Journal of Thermal Biology 34 (2009) 138–143

Contents lists available at ScienceDirect

Journal of Thermal Biology

journal homepage: www.elsevier.com/locate/jtherbio

The variance of incubation temperatures does not affect the phenotype of hatchlings in a colubrid snake, *Xenochrophis piscator*

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ARTICLE INFO

Article history:
Received 3 September 2008
Accepted 16 December 2008

Keywords:
Colubridae
Xenochrophis piscator
Egg incubation
Temperature
Hatchling phenotype

ABSTRACT

It has been documented in some reptiles that fluctuating incubation temperatures influence hatchling traits differently than constant temperatures even when the means are the same between treatments; yet whether the observed effects result from the thermal variance, temperature extremes or both is largely unknown. We incubated eggs of the checkered keelback snake *Xenochrophis piscator* under one fluctuating (Ft) and three constant (24, 27 and 30 °C) temperatures to examine whether the variance of incubation temperatures plays an important role in influencing the phenotype of hatchlings. The thermal conditions under which eggs were incubated affected a number of hatchling traits (wet mass, SVL, tail length, carcass dry mass, fatbody dry mass and residual yolk dry mass) but not hatching success and the sex ratio of hatchlings. Body sizes were larger in hatchlings from incubation temperatures of 24 and 27 °C compared with the other two treatments. Hatchlings from the four treatments could be divided into two groups: one included hatchlings from the 24 and 27 °C treatments, and the other included hatchlings from the 30 °C and Ft treatments. In the Ft treatment, the thermal variance was not a significant predictor of all examined hatchling traits, and incubation length was not correlated with the thermal variance when holding the thermal mean constant. The results of this study show that the mean rather than the variance of incubation temperatures affects the phenotype of hatchlings.

1. Introduction

Incubation temperature affects many aspects of development in reptiles, and inappropriate incubation temperatures have detrimental effects on embryos and hatchlings. For example, temperatures below and above the range of optimal incubation temperatures can dramatically decrease hatchability, and extremes of temperature can have adverse effects on a wide variety of hatchling phenotypes including morphology, behavior, physiology, growth and survivability (Birchard, 2004; Deeming, 2004; Booth, 2006). Most studies in this field have been based on oviparous reptile species where eggs are incubated at constant temperatures. However, as temperatures in natural nests, shallow nests in particular, are rarely constant but vary on a daily and seasonal basis over the course of incubation, the thermal effects demonstrated in constant-temperature incubations often do not realistically reflect what occurs in nature (Ackerman and Lott, 2004; Booth, 2006).

Recent studies have paid increasingly more attention to the developmental plasticity under incubation thermal conditions mimicking those in natural nests (Birchard, 2004). These studies are often designed to test the hypothesis that not only the thermal mean but also the way in which incubation temperatures vary around any given mean level can have important effects on embryos and hatchlings in reptiles, but they do not reach any consistent conclusion regarding the influence of thermal fluctuation on hatchling traits. For example, fluctuating temperatures influence hatchling size and mass differently than constant temperatures with the same mean temperature in some species but not in others (Lin et al., 2008 and included references). This inconsistency, though likely reflecting to some extent the differences in the temperature protocols used, raises a question of whether the thermal variance, a measure of thermal variability, in natural nests or fluctuating-temperature incubations plays an important role in determining the phenotype of hatchling reptiles. In species where fluctuating incubation temperatures affect hatchling phenotypes differently than constant temperatures with the same mean, that effect could be due to either the thermal variance *per se*, or the fact that thermal fluctuations result in exposure of eggs to extreme temperatures adversely affecting embryogenesis (Shine, 2005). In species where incubation temperatures fluctuating within some thresholds do not have

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doi:10.1016/j.jtherbio.2008.12.002

any differential effects on embryogenesis, we hypothesize that hatchling phenotype cannot be altered by the thermal variance but by the extreme temperatures experienced by embryos in thermally variable environments.

Here, we describe a study incubating eggs of *Xenochrophis piscator* (checkered keelback snake) under one fluctuating and three constant-temperature regimes (see below for details) to test our hypothesis. This medium-sized (up to 940 mm snout-vent length, SVL) colubrid snake shows a preference for aquatic habitats, and is one of most conspicuous reptile species in the southern provinces of China (Zhao, 1998). Females lay a single clutch of 11–88 pliable-shelled eggs per breeding season between May and June in shallow nests where temperatures vary appreciably in response to short- and long-term oscillations in environmental temperature (Zhao, 1998; Ji et al., 2001). Incubation temperatures higher than 32 °C are lethal to *X. piscator* embryos, whereas temperatures within the range of 24–30 °C do not have any differential effects on hatchability and any examined hatchling trait (Ji et al., 2001).

