Do fluctuations in incubation temperature affect hatchling quality in the Chinese soft-shelled turtle *Pelodiscus sinensis*?

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ABSTRACT

The Chinese soft-shelled turtle *Pelodiscus sinensis* is the most common turtle species sold in China. In most turtle farms *P. sinensis* eggs are incubated at fluctuating temperatures. To examine if fluctuations in incubation temperature affect hatching success, incubation length and hatchling quality, we incubated *P. sinensis* eggs in five incubators: one set at 30 °C, two ramp-programmed at 30 $\pm$ 3 °C and $\pm$ 5 °C; the remaining two step-programmed at 30 $\pm$ 3 °C and $\pm$ 5 °C. Temperatures changed at 12 h (+) and 12 h (−) in the ramp-programmed incubators, and at 6 h intervals in an order of lowest–mean–highest–mean levels in the step-programmed incubators. Size-related (carapace length, carapace width and mass) rather than performance-related (swimming speed and early growth) hatchling phenotypes differed among the five treatments. Eggs in ramp-programmed incubators overall produced larger hatchlings than did those in step-programmed incubators, and eggs at 30 °C produced larger hatchlings than did those incubated at fluctuating temperatures. However, temperature-induced variation in hatchling size was not a significant source of variation in post-hatching growth, and the pattern of fluctuations in incubation temperature was less important for turtles reared in the laboratory. We suggest that the most important thing that should be done by farmers is to avoid exposure of *P. sinensis* eggs to the temperatures potentially lethal to embryos rather than to incubate them at stable temperatures.

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1. Introduction

The exploitation of turtles has been a traditional activity for many centuries in China, where people use turtles as food, pets and in traditional medicine (Chen et al., 2009). China currently consumes much more tons of turtles than any other country in the world, and the long-term unsustainable exploitation of wild turtles has resulted in "a turtle crisis" in the country. Increasingly great efforts have been made by Chinese entrepreneurs over the past four decades to satisfy increasing demand for turtles with farm-raised individuals, instead of wild-caught ones (Shi et al., 2008). Nonetheless, wild turtles continue to be caught and sent to market in large number, and the majority of the wild-caught turtles are exported illegally to China from several countries in Southeast Asia where turtle species richness is still high (Buhlmann et al., 2009). The traditionally most common turtle species sold in China is the Chinese soft-shelled turtle *Pelodiscus* (*Trionyx*) *sinensis*, so much so that ever since wild stocks became depleted in 1970's, over a thousand farms have been established nationwide to raise turtles of this species for their meat (Shi et al., 2008).

How to increase hatching success and how to produce high-quality hatchlings are the two things greatly concerned in turtle aquaculture (Du et al., 2007, 2010; Zhu et al., 2006). As in many other oviparous reptiles (Birchard, 2004; Booth, 2006; Deeming, 2004), incubation temperature affects hatching success and hatchling phenotype (and thus, hatchling quality) in *P. sinensis* (Choo and Chou, 1987; Du and Ji, 2001, 2003; Ji et al., 2003). Exposure of *P. sinensis* eggs to temperatures outside the range of 24–34 °C for prolonged periods of time dramatically decreases hatching success and also results in the production of less developed (and thus smaller-than-usual) and even abnormal hatchlings (Choo and Chou, 1987; Du and Ji, 2001, 2003; Ji et al., 2003). Earlier studies on *P. sinensis* also show that incubation temperatures within the range of 24–34 °C do not have any differential effects on hatching success and hatchling phenotype, and that exposure of eggs to the temperature as high as 36 °C for brief periods of time may not necessarily increase embryonic mortality (Choo and Chou, 1987; Du and Ji, 2001, 2003; Ji et al., 2003).

In most turtle farms *P. sinensis* eggs are incubated onsite or in indoor facilities where temperatures vary naturally, with the mean level and magnitude of thermal fluctuations depending on when and where eggs are incubated. Previous studies have shown that fluctuations in incubation temperature affect incubation length and/or hatchling phenotype in some reptilian species, but not in others (Andrewartha et al., 2010; Li et al., 2013; Lin et al., 2008; Löwenborg

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et al., 2012; Lu et al., 2009). This inconsistency, though likely reflecting to some extent the differences in the temperature protocols used, the among-species differences in embryonic thermosensitivity, and/or the confounding influence of maternal and genetic factors on phenotypic expression, raises a question of whether the pattern of fluctuations in incubation temperature influences hatchling phenotype. In species where fluctuating temperatures affect hatchling phenotype differently than constant temperatures, that effect could be because of the thermal fluctuation *per se* or the fact that thermal fluctuations may result in exposure of eggs to extreme temperatures that increase embryonic mortality and/or result in major phenotypic modifications (Les et al., 2009; Li et al., 2013; Lin et al., 2008; Lu et al., 2009). Thus, to answer the question mentioned above, we need to examine whether and, if so, how temperature fluctuations affect hatchling phenotype.

