Does the hydric environment affect the incubation of small rigid-shelled turtle eggs?

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ABSTRACT

Hydric environments are hypothesized to have minor effects on the embryonic development of rigid-shelled turtle eggs due to the low water permeability of the eggshell. However, the water reserve in the eggs may still influence their resistance to environmentally induced dehydration. We incubated rigid-shelled turtle eggs (*Pelodiscus sinensis*) on different moist substrates (from -12 to -750 kPa) to test the hypothesis that small rigid-shelled eggs would be sensitive to hydric environments. The hydric treatment significantly affected the incubation period, with eggs incubated in the moistest and driest substrates taking longer to hatch than those on the medium-moisture substrates. Hatching success was slightly lower for eggs incubated in dry conditions than those in wet conditions, but the difference was not statistically significant. The heart rates of early embryos were lower on moist substrates than those on dry substrates, but this difference disappeared in late embryos. Hatchlings from the moistest substrate were larger (in carapace length and width) and heavier than those from drier substrates. However, the dry body mass of the hatchlings did not differ among the hydric treatments. The functional performance (righting response) of the hatchlings was affected by the hydric environment. The time to right was shorter for the hatchlings from the substrate of -12 kPa than those from -220 kPa. These results are consistent with the hypothesis that the hydric environment may significantly affect developing embryos and the resulting hatchlings in turtle species, such as *P. sinensis*, with small rigid-shelled eggs.

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1. Introduction

The phenotypes of offspring are determined not only by the genetic basis derived from their parents but also by environmental factors experienced during development (Via and Lande 1985). The phenotypic variation induced by the developmental environment (e.g., developmental plasticity) may impose selection on a number of ecological processes in both mother and offspring, e.g., female nest-site selection and hatchling survival (Via et al. 1995; Birchard and Deeming 2004; Shine 2004). Developmental plasticity is especially prevalent in oviparous species, whose embryogenesis is completed outside of the mother's body, and is thus strongly affected by environmental factors (Deeming 2004). Therefore, the consideration of developmental plasticity is critical to an understanding of the adaptation of these species to their environment.

Developmental plasticity in oviparous reptiles has attracted increasing attention from ecologists (See review by Shine 2004). Despite the large quantity of literature on the phenotypic responses of the embryonic development and hatchling traits of reptiles to environmental factors such as moisture (Deeming 2004), conclusions about hydric effects on egg incubation are not universal among species. Significant effects are found in certain species of reptiles but not in others (Packard 1999; Ji and Du 2001; Booth 2002; Shine and Brown 2002; Brown and Shine 2006). This among-species discrepancy may reflect interspecific variation in the embryonic response to the hydric environment due to the diversity of egg traits such as the eggshell and the water content. Oviparous reptiles produce eggs with either a rigid or a flexible shell (Packard and DeMarco 1991). The difference in water permeability between rigid and flexible shells is profound due to the differences in physical properties between these types of shells. A rigid shell has a thick mineral layer and is relatively impermeable to water vapor, whereas a flexible shell has a thin mineral layer and is relatively permeable to water vapor (Packard 1991; Thompson and Speake 2004). Consequently, embryonic development in rigid-shelled eggs relies primarily on the availability of sufficient water in the eggs at oviposition and is generally insensitive to the external hydric environment (Packard 1999; Booth 2002; Booth and Yu 2009), whereas embryonic development in flexible-shelled eggs relies primarily on the water absorbed during incubation and is relatively sensitive to the hydric environment (Ji and Braña 1999; Du and Shine 2008). Nevertheless, the water content of eggs would affect the sensitivity of embryonic development of rigid-shelled eggs to external hydric environments, with small eggs less resistant to environmentally induced dehydration than large eggs (Packard 1999). In this case, one might expect that small rigid-shelled eggs would tend to be

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sensitive to the hydric environment. However, this hypothesis has not been tested empirically.

