The cost of behavioral thermoregulation is well recognized in the posthatching stage of ectothermic vertebrates. However, thermoregulatory behavior was discovered only recently in turtle embryos, and the cost of this interesting behavior remains unknown. We manipulated the intensity of thermoregulatory behavior in turtle embryos (*Pelodiscus sinensis*) and determined the hatching success, body mass, and righting response to assess the energetic cost associated with this behavior. Hatchlings from embryos that had experienced intensive behavioral thermoregulation were smaller and contained less energy than those from the control group, which indicates that behavioral thermoregulation by turtle embryos incurs energetic costs. Nonetheless, the smaller hatchings did not exhibit a lower hatching success or a slower righting response, suggesting that the cost incurred by behavioral thermoregulation is relatively low in turtle embryos.

**Key words:** ectothermic, embryonic development, hatching success, righting response, thermal adaptation, thermoregulatory behavior.

**INTRODUCTION**

Thermoregulatory behavior is widespread in the posthatching stage of ectothermic vertebrates, and the benefits and costs of this behavior have been well known for decades (e.g., Huey and Slatkin 1976; Angilletta and Werner 1998; Blosin-Demers and Weatherhead 2001; Herczeg et al. 2008). However, behavioral thermoregulation was assumed not to occur in animal embryos because embryos appear to be trapped inside an immobile egg with little opportunity to regulate their own temperature. In contrast, recent studies demonstrated that turtle embryos can move inside the egg to exploit warmer regions, suggesting that behavioral thermoregulation is not restricted to the posthatching stage, but may also be an important tactic during the embryonic stages of the life cycle (Du et al. 2011; Zhao et al. 2013). Nonetheless, the cost of this interesting behavior remains unknown in embryos. The apparent cost of behavioral thermoregulation by embryos is the energetic cost associated with embryonic locomotion.

Ideally, a comparison between embryos with or without thermoregulatory behavior would be perfect for determining the cost of thermoregulation. However, constraining the thermoregulatory behavior of an embryo is operationally challenging, if not impossible. Given that turtle embryos move inside an egg to follow the heat source, the intensity of the thermoregulatory behavior can be manipulated by moving the position of the heat source; thereby, providing an alternative method of evaluating the cost of thermoregulation (Du et al. 2011). In this study, we manipulated the intensity of thermoregulatory behavior in embryos of the Chinese soft-shelled turtle (*Pelodiscus sinensis*) and determined the hatching success, body mass, and righting response of hatchlings to assess the energetic cost associated with this behavior.

Given that the total energy contained within an egg is finite, any increase in energy expense during embryonic development (e.g., more energy spent during incubation due to behavioral thermoregulation) may lead to a decrease in energy conversion into hatchlings. We thus hypothesized that hatchlings from embryos that experienced intensive behavioral thermoregulation would be smaller than those from embryos in the control group, if this behavior incurs energetic costs. Moreover, hatchlings would have a lower hatching success as well as a slower righting response from embryos that experienced intensive behavioral thermoregulation than those from embryos in the control group, if the cost of behavioral thermoregulation is high.

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MATERIALS AND METHODS

Egg collection and incubation

The Chinese soft-shelled turtle (P. sinensis) lays clutches of small rigid-shelled eggs from May to August in eastern China. We collected 92 freshly laid and fertilized eggs from a private farm in Hangzhou City, Zhejiang Province, China. The eggs were weighed (± 1mg) using an electronic balance (Mettler Toledo AB133-S) (average egg mass = 2.751g) and incubated individually in 80-mL jars containing moist vermiculite (~220 kPa) in a temperature-controlled room at 26 °C. The jars were assigned randomly to 2 thermal treatments with different behavioral thermoregulation regimes for the embryos. In the control group, the jars were heated to 30 °C from one direction by electronic heating mats (75 W, 500 × 450 mm).

In the thermoregulation group, the jars were heated to 30 °C, but heating was initiated with all the heating mats on one side, before switching them to the opposite side every 3 days. In this experimental design, the embryos experienced similar thermal environments (which was verified by the similar incubation period with the 2 treatments; see Results), but different intensities of behavioral thermoregulation because the embryos in the thermoregulation group moved more frequently than those in the control group.

Hatching success, incubation period, and hatchling traits

After emergence, hatchlings were collected and weighed (± 0.001 g). The righting responses, an important fitness index of turtles (Delmas et al. 2007), was assessed in a temperature-controlled room at 28 °C. Each turtle was placed upside down in an open area (250 × 200 × 40 mm) and tested 5 times. A digital camera (Sony DCR-SR220E) was used to record the trials. The time required by a turtle to right itself after it began to move (“time to right” sensu Delmas et al. 2007) was collected a posteriori using the videotapes. We defined all individuals that managed to right in at least one test as the successful in righting response, and those individuals that failed in all the 5 tests as the unsuccessful one. Consequently, in each treatment, we had the number of both successful and unsuccessful individuals in righting response, which enabled us to use a contingency table to test the influence of experimental treatment on the righting success.

