

# Phenotypic Responses of Hatchlings to Constant Versus Fluctuating Incubation Temperatures in the Multi-banded Krait, *Bungarus multicinctus* (Elapidae)

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Most studies on egg incubation in reptiles have relied on constant temperature incubation in the laboratory rather than on simulations of thermal regimes in natural nests. The thermal effects on embryos in constant-temperature studies often do not realistically reflect what occurs in nature. Recent studies have increasingly recognized the importance of simulating natural nest temperatures rather than applying constant-temperature regimes. We incubated *Bungarus multicinctus* eggs under three constant and one fluctuating-temperature regimes to evaluate the effects of constant versus fluctuating incubation temperatures on hatching success and hatchling phenotypes. Hatching success did not differ among the four treatments, and incubation temperature did not affect the sexual phenotype of hatchlings. Incubation length decreased as incubation temperature increased, but eggs incubated at fluctuating temperatures did not differ from eggs incubated at constant temperatures with approximately the same mean in incubation length. Of the hatchling phenotypes examined, residual yolk, fat bodies and locomotor performance were more likely affected by incubation temperature. The maximal locomotor speed was fastest in the fluctuating-temperature and 30°C treatments and slowest in the 24°C treatment, with the 27°C treatment in between. The maximal locomotor length was longest in the fluctuating-temperature treatment and shortest in the 24°C and 27°C treatments, with the 30°C treatment in between. Our results show that fluctuating incubation temperatures do not influence hatching success and hatchling size and morphology any differently than constant temperatures with approximately the same mean, but have a positive effect on locomotor performance of hatchlings.

**Key words:** Elapidae, *Bungarus multicinctus*, egg, incubation, hatching success, temperature, hatchling phenotype

## INTRODUCTION

Temperature exerts profound effects on embryonic development in many vertebrate and invertebrate taxa. In reptiles, for example, the thermal environments experienced by an embryo during incubation can affect not only offspring survivorship but also a number of morphological, physiological and behavioral phenotypes of the hatchling. Some temperature-induced phenotypic changes can have long-term consequences to the hatchlings (Webb and Cooper-Preston, 1989; Burger, 1991, 1998; Johnston *et al.*, 1996; Deeming, 2004). In species with temperature-dependent sex determination, incubation or gestation temperature also determines the hatchling's sexual phenotype (Bull, 1980; Janzen and Paukstis, 1991; Robert and Thompson, 2001).

Unlike viviparous females that have the potential to manipulate their offspring's phenotypes by behavioral thermoregulation, oviparous females do not control thermal conditions of their eggs (except for those brooding eggs; Wang, 1966; Shine *et al.*, 1997a, b). Consequently, a female's choice of nest site can have profound effects on her offspring's phenotypes.

Most studies on the temperature effects on embryogenesis in reptiles have been based on oviparous species in which eggs are incubated at constant temperatures in the laboratory. Eggs in natural nests, however, are subjected to fluctuating temperatures on a daily and seasonal basis during the course of incubation (Overall, 1994; Shine and Harlow, 1996; Shine *et al.* 1997a, b; Valenzuela, 2001). Whereas incubation of eggs at constant temperatures allows examination of hatching success and offspring phenotypes at any given temperature, the thermal effects on hatchling phenotype demonstrated in such an experimental approach often do not realistically reflect what occurs in nature. For

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example, eggs of *Eumeces chinensis* (Chinese skink) cannot be incubated successfully at constant temperatures higher than 32°C, but temporary exposure of eggs to temperatures higher than this upper threshold for brief periods has no noticeable adverse effects on hatching success and size, morphology and locomotor performance of hatchlings (Ji and Zhang, 2001; Chen *et al.*, 2003).

Recent studies in this field have increasingly recognized the importance of simulating natural nest temperatures rather than applying constant-temperature regimes (*e.g.*, Overall, 1994; Shine and Harlow, 1996; Shine *et al.*, 1997a, b; Valenzuela, 2001). Research in the past decade shows that both the mean and the variance of incubation temperatures can have important effects on embryogenesis in a number of reptilian taxa, but the nature and extent of the influence and mechanism of fluctuating versus constant temperatures on developing embryos remains unclear. For example, fluctuating temperatures influence incubation length differently than constant temperatures with the same mean in some reptilian species (Overall, 1994; Shine and Harlow, 1996; Asmore and Janzen, 2003), but not in others (Georges *et al.*, 1994; Andrews *et al.*, 2000; Webb *et al.*, 2001).