Traditional egg-incubation experiments focus on the effects of incubation environments on hatchling traits, with less emphasis on embryos *per se* due to logistical difficulties. Recent advances in methodology provide an opportunity to elucidate the physiological basis of environmental effects on developing embryos (Du et al. 2010b). An interesting aspect of hydric effects on reptilian eggs is that eggs incubated on wet substrates tend to take longer to hatch (Packard 1999; Brown and Shine 2006). The physiological mechanisms underlying the variation in incubation period have not been determined but may be related to (1) different rates of embryonic development, i.e., embryos on a wet substrate develop more slowly than those on a dry substrate; and (2) a facultative shift in the degree of embryogenesis completed prior to hatching (Shine and Olsson 2003; Du et al. 2010b). Given that heart size, heart rate, and stroke volume are related to cardiac output, an important determinant of the rate of embryonic development (Kam 1993; Burggren and Keller 1997; Pearson et al. 2000), differences in heart rate or heart size would be expected if the embryos have different developmental rates under various hydric treatments. Alternatively, a wet substrate could induce eggs to delay hatching because a longer period of embryonic development within the egg facilitates energy conversion from yolk to hatchling tissues (Booth and Yu 2009). If the embryos adopt this avenue of physiological regulation, eggs from a wet substrate would produce larger hatchlings with small residual yolk than their counterparts from a dry substrate.

In the present study, we experimentally incubated small rigid-shelled turtle eggs (*Pelodiscus sinensis*) at a range of substrate water contents (from -12 to -750 kPa) to quantify the hydric effects on embryonic heart rates, incubation periods, hatchling body sizes, heart mass, and performance. We applied this system to answer two questions. Are small rigid-shelled eggs sensitive to the hydric environment? Is the moisture-induced variation in the incubation period due to the different developmental rate of embryos (reflected by different heart rate) or the facultative shift in the relative amount of development that occurs within the egg (reflected by the ratio of tissue to yolk in the hatchling)?

2. Materials and methods

2.1. Egg collection and incubation

In May 2009, a total of 77 freshly laid *P. sinensis* eggs (fertilized eggs with a white patch on the shell surface, average egg mass $= 2.9$ g) were collected from a private farm in Hangzhou city of Zhejiang Province, China. The eggs were weighed to ± 1 mg using an electronic balance (Mettler Toledo AB135-S) and individually numbered with a pencil on the eggshell for later identification. Because the maternity of the eggs was unknown, we randomly assigned these eggs to different treatments to minimize maternal effects. The eggs were individually incubated (half buried) in a 60-mL jar filled with vermiculite at four different levels of moisture: -12 (2 g water/1 g vermiculite), -220 (1 g water/1 g vermiculite), -500 (0.63 g water/1 g vermiculite), and -750 (0.38 g water/1 g vermiculite) kPa, following an empirically derived calibration curve linking the water potential to the mass ratio of water to dry vermiculite (M. Thompson, unpublished data) and previous studies (Ji and Braña 1999). The jars were covered with plastic wrap (sealed with a rubberband) and then placed in an FPQ incubator (Ningbo Life Science and Technology Ltd, China) at a constant temperature of 28 °C, an optimal incubation temperature for this species (Du and Ji 2003). Each jar with moist vermiculite was weighed at the beginning of the experiment and was then reweighed twice a week after removing the egg. Water was added to balance the water loss due to evaporation and absorption by the eggs and thereby maintain the water potential of the incubation substrate at a relatively constant level.

In May 2012, we further determined the influence of hydric condition on water exchange of *P. sinensis* eggs during incubation. A total of 102 freshly laid eggs (average egg mass $= 4.3$ g) were incubated at the four hydric treatments of -12, -220, -500, and -750 kPa. We weighed the eggs once a week to estimate the water exchange during incubation. The setup and procedure of egg incubation in 2012 were the same as those in 2009.

2.2. Heart rate of embryos

We measured the heart rates of embryos twice during incubation (days 10 and 30). The heart rates (beats per minute, bpm) were measured at 28 °C using an infrared heart rate monitor (Buddy system, Avian Biotech; see detailed procedures in Du et al. 2009 and Du et al., 2010b).

2.3. Incubation period, hatchling morphology and performance

Toward the end of incubation, the jars were monitored once a day for newly emerging hatchlings. The days elapsed between the beginning of incubation and the emergence of the hatchlings was recorded as the incubation period. After emergence, the hatchlings were maintained in the cup until the yolk had been entirely absorbed. The turtles were then weighed and individually kept in cages placed in a temperature-controlled room at 28 $\pm$ 1 °C and with a 12-h light/12-h dark cycle. One week later, we assessed the righting response, a fitness index in freshwater turtles, in the temperature-controlled room. Each turtle was placed upside down in an open area ($250 \times 200 \times 40$ mm). A digital camera (SONY, DCR-SR220E) was used to record the trials. Each turtle was tested five times, and the time to right (defined as the time required for a turtle to right itself after it began to move; Delmas et al., 2007) was collected *a posteriori* from the videotapes. After that the turtles were euthanized, and oven dried at 65 °C for 48 h to determine hatchling dry mass.

