

Effects of Constant and Fluctuating Temperatures on Egg Survival and Hatchling Traits in the Northern Grass Lizard (*Takydromus septentrionalis*, Lacertidae)

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ABSTRACT To understand how nest temperatures influence phenotypic traits of reptilian hatchlings, the effects of fluctuating temperature on hatchling traits must be known. Most investigations, however, have only considered the effects of constant temperatures. We incubated eggs of *Takydromus septentrionalis* (Lacertidae) at constant (24°C, 27°C, 30°C and 33°C) and fluctuating temperatures to determine the effects of these thermal regimes on incubation duration, hatching success and hatchling traits (morphology and locomotor performance). Hatching success at 24°C and 27°C was higher, and hatchlings derived from these two temperatures were larger and performed better than their counterparts from 30°C and 33°C. Eggs incubated at fluctuating temperatures exhibited surprisingly high hatching success and also produced large and well-performed hatchlings in spite of the extremely wide range of temperatures (11.6–36.2°C) they experienced. This means that exposure of eggs to adversely low or high temperatures for short periods does not increase embryonic mortality. The variance of fluctuating temperatures affected hatchling morphology and locomotor performance more evidently than did the mean of the temperatures in this case. The head size and sprint speed of the hatchlings increased with increasing variances of fluctuating temperatures. These results suggest that thermal variances significantly affect embryonic development and phenotypic traits of hatchling reptiles and are therefore ecologically meaningful. *J. Exp. Zool.* 305A:47–54, 2006. © 2005 Wiley-Liss, Inc.

Phenotypic variation generates fitness differentials on which natural selection may act, and is thus a focus of evolutionary biology (Futuyma, '97). For a given phenotypic trait, it is not only coded by the underlying genes, but is also significantly affected by the environment. Therefore, environmental conditions can induce substantial phenotypic variation in animals. In oviparous reptiles, for example, most of the embryonic development occurs outside the mother's body, and incubation environments can greatly affect offspring phenotypes (Deeming, 2004). Temperature is undoubtedly one of the most important environmental factors, and profoundly affects incubation duration (IND), hatching success and hatchling traits such as morphology, behavior, growth and gender (Burger, '90; Booth and Thompson, '91; Deeming and Ferguson, '91; Janzen and Paukstis, '91). In some instances, there are differences in the way that different reptiles are affected by incubation

temperature (Deeming, 2004). For example, hatchling lizards are faster from low-to-moderate incubation temperatures and slower from high temperatures (Van Damme et al., '92; Phillips and Packard, '94; Brana and Ji, 2000; Ji and Zhang, 2001; Du et al., 2003). In contrast, turtles are fastest from intermediate incubation temperatures and are slowest at both extremes (Du et al., 2003; Deeming, 2004).

Although temperatures fluctuate considerably on a daily and seasonal basis in natural nests (Shine and Harlow, '96; Valenzuela, 2001), most

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knowledge of the temperature effects on embryonic development and hatchling traits comes from studies where eggs are incubated at constant temperatures, and how fluctuating temperatures influence reptilian embryos and hatchlings is poorly known to date (Shine and Harlow, '96; Shine et al., '97; Ashmore and Janzen, 2003). Some investigators have tried to reveal the influence of natural incubation environments on hatchling phenotypes by conducting egg incubation experiments in natural nests (e.g., Packard et al., '93; Shine et al., '97; Shine et al., 2003). However, it is often unfeasible to detect temperature effects in natural incubation for at least two reasons. The first is that not only temperature but also moisture varies enormously in natural nests, and this makes it difficult to discriminate temperature effects from moisture effects. The second is the difficulty in locating nest sites of reptiles, especially in lizards. Given these difficulties, alternative methods have been developed to detect the effect of fluctuating incubation temperatures on reptilian eggs and hatchling traits. One approach is to mimic the natural cyclicality of temperatures using programmable incubators in the laboratory (e.g., Shine and Harlow, '96; Webb et al., 2001; Ashmore and Janzen, 2003). Alternatively, eggs may be incubated in an outdoor incubator in which eggs experience fluctuating temperatures but identical water potentials. By using the latter approach one may remove the background noise resulting from variation in nest moisture, so that the effects of fluctuating temperatures can be examined (Castilla and Awallow, '96; Du et al., 2003). Results from these studies suggest that both the variances and the means of incubation temperatures significantly affect developmental rates and hatchling traits (Shine and Harlow, '96; Shine et al., '97). Thus, fluctuating-temperature incubation provides a way to look at how nest temperatures affect phenotypic traits of hatchling reptiles.