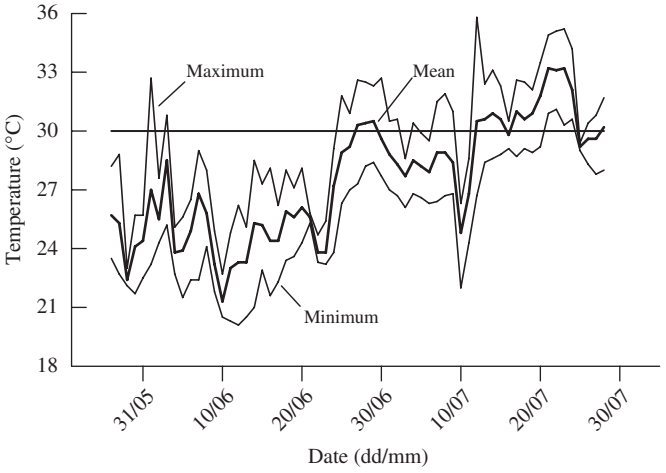

Fig. 1. Temporal variation in daily minimal, mean and maximal temperatures experienced by *X. piscator* eggs incubated at fluctuating temperatures. Daily mean temperatures ranged from 26.7 to 29.2 °C with an average of 27.7 ± 0.2 °C. Exposure of *X. piscator* eggs to temperatures higher than 30 °C (the horizontal line) for a prolonged period may lead to production of less developed hatchlings.

2. Materials and methods

2.1. Egg collection and incubation

Twenty-one gravid females (635–886 mm snout-vent length) were collected in mid-May 2005 from Yongzhou (26°25′N, 111°36′E), Hunan, central China. Females were transported to our laboratory in Hangzhou, where they were housed individually in 60 cm × 60 cm × 60 cm (length × width × height) wire cages placed in an indoor animal holding facility at temperatures (24–28 °C) optimal for embryonic development (Ji et al., 2001). Food [rice frogs (*Rana limnocharis*) and Chinese loaches (*Paramisgumus dabryanus*)] was provided ad libitum. The cages were checked at least twice daily for freshly laid eggs as soon as the first female laid eggs, such that eggs could be always collected, measured and weighed within a few hours after oviposition. The viability or fertility of freshly laid eggs was identified by the presence of an embryonic disc using a spotlight. SVL, tail length and body mass were taken for each post-oviposition female. Eggs were individually measured to 0.01 mm for length and width with a Mitutoyo digital caliper and weighed to 1 mg on a Mettler top loading balance.

A total of 310 eggs, 14–16 from each of the 21 clutches, were divided as equally as possible among the four treatments. Eggs were individually incubated in covered plastic jars (100 ml) with known amounts of vermiculite and water at about −220 kPa water potential (1 g dried vermiculite/1 g water; Ji and Braña, 1999). One-third of the egg was buried lengthwise in the substrate, with the surface near the embryo exposed to air inside the jar. Jars were weighed at 5-day intervals, and water was added into the substrate to compensate for evaporative losses and water taken up by eggs.

A total of 235 jars were assigned to three Shellab incubators (Sheldon MFG Inc., USA), with incubation temperatures set at 24, 27 and 30(±0.3) °C, respectively. The location of the jars was altered daily according to a predetermined schedule to compensate for possible undetected thermal gradients within incubators. The remaining 75 jars [hereafter the Ft (fluctuating-temperature) treatment] were buried approximately 20 cm below the ground surface in a 60 cm × 60 cm × 30 cm soil-constructed chamber in the bush-covered backyard of our laboratory, thereby mimicking thermal conditions in natural nests. Temperatures recorded hourly using a Tinytalk datalogger (Gemini Pty, Australia) present within the chamber varied daily and seasonally (Fig. 1); the maximum magnitude of daily thermal variation, the mean, the lowest and the highest temperatures were 15.7, 27.7, 20.1 and 35.8 °C, respectively.

2.2. Size, mass and composition of hatchlings

Incubation length, measured as the time between oviposition and pipping, was recorded for each egg. Hatchlings were collected, measured to 1 mm for SVL and tail length, and weighed to 1 mg immediately after they emerged from the eggs. Hatchlings were then euthanized by freezing to −15 °C for determination of composition and sex. The killed hatchlings were separated into carcass, residual yolk and fat bodies. The three hatchling components were dried to constant mass in an oven at 65 °C, weighed to 0.1 mg and preserved frozen for later analyses. We determined the sex of hatchlings by pressing on both sides of the ventral tail base with forceps to record the presence or absence of hemipenes; hatchlings with everted hemipenes were recorded as males.