2.4. Data analysis

The normality of distributions and the homogeneity of variances were tested with a Kolmogorov–Smirnov test and a Bartlett's test, respectively. We conducted a G test to determine the effect of the hydric environment on hatching success. An analysis of variance (ANOVA) and a repeated-measures ANOVA were used to determine the influence of the hydric environment on the incubation period and heart rates of embryos, and water exchange during incubation. An analysis of covariance (ANCOVA) or MANCOVA, with initial egg mass or hatchling carapace length as the covariate, was used to analyze hydric effects on the body size and righting response of hatchlings. A Tukey post hoc multiple comparisons test was used to detect differences among treatments.

3. Results

During incubation, eggs incubated at -12 kPa gained a small amount of water, whereas eggs incubated at drier conditions from -220 kPa to -750 kPa lost water ($F_{18,588} = 24.11$, $P < 0.0001$). Water loss by the eggs increased as substrate moisture decreased (Fig. 1).

Hatching success was not affected by the hydric treatment ($G = 0.55$, $df = 3$, $P > 0.05$), with 85%, 69%, 62% and 61% success for eggs incubated on substrates with a water potential (WP) of -12, -220, -500 and -750 kPa, respectively. The incubation period differed among different hydric treatments ($F_{3,47} = 3.99$, $P = 0.01$), with longer periods for hatchlings from the moistest (-12 kPa, 52.5 ± 0.5 d) and driest (-750 kPa, 52.1 ± 0.6 d) substrates than from the two medium substrates (-220 and -500 kPa, 50.5 ± 0.8 and 50.3 ± 0.6 d).
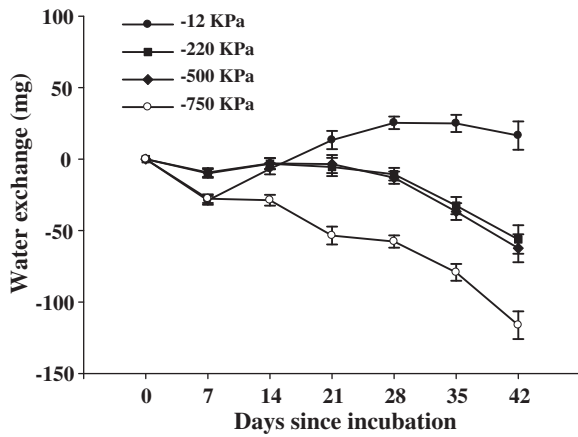
Fig 1. Water exchange of *Pelodiscus sinensis* eggs incubated at different hydric environments. Data are expressed as least square mean ± SE, adjusted for initial egg mass (standardized to 4.285 g) by ANCOVA with initial egg mass as the covariate. The sample sizes for the treatments of −12, −200, −500 and −750 kPa are 26, 26, 25 and 25, respectively.

A repeated-measures ANOVA indicated that heart rates were significantly affected by the hydric environment ($F_{3,47} = 14.77$, $P < 0.0001$) and differed at different stages of development ($F_{1,47} = 142.4$, $P < 0.0001$). Given the significant effect of the interaction between the hydric environment and developmental stage on heart rates ($F_{3,47} = 7.82$, $P < 0.001$), we further conducted separate ANOVAs on heart rates at each developmental stage. The hydric environment significantly affected the heart rates of embryos on day 10 ($F_{3,47} = 19.85$, $P < 0.0001$) but not on day 30 ($F_{3,47} = 0.59$, $P = 0.62$). The heart rates of early embryos from moist substrates were lower than those of embryos from dry substrates (Fig. 2).

The hatchlings from the moistest substrate were larger (in carapace length and width) than those from drier substrates ($F_{6,90} = 4.42$, $P < 0.001$; Fig. 3A, B). Similarly, the fresh body mass of the hatchlings from the moistest substrate was greater than that of their counterparts from drier substrates ($F_{3,46} = 21.79$, $P < 0.0001$; Fig. 3C). However, the dry body mass of the hatchlings did not differ among the hydric treatments ($F_{3,24} = 1.31$, $P = 0.29$; Fig. 3D). The functional performance (righting response) of the hatchlings was affected by the hydric environment ($F_{3,34} = 2.95$, $P < 0.05$). The time to right was shorter for hatchlings from the −12-kPa substrate than for those from the −220-kPa substrate (Fig. 4).
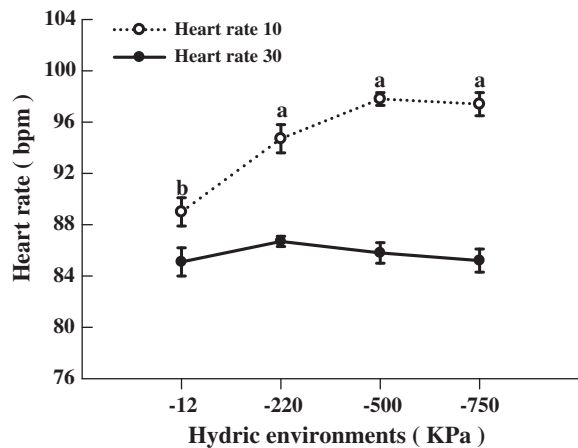