In this study, we evaluate phenotypic responses of hatchling Chinese soft-shelled turtles to constant *versus* fluctuating incubation temperatures using a temperature equivalent design to hold the thermal mean constant. We chose this turtle for study not only because it is of economical importance but also because it has a known range (24–34 °C) of constant temperatures at which successful embryonic development can take place (Choo and Chou, 1987; Du and Ji, 2001, 2003; Ji et al., 2003). Data from this study will be useful in designing optimal temperature protocols for incubation of *P. sinensis* eggs.

2. Materials and methods

2.1. Egg collection and incubation

A total of 144 freshly laid eggs (4.57–7.24 g) with a white patch (indicative of fertilization) on the shell from unknown mothers were collected in mid-July 2011 from a turtle farm in Baoying, Jiangsu, eastern China. Eggs were brought to our laboratory in Nanjing, where they were randomized to minimize the possible family effect, numbered individually with a pencil, and weighed on a Mettler balance to nearest 1 mg. Eggs were individually placed in covered 100 ml plastic jars with known amounts of vermiculite and water at about −220 kPa water potential (1 g dried vermiculite/1 g water; Ji et al., 2003). All eggs were half-buried in the substrate, with the white patch kept upward. We randomly assigned jars among five Binder KB incubators (Binder, Germany): one set constant at 30 °C (the C treatment); two ramp-programmed at 30 ± 3 °C (the R3 treatment) and ± 5 °C (the R5 treatment); the remaining two step-programmed at 30 ± 3 °C (the S3 treatment) and ± 5 °C (the S5 treatment) (Figs. 1A, B). Temperatures changed at 12 h (+) and 12 h (−) in the ramp-programmed incubators (Fig. 1A), and at 6 h intervals in an order of lowest–mean–highest–mean levels in the step-programmed incubators (Fig. 1B). Thermal fluctuations were monitored with Tinytalk temperature loggers (Gemini Pty, Australia) programmed to record temperature once every 30 min on 12 consecutive days. We moved jars among shelves every other day to compensate for possible thermal gradients within the incubator. Water potential was adjusted at 5-day intervals by weighing jars, and water was added as necessary to compensate for evaporative losses and water taken up by eggs.

2.2. Measurement of hatchling phenotypes

Hatchlings were collected, weighed and measured for carapace length and width less than 6 h post-hatching. All hatchlings had fully un-curved at hatching, thus allowing accurate measurements of carapace length and width. Incubation length was defined as the time period between oviposition and pipping. All hatchlings were measured for locomotor performance less than 24 h post-hatching. We conducted all locomotor trials at the body temperature of 30 °C, which was achieved by placing the hatchlings in water at 30 °C for 30 min prior to testing. Locomotor performance was assessed by chasing the hatchlings along a 2000 × 200 × 400 (length × width × height) mm bath
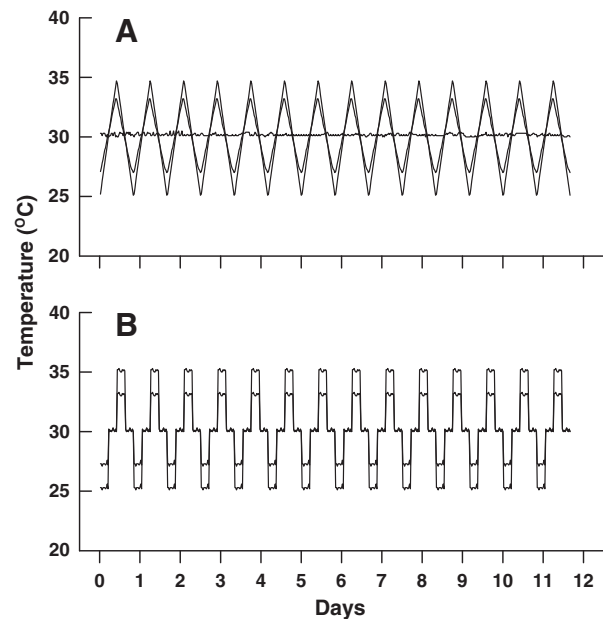

Fig. 1. Representative temperature profiles in a 12 day period for the five treatments. A: the C, R3 and R5 treatments; B: the S3 and S5 treatments. C treatment: eggs in an incubator set at 30 °C; R3 and R5 treatments: eggs in two incubators ramp-programmed at 30 ± 3 °C and ± 5 °C, respectively; S3 and S5 treatments: eggs in two incubators step-programmed at 30 ± 3 °C and ± 5 °C, respectively. Temperatures changed at 12 h (+) and 12 h (−) in the R3 and R5 treatments, and at 6 h intervals in an order of lowest–mean–highest–mean levels in the S3 and S5 treatments.

filled with water to a height of 100 mm, which allowed vertical filming with a NV-DS77 digital video camera (Panasonic, Japan). The water temperature was maintained at 30 °C *via* a water bath heater fixed to a metal stand. A WMZ-3 digital thermometer (Shanghai Medical Instrument, China) confirmed that the water temperature did not vary more than 0.5 °C during trials. Each hatchling was tested twice with a 30 min rest between the two successive trials and, during the resting interval, it was placed back in water at 30 °C. The tapes were later examined with MGI VideoWave III software for PC (MGI Software, Canada) for swimming speed in the fastest 250 mm interval and the maximal swimming distance traveled without stopping.