In this study, we incubated eggs of *Bungarus multicinctus* (multi-banded krait) under three constant and one fluctuating temperature regimes to evaluate the influence of constant versus fluctuating temperatures on hatching success and hatchling phenotypes. The species is a medium-large sized (up to 1,800 mm total length) oviparous nocturnal elapid snake that ranges from the southern provinces of China (including Taiwan and Hainan) to Burma, Laos and northern Vietnam (Zhao, 1998). The snakes show a preference for habitats near aquatic areas from lowlands along coastal regions to mountains at elevations up to 1,300 m (Zhao, 1998). Females lay a single clutch of pliable-shelled eggs per breeding season (June-August) in relatively shallow nests where temperatures vary appreciably in response to short-term environmental variation in thermal flux (Huang, 1998). The species is an ideal model system to investigate phenotypically plastic responses of embryos to thermal fluctuations. Our study focuses on three questions: (1) Does brief exposure of eggs to extreme temperatures that are harmful or potentially lethal to embryos have any adverse effects on hatching success and hatchling phenotypes? (2) What hatchling phenotypes are more likely to be affected by incubation temperature, and to what extent? (3) Do fluctuating temperatures influence developing embryos differently than constant temperatures?

## MATERIALS AND METHODS

### Egg collection and treatment

Fifteen gravid females were obtained in late June 2001 from a private hatchery in Yongzhou (25°26'N, 111°08'E), Hunan, China. The snakes were brought to our laboratory in Hangzhou, where they were housed individually in 60×60×50 (length×width×height) cm wire-cages placed in a room where temperatures were controlled within the range (26–30°C) that was deemed optimal for activity of the species (Huang, 1998). We checked the cages at least twice daily after the first female laid her eggs to ensure that eggs were collected within 6 hours after oviposition. Snout-vent length (SVL), tail length and body mass were recorded for each postpartum female. Eggs were individually measured for length and

width with a Mitutoyo digital caliper and weighed on a Mettler balance. A total of 60 eggs from 15 clutches of four eggs each were incubated systematically (such that eggs from any one clutch were distributed equally among treatments) under the four temperature regimes, and the remaining eggs were returned to the hatchery together with postpartum females.

Eggs were individually incubated in covered plastic jars (150 ml) containing known amounts of vermiculite and distilled water at approximately –220 water potential (1 g dried vermiculite / 1 g water) (Ji and Braña, 1999). One-third of the egg was buried lengthwise in the incubating substrate, with the surface near the embryo exposed to air inside the jar. Jars were weighed on alternate days, and distilled water was added evenly into substrates when necessary to compensate for evaporative losses and water absorbed by eggs, thereby maintaining the substrate water-potential constant.

A total of 45 jars, containing one egg each and covered with perforated lids, were equally assigned to three Shellab incubators (Sheldon MFG Inc, USA), with incubation temperatures set at 24, 27 and 30 (±0.3)°C, respectively. These temperatures were chosen because temperatures lower than 24°C or higher than 30°C can exert appreciably adverse effects on hatching success and hatchling phenotypes in most species of snakes we have studied (Ji and Du, 2001a, 2001b; Ji *et al.*, 2001; Chen and Ji, 2002; Du and Ji, 2002; Zhang and Ji, 2002; Lin and Ji, 2004). We moved jars among the shelves in the incubator daily according to a predetermined schedule to minimize any effects of thermal gradients inside the incubator. Eggs were weighed every five days, and the final mass was taken for each egg one day prior to hatching.

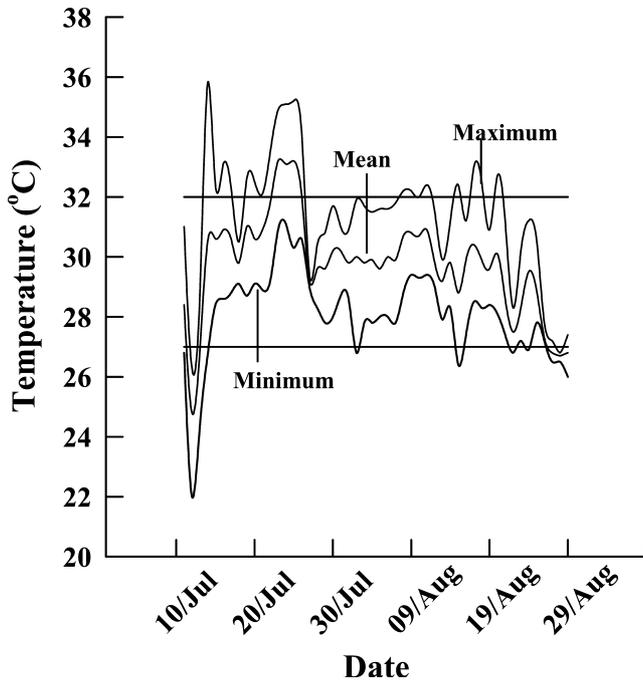
The remaining 15 jars of eggs (hereafter the Ft treatment) were buried 50 cm below the ground surface in a 60×60×30 cm chamber in the bush-covered backyard of our laboratory, thereby simulating thermal conditions in natural nests (Huang, 1998). A Tinytalk datalogger (Gemini Pty, Australia) programmed to record temperature every one hour was placed in the chamber throughout the incubation period, so that temporal variation in temperature inside the chamber was automatically recorded. Temperatures within the chamber varied daily and seasonally; the maximum magnitude of the diel thermal variation was 9.1°C, the mean temperature 30.1°C, and the lowest and the highest temperatures 22.0°C and 35.8°C, respectively (Fig. 1).