Fig. 2. Mean heart rates of turtle embryos (*Pelodiscus sinensis*) from eggs maintained in different hydric environments and measured at days 10 and 30 of incubation and a temperature of 28 °C. Data are expressed as mean ± SE. The sample sizes for the treatments of −12, −200, −500 and −750 kPa are 17, 11, 12, and 11, respectively.
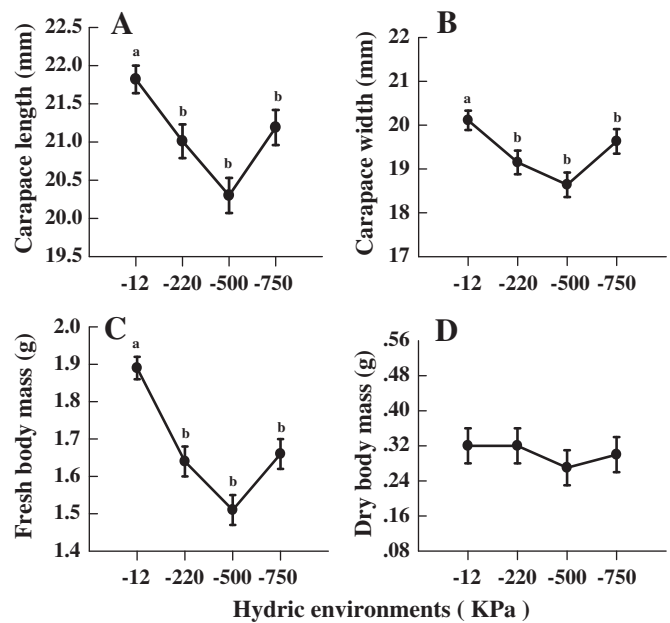

Fig 3. Carapace size (A, B) and body mass (C, D) of *P. sinensis* hatchlings from eggs incubated at different hydric environments. The sample sizes for the treatments of −12, −200, −500 and −750 kPa are 17, 11, 12, and 11, respectively, but the dry body mass was only determined for 7 individuals from each treatment. An ANCOVA was employed to test for the hydric effect, with initial egg mass as the covariate. Data are expressed as least square mean ± SE. Means with different superscripts differ significantly (Tukey test).

4. Discussion

The hydric environment significantly influenced the heart rates of embryos on day 10 of incubation, incubation period, and hatchling body size and self-righting time of the Chinese soft-shelled turtle, *P. sinensis*. These results are not consistent with the view that hydric environments have little effect on the incubation of rigid-shelled turtle eggs and the quality of the resulting hatchlings (Packard and Packard 1990; Packard 1999). Given that this view is based on evidence from relatively large eggs (>9 g), whereas the result of our study is based on small eggs (3–4 g), these results suggest that small rigid-shelled eggs are more sensitive to hydric environments than are large eggs.
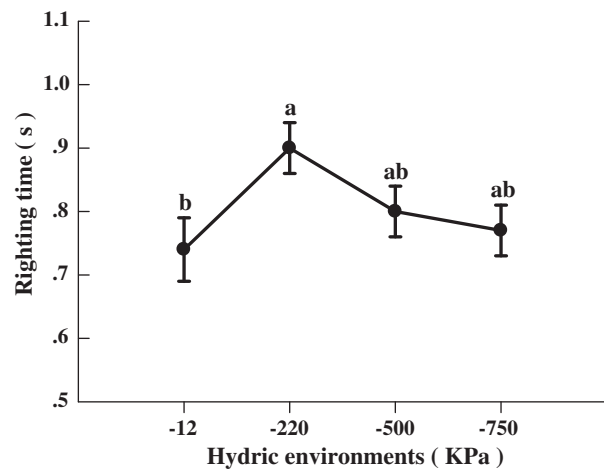

Fig 4. The influence of hydric environments during incubation on the righting time of hatchlings in turtles (*Pelodiscus sinensis*). An ANCOVA was employed to test for the hydric effect, with hatchling carapace length as the covariate. Data are expressed as least square mean ± SE. The sample sizes for the treatments of −12, −200, −500 and −750 kPa are 13, 7, 10, and 11, respectively.