After measurement, 16 turtles from the control group and 18 from the thermoregulation group were euthanized and separated into the carcass and residual yolk. These components of hatchlings were dried in an oven (55 °C) to a constant mass, weighed, and preserved for later determinations of energy density using an adiabatic bomb calorimeter (Parr 6300 Calorimeter; Parr Instrument Co., Moline, IL). The energy density was expressed as kJ per gram of dry body mass. The energy content of hatchlings was calculated as hatchling wet mass × (1 – water content) × energy density.

Data analysis

We used contingency tables to determine the effect of behavioral thermoregulation by embryos on the hatching success and righting success of hatchlings, and one-way ANOVA to test the effect of behavioral thermoregulation on the incubation period, and body components, energy density, and righting response of hatchlings. One-way ANCOVA (analysis of covariance) was used to test the effect of behavioral thermoregulation by embryos on the hatchling mass and energy content using the initial egg mass as a covariate. Data are presented as means ± 1 standard error and significance differences were assumed if P < 0.05.

RESULTS

The hatching success of eggs did not differ significantly between the thermoregulation (40/49, 81.6%) and control (36/43, 83.7%) groups (X² = 0.01, df = 1, P = 0.94). The intensity of behavioral thermoregulation also did not affect the incubation period (F1,175 = 0.06, P = 0.81), that is, hatchlings from the thermoregulated eggs emerged at 57.1 ± 0.5 days (n = 40), whereas those from the control eggs emerged at 57.3 ± 0.6 days (n = 36).

Behavioral thermoregulation by embryos significantly affected the hatchling body mass (Table 1), and hatchlings generated from the thermoregulation group were smaller than those from the control group (Figure 1a). The water content and energy density of the hatchlings did not differ between the 2 groups (Table 2), but hatchlings from the thermoregulated group contained less energy (0.56 kJ, 7.6% of total energy) than those from the control group (Table 1; Figure 1b). In contrast to the effect on body mass, behavioral thermoregulation by embryos did not affect the energy allocation among the body components of the hatchlings (Table 2).

A total of 5 hatchlings could not right themselves within 5 min, being 1 from the control group (n = 36) and 4 from the thermoregulation group (n = 40). Behavioral thermoregulation by embryos did not affect the righting success rate (X² = 1.61, df = 1, P = 0.21) or the righting time of hatchlings (thermoregulation: 0.70 ± 0.05 s, n = 36 vs. control: 0.76 ± 0.05 s, n = 35; F1,69 = 0.78, P = 0.38).

DISCUSSION

As found in the posthatching stage of ectothermic vertebrates, our study first demonstrated that behavioral thermoregulation by turtle embryos incurred energetic costs. The energetic cost led to the generation of smaller hatchlings from embryos that experienced intensive behavioral thermoregulation, which confirmed our first hypothesis. Nonetheless, the smaller hatchlings did not exhibit a lower hatching success or any decreased performance in terms of their righting response, which refutes our second hypothesis.

<table>
<thead>
<tr>
<th>Table 1</th>
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<tbody>
<tr>
<td>Result of ANCOVA (with egg mass as a covariate) on the body mass and energy contents of hatchling soft-shelled turtles (Pelodiscus sinensis) incubated at different treatments of behavioral thermoregulation</td>
</tr>
<tr>
<td>Hatchling traits</td>
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<tr>
<td>Body mass</td>
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Figure 1
Body mass (BM) and energy contents (EC) of hatchling soft-shelled turtles (*Pelodiscus sinensis*) incubated at different treatments of behavioral thermoregulation. Hatchlings from the thermoregulation group were smaller and contained less energy than those from the control group, when egg mass (EM) was controlled. For BM, the regression equations of control and thermoregulation group are BM = 0.42 EM + 0.87 and BM = 0.53 EM + 0.47, respectively (a). For EC, the regression equations of control and thermoregulation group are BM = 1.53 EM + 3.14 and BM = 1.86 EM + 1.66, respectively (b).