We extracted non-polar lipids from dried samples in a Soxhlet apparatus for 5.5 h using absolute ether as solvent. The amount of lipids in each sample was determined by subtracting the lipid-free dry mass from the total sample dry mass. The total lipid in a hatchling was calculated as the sum of the lipids in its carcass, residual yolk and fat bodies. We determined energy density of dried samples using a WGR-1 adiabatic bomb calorimeter (Changsha Bente Instruments, China). We determined ash content in each dried sample using a muffle furnace at 700 °C for a minimum of 12 h and weighing to 0.1 mg the remaining ash.

2.3. Data analysis

All data were analyzed using the statistics package Statistica (version 5.0 for PC). Data on hatchlings of the same sex were blocked by clutch to avoid pseudo-replication. We used the *G* test to examine whether hatchability and the sex ratio of hatchlings differed among the four treatments. Between-sex differences in incubation length within each treatment and differences in mean egg mass among the four treatments were examined using one-way analysis of variance (ANOVA). We used Kruskal–Wallis test to examine the differences in incubation length among the four treatments. A partial correlation analysis was used to examine the

Table 1
Effects of incubation thermal environments on incubation length, hatching success and sex ratio of hatchlings.

Thermal treatments[a] (°C)	Incubated eggs	Incubation length[b] (d)	Hatching success (%)	Sex ratio (♀♀/♂♂)
Fluctuating temperature	75	45.2±0.7 (37.9–52.0)	81.3 (61/75)	24/37
24	74	67.9±0.2 (65.9–70.3)	77.0 (57/74)	22/35
27	78	48.8±0.2 (45.2–50.8)	88.5 (69/78)	33/36
30	83	36.7±0.2 (34.8–39.0)	74.7 (62/83)	33/29

Data on incubation length are expressed as mean±SE (range).
[a] The overall mean temperature in the Ft treatment was 27.7 °C.
[b] Data from the same clutch are blocked.

relationships between the selected pairs of variables while holding the remaining relevant variables constant. We used a multivariate analysis of variance (MANOVA) to examine the effects of the same factors (sex and incubation treatment) and their interaction on the examined hatchling traits. Prior to this analysis, the assumption of homogeneity of variances was tested at univariate (Bartlett's test) and multivariate (Box's *M* test) levels. A principal component analysis was used to investigate the possible existence of space characteristic of hatchlings from different incubation thermal environments. Throughout the paper, values are presented as mean±SE, and the significance level is set at $\alpha = 0.05$.

3. Results

The 21 females laid clutches ranging in size from 31 to 82 (mean 56.7±0.3) between 27 May and 20 June. Clutch size was positively correlated with maternal SVL ($r^2 = 0.56$, $F_{1,19} = 24.46$, $P<0.0001$), whereas the mean egg mass of clutches, ranging from 1.78 to 3.16 g (mean 2.37±0.06), was not ($r^2 = 0.16$, $F_{1,19} = 3.55$, $P = 0.075$).

Eggs incubated under the four thermal regimes did not differ from each other in mean mass (one-way ANOVA; $F_{3,124} = 0.35$, $P = 0.788$). Hatching success ($G = 5.81$, $df = 3$, $P>0.10$) and the sex ratio of hatchlings did not differ among the four treatments ($G = 3.66$, $df = 3$, $P>0.25$) (Table 1). Incubation length was independent of egg mass (simple linear regression; all $P>0.097$), and did not differ between the sexes within each treatment (one-way ANOVA; all $P>0.299$). Incubation length differed among the four treatments ($H_{3,\ N=128} = 107.42$, $P<0.0001$), with the mean value shortened by 19.1 days from 24 to 27 °C and by 12.1 days from 27 to 30 °C (Table 1). We found in the Ft treatment that incubation length was negatively correlated with the thermal mean over the course of incubation ($F_{1,34} = 511.22$, $P<0.0001$). A partial correlation analysis revealed that incubation length was not correlated with the thermal variance [the standard deviation squared, ranging from 7.7 to 12.3 (mean 9.8±0.2)] when holding the thermal mean constant ($r = -0.16$, $t = 0.933$, $df = 33$, $P = 0.358$). Embryos developing under fluctuating temperatures developed slightly but significantly faster than at an equivalent constant temperature within the range of the fluctuating temperatures (paired-sample *t*-test; $t = 11.37$, $df = 35$, $P<0.0001$), with the expected incubation lengths being on average shortened by 0.3 days in the Ft treatment (Fig. 2).