In this study, we incubated eggs of *Takydromus septentrionalis* (Lacertidae) at both constant and fluctuating temperatures to examine the effects of these thermal regimes on IND, hatching success and hatchling traits (morphology and locomotor performance). We assessed the effects of fluctuating temperature by incubating eggs in an outdoor incubator and the effect of constant temperature by incubating eggs in four incubators inside which temperatures were controlled at 24°C, 27°C, 30°C and 33°C, respectively. Our aims are to (1) examine the range of viable incubation tempera-

tures in *T. septentrionalis* and (2) evaluate the effects of the means and the variances of fluctuating temperatures on embryonic development and hatchling traits.

MATERIALS AND METHODS

Study species

The northern grass lizard, *T. septentrionalis*, is a small (up to 80 mm snout-vent length (SVL)), diurnal, oviparous lizard, which is widely distributed in the central and southern provinces of China (Zhao and Adler, '93). Female *T. septentrionalis* lay clutches of one-five eggs from early April to August. A former work on this species has indicated that constant temperatures can significantly affect hatching success, hatchling size and sprint speed (Lin and Ji, '98).

Methods

In April 1999, we captured adult *T. septentrionalis* by hand from Zhejiang, eastern China. Lizards were transported to our laboratory in Hangzhou, where they were housed in 60 × 40 × 30 cm³ terraria (ten females and five males per terrarium) filled with sand and grass. A 60 W light bulb was suspended 15 cm above the terrarium floor as a supplementary heating source from 0700 to 1700 hr. Food (larvae of *Tenebrio molitor*) and water (containing mixed vitamins and minerals) were provided ad libitum. Females with shelled oviductal eggs were individually transferred into 20 × 15 × 20 cm³ glass terraria filled with 2 cm-depth moist sand. These small terraria were checked at least five times daily for freshly laid eggs. All eggs were weighed (± 0.001 g) promptly so as to minimize the egg mass change due to loss or gain of water.

We obtained a total of 620 eggs which were produced by 190 females. These eggs were incubated in vermiculite-filled containers that were placed either in four incubators at constant temperatures of 24°C, 27°C, 30°C and 33°C or in an outdoor chamber buried (300 mm below the ground surface) in the backyard of our laboratory. Eggs from individual clutches were assigned as equally as possible to different temperature treatments, thereby minimizing the family effect. The water potential of vermiculite was controlled constant at -12 kPa (Lin and Ji, '98). We weighed all containers daily and added water to compensate for evaporative losses and water absorbed by eggs. The containers holding eggs incubated at

constant temperatures were moved daily among shelves according to a predetermined schedule to minimize the potential effects of the thermal gradient inside the incubator. Temperatures inside the outdoor chamber were taken four times daily at 0600, 1200, 1800 and 2200 hr. The overall mean temperature in the outdoor chamber during the experimental period was 24.2°C, ranging from 11.6°C to 35.0°C.

We had checked the containers at least five times a day since the first hatchling appeared. Each hatchling was weighed, and a sample of hatchlings was randomly selected to determine their locomotor performance. Because locomotor performance is highly sensitive to body temperature in reptiles, we performed trials after placing hatchlings in an incubator at 30°C for 30 min prior to testing (Lin and Ji, '98; Du et al., 2003). We assessed locomotor capacity by chasing the lizards along a 1.5 m racetrack with a paintbrush. The locomotor performance of each lizard was recorded with a Panasonic video camera. Videotapes were then examined for sprint speed in the fastest 150 mm interval and maximum distance in a dash.