### Incubation length and hatchling phenotypes

The duration of incubation, measured as the number of days to pipping, was recorded for each egg. Hatchlings were measured for mass, SVL and tail length at hatching, and were then used to evaluate the effects of incubation temperature on locomotor performance. Because locomotor performance is sensitive to variation in body temperature in reptiles, we conducted all hatchling performance trials at the body temperature of 30°C. This was achieved by placing experimental hatchlings in an incubator for one hour prior to testing. Locomotor performance was assessed by chasing hatchlings along a 100×15×20 (diameter×width×height) cm circular racetrack, which allowed vertical filming with a Panasonic NV-DS77 digital video camera. The tapes were later examined with a computer using MGI VideoWave III software (MGI Software Co., Canada) for the speed in the fastest 25-cm interval (hereafter the maximal speed) and the maximal distance traveled without stopping (*i.e.*, the maximal interval between two consecutive pauses; hereafter the maximal length).

### Hatchling composition

After they were examined for locomotor performance, hatchlings were 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 components of the hatchling were dried in an oven (60°C) to constant mass, weighed and preserved frozen for later analyses. We determined the sex of



**Fig. 1.** Temporal variation in daily minimal, mean and maximal temperatures experienced by *B. multicinctus* eggs incubated under fluctuating temperatures. Daily mean temperatures ranged from 24.8–33.2°C with an average of 30.1±0.2°C, daily minimal temperatures ranged from 22.0–33.1°C with an average of 28.0±0.2°C, and daily maximal temperatures ranged from 26.3–35.8°C with an average of 31.4±0.3°C. Exposure of eggs to temperatures higher than 32°C (the upper horizontal line) for a prolonged period markedly reduces hatching success in other species of snakes found in the geographic range of *B. multicinctus* (see text for citations), and exposure of eggs to temperatures lower than 27°C (the lower horizontal line) dramatically reduces locomotor performance of hatchlings in *B. multicinctus* (see Fig. 4 of this study).

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 a minimum of 5.5 h using absolute ether as the 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 the energy density of each dried sample using a WGR-1 adiabatic bomb calorimeter (Bente Instruments, China). We determined ash content in dried samples by burning them in a muffle furnace at 700°C for a minimum of 12 h and weighing the remaining ash.

#### Data analysis

All data were tested for normality (Kolmogorov–Smirnov test) and homogeneity of variances (Bartlett test), and  $\log_e$  transformations were performed when necessary to satisfy the assumptions for parametric tests. Parametric analyses were used to analyze data when the assumptions for these analyses were met; otherwise, non-parametric analyses were used. The significance level was set at  $\alpha=0.05$ . A principal-component analysis (varimax rotation) was used to investigate the possible existence of space characteristic of hatchlings from different incubation thermal environments. Values are presented as mean±1 standard error.