Following locomotor trials, hatchlings were maintained under identical laboratory conditions to measure post-hatching growth during the first 60 day post-hatching. We individually marked hatchlings with toe-clipping, and then randomly moved them into one of ten 400 × 300 × 150 mm plastic containers filled with water to a height of 100 mm and placed in a room at 28 ± 1 °C. The room light was on a cycle of 12 h light: 12 h dark. Commercially sold food (10% water, 60% proteins, 5% lipids, 5% carbohydrates and 20% minerals) was provided in excess and spread throughout the cage, so that the hatchlings had free access to food. The hatchlings were weighed again at 60 days of age, and then returned to the turtle farm in Baoying.

2.3. Statistical analyses

All statistical analyses were performed in STATISTICA 6.0 (StatSoft, Inc.; Tulsa, USA). We used G test to examine the differences in hatchling success among the five temperature treatments, and linear regression analysis to examine whether a hatchling variable was related to egg mass. We used multivariate analysis of covariance (MANCOVA) or one-way analysis of covariance (ANCOVA) with egg mass as the covariate and one-way ANOVA to examine the effects of incubation treatment on the traits examined. Tukey's test was done on the traits that differed among the five treatments. Prior to using parametric analyses, data were tested for homogeneity of variances

using Bartlett's test (at the univariate level) and/or Box's M test (at the multivariate level), and for normality using the Kolmogorov–Smirnov test. Values are presented as mean ± standard error (SE), and statistical significance is set at $P < 0.05$.

3. Results

A total of 92 eggs hatched. Incubation length differed among the five treatments (ANOVA; $F_{4, 87} = 2.58$, $P = 0.043$), so did hatching success ($G = 97.24$, $df = 4$, $P < 0.001$). The difference in incubation length was significant only between the R3 and R5 treatments (Tukey's test, $P = 0.031$), with eggs in the R3 treatment hatched earlier than did those in the R5 treatment by an average of 2.4 days (Table 1). Hatching successes were much higher in the C (96.7%), R3 (96.4%) and S3 (92.3%) treatments than in the R5 (23.3%) and S5 (16.7%) treatments (Table 1).

Hatchling size (carapace length and width) and mass were positively related to egg mass within each treatment (linear regression analysis; all $P < 0.028$). These three hatchling variables differed among the five treatments after accounting for egg mass with MANCOVA (Wilks' $\lambda = 0.179$, $df = 12$, 222, $P < 0.0001$). The egg size-free mean values for hatchling size and mass were greatest in the C treatment and smallest in the R5 and S5 treatments, with the R3 and S3 treatments in between (Fig. 2). Neither the maximal swimming distance nor swimming speed was related to egg mass within each treatment (linear regression analysis; all $P > 0.166$). The maximal swimming distance differed among the five treatments (ANOVA; $F_{4, 87} = 4.57$, $P < 0.003$), with the mean value being greater in the C and R3 treatments than in the R5 and S5 treatments (Fig. 3). Swimming speed did not differ significantly among the five treatments (ANOVA; $F_{4, 87} = 1.61$, $P = 0.180$). Data pooled for the five treatments showed that swimming speed ranged from 139 to 615 mm/s, with an overall mean speed of 294 mm/s. Body mass measured at 60 days of age, which was positively related to egg mass within each treatment (all $P < 0.005$), did not differ among the five treatments after accounting for egg mass with ANCOVA ($F_{1, 86} = 0.20$, $P = 0.937$). Data pooled for the five treatments showed that the mean body mass (12.7 g) at 60 days of age was approximately 9.1 g greater than the mean body mass (3.6 g) at hatching.

4. Discussion

Temperature affects many biological processes in organisms (Angilletta, 2009; Birchard, 2004; Hochochka and Somero, 2002; Johnston et al., 1996; Lindström, 1999). It has been documented in a wide range of oviparous reptiles that high temperatures affect embryonic development differently than low temperatures. For example, eggs exposed to extremely high temperatures either fail to hatch or produce less developed, poorly performed and even abnormal hatchlings; low incubation temperatures, although slowing or even arresting embryonic development, often have no adverse effects on hatching success and hatchling traits (Bell et al., 2013; Booth, 2006; Li et al., 2013). In this study, only eggs in the R5 and S5 treatments were daily exposed to temperatures up to the level of 35 °C, which

Table 1
Effects of incubation thermal environment on incubation length [expressed as mean ± SE (range)] and hatching success. Mean incubation lengths with different superscripts differ significantly (Tukey's test, $\alpha = 0.05$).