We euthanized hatchlings by freezing, and then took the following measurements: SVL, tail length, head length and width, limb length and the number of ventral scales. After measurements, these killed hatchlings were oven dried to constant mass at 65°C and weighed.

Two-way analysis of covariance (ANCOVA) with initial egg mass as the covariate was used to investigate the effects of incubation temperature and sex on the size and mass of hatchlings. To determine whether incubation temperature and sex affect the morphology and locomotor performance of hatchlings, we performed a two-way ANCOVA with hatchling SVL as the covariate. For those traits that are not related to egg size or hatchling SVL, analysis of variance (ANOVA) was used to test for temperature effects. Tukey's post-hoc multiple comparisons were used to distinguish between means of significantly affected traits.

Eggs incubated in the outdoor incubator were produced at different days, allowing us to calculate the mean and variance of incubation temperatures for each egg in the outdoor incubator. Nonlinear estimation was used to calculate the regression function between mean temperature and IND of these eggs. Partial correlation was used to determine relationships between the means and variances of fluctuating temperatures and hatchling traits.

RESULTS

For those eggs incubated at constant temperatures, initial egg mass did not affect the IND (all $P > 0.45$), whereas temperatures significantly affected IND (ANOVA— $F_{3,380} = 3,769.6$ $P < 0.0001$). IND increased nonlinearly with decreasing temperature. With every 3°C decrease in temperature from 33°C to 24°C, IND increased 3.6, 5.4 and 12.9 days, respectively (Table 1). Hatching success statistically differed between different temperatures (G -test, $G = 18.08$, $df = 3$, $P < 0.01$). Hatching success of eggs incubated at 30°C and 33°C was substantially lower than that of eggs incubated at 24°C and 27°C. The sex ratio of the hatchlings differed between temperature treatments (G -test, $G = 11.12$, $df = 3$, $P < 0.05$), with eggs incubated at 33°C producing more males (Table 1).

For those eggs incubated in the outdoor incubator, IND was negatively correlated with mean temperature ($IND = 455.47 - 30.37 \times T + 0.56 \times T^2$, $r = -0.81$, $df = 53$, Fig. 1), but not with the variances of temperatures ($r = 0.24$, $df = 53$, $P = 0.06$). The mean IND of eggs incubated in the outdoor incubator was 46.7 days (range from 40.6 to 49.9 d). Even though these eggs had experienced short periods of extremely low and high temperatures (11.6–36.2°C), the hatching success [91.5% (54/59)] was surprisingly high.

The SVL and body mass of hatchlings from different incubation treatments were positively correlated with initial egg mass (all $P < 0.05$);

TABLE 1. Incubation length, hatching success and sex ratio of hatchlings at different constant temperatures in the northern grass lizard, *Takydromus septentrionalis*

Incubation temperature (°C)	Hatching success (%)	Incubation duration (d)	Sex ratio (♀/♂)
24	87.8 (115/131)	45.4 ± 0.1 (42.5–49.8)	0.76 (44/58)
27	90.5 (152/168)	32.5 ± 0.1 (29.9–35.3)	1.13 (70/62)
30	67.7 (90/133)	27.1 ± 0.1 (24.9–30.6)	1.00 (22/22)
33	35.1 (27/77)	23.5 ± 0.2 (22.0–26.3)	0.26 (5/19)

Data on incubation duration are expressed as mean ± SE (range).