The variance of incubation temperatures in the Ft treatment was not a significant predictor of all examined hatchling traits in both sexes (simple linear regression; all $P>0.142$). The examined hatchling traits were overall affected by both sex (Wilks' $\lambda = 0.306$, $df = 10$, 111, $P<0.0001$) and incubation treatment (Wilks' $\lambda = 0.165$, $df = 30$, 326, $P<0.0001$), but not by the interaction between the two factors (Wilks' $\lambda = 0.763$, $df = 30$, 326, $P = 0.397$). Female hatchlings were larger in SVL but smaller

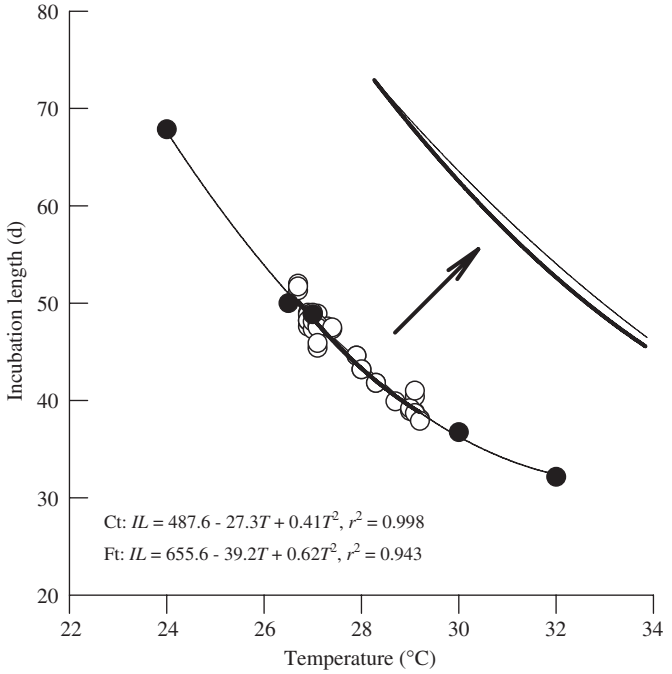

Fig. 2. The curvilinear regressions of incubation length on mean incubation temperature. Solid dots and bold lines represent eggs incubated at constant temperatures, and data supporting the two asterisk-marked dots are from an earlier study of *X. piscator* (Ji et al., 2001); open dots represent eggs incubated at fluctuating temperatures. Regression equations, curvilinear lines and an enlarged view of the difference between the two lines within the range of the fluctuating temperatures are given in the figure.

in tail length than male hatchlings from the same-sized egg (both $P<0.001$; Table 2). Hatchling wet mass was greatest in the 24 and 27 °C treatments and smallest in the 30 °C treatment, with the Ft treatment in between; SVL and tail length were both greatest in the 24 °C treatment and smallest in the 30 °C treatment, with the Ft and 27 °C treatments in between; carcass dry mass and fatbody dry mass were greater in the 24 and 27 °C treatments than in the other two treatments; residual yolk dry mass was greatest in the Ft treatment and smallest in the 24 and 27 °C treatments, with the 30 °C treatment in between (Tukey's *post hoc* test, all $P<0.03$; Table 2). The remaining hatchling traits, including dry body mass, lipid mass, energy contents and ash mass, were not affected by sex and by incubation treatment (Tukey's *post hoc* test, all $P>0.119$; Table 2). A series of partial correlation analysis revealed that (1) hatchling SVL was positively correlated with carcass dry mass ($r = 0.48$, $t = 6.06$, $df = 124$, $P<0.0001$) but not with residual yolk dry mass and fatbody dry mass (both $P>0.493$); (2) carcass dry mass was positively correlated with both residual yolk dry mass ($r = 0.27$, $t = 3.07$, $df = 124$, $P<0.003$) and fatbody dry mass ($r = 0.59$, $t = 8.06$, $df = 124$, $P<0.0001$); and (3) residual yolk dry mass was negatively correlated with fatbody dry mass ($r = -0.34$, $t = 4.01$, $df = 124$, $P<0.0002$).

Table 2
Size, morphology and composition of hatchlings incubated under different thermal conditions.