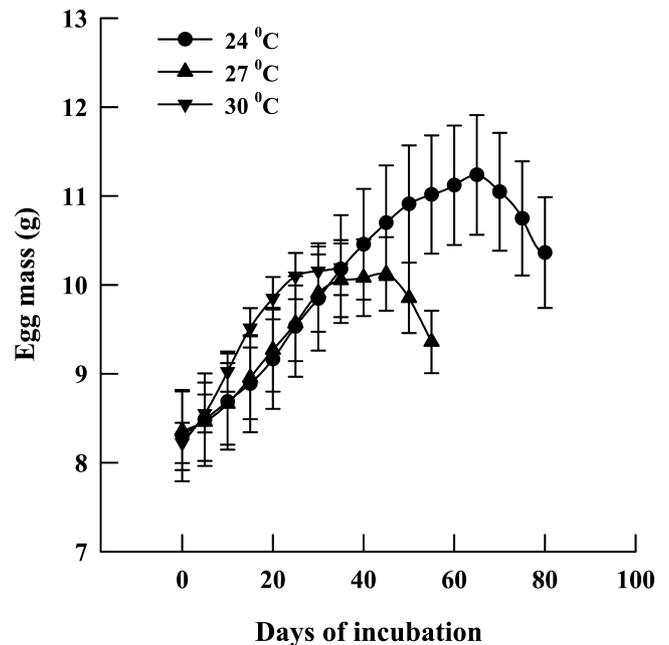
## RESULTS

Females laid eggs from 11–16 July. Descriptive statistics of female reproductive traits are given in Table 1. Both clutch size ( $r^2=0.72$ ,  $F_{1, 13}=34.77$ ,  $P<0.001$ ) and clutch mass ( $r^2=0.68$ ,  $F_{1, 13}=27.70$ ,  $P<0.001$ ) were positively correlated with female SVL, whereas mean egg mass of clutches was not ( $r^2=0.15$ ,  $F_{1, 13}=2.22$ ,  $P=0.160$ ).

Mean values for initial egg mass did not differ significantly among the four treatments ( $F_{3, 48}=0.49$ ,  $P=0.693$ ). Temporal variation in egg mass was very pronounced (repeated measures ANOVA, all  $P<0.0001$ ), with all eggs in the constant-temperature treatments gaining net mass during incubation because of absorption of water (Fig. 2). One-way ANCOVA with initial egg mass as the covariate showed that mean values for final egg mass differed among the three constant-temperature treatments ( $F_{2, 34}=4.78$ ,  $P=0.015$ ), with eggs incubated at 24°C and 30°C gaining significantly more mass than did those incubated at 27°C (Tukey's post-hoc test, both  $P<0.022$ ) (Fig. 2).

**Table 1.** Descriptive statistics for female reproductive traits of *Bungarus multicinctus* ( $N=15$ )

	Mean	SE	Range
Snout-vent length (mm)	923.6	26.1	815.0–1160.0
Tail length (mm)	124.6	3.8	90.0–150.0
Postpartum body mass (g)	138.9	14.5	60.0–253.0
Clutch size	8.1	0.6	5–12
Clutch mass (g)	68.7	6.1	32.1–113.9
Egg mass (g)	8.3	0.4	5.3–10.8
Egg length (mm)	39.2	1.1	33.0–48.9
Egg width (mm)	18.2	0.5	14.7–21.5



**Fig. 2.** Temporal variation in mass of *Bungarus multicinctus* eggs incubated at the three constant temperatures. Data are expressed as mean±SE.

Hatching success did not differ among the four treatments ( $G=0.15$ ,  $df=3$ ,  $P>0.95$ ) (Table 2). Within each treatment, incubation length was not correlated with initial egg mass (all  $P>0.10$ ), and one-way ANOVA revealed that incubation length differed considerably among treatments ( $F_{3, 48}=1796.46$ ,  $P<0.0001$ ). Mean values for incubation length did not differ between eggs incubated at fluctuating temperatures and at constant temperatures with nearly the same mean (30°C; Tukey's post-hoc test,  $P>0.98$ ), whereas constant-temperature treatments at 24, 27 and 30°C differed significantly from each other in developmental duration ( $P<0.0001$  in all three pairwise comparisons). Incubation lengths were on average shortened by 23.4 days from 24°C to 27°C, and by 20.6 days from 27°C to 30°C (Table 2).

Two hatchlings, one incubated at 24°C and the other at 27°C, exhibited slight tail malformation, but the frequency of deformity was independent of treatments ( $G=2.77$ ,  $df=3$ ,  $P>0.25$ ) (Table 2). Although more female hatchlings (33 females versus 19 males) were produced in this study, the sex ratio of hatchlings did not differ significantly among the four treatments ( $G=3.64$ ,  $df=3$ ,  $P>0.10$ ) (Table 2), thus indicating that incubation temperature does not affect the sexual phenotype in *B. multicinctus*.

Male hatchlings had longer tails than did females (ANCOVA with initial egg mass as the covariate,  $F_{1, 49}=19.20$ ,  $P<0.0001$ ), whereas all other examined hatchling traits did not differ between sexes (ANCOVAs for size and composition and ANOVAs for locomotor performance, all  $P>0.10$ ). Most examined traits related to size and composi-

tion of hatchlings did not differ among the four treatments (Table 3). Nonetheless, hatchlings from higher incubation temperatures (27°C, 30°C and Ft treatments) characteristically had larger residual yolks than did those from eggs incubated at 24°C (Table 3). Fat body dry mass also differed significantly among the four treatments, but the differences were in fact very slight (Table 3).

