate, local information on the risk of mortality to make instantaneous

behavioral decisions about hatching (Warkentin 1995). Such examples suggest that embryos of a diverse array of animal taxa may be capable not only of detecting fitness-relevant cues in their nest environment but also of responding to such cues behaviorally in ways that enhance organismal fitness. The lack of published evidence for such abilities may be due not to their absence but to a lack of investigation.

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