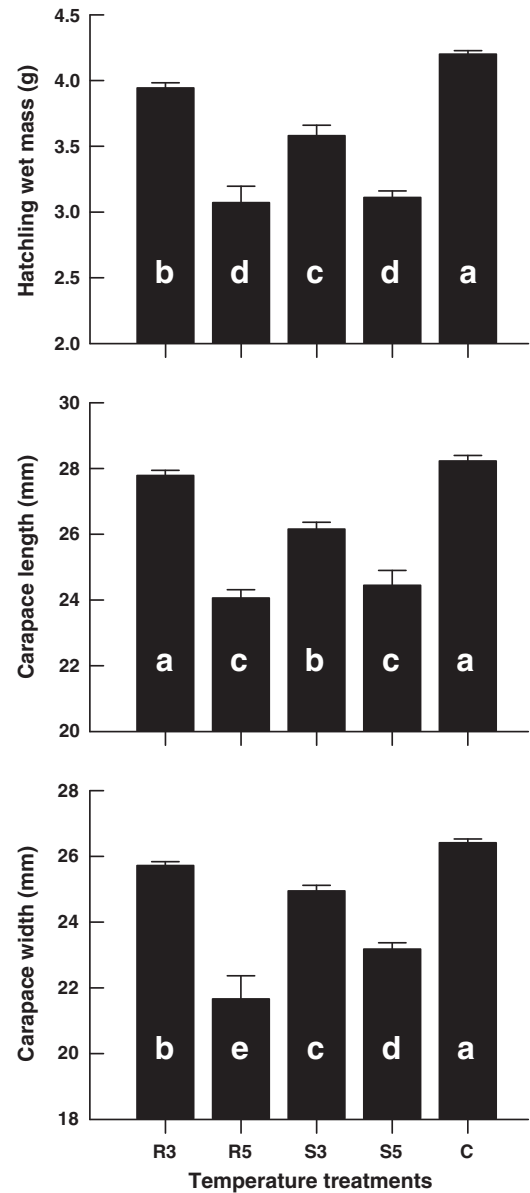| Temperature treatments	Number of eggs incubated	Incubation length (days)	Hatching success (%)
R3	28	48.9[b] ± 0.3 (48.0–52.0)	96.4 (27/28)
R5	30	51.3[a] ± 0.5 (50.0–53.0)	23.3 (7/30)
S3	26	49.1[ab] ± 0.3 (47.0–53.0)	92.3 (24/26)
S5	30	50.2[ab] ± 0.7 (49.0–52.0)	16.7 (5/30)
C	30	49.6[ab] ± 0.4 (48.0–53.0)	96.7 (29/30)

Fig. 2. Adjusted mean values (+SE) for wet mass, carapace length, carapace width of hatchlings from eggs incubated in the five temperature regimes. Egg mass was set at the overall mean level (5.80 g). Adjusted means with different letters differ significantly (Tukey's test, $\alpha = 0.05$, a > b > c > d). See Fig. 1 for definitions for the C, R3, R5, S3 and S5 treatments.

is 1 °C above the upper threshold (34 °C) for successful embryonic development during constant-temperature incubation (Choo and Chou, 1987; Du and Ji, 2001, 2003; Ji et al., 2003). Hatching successes dramatically decreased in the R5 and S5 treatments (Table 1). Moreover, eggs in these two treatments both produced smaller hatchlings with poor locomotor performance (Figs. 2 and 3). These results are consistent with the findings from earlier studies of oviparous reptiles where eggs incubated at high temperatures either fail to hatch or produce small, poorly performed and thus low-quality hatchlings as compared with those incubated at relatively low to moderate temperatures (Burger, 1990; Du et al., 2007; Gutzke et al., 1987; Li et al., 2013; Van Damme et al., 1992). The way in which temperature fluctuated differed between the R5 (in a sinusoidal way) and S5 (in a saltatorial way) treatments. However, this difference was not significant for the end result of egg incubation because: (1) the mean incubation length did not differ between the two treatments; (2) hatching success, although apparently higher in the R5 treatment (23.3%) than
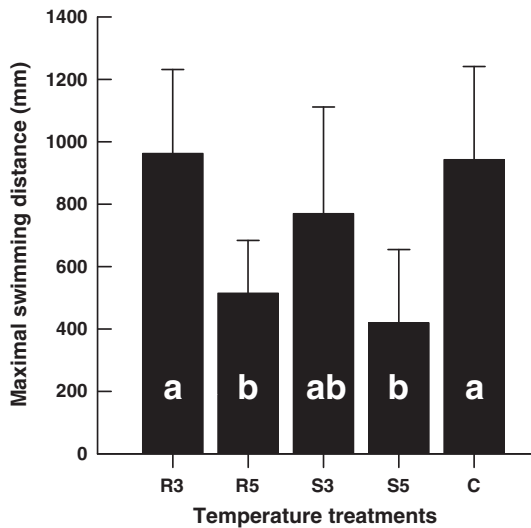
Fig. 3. Mean values (+SE) for the maximal swimming distance of hatchlings from eggs incubated in the five temperature regimes. Means with different letters differ significantly (Tukey's test, α = 0.05, a > b). See Fig. 1 for definitions for the C, R3, R5, S3 and S5 treatments.

in the S5 treatment (16.7%), did not differ significantly between the two treatments ($G = 0.42$, $df = 1$, $P > 0.25$); and (3) only carapace width differed between the two treatments, whereas all other examined hatchling traits (body mass, carapace length, locomotor performance and early growth) did not.