A principal-component analysis resolved two components (with eigenvalues $\geq 1$ ) from ten size (initial egg mass)-free hatchling variables, accounting for 75.2% of the variation in the original data (Table 4). The first component (52.7% variance explained) had high positive loading for size-free values of wet mass, dry mass, energy contents, lipid mass, ash mass and carcass dry mass of hatchlings, and the second component (22.5% variance explained) had high negative loading for size-free values of SVL and tail length (Table 4). Hatchlings from different incubation temperatures differed in their scores on the second axis (ANOVA,  $F_{3, 48}=3.26$ ,  $P=0.029$ ) but not on the first axis (ANOVA,  $F_{3, 48}=1.53$ ,  $P=0.218$ ) (Fig. 3).

Neither maximal locomotor speed nor maximal locomotor length was correlated with hatchling SVL (both  $P>0.05$ ) in this study. One-way ANOVA revealed that both the maximal speed ( $F_{3, 48}=82.95$ ,  $P<0.0001$ ) and the maximal length ( $F_{3, 48}=20.18$ ,  $P<0.0001$ ) differed among the four treatments. The maximal speed was fastest in the Ft and 30°C treatments, slowest in the 24°C treatment, and intermediate in the 27°C (Fig. 4). The maximal length was longest in the Ft treatment and shortest in the 24°C and 27°C treatments, with the 30°C treatment in between (Fig. 4).

**Table 2.** Effects of incubation temperature on duration of incubation, hatching success, sex ratio and abnormality of hatchlings. Data on duration of incubation are expressed as mean $\pm$ SE (range). Ft: the fluctuating temperature treatment

Thermal treatment (°C)	Number of incubated eggs	Duration of incubation (d)	Hatching success (%)	Sex ratio (♀♀ / ♂♂)	Abnormality (%)
24	15	85.0 $\pm$ 0.6 (81.7–90.7)	86.7 (13 / 15)	(10 / 3)	6.7 (1 / 15)
27	15	61.6 $\pm$ 0.5 (58.0–64.3)	80.0 (12 / 15)	(5 / 7)	6.7 (1 / 15)
30	15	41.0 $\pm$ 0.5 (38.7–43.4)	86.7 (13 / 15)	(9 / 4)	0 (0 / 15)
Ft	15	41.0 $\pm$ 0.4 (39.1–43.5)	93.3 (14 / 15)	(9 / 5)	0 (0 / 15)

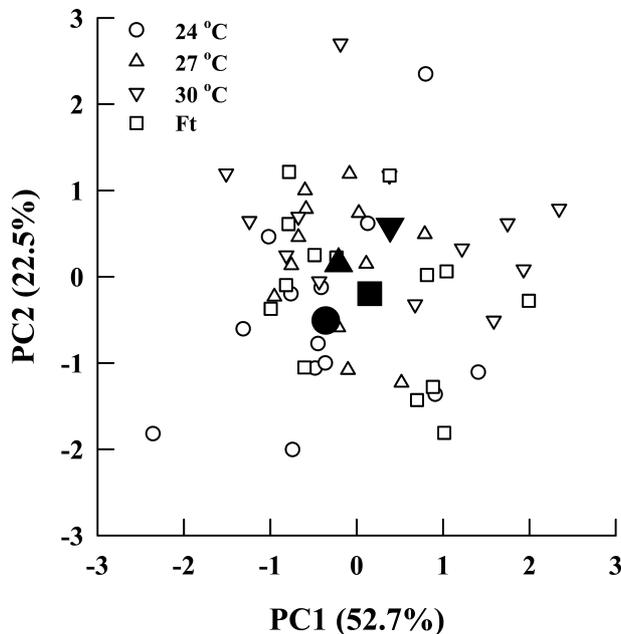
**Table 3.** Size, mass and composition of hatchlings from eggs incubated under different temperature regimes. Data are expressed as mean $\pm$ SE. F values of ANOVA (for initial egg mass) or ANCOVAs (for all other variables, with initial egg mass as the covariate) and significance levels are indicated in the table.