Variable	Thermal treatments							
	Fluctuating temperature		24 °C		27 °C		30 °C	
	♀♀	♂♂	♀♀	♂♂	♀♀	♂♂	♀♀	♂♂
N	15	21	12	17	17	18	13	15
Egg mass (g)	2.34±0.04 1.96–2.70	2.34±0.05 2.07–2.88	2.36±0.06 2.08–2.80	2.34±0.04 2.11–2.69	2.37±0.04 2.18–2.79	2.39±0.08 2.10–3.44	2.34±0.04 2.09–2.59	2.33±0.07 1.96–2.93
Snout–vent length (mm)	128.0±1.9 111.0–139.0	122.7±1.2 110.0–130.0	132.6±1.3 125.7–140.8	126.5±0.7 121.5–131.6	129.9±.9 108.0–140.0	122.4±1.9 104.0–138.5	120.0±2.4 97.0–130.2	119.5±2.1 105.0–133.0
Tail length (mm)	39.7±0.7 33.0–43.0	44.3±0.5 41.0–47.0	44.0±0.6 40.0–47.0	48.9±0.6 45.9–52.0	41.5±0.9 28.0–45.0	45.2±0.8 36.0–51.0	36.7±1.2 26.0–44.0	41.8±1.0 36.0–48.0
Wet body mass (g)	1.64±0.04 1.34–1.94	1.64±0.03 1.36–1.88	1.71±0.04 1.49–1.98	1.72±0.03 1.42–1.93	1.69±0.05 1.04–1.99	1.73±0.04 1.45–2.27	1.52±0.05 1.24–1.78	1.58±0.05 1.36–2.10
Dry body mass (mg)	389.5±13.4 259.4–485.9	391.3±9.9 263.3–466.0	399.5±9.3 353.8–464.4	403.3±8.8 349.5–456.0	406.5±7.9 357.5–472.9	414.7±18.6 277.4–680.3	369.9±11.8 296.3–438.8	398.7±16.0 269.3–552.2
Carcass dry mass (mg)	266.7±7.8 214.9–310.0	272.9±5.1 218.2–306.4	306.8±5.8 273.8–345.1	306.8±5.3 266.5–342.7	301.0±7.8 200.1–347.4	307.1±8.6 232.2–401.9	273.0±6.5 228.5–308.6	284.8±9.3 223.8–376.1
Yolk dry mass (mg)	99.8±8.3 32.2–148.9	94.7±6.7 40.3–147.6	62.8±4.0 38.4–84.9	67.4±4.6 35.6–116.7	68.2±3.9 41.0–97.8	66.4±5.2 32.5–114.4	69.7±6.5 20.3–99.3	86.3±8.7 28.2–144.1
Fatbody dry mass (mg)	19.9±1.6 6.6–27.0	23.6±1.8 4.8–40.6	30.0±1.8 20.7–40.3	29.1±1.7 16.2–44.0	32.8±2.1 11.9–49.7	30.7±2.2 12.7–48.0	26.6±2.6 9.3–38.1	28.2±2.0 17.3–47.0
Hatchling lipids (mg)	71.5±3.4 40.8–91.8	74.5±2.7 35.6–95.3	77.0±2.6 64.3–94.9	76.8±3.0 49.2–95.9	74.6±3.1 44.6–98.9	76.0±4.7 40.8–134.7	68.9±3.4 48.4–87.8	76.0±4.1 42.6–108.1
Hatchling energy (kJ)	8.42±0.34 5.15–10.64	8.51±0.25 5.35–10.56	8.76±0.22 7.76–10.13	8.87±0.22 7.6–10.47	9.06±0.17 7.87–10.64	9.18±0.45 5.42–15.45	8.27±0.32 6.42–10.11	8.72±0.39 5.49–12.53
Hatchling ash (mg)	57.7±1.6 43.3–71.3	58.2±1.2 43.1–65.7	59.7±1.3 52.9–69.0	59.9±1.1 53.4–67.4	57.8±1.5 40.7–67.2	60.0±2.3 47.3–93.4	53.4±1.6 45.1–62.0	58.4±2.1 40.6–76.9

Data are expressed as mean±SE and range.

A principal component analysis resolved two components (with eigenvalues $\geqslant 1$) from 10 hatchling variables, accounting for 71.1% of the variation in the original data (Table 3). The first component (52.6% variance explained) had high positive loading for egg size-free values of wet body mass, dry body mass, carcass dry mass, lipid mass, energy contents and ash mass, and the second component (18.5% variance explained) had high negative loading for the egg size-free value of residual yolk dry mass (Table 3). Hatchlings incubated under different thermal conditions differed in their scores on the first (ANOVA, $F_{3,124}=6.00$, $P<0.001$; Ft^{bc}, 24^{a}, 27^{ab}, 30^{c}, Tukey's *post hoc* test, $a>b>c$) and second axes (ANOVA, $F_{3,124}=15.77$, $P<0.0001$; Ft^{b}, 24^{a}, 27^{a}, 30^{b}, Tukey's *post hoc* test, $a>b$), and could be divided into two groups: one group included hatchlings from the 24 and 27 °C treatments, and the other included hatchlings from the Ft and 30 °C treatments (Fig. 3).

4. Discussion

Female hatchlings were larger in SVL but smaller in tail length than males from the same-sized egg (Table 2), indicating that, as in other snakes such as *Naja atra* (Chinese cobra; Ji and Du, 2001b; Lin et al., 2008), *Ptyas korros* (gray ratsnake; Du and Ji, 2002), *Deinagkistrodon acutus* (five-paced pit-viper; Lin et al., 2005) and *Bungarus multicintus* (multi-banded krait; Ji et al., 2007), sexual dimorphism occurs at hatching in *X. piscator*. The other examined phenotypic traits were affected neither by sex nor by the sex × temperature interaction. The thermal conditions under which *X. piscator* eggs were incubated affected a number of hatchling traits examined (wet mass, SVL, tail length, carcass dry mass, fatbody dry mass and residual yolk dry mass) but not hatching success and the sexual phenotype of hatchlings. Hatchlings incubated under the four thermal regimes did not differ from each other in energy and lipid contents, suggesting that *X. piscator* is amongst snake species where embryos can complete development at nearly the same energy expenditure over a wide range of incubation temperatures (Ji and Du, 2001a, b; Chen and Ji, 2002; Du and Ji, 2002, 2008; Lin and Ji, 2004; Lin et al., 2005).