Temperatures in the other three treatments either were maintained constant (the C treatment) at 30 °C or varied sinusoidally (the R3 treatment) and saltatorially (the S3 treatment) within the range of 27–33 °C (Fig. 1B). In none of the three treatments were eggs exposed to temperatures unsuitable for embryonic development because constant temperatures within the range of 24–34 °C are suitable for incubation of *P. sinensis* eggs without any differential effects on hatching success and hatchling phenotype (Choo and Chou, 1987; Du and Ji, 2001, 2003; Ji et al., 2003). However, hatchling size

and mass were greatest in the C treatment and smallest in the S3 treatment, with the R3 treatment in between (Fig. 2). These observations tell us that fluctuations in incubation temperature can affect size-related hatchling traits in *P. sinensis*. Eggs in the three treatments did not differ from each other in incubation length (Table 1) and hatching success ($G = 0.66$, $df = 2$, $P > 0.50$); hatchlings produced in the three treatments did not differ from each other in locomotor performance (Fig. 2) and early growth. These results, together with the adverse effects on egg incubation found in the R5 and S5 treatments allow us to draw the following two conclusions: (1) the way and magnitude of temperature fluctuations do not influence incubation length in *P. sinensis*; and (2) fluctuations in incubation temperature do not influence hatching success and performance-related hatchling traits in *P. sinensis* as long as eggs are not exposed to temperatures above the upper threshold for successful embryonic development.

Earlier hatching can be translated into a longer growth period prior to the first winter. Thus, incubation length should be a fitness-related trait. As in a wide range of reptile taxa (Li et al., 2013; Lin et al., 2010 and references therein), incubation length increases at an ever-increasing rate as temperature decreases in *P. sinensis* (Choo and Chou, 1987; Du and Ji, 2003). Such a pattern of the relationship between incubation length and temperature allows the expectation that eggs should hatch earlier if they are incubated at constant rather than fluctuating temperatures with the same mean, because the decelerated developmental rates at temperatures below a given (or central) level often cannot be equally canceled out by the accelerated developmental rates at temperatures above that level (Li et al., 2013; Lin et al., 2008; Löwenborg et al., 2012; Lu et al., 2009; but see also Du and Shine, 2010), and this is especially true in species without temperature compensation in growth or development during embryonic development (Booth, 1998). In contrast to this expectation, *P. sinensis* eggs incubated at constant temperatures do not hatch earlier than those incubated at fluctuating temperatures with the same mean (Tables 1 and 2). Previous studies on the effect of fluctuating temperatures on incubation length in reptiles have generated contradictory results: fluctuating temperatures do result in increased or decreased incubation length in some species, but have no role in influencing this trait in others (Table 2). Of the

Table 2
The influence of constant *versus* fluctuating temperatures on incubation length in oviparous reptiles. TED: eggs are incubated at constant *versus* fluctuating temperatures using a temperature equivalent design; NAT: eggs are incubated at constant *versus* naturally fluctuating temperatures with the same mean; (+): eggs hatch earlier if they are incubated at constant not fluctuating temperatures; (−): eggs hatch later if they are incubated at constant not fluctuating temperatures; (0): eggs incubated at constant and fluctuating temperatures do not differ in incubation length.

Taxa Species	Incubation methods (influence)	References
Turtles		
Apalone mutica (smooth soft-shelled turtle)	TED (+)	Ashmore and Janzen (2003)
Chinemys reevesii (Chinese three-keeled pond turtle)	TED (+)	Du et al. (2009)
Chrysemys picta (painted turtle)	TED (+)	Les et al. (2007)
Pelodiscus sinensis (Chinese soft-shelled turtle)	TED (0)	This study
	NAT (0)	Du and Ji (2003); Ji et al. (2003)
Trachemys scripta (red-eared slider turtle)	TED (+)	Les et al. (2007)
Lizards		
Bassiana duperreyi (eastern three-lined skink)	TED (0/−)	Shine (2004); Du and Shine (2010)
Eremias argus (Mongolian racerunner)	NAT (+)	Hao et al. (2006)
Eumeces chinensis (Chinese skink)	TED (0)	Du et al. (2005)
Heteronotia binoei (Bynoe's gecko)	TED (+)	Andrewartha et al. (2010)
Lacerta agilis (sand lizard)	TED (+)	Li et al. (2013)
Leiolepis reevesii (Reevese's butterfly lizard)	NAT (0)	Lin et al. (2007)
Podarcis muralis (common wall lizard)	TED (+)	Braña and Ji (2007)
Sceloporus undulatus (eastern fence lizard)	TED (0)	Andrews et al. (2000)
Takydromus septentrionalis (northern grass lizard)	NAT (0)	Du and Ji (2006)
Snakes		
Bungarus multicinctus (multi-banded krait)	NAT (0)	Ji et al. (2007)
Elaphe obsolete (black ratsnake)	TED (+)	Patterson and Blouin-Demers (2008)
Naja atra (Chinese cobra)	NAT (−)	Lin et al. (2008)
Natrix natrix (grass snake)	Nat (+)	Löwenborg et al. (2012)
Rhabdophis tigrinus lateralis (red-necked keelback snake)	NAT (0)	Chen and Ji (2002)
Xenochrophis piscator (checkered keelback snake)	NAT (+)	Lu et al. (2009)