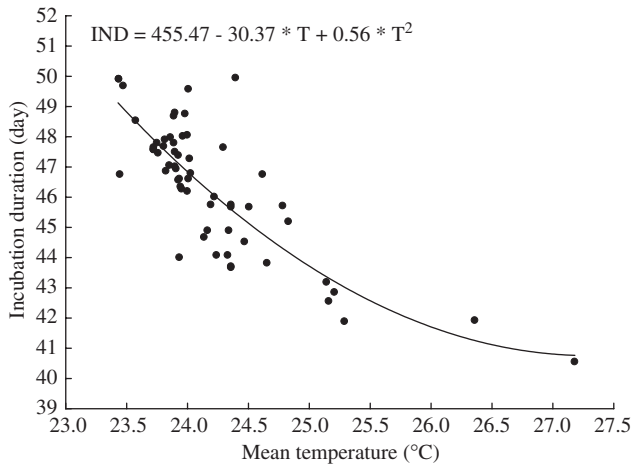


Fig. 1. Relationship between incubation length and the means of fluctuating temperatures.

ANCOVA with initial egg mass as the covariate was thus applied to test the influence of temperature and sex. This analysis showed that incubation temperature significantly affected SVL ($F_{4,223} = 5.83$, $P < 0.0001$; Fig. 2A) and wet body mass of hatchlings ($F_{4,223} = 15.60$, $P < 0.0001$; Fig. 2B), but did not affect dry body mass ($F_{4,223} = 0.51$, $P > 0.05$; Fig. 2C). In contrast, these three traits did not differ between the sexes (All $P > 0.05$). Furthermore, no interaction between temperature and sex suggested that there was no between-sex difference in temperature effects on body size. Tukey's test indicated that the hatchlings incubated at 24°C, 27°C and fluctuating temperatures were larger than those incubated at 30°C and 33°C (Fig. 2).

When performing ANCOVAs with SVL as the covariate to control for the differences in hatchling SVL, we found that incubation temperature significantly affected all hatchling traits involved (Table 2). Hatchlings from 24°C, 27°C and 30°C had longer tails than those from 33°C and fluctuating temperatures. Head size and the number of ventral scales were greater for hatchlings from low temperatures (24°C and 27°C) and fluctuating temperatures than from high temperatures (30°C and 33°C), whereas forelimb and hindlimb lengths were significantly longer for hatchlings from 33°C than those incubated at 27°C (Tukey's test). Male hatchlings had significantly larger head length than females, whereas females had on average 1.5 more ventral scales than males (Table 2).

To further understand the influence of fluctuating temperatures on hatchling traits, we employed