Embryonic turtles in species with flexible-shelled eggs incubate longer in wet environments than in dry ones (Packard 1999). Our study indicates that this phenomenon also occurs in species with rigid-shelled eggs. The potential mechanisms underlying the longer incubation period in wet environments may be associated with a slower rate of embryonic development (Du et al. 2010b). However, this supposition contradicts the view that embryos in wet conditions have access to more water, have higher rates of metabolism, and grow faster than embryos in dry conditions (Miller and Packard 1992; Packard 1999). We found that heart rates were lower in wet environments than those in dry environments in early (day 10 of incubation) but not late (day 30 of incubation) embryos in *P. sinensis*. This finding suggests that wet environments could retard the differentiation rather than the growth of embryos because differentiation (e.g., organogenesis) occurs at early developmental stages and growth at late developmental stages in embryonic reptiles (Andrews 2004). However, it is noteworthy that some ecological factors may affect the relationship between heart rate and developmental rate in certain circumstances, despite this relationship is generally positive in reptiles (Du et al. 2010c; Du et al. 2011). For example, adverse environments such as hypoxia and dehydration may induce facultative increase in heart rate, but retard embryonic development (Packard and Packard 2002; Du et al. 2010a). Similarly, *P. sinensis* eggs incubated at the dry condition of −750 kPa had higher heart rate but longer incubation period. Alternatively, given that heart rate declined as embryos developed (Fig. 2), the lower heart rate in wet conditions could simply be a result of advanced development of the early embryos. If this explanation stands, other physiological mechanisms than slow development rate would be involved in the longer incubation period of eggs incubated at wet conditions. The longer incubation period in wet environments may be due to delayed hatching, because prolonged development within the egg facilitates more yolk being converted to hatchling tissues, and thus a larger hatchling (Booth and Yu 2009). Our results were consistent with the expectation that hatchlings from wet conditions would be larger than their counterparts from drier conditions (Fig. 3).

The hatchlings from the *P. sinensis* eggs incubated in wet conditions were not only larger and heavier but also showed better performance than those from eggs incubated in drier conditions. The effect of hydric effects on hatchling size has been evaluated both in turtles (Packard 1999; Booth 2002) and squamates (Ji and Braña 1999; Du and Shine 2008). Generally, the wet body mass of hatchlings from moist conditions is larger than that from dry conditions, but the dry body mass of hatchlings does not differ among the hydric treatments. This result suggests that the difference in wet body mass resulted from the difference in the water content of the hatchlings (Ji and Braña 1999; Packard 1999; this study). In contrast, the effects of the hydric environment on fitness-related traits (e.g., functional performance, growth and survival) have only been determined in a limited number of species of reptiles (Deeming 2004). Future studies of these characteristics would greatly extend our knowledge of the ways in which the hydric environment during incubation produces long-term effects on offspring fitness.

Note that despite the significant effects of the hydric environment on embryonic development and hatchling traits, *P. sinensis* embryos can still develop successfully into healthy hatchlings in extremely dry environments. Eggs incubated on the driest substrate (−750 kPa) did lose water during incubation (Fig. 1) and took longer to hatch, but eventually produced hatchlings with a body size and functional performance similar to those from medium-moist substrates. This finding suggests that *P. sinensis* embryos may modulate development in response to extremely dry conditions to generate healthy hatchlings. The resistance to a dry environment may make the eggs more capable of surviving the extreme conditions and thus be an adaptive strategy of embryos to unpredictable dry environments. The physical structures of the rigid eggshells may play an important role in this resistance, given that

there is no difference in the albumen (water content) between rigid and flexible chelonian eggs (Deeming and Whitfield 2010). The rigid-shelled eggs have a thick mineral layer to minimize the water loss from the eggs to the environment during incubation (Packard 1991; Thompson and Speake 2004). In our study, for example, water loss was less than 3 percent of the total egg mass even at the driest condition. By contrast, embryos in flexible-shelled eggs adopt different pathways to resist dry conditions due to the high water permeability of the eggshell. They are able to accumulate water reserves during a wet period (e.g., a rainy period) and retain that moisture as a buffer during subsequent dry periods (Brown and Shine 2005).

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