other 11 reptile species where eggs have been incubated at constant *versus* fluctuating temperatures using a temperature equivalent design with some fixed extremes of temperature fluctuation, only three lizards are similar to *P. sinensis* in that eggs incubated at constant temperatures do not differ from those at fluctuating temperatures with the same mean in incubation length (Table 2). Earlier studies show that constant temperatures do not affect incubation length differently than naturally fluctuating temperatures with the same mean in *P. sinensis* (Du and Ji, 2003; Ji et al., 2003). Of the other eight reptile species where eggs have been incubated at constant (or relatively constant) *versus* naturally fluctuating temperatures, four species, two lizards and two snakes, are similar to *P. sinensis*, and the other four species show that fluctuating temperatures result in increased or decreased incubation length (Table 2). That embryonic cardiac output (and thus, the rate of embryonic development; Du et al., 2011) is increased more by a diel increase in temperature than it is decreased by a diel fall in temperature explains why *Bassiana duperreyi* eggs hatch earlier if incubated at fluctuating rather than constant temperatures (Du and Shine, 2010). Future work could usefully investigate whether a similar physiological mechanism also works in *P. sinensis* to seek a possible explanation for why fluctuating temperatures do not affect incubation length in the turtle.

In summary, our data show that the pattern of fluctuations in incubation temperature affects size-related hatchlings traits in *P. sinensis*, as revealed by the fact that eggs in the ramp-programmed incubators where temperatures fluctuated in a sinusoidal way produced larger hatchlings than do those in the step-programmed incubators where temperatures fluctuated in a saltatorial way. Our data provide an inference that *P. sinensis* eggs incubated at constant temperatures would produce larger hatchlings than those incubated at fluctuating temperatures with the same mean. Offspring size is a highly fitness-related trait in many animals as larger offspring may have selectively more advantages than smaller ones, and this is especially true for animals living in the wild (Ferguson and Fox, 1984; Janzen et al., 2000a,b; Ji et al., 2009; McGinley et al., 1987; Sargent et al., 1987). However, the size advantage could be substantially reduced if animals are maintained in the laboratory or other artificial environments with unlimited resources and suitable thermal environments where an individual's performance can be expressed to an extent much closer to its physiological or genetic potential. In the present study, hatchling size differed among the five treatments, whereas early growth did not after accounting for egg size. These findings confirm the earlier conclusion drawn in a long-term study of *P. sinensis* that temperature-induced variation in hatchling size is not a significant source of variation in post-hatching growth (Ji et al., 2003). The Chinese soft-shelled turtle is believed to be among reptile species where a small hatchling with a high physiological potential can compensate for its small size by catch-up growth during subsequent post-hatching growth (Ji et al., 2003). Thus, although fluctuations in incubation temperature can affect hatchling size at hatching, such an influence could be less important at least for turtles reared in the laboratory. We suggest that the most important thing that should be done by turtle farmers is to avoid exposure of *P. sinensis* eggs to the temperatures potentially lethal to embryos rather than to incubate them at stable temperatures that cannot be achieved without increasing energy input and thus the costs of turtle aquaculture associated with the increased energy payment.

Acknowledgments

References

Andrewartha, S.J., Mitchell, N.J., Frappell, P.B., 2010. Does incubation temperature fluctuation influence hatchling phenotypes in reptiles? A test using parthenogenetic geckos. Physiological and Biochemical Zoology 83, 597–607.

Andrews, R.M., Mathies, T., Warner, D.A., 2000. Effect of incubation temperature on morphology, growth, and survival of juvenile *Sceloporus undulatus*. Herpetological Monographs 14, 420–431.

Angilletta, M.J., 2009. Thermal Adaptation: A Theoretical and Empirical Synthesis. Oxford University Press, Oxford.

Ashmore, G.M., Janzen, F.J., 2003. Phenotypic variation in smooth softshell turtles (*Apalone mutica*) from eggs incubated in constant *versus* fluctuating temperatures. Oecologia 134, 182–188.

Bell, K., Blomberg, S., Schwarzkopf, L., 2013. Detrimental influence on performance of high temperature incubation in a tropical reptile: is cooler better in the tropics? Oecologia 171, 83–91.

Birchard, G.F., 2004. Effects of incubation temperature. In: Deeming, D.C. (Ed.), Reptilian Incubation: Environment, Evolution, and Behaviour. Nottingham University Press, Nottingham, pp. 103–123.