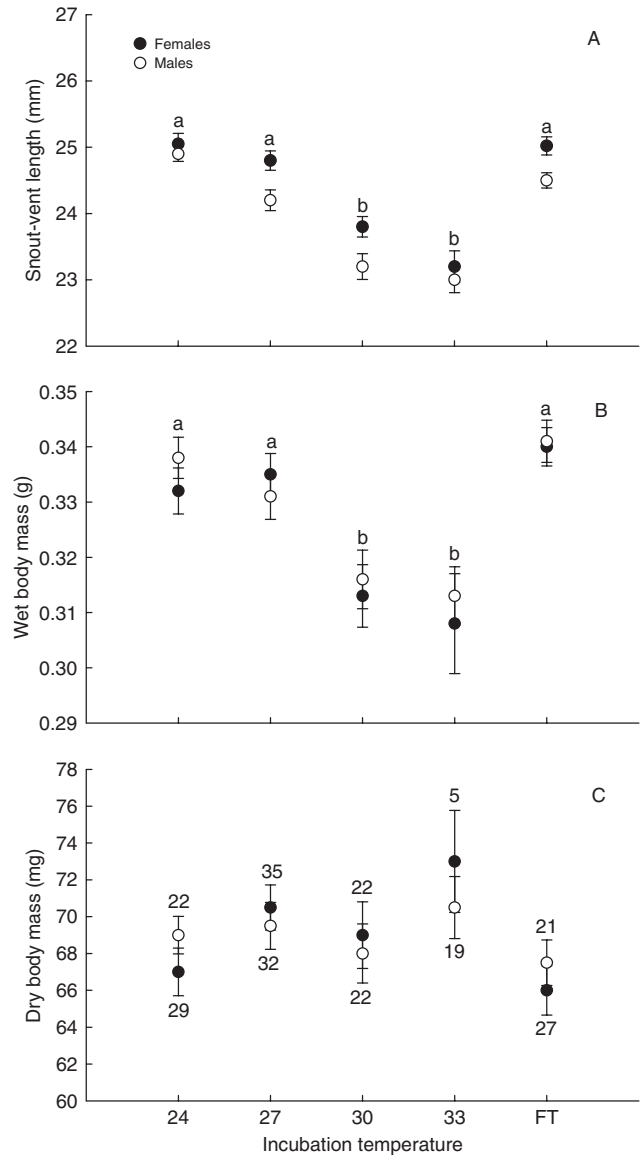


Fig. 2. Effects of incubation temperature and sex on body mass and length of hatchlings in the northern grass lizard, *Takydromus septentrionalis*. Data are expressed as adjusted mean \pm SE. A two-way ANCOVA was employed to test for the temperature and sex effects, with initial egg mass as the covariate that was set at 0.288 g. Sample sizes are shown as numbers above (females) and under (males) the error bar in the lower graph, and apply to all graphs within this figure. Means with different superscripts in the upper two graphs are statistically different (Tukey's test). FT = fluctuating temperature.

partial correlation analysis between hatchling traits and the means and variances of fluctuating temperature. Before conducting the analysis, we removed effects of egg size on hatchling size by calculating residual values of SVL and body mass against initial egg mass, and the influence of

TABLE 2. Effects of incubation temperature and sex on morphological traits of hatchlings in the northern grass lizard, *Takydromus septentrionalis*

Incubation temperature (°C)	Sex	Sample size	Tail length (mm)	Head length (mm)	Head width (mm)	Forelimb length (mm)	Hindlimb length (mm)	Ventral scale
24	♀	22	49.5 ± 0.3	6.62 ± 0.02	4.29 ± 0.02	6.01 ± 0.06	7.21 ± 0.07	27.9 ± 0.2
	♂	29	49.5 ± 0.4	6.75 ± 0.03	4.31 ± 0.02	6.10 ± 0.05	7.28 ± 0.06	26.3 ± 0.2
27	♀	25	49.3 ± 0.4	6.56 ± 0.02	4.22 ± 0.02	5.98 ± 0.04	7.19 ± 0.05	28.0 ± 0.2
	♂	22	49.0 ± 0.4	6.71 ± 0.02	4.25 ± 0.02	6.02 ± 0.05	7.26 ± 0.06	26.6 ± 0.1
30	♀	22	50.1 ± 0.7	6.46 ± 0.03	4.15 ± 0.04	6.16 ± 0.07	7.36 ± 0.07	26.9 ± 0.4
	♂	21	48.9 ± 0.6	6.54 ± 0.04	4.13 ± 0.03	6.13 ± 0.10	7.38 ± 0.09	26.4 ± 0.4
33	♀	5	47.9 ± 1.3	6.35 ± 0.06	4.12 ± 0.04	6.29 ± 0.09	7.49 ± 0.12	26.8 ± 0.5
	♂	19	48.1 ± 0.5	6.53 ± 0.05	4.13 ± 0.04	6.22 ± 0.08	7.43 ± 0.08	25.6 ± 0.4
Fluctuating temperatures	♀	21	47.3 ± 0.4	6.60 ± 0.03	4.23 ± 0.03	5.98 ± 0.06	7.17 ± 0.08	27.8 ± 0.2
	♂	27	48.2 ± 0.4	6.74 ± 0.03	4.26 ± 0.03	6.07 ± 0.08	7.09 ± 0.07	26.1 ± 0.3
Temperature ($F_{4,202}$)			4.27** 24 ^a , 27 ^a , 30 ^a , 33 ^c , F^c	8.47*** 24 ^a , 27 ^b , 30 ^c , 33 ^c , F^{ab}	7.56*** 24 ^a , 27 ^b , 30 ^c , 33 ^c , F^{ab}	3.82** 24 ^{ab} , 27 ^b , 30 ^{ab} , 33 ^a , F^b	4.73** 24 ^{abc} , 27 ^{bc} , 30 ^{ab} , 33 ^a , F^c	2.68* 24 ^{ab} , 27 ^a , 30 ^{bc} , 33 ^c , F^{ab}
Sex ($F_{1,202}$)			0.04 ^{NS}	22.90*** ♂ > ♀	0.19 ^{NS}	0.25 ^{NS}	0.35 ^{NS}	26.89* ♂ < ♀
Interaction ($F_{4,202}$)			0.86 ^{NS}	0.77 ^{NS}	0.48 ^{NS}	0.37 ^{NS}	0.26 ^{NS}	0.50 ^{NS}