	Thermal treatment (°C)				
	24	27	30	Ft	
N	13	12	13	14	
Initial egg mass (g)	8.3 $\pm$ 0.5 (5.1–10.8)	8.4 $\pm$ 0.4 (5.8–10.6)	8.2 $\pm$ 0.2 (7.1–10.2)	8.8 $\pm$ 0.3 (7.7–11.4)	0.58 <sup>NS</sup>
Snout-vent length (mm)	242.5 $\pm$ 6.1 (208–275)	240.0 $\pm$ 3.6 (219–255)	235.2 $\pm$ 2.3 (220–253)	248.0 $\pm$ 3.1 (227–262)	2.19 <sup>NS</sup>
Female tail length (mm)	39.2 $\pm$ 1.1 (35.0–45.0)	38.1 $\pm$ 0.5 (36.6–39.8)	37.2 $\pm$ 0.7 (34.0–40.0)	38.1 $\pm$ 0.6 (35.0–41.0)	2.14 <sup>NS</sup>
Male tail length (mm)	42.3 $\pm$ 2.0 (39.0–46.0)	40.7 $\pm$ 0.9 (35.7–43.0)	39.5 $\pm$ 1.0 (37.0–42.0)	41.2 $\pm$ 0.4 (40.0–42.0)	
Hatchling wet mass (g)	6.6 $\pm$ 0.5 (3.9–8.8)	6.5 $\pm$ 0.3 (4.5–8.0)	6.6 $\pm$ 0.2 (4.5–7.9)	7.1 $\pm$ 0.2 (5.6–8.2)	1.25 <sup>NS</sup>
Hatchling dry mass (g)	1.71 $\pm$ 0.11 (0.83–2.06)	1.78 $\pm$ 0.10 (1.13–2.23)	1.87 $\pm$ 0.06 (1.53–2.29)	1.91 $\pm$ 0.09 (1.55–2.67)	1.95 <sup>NS</sup>
Hatchling energy (KJ)	42.4 $\pm$ 2.7 (20.1–50.5)	44.4 $\pm$ 2.4 (28.6–54.7)	46.9 $\pm$ 1.7 (38.3–59.0)	47.8 $\pm$ 2.2 (38.9–67.4)	2.26 <sup>NS</sup>
Hatchling lipids (g)	0.48 $\pm$ 0.03 (0.21–0.61)	0.49 $\pm$ 0.02 (0.35–0.59)	0.54 $\pm$ 0.02 (0.43–0.74)	0.55 $\pm$ 0.03 (0.45–0.79)	2.51 <sup>NS</sup>
Hatchling ash (mg)	173.1 $\pm$ 10.7 (93.9–226.2)	183.8 $\pm$ 10.4 (116.7–232.4)	184.7 $\pm$ 5.0 (155.6–210.7)	190.2 $\pm$ 7.0 (158.4–254.0)	1.61 <sup>NS</sup>
Carcass dry mass (g)	1.01 $\pm$ 0.06 (0.64–1.27)	0.96 $\pm$ 0.05 (0.70–1.22)	0.98 $\pm$ 0.02 (0.79–1.10)	1.07 $\pm$ 0.04 (0.85–1.30)	0.92 <sup>NS</sup>
Fat body dry mass (g)	0.26 $\pm$ 0.02 (0.12–0.34)	0.23 $\pm$ 0.01 (0.16–0.27)	0.25 $\pm$ 0.01 (0.18–0.35)	0.29 $\pm$ 0.02 (0.23–0.45)	3.05 <sup>*</sup> ; 24 <sup>ab</sup> , 27 <sup>b</sup> , 30 <sup>ab</sup> , F <sup>a</sup>
Residual yolk dry mass (g)	0.44 $\pm$ 0.05 (0.08–0.70)	0.59 $\pm$ 0.05 (0.27–0.84)	0.64 $\pm$ 0.04 (0.43–0.85)	0.54 $\pm$ 0.04 (0.41–0.91)	6.75 <sup>***</sup> ; 24 <sup>b</sup> , 27 <sup>a</sup> , 30 <sup>a</sup> , F <sup>ab</sup>

Symbols immediately after F values represent significant levels: NS  $P>0.05$ , \*  $P<0.05$ , and \*\*\*  $P<0.001$ . Means corresponding to temperature treatments with different superscripts differ significantly (Tukey's test,  $\alpha=0.05$ ,  $a>b$ )

**Table 4.** Loading of the first two axes of a principal component analysis on ten hatchling variables. 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 are in bold