The differences in hatchling wet mass among the four treatments primarily resulted from variation in the water content, because hatchlings from the four treatments did not differ from each other in dry mass. Development conditions of carcass

Table 3
Loading of the first two axes of a principal component analysis on 10 variables of hatchling traits.

	Factor loading	
	PC 1	PC 2
Snout-vent length	0.514	0.454
Tail length	0.637	0.410
Wet body mass	**0.813**	0.214
Dry body mass	**0.917**	−0.346
Carcass dry mass	**0.836**	0.390
Residual yolk dry mass	0.246	**−0.879**
Fat body dry mass	0.539	0.385
Hatchling lipids	**0.709**	−0.249
Hatchling ash	**0.900**	−0.200
Hatchling energy	**0.855**	−0.364
Variance explained (%)	52.6	18.5

Size effects are removed in all cases by using residuals from the regressions on initial egg mass.
Variables with the main contribution to each factor in bold face font.

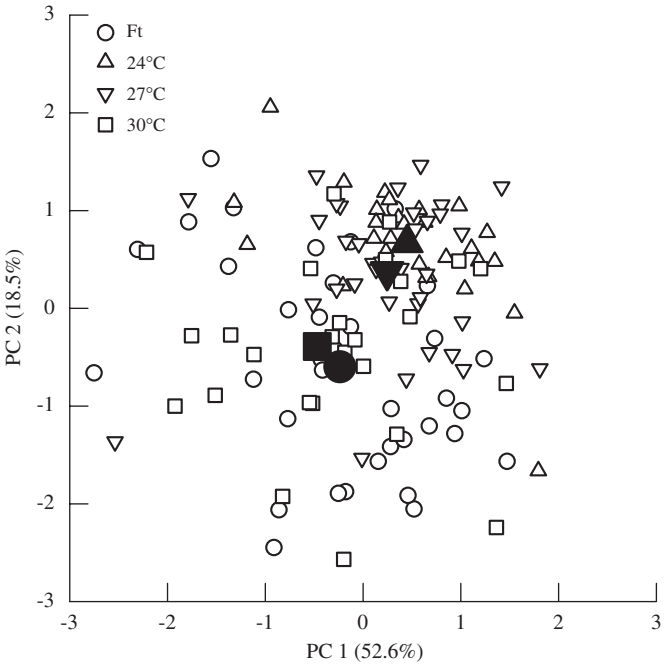

Fig. 3. Positions of *X. piscator* hatchlings from different temperature treatments (symbols on the top left corner) in the space defined by the first two axes of a principal component analysis based on 10 hatchling variables. Effects of egg size were removed using residuals from the regressions of corresponding variables on initial egg mass. Larger black symbols show the mean values of scores on the two axes.

differed among the four treatments, with hatchlings incubated at 24 and 27 °C having larger carcasses [and thus, larger body dimensions (SVL and tail length)] but smaller residual yolks (Table 2). This pattern is consistent with that reported for other reptiles so far studied where incubation at low and medium temperatures results in a greater amount of yolk converted to somatic tissue such that hatchlings incubated at these temperatures tend to have larger carcasses but smaller residual yolks (Lin et al., 2008 and included references). Residual yolk can be used by hatchling snakes for maintenance and somatic tissue growth during their first post-hatching days, with the depletion of residual yolk always followed by a subsequent carcass growth (Ji et al., 1997, 1999; Ji and Sun, 2000). Such an "ebb and flow" relationship between carcass and residual yolk is very similar to that between embryo and yolk seen during embryogenesis (Cai et al., 2007). In *X. piscator*, it is possible for (small) hatchlings incubated at high temperatures to increase their linear dimensions (SVL and tail length) by using residual yolk (Ji et al., 2001), but this entails additional energetic costs associated with carcass growth (Cai et al., 2007). In the present study, embryos in the 30 °C and Ft treatments completed development at nearly the same energy expenditure as did those at the two lower incubation temperatures but produced smaller hatchlings with smaller carcasses, suggesting that embryos in the former two treatments would consume more energy to grow to the size of embryos developing at 24 and 27 °C.