Data are expressed as adjusted mean ± SE. A two-way ANCOVA was employed to test for the temperature and sex effects, with hatchling SVL as the covariate that was set at 24.68 mm. Symbols immediately after F values represent significant level. NS $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$. Means with different superscripts differ significantly (Tukey's test, a > b > c).

TABLE 3. Relationships between the means and variances of fluctuating incubation temperatures and hatchling morphology (partial correlation)

	Sample size	Mean temperature	Variance of temperatures
Snout-vent length	48	-0.072 ^{NS}	0.216 ^{NS}
Body mass	48	-0.141 ^{NS}	0.105 ^{NS}
Tail length	48	-0.093 ^{NS}	0.214 ^{NS}
Head length	48	-0.179 ^{NS}	-0.338**
Head width	48	-0.192 ^{NS}	-0.301*
Forelimb length	48	-0.012 ^{NS}	0.214 ^{NS}
Hindlimb length	48	-0.204 ^{NS}	0.053 ^{NS}
Ventral scales	48	0.105 ^{NS}	-0.207 ^{NS}

Symbols immediately after correlation coefficients represent significant level. NS $P > 0.05$, * $P < 0.05$, ** $P < 0.01$.

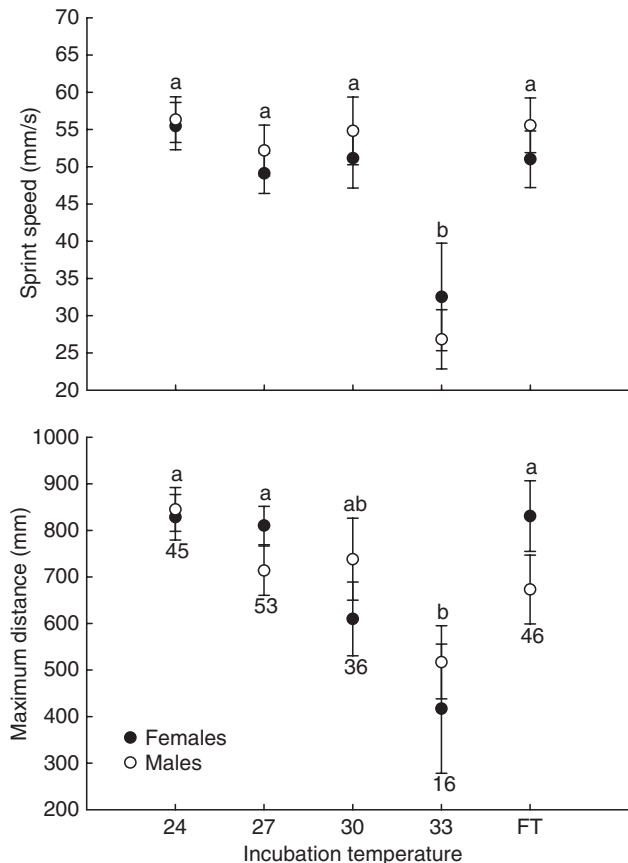


Fig. 3. Effects of temperature on locomotor capacity of hatchlings in northern grass lizard, *Takydromus septentrionalis*. Data are expressed as adjusted mean \pm SE. A two-way ANCOVA was employed to test for the temperature and sex effects, with hatchling snout-vent length as the covariate that was set at 24.61 mm. Numbers under the error bar in the lower graph are sample sizes, and apply to all graphs within this figure. Means with different superscripts in graphs are statistically different (Tukey's test). FT = fluctuating temperature.

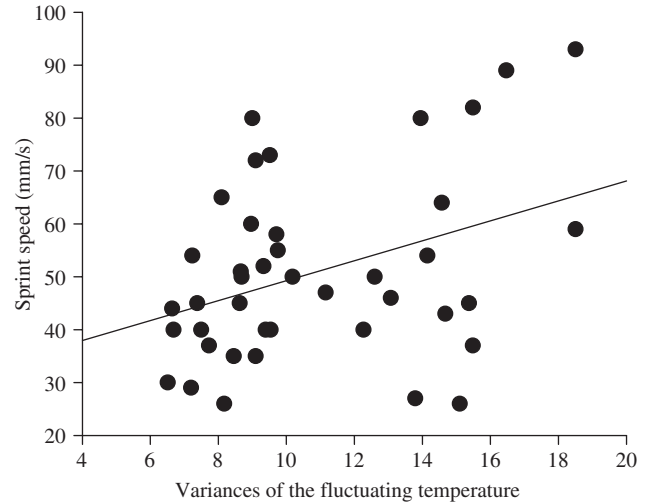


Fig. 4. Sprint speed of hatchlings improves with increasing variance of the fluctuating temperature.

hatchling body size on head length, the number of ventral scales, limb length and tail length by calculating residual values of these traits against hatching SVL. This analysis showed that the mean temperature did not significantly affect any hatchling trait, whereas the variances of fluctuating temperatures negatively correlated with head size (Table 3).