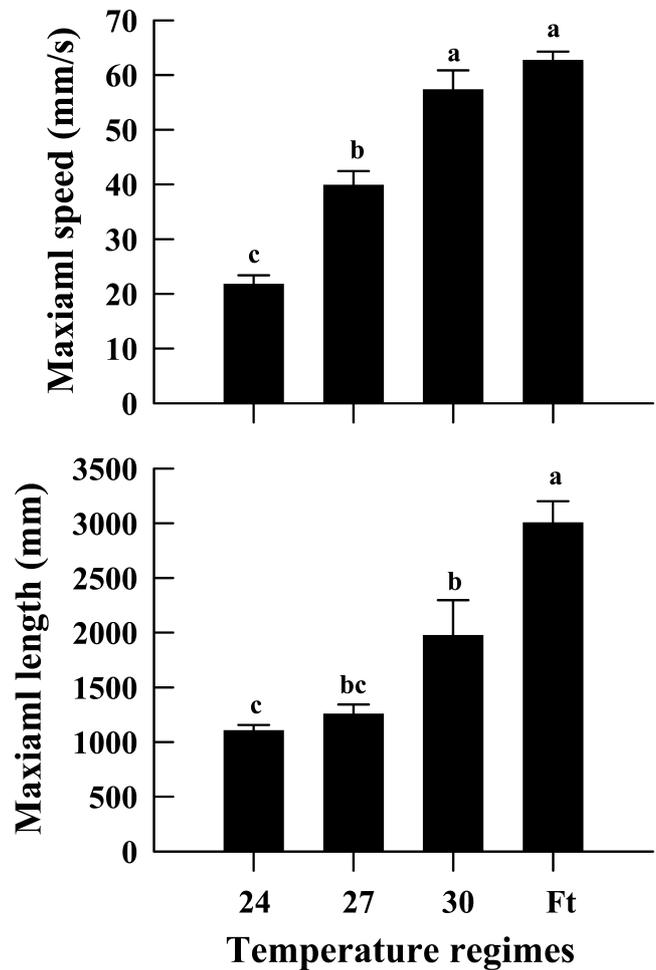
	Factor loading	
	PC 1	PC 2
Snout-vent length	0.189	<b>-0.889</b>
Tail length	-0.243	<b>-0.722</b>
Wet body mass	<b>0.733</b>	-0.377
Dry body mass	<b>0.961</b>	0.218
Hatchling energy	<b>0.858</b>	0.395
Hatchling lipids	<b>0.95</b>	0.287
Hatchling ash	<b>0.902</b>	0.02
Carcass dry mass	<b>0.701</b>	-0.41
Fat body dry mass	0.581	0.003
Residual yolk dry mass	0.66	0.588
Variance explained (%)	52.7	22.5



**Fig. 3.** Positions of hatchlings from eggs incubated under different temperature regimes (symbols on the left top corner) in the space defined by the first two axes of a principal-component analysis based on ten egg size-adjusted 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.

## DISCUSSION

Exposure of reptilian eggs to low incubation temperatures slows or arrests embryonic development with little effect on embryos. Extremely high temperatures substantially increase embryonic abnormality and mortality (Sexton and Marion, 1974; Andrews and Rose, 1994; Andrews *et al.*, 1997; Ji *et al.*, 2003; Du and Ji, 2006). Previous studies of snakes in sympatry with *B. multicinctus* in the southern provinces of China show that hatching success decreases markedly at temperatures higher than 32°C (Ji and Du, 2001a, b;



**Fig. 4.** Mean values (+SE) for the maximal speed and the maximal length of hatchlings from eggs incubated under different temperature regimes. Means with different superscripts differ significantly (Tukey's post-hoc test,  $\alpha=0.05$ , a>b>c).

Ji *et al.*, 2001; Chen and Ji, 2002; Du and Ji, 2002; Zhang and Ji, 2002; Lin and Ji, 2004). In this study, only eggs incubated at fluctuating temperatures had exposure to temperatures higher than 32°C, which occurred mostly during the first one-third of incubation (Fig. 1). However, hatching success was high (93.3%) in the Ft treatment (Table 2). This finding signifies that temporary exposure of *B. multicinctus* eggs to extreme high temperatures does not necessarily increase embryonic mortality and, thus, provides support for the prediction that incubating reptilian eggs at fluctuating temperatures at least has one advantage in widening the range of temperatures yielding viable hatchlings (Chen and Ji, 2002; Chen *et al.*, 2003; Du and Ji, 2003, 2006; Ji *et al.*, 2003).

Incubation of *B. multicinctus* eggs at constant temperatures revealed that incubation length decreased as incubation temperature increased. Such a pattern is widespread in reptiles, although the incubation length at a given temperature differs among species that differ in egg size or embryonic stage at oviposition. For example, the embryonic stage at oviposition, approximately stage 27 according to the classic paper by Zehr (1962), does not differ appreciably between *B. multicinctus* and *Naja atra* (Chinese cobra), but

incubation lengths at identical temperatures are shorter in *B. multicinctus* than in *N. atra* (92.4 d at 24°C and 46.1 d at 30°C; Ji and Du, 2001a) largely because females of the latter species lay much larger eggs (11.9–29.1 g; Ji and Wang, 2005). In *Deinagkistrodon acutus* (five-paced pit-viper), however, relatively short incubation lengths (36.4 d at 24°C and 15.7 d at 30°C; Lin *et al.*, 2005) result primarily from females retaining eggs for a much longer period before oviposition (Lin *et al.*, 2005). In this study, both the mean and the variance of incubation length did not differ between eggs incubated at fluctuating temperatures and at 30°C (Table 2), thus signifying that, as in *Caretta caretta* (loggerhead sea turtle; Georges *et al.*, 1994), *Sceloporus undulatus* (eastern fence lizard; Andrews *et al.*, 2000) and *Tropidonophis mairii* (keelback snake; Webb *et al.*, 2001), fluctuating temperatures do not influence incubation length any differently than constant temperatures with approximately the same mean (30°C) in *B. multicinctus*. In other reptilian species, however, thermal fluctuations may either increase (Ashmore and Janzen, 2003; Hao *et al.*, 2006) or reduce incubation length (Overall, 1994; Shine and Harlow, 1996). It seems that eggs of different reptilian species may respond differentially to the variance of incubation temperatures.