Booth, D.T., 1998. Incubation of turtle eggs at different temperatures: do embryos compensate for temperature during development? Physiological Zoology 71, 23–26.

Booth, D.T., 2006. Influence of incubation temperature on hatchling phenotype in reptiles. Physiological and Biochemical Zoology 79, 274–281.

Braña, F., Ji, X., 2007. The selective basis for increased egg retention: early incubation temperature determines hatchling phenotype in wall lizards. Biological Journal of the Linnean Society 92, 441–447.

Buhlmann, K.A., Akre, T.S.B., Iverson, J.B., Karapatakis, D., Mittermeier, R.A., Georges, A., Rhodin, A.G.J., Van Dijk, P.P., Gibbons, J.W., 2009. A global analysis of tortoise and freshwater turtle distributions with identification of priority conservation areas. Chelonian Conservation and Biology 8, 116–149.

Burger, J., 1990. Effects of incubation temperature on behavior of young black racers (*Coluber constrictor*) and king snakes (*Lampropeltis getulus*). Journal of Herpetology 24, 158–163.

Chen, H.L., Ji, X., 2002. The effects of thermal environments on duration of incubation, hatching success and hatchling traits in a colubrid snake *Rhabdophis tigrinus lateralis* (Boie). Acta Ecologica Sinica 22, 1850–1858.

Chen, T.H., Chang, H.C., Lue, K.Y., 2009. Unregulated trade in turtle shells for Chinese traditional medicine in East and Southeast Asia: the case of Taiwan. Chelonian Conservation and Biology 8, 11–18.

Choo, B.L., Chou, L.M., 1987. Effect of temperature on the incubation period and hatchability of *Trionyx sinensis* Wiegmann eggs. Journal of Herpetology 21, 230–232.

Deeming, D.C., 2004. Post-hatching phenotypic effects of incubation in reptiles. In: Deeming, D.C. (Ed.), Reptilian Incubation: Environment, Evolution, and Behaviour. Nottingham University Press, Nottingham, pp. 211–228.

Du, W.G., Ji, X., 2001. Influence of incubation temperature on embryonic use of material and energy in the Chinese soft-shelled turtle (*Pelodiscus sinensis*). Acta Zoologica Sinica 47, 512–517.

Du, W.G., Ji, X., 2003. The effects of incubation thermal environments on size, locomotor performance and early growth of hatchling soft-shelled turtles, *Pelodiscus sinensis*. Journal of Thermal Biology 28, 279–286.

Du, W.G., Ji, X., 2006. Effects of constant and fluctuating temperatures on egg survival and hatchling traits in the northern grass lizard (*Takydromus septentrionalis*, Lacertidae). Journal of Experimental Zoology Part A 305, 47–54.

Du, W.G., Shine, R., 2010. Why do the eggs of lizards (*Bassiana duperreyi*: Scincidae) hatch sooner if incubated at fluctuating rather than constant temperatures? Biological Journal of the Linnean Society 101, 642–650.

Du, W.G., Shou, L., Shen, J.Y., Lu, Y.W., 2005. Influence of fluctuating incubation temperatures on hatchling traits in a Chinese skink, *Eumeces chinensis*. Herpetological Journal 15, 139–142.

Du, W.G., Hu, L.J., Lu, J.L., Zhu, L.J., 2007. Effects of incubation temperature on embryonic development rate, sex ratio and post-hatching growth in the Chinese three-keeled pond turtle, *Chinemys reevesii*. Aquaculture 272, 747–753.

Du, W.G., Shen, J.W., Wang, L., 2009. Embryonic development rate and hatchling phenotypes in the Chinese three-keeled pond turtle (*Chinemys reevesii*): the influence of fluctuating temperature *versus* constant temperature. Journal of Thermal Biology 34, 250–255.

Du, W.G., Wang, L., Shen, J.W., 2010. Optimal temperatures for egg incubation in two Geoemydid turtles: *Ocadia sinensis* and *Mauremys mutica*. Aquaculture 305, 138–142.

Du, W.G., Ye, H., Zhao, B., Pizzatto, L., Ji, X., Shine, R., 2011. Patterns of interspecific variation in the heart rates of embryonic reptiles. PLoS One 6 (12), e29027.

Ferguson, G.W., Fox, S.F., 1984. Annual variation of survival advantage of large juvenile side-blotched lizards, *Uta stansburiana*: its causes and evolutionary significance. Evolution 38, 342–349.

Gutzke, W.H.N., Packard, G.C., 1987. Influence of the hydric and thermal environments on eggs and hatchlings of bull snakes *Pituophis melanoleucus*. Physiological Zoology 60, 9–17.

Hao, Q.L., Liu, H.X., Ji, X., 2006. Phenotypic variation in hatchling Mongolian racerunners (*Eremias argus*) from eggs incubated at constant *versus* fluctuating temperatures. Acta Zoologica Sinica 52, 1049–1057.

Hochochka, P.W., Somero, G.N., 2002. Biochemical Adaptation. Oxford University Press, Oxford.