Hatchlings incubated at 24 and 27 °C did not differ in any examined trait, suggesting that these two temperatures do not have any differential effects on hatchling phenotypes in *X. piscator*. Body sizes were larger in hatchlings from incubation temperatures of 24 and 27 °C compared with 30 °C, suggesting that the temperature of 30 °C is suboptimal for incubation of *X. piscator* eggs. The upper threshold over which incubation temperatures are suboptimal differs among species, being broadly correlated with a species' natural incubation environment (Booth, 2004; Deeming, 2004; Lin et al., 2005). For example, eggs can be incubated at 30 °C without any important adverse effects on hatchling phenotypes in *Elaphe carinata* (king ratsnake; Ji and Du, 2001a), *E. taeniura* (stripe-tailed ratsnake; Du and Ji, 2008), *Rhabdophis tigrinus lateralis* (red-necked keelback snake; Chen and Ji, 2002) and *B. multicintus* (Ji et al., 2007) using relatively warm habitats. However, the upper threshold (28 °C) is lower in *D. acutus* using relatively cool habitats near mountain streams (Lin et al., 2005).

Most studies applying natural or fluctuating-temperature incubation show that fluctuating temperatures influence hatchling traits differently than constant temperatures even when the means are the same between treatments (Lin et al., 2008 and included references). Nonetheless, whether the observed effects result from the thermal variance, temperature extremes, or both remains largely unknown due to the paucity of studies examining the ways that specific attributes of incubation thermal regimes affect hatchling phenotypes. In the present study, hatchlings from the Ft treatment were more similar to hatchlings incubated at 30 °C than to hatchlings incubated at 27 °C, although the mean temperature (27.7 ± 0.2 °C) in the Ft treatment was much closer to 27 °C. Does this result mean that the addition of the thermal variance in the Ft treatment can affect hatchling phenotypes? To answer this question, we need to look at whether the thermal variance *per se* is a significant source of variation in hatchling phenotypes.

In the Ft treatment, incubation temperatures higher than 30 °C accounted for 16–48% of total temperature recordings (Fig. 1), and the thermal variance varied from 7.7 to 12.3. In opposition to what is expected, the thermal variance was not a significant predictor of all hatchling traits examined in this study. Hatchlings from the 30 °C and Ft treatments had smaller body sizes as compared with those from the 24 and 27 °C treatments. This temperature-induced modification of body size is highly consistent with the finding from an earlier study of *X. piscator* where eggs incubated at high temperatures produced hatchlings with shorter total lengths as compared with those incubated at moderate temperatures (Ji et al., 2001). This consistency suggests that incubation at 30 °C or higher can substantially modify hatchling size in *X. piscator* but does not provide any evidence showing the importance of the thermal variance in determining this hatchling phenotype. Taken together, our data show that the variance of incubation temperatures is if any less important in determining

the phenotype of *X. piscator* hatchlings. This result is consistent with that reported for other reptiles such as *R. t. lateralis* (Chen and Ji, 2002), *N. atra* (Lin et al., 2008), *Eumeces chinensis* (Chinese skink; Chen et al., 2003), *Eremias argus* (Mongolian racerunner; Hao et al., 2006) and *Pelodiscus sinensis* (Chinese softshell turtle; Ji et al., 2003). In these species, thermal fluctuations during incubation have no role in modifying hatchling traits as long as eggs are not exposed to extreme temperatures for long periods of time.

It is widespread among reptiles that incubation length increases at an ever-increasing rate as temperature decreases within the range where successful development can take place (Birchard, 2004). Such a pattern provides an inference that eggs incubated at fluctuating temperatures should take a longer time to hatch than those at constant temperatures with the same mean. Interestingly, however, fluctuating temperatures result in longer incubation lengths relative to constant temperatures in some species (Shine, 2004a; Hao et al., 2006; Braña and Ji, 2007; Les et al., 2007) but not in others (Andrews et al., 2000; Shine, 2004b; Du and Ji, 2006; Ji et al., 2007; Lin et al., 2007, 2008). In the present study, incubation length decreased by approximately 19 days as temperature increased from 24 to 27 °C and by approximately 12 days as temperature increases from 27 to 30 °C (Table 1), showing a pattern similar to the aforementioned one. However, contrary to what was expected the Ft treatment resulted in shorter incubation periods relative to the constant treatment with the same mean temperature (Fig. 2). This result suggests that, as in *Bassiana duperreyi* (three-lined skink; Shine, 2004b) and *N. atra* (Lin et al., 2008), incubation at stable temperatures may lead to delayed hatching in *X. piscator*. In the Ft treatment, incubation length was not correlated with the thermal variance when holding the thermal mean constant. Thus, although thermal fluctuations during incubation play an important role in influencing incubation length, the thermal variance is not a determinant of this thermally sensitive trait.

Acknowledgments

We thank Hui-Li Chen and Ling Zhang for their assistance in the laboratory. The experiment complied with the current laws on animal welfare and research in China. Financial support was provided by the grants from Natural Science Foundation of China (30370229) and Nanjing Normal University.