The finding that more yolk remained unutilized at hatching when eggs were incubated at higher temperatures has been reported for nearly all reptiles studied to date (Beuchat, 1988; Phillips *et al.*, 1990; Phillips and Packard 1994; Ji and Braña 1999; Ji and Du, 2001a, b; Ji and Zhang, 2001; Ji *et al.*, 2001, 2002; Chen and Ji, 2002; Du and Ji, 2002; Lin and Ji, 2004; Lin *et al.*, 2005). Statistically, fat-body dry mass differed among treatments, but the differences were in fact very slight (Table 3). Except for the effect of incubation temperature on residual yolk and fat bodies, little variation was detected among measures of size, morphology and composition of hatchlings across the four temperature treatments (Table 3). Therefore, our results are generally consistent with findings from similar studies on reptile egg incubation in confirming the existence of a range of temperatures within which there are no detectable differential effects of incubation temperature on hatchling size and morphology (e.g., Van Damme *et al.*, 1992, Ji and Braña, 1999; Braña and Ji, 2000; Ji and Du, 2001a; Ji and Zhang, 2001; Ji *et al.*, 2001, 2002). For example, no appreciable thermal effects on size and morphology of hatchlings are detectable within the range of 24–30°C in most species of snakes found in the range occupied by *B. multicinctus*, including *Dinodon rufozonatum* (red-banded wolf snake; Ji *et al.*, 1999; Zhang and Ji, 2002), *Elaphe carinata* (king ratsnake; Ji and Du, 2001b), *Ptyas korros* (gray ratsnake; Du and Ji, 2002), *Ptyas mucosus* (mucous ratsnake; Lin and Ji, 2004) and *Rhabdophis tigrinus lateralis* (red-necked keelback; Chen and Ji, 2002).

Incubation temperatures within the range of 24–30°C also exerted no differential effects on size and morphology but modulated locomotor performance (both the maximal speed and the maximal length) of hatchlings in this study, with hatchlings from higher incubation temperatures performing better than did their sibs from lower incubation temperatures (Fig. 4). This finding is interesting, because it suggests that incubation temperatures, while exerting no noticeable effects on morphological phenotypes, can have

remarkable effects on locomotor performance of hatchlings in *B. multicinctus*. It is worth noting that hatchlings from eggs incubated at fluctuating temperatures performed better in the racetrack than those from constant temperatures with the same means in this study (Fig. 4). This result suggests that exposure of eggs to fluctuating temperatures has a positive effect on locomotor performance of hatchlings in *B. multicinctus*, and is therefore consistent with findings from two recent studies on *Apalone mutica* (smooth soft-shelled turtle; Ashmore and Janzen, 2003) and *Takydromus septentrionalis* (northern grass lizard; Du and Ji, 2006) in which increased thermal variance during embryonic development improved locomotor performance of hatchlings.

Taken together, our results show that temporary exposure of *B. multicinctus* eggs to extreme high temperatures that are harmful to embryos does not have adverse effects on hatching success and hatchling phenotypes. Of the hatchling phenotypes examined, residual yolk, fat bodies and locomotor performance are more likely to be affected by incubation temperature. Fluctuating incubation temperatures do not influence hatching success and size and morphology of hatchlings any differently than constant temperatures with approximately the same mean do, but have an overall positive effect on locomotor performance of hatchlings. Temperatures over the range of 24–30°C have no effect on the sexual phenotype of hatchlings, suggesting that *B. multicinctus* is a GSD (genetic sex determination) snake.

#### ACKNOWLEDGMENTS

We are grateful to Hui-Li Chen, Li-Hua Li, Guo-Qiao Wang, Hong-Liang Lu, Gang Shen and Yan Sun for their help during the research. The experiment complied with the current laws on animal welfare and research in China, and was conducted under authority of the Provincial Bureaus of Zhejiang and Hunan. This project was supported by the grants from the Zhejiang Provincial Natural Science Foundation (RC97019), Nanjing Normal University and the local government of Zhejiang Province for the Key Discipline of Zoology.

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