Janzen, F.J., Tucker, J.K., Paukstis, G.L., 2000a. Experimental analysis of an early life-history stage: avian predation selects for larger body size of hatchling turtles. Journal of Evolutionary Biology 13, 947–954.

Janzen, F.J., Tucker, J.K., Paukstis, G.L., 2000b. Experimental analysis of an early life-history stage: selection on size of hatchling turtles. Ecology 81, 2275–2280.

Ji, X., Chen, F., Du, W.G., Chen, H.L., 2003. Incubation temperature affects hatchling growth but not sexual phenotype in the Chinese soft-shelled turtle *Pelodiscus sinensis*. Journal of Zoology 261, 409–416.

Ji, X., Gao, J.F., Han, J., 2007. Phenotypic responses of hatchling multi-banded kraits (*Bungarus multicintus*) to constant *versus* fluctuating incubation temperatures. Zoological Science 24, 384–390.

Ji, X., Du, W.G., Qu, Y.F., Lin, L.H., 2009. Nonlinear continuum of egg size-number trade-offs in a snake: is egg-size variation fitness related? Oecologia 159, 689–696.

Johnston, I.A., Vieira, V.L.A., Hill, J., 1996. Temperature and ontogeny in ectotherms: Muscle phenotype in fish. In: Johnston, I.A., Bennett, A.F. (Eds.), Animals and Temperature: Phenotypic and Evolutionary Adaptation. Cambridge University Press, Cambridge, pp. 153–181.

Les, H.L., Paitz, R.T., Bowden, R.M., 2007. Experimental test of the effects of fluctuating incubation temperatures on hatchling phenotype. Journal of Experimental Zoology Part A 307, 274–280.

Les, H.L., Paitz, R.T., Bowden, R.M., 2009. Living at extremes: development at edges of viable temperature under constant and fluctuating conditions. Physiological and Biochemical Zoology 307, 274–280.

Li, H., Zhou, Z.S., Ding, G.H., Ji, X., 2013. Fluctuations in incubation temperature affect incubation duration but not morphology, locomotion and growth of hatchlings in the sand lizard *Lacerta agilis* (Lacertidae). Acta Zoologica (Stockholm) 94, 11–18.

Lin, C.X., Du, Y., Qiu, Q.B., Ji, X., 2007. Relatively high but narrow incubation temperatures in lizards depositing eggs in warm and thermally stable nests. Acta Zoologica Sinica 53, 437–445.

Lin, L.H., Li, H., An, H., Ji, X., 2008. Do temperature fluctuations during incubation always play an important role in shaping the phenotype of hatchling reptiles? Journal of Thermal Biology 33, 193–199.

Lin, L.H., Ma, X.M., Li, H., Ji, X., 2010. Phenotypic variation in hatching Chinese ratsnakes (*Zaocys dhumnades*) from eggs incubated at constant temperatures. Journal of Thermal Biology 35, 28–33.

Lindström, J., 1999. Early development and fitness in birds and mammals. Trends in Ecology & Evolution 14, 343–348.

Löwenborg, K., Gotthard, K., Hagman, M., 2012. How a thermal dichotomy in nesting environments influences offspring of the world's most northerly oviparous snake, *Natrix natrix* (Colubridae). Biological Journal of the Linnean Society 107, 833–844.

Lu, H.L., Hu, R.B., Ji, X., 2009. The variance of incubation temperatures is not important in influencing the phenotype of hatchlings in an aquatic snake, *Xenochrophis piscator* (Colubridae). Journal of Thermal Biology 34, 138–143.

McGinley, M.A., Temme, D.H., Geber, M.A., 1987. Parental investment in offspring in variable environments: theoretical and empirical considerations. American Naturalist 130, 370–398.

Patterson, L.D., Blouin-Demers, G., 2008. The effect of constant and fluctuating incubation temperatures on the phenotype of black ratsnakes (*Elaphe obsoleta*). Canadian Journal of Zoology 86, 882–889.

Sargent, R.C., Taylor, P.D., Gross, M.R., 1987. Parental care and the evolution of egg size in fishes. American Naturalist 129, 32–46.

Shi, H.T., Parham, J.F., Fan, Z.Y., Hong, M.L., Yin, F., 2008. Evidence for the massive scale of turtle farming in China. Oryx 42, 147–150.

Shine, R., 2004. Seasonal shifts in nest temperature can modify the phenotypes of hatchling lizards, regardless of overall mean incubation temperature. Functional Ecology 18, 43–49.

Van Damme, R., Bauwens, D., Braña, F., Verheyen, R.F., 1992. Incubation temperature differentially affects hatching time, egg survival, and hatchling performance in the lizard *Podarcis muralis*. Herpetologica 48, 220–228.

Zhu, X.P., Wei, C.Q., Zhao, W.H., Du, H.J., Chen, Y.L., Gui, J.F., 2006. Effects of incubation temperatures on embryonic development in the Asian yellow pond turtle. Aquaculture 259, 243–248.