Table 2
The energy density and body components of hatchling soft-shelled turtles (*Pelodiscus sinensis*) incubated at different treatments of behavioral thermoregulation

<table>
<thead>
<tr>
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<th>Thermoregulation (<em>n</em> = 18)</th>
<th>Control (<em>n</em> = 16)</th>
<th>Anova</th>
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</thead>
<tbody>
<tr>
<td>Hatchling mass (g)</td>
<td>1.901 ± 0.053</td>
<td>1.955 ± 0.060</td>
<td><em>F</em>₁,₁₃ = 0.489, <em>P</em> = 0.49</td>
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<td>Water content (%)</td>
<td>83.13 ± 0.43</td>
<td>82.21 ± 0.45</td>
<td><em>F</em>₁,₁₃ = 2.161, <em>P</em> = 0.15</td>
</tr>
<tr>
<td>Energy density (kJ/g)</td>
<td>20.64 ± 0.22</td>
<td>20.22 ± 0.24</td>
<td><em>F</em>₁,₁₃ = 1.445, <em>P</em> = 0.24</td>
</tr>
<tr>
<td>Carcass wet mass (g)</td>
<td>1.895 ± 0.057</td>
<td>1.916 ± 0.063</td>
<td><em>F</em>₁,₁₃ = 0.064, <em>P</em> = 0.80</td>
</tr>
<tr>
<td>Carcass dry mass (g)</td>
<td>0.318 ± 0.012</td>
<td>0.345 ± 0.014</td>
<td><em>F</em>₁,₁₃ = 2.421, <em>P</em> = 0.13</td>
</tr>
<tr>
<td>Fresh residual yolk (g)</td>
<td>0.010 ± 0.002</td>
<td>0.008 ± 0.002</td>
<td><em>F</em>₁,₁₃ = 0.192, <em>P</em> = 0.66</td>
</tr>
<tr>
<td>Dry residual yolk (g)</td>
<td>0.003 ± 0.001</td>
<td>0.003 ± 0.001</td>
<td><em>F</em>₁,₁₃ = 0.034, <em>P</em> = 0.86</td>
</tr>
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</table>

Data are expressed as mean ± standard error. The energy density of hatchlings is calculated as energy per gram of dry body mass.
The cost of behavioral thermoregulation differs between embryos and free-living animals in the posthatching stage, although this behavior is generally beneficial in both life stages. After hatching, the animals may behaviorally regulate their body temperature by changing the timing of their daily and seasonal activities, and by selecting optimal thermal habitats (Stevenson 1985). The increased energy expenditure due to movement during thermoregulation may reduce the growth rate of hatchling lizards (Brewster et al. 2013). In addition to the energetic cost of locomotion during behavioral thermoregulation, the costs associated with the time and energy directed from other activities such as foraging, avoiding predation, and mate searching are also common during the posthatching stage (Huey and Slatkin 1976; Herczeg et al. 2008).

Unlike animals at the posthatching stage, embryos had a guaranteed food supply (in the yolk) to fuel their development until hatching (Ar et al. 2004), and they engaged in none of the social activities found in the posthatching stage. Therefore, the costs of directing time and energy from other activities to thermoregulation should be regarded as minimum for the embryos. However, the heat source in our experiment shifted every 3 days, so turtles had to readjust their position 29 times (incubation period divided by 3) during incubation, and this behavior reduced their energy content about 290 J as we calculated. The energetic cost of behavioral thermoregulation by embryos probably may have negative effects on hatching fitness. In fact, the embryos produced relatively small hatchlings due to the energetic costs associated with behavioral thermoregulation, but these hatchlings did not exhibit inferior functional performance (righting response). Previous studies also confirmed that the body mass at hatching does not affect the locomotor performance or long-term posthatching growth in this species (Du and Ji 2003; Ji et al. 2003) as well as in some other turtles (Rhen and Lang 1995; Congdon et al. 1999). Therefore, the energetic cost associated with locomotion is probably the main cost incurred by behavioral thermoregulation in embryos of ectothermic vertebrates, and the cost of behavioral thermoregulation is lower for embryos than posthatching individuals.

The costs and benefits of behavioral thermoregulation by embryos are probably complex in nature due to the diversity of thermal environments in nests, although these data are currently limited. The cost–benefit model of thermoregulation suggests that ectothermic animals such as lizards should modify their thermoregulatory strategy not only based on the costs and benefits associated with this behavior but also according to the thermal quality of the environment (Huey and Slatkin 1976). A number of studies of animals in the posthatching stage have verified the predictions of this model (Grant and Dunham 1988; Herczeg et al. 2008). If the cost–benefit model is also applicable to embryos, exploring the implications of this model would be insightful. For example, the thermal conditions of embryos may vary geographically (e.g., eggs in southern vs. northern localities) and even over small spatial scales inside a nest (e.g., eggs at the top vs. bottom of the nest) (Thompson 1988; Ackerman and Lott 2004). Thus, it would be of great interest to determine whether the cost of behavioral thermoregulation differs among embryos in various thermal conditions.

**FUNDING**

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