References

Ackerman, R.A., Lott, D.B., 2004. Thermal, hydric and respiratory climate of nests. In: Deeming, D.C. (Ed.), Reptilian Incubation: Environment, Evolution, and Behaviour. Nottingham University Press, Nottingham, pp. 15–43.

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

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., 2004. Artificial incubation. In: Deeming, D.C. (Ed.), Reptilian Incubation: Environment, Evolution, and Behaviour. Nottingham: Nottingham University Press, Nottingham, pp. 253–263.

Booth, D.T., 2006. Influence of incubation temperature on hatchling phenotype in reptiles. Physiol. Biochem. Zool. 79, 274–281.

Braña, F., Ji, X., 2007. The selective basis for increased egg retention: early incubation temperature determines hatchling phenotype in wall lizards. Biol. J. Linn. Soc. 92, 441–447.

Cai, Y., Zhou, T., Ji, X., 2007. Embryonic growth and mobilization of energy and material in oviposited eggs of the red-necked keelback, *Rhabdophis tigrinus lateralis*. Comp. Biochem. Physiol. A 147, 57–63.

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 Ecol. Sin. 22, 1850–1858.

Chen, X.-J., Lin, Z.-H., Ji, X., 2003. Further studies on influence of temperature on egg incubation in the Chinese skink, *Eumeces chinensis*. Zool. Res. 24, 21–25.

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. 229–251.

Du, W.-G., Ji, X., 2002. Influence of incubation temperature on hatching success and hatchling traits in a colubrid snake, *Ptyas korros*. Acta Ecol. Sin. 22, 548–553.

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). J Exp. Zool. A 305, 47–54.

Du, W.-G., Ji, X., 2008. The effects of incubation temperature on hatching success, embryonic use of energy and hatchling morphology in the stripe-tailed ratsnake *Elaphe taeniura*. Asiat. Herpetol. Res. 11, 24–30.

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 Zool. Sin. 52, 1049–1057.

Ji, X., Braña, F., 1999. The influence of thermal and hydric environments on incubating eggs and embryonic use of energy and nutrients in the wall lizard *Podarcis muralis*. Comp. Biochem. Physiol. A 124, 205–213.

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*. J. Zool. London 261, 409–416.

Ji, X., Du, W.-G., 2001a. The effects of thermal and hydric environments on hatching success, embryonic use of energy and hatchling traits in a colubrid snake, *Elaphe carinata*. Comp. Biochem. Physiol. A 129, 461–471.

Ji, X., Du, W.-G., 2001b. The effects of thermal and hydric conditions on incubating eggs and hatchling traits in the cobra *Naja naja atra*. J. Herpetol. 35, 186–194.

Ji, X., Du, W.-G., Xu, X.-F., 2001. Influence of thermal and hydric environments on incubating eggs and resultant hatchlings in a colubrid snake (*Xenochrophis piscator*). Acta Zool. Sin. 47, 45–52.

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

Ji, X., Sun, P.-Y., 2000. Embryonic use of energy and post-hatching yolk in the gray rat snake, *Ptyas korros* (Colubridae). Herpetol. J. 10, 13–17.

Ji, X., Sun, P.-Y., Fu, S.-Y., Zhang, H.-S., 1997. Utilization of energy and nutrients in incubating eggs and post-hatching yolk in a colubrid snake, *Elaphe carinata*. Herpetol. J. 7, 7–12.

Ji, X., Xu, X.-F., Lin, Z.-H., 1999. Influence of incubation temperature on characteristics of *Dinodon rufozonatum* (Reptilia: Colubridae) hatchlings, with comments on the function of residual yolk. Zool. Res. 20, 343–346.

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

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 Zool. Sin. 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? J. Therm. Biol. 33, 193–199.

Lin, Z.-H., Ji, X., 2004. Reproductive output and effects of incubation thermal environments on hatchling phenotypes of mucous rat snakes *Ptyas mucosus*. Acta Zool. Sin. 50, 541–550.

Lin, Z.-H., Ji, X., Luo, L.-G., Ma, X.-M., 2005. Incubation temperature affects hatching success, embryonic expenditure of energy and hatchling phenotypes of a prolonged egg-retaining snake, *Deinagkistrodon acutus* (Viperidae). J. Therm. Biol. 30, 289–297.

Shine, R., 2004a. Incubation regimes of cold-climate reptiles: the thermal consequences of nest-site choice, viviparity and maternal basking. Biol. J. Linn. Soc. 83, 145–155.

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

Shine, R., 2005. Life-history evolution in reptiles. Annu. Rev. Ecol. Evol. Syst. 36, 23–46.

Zhao, E.-M., 1998. *Xenochrophis* Günther. In: Zhao, E.-M., Huang, M.-H., Zong, Y. (Eds.), Fauna Sinica, Reptilia (Squamata: Serpentes), vol. 3. Science Press, Beijing, pp. 322–327.