An ANCOVA with hatchling SVL as a covariate was used to test for the effect of temperature and sex on locomotor performance. Incubation temperature significantly affects both sprint speed ($F_{4,187} = 4.11$, $P < 0.01$) and maximal distance in a dash ($F_{4,187} = 3.68$, $P < 0.01$) of hatchlings, but between-sex difference and interaction between temperature and sex were not significant in locomotor performance (all $P > 0.4$). Hatchlings from 33°C had lower sprint speed and maximal distance in a dash than their counterparts from the other temperatures (Fig. 3). For those hatchlings from fluctuating temperatures, mean temperature did not affect sprint speed ($r = -0.03$, $df = 46$, $P = 0.85$) or maximum distance ($r = -0.04$, $df = 46$, $P = 0.80$), whereas the variances of the temperatures positively correlated with sprint speed ($r = 0.32$, $df = 46$, $P < 0.05$; Fig. 4) but not with maximum distance ($r = 0.11$, $df = 46$, $P = 0.48$).

DISCUSSION

Our constant-temperature incubation indicates that temperatures significantly affect hatchling traits in *T. septentrionalis*. The hatchlings from

intermediate and low temperatures are larger and perform better than those from high temperatures. This result is consistent with the general pattern of the temperature effects in lizards that hatchlings are faster from low-to-moderate incubation temperatures and slower from high temperatures (Deeming, 2004). Incubation temperature also modified other hatchling traits such as head size, limb length and the number of ventral scales (Table 2). Of these traits, head size and limb length have been proved to have functional links with foraging success and predator avoidance (Webb, '86; Losos, '90), but the ecological significance of temperature-induced variation in morphological traits is largely unrevealed to date. Thus, long-term field studies on growth and survival of hatchlings from different incubation temperatures would be crucial for future studies in this field (Andrews et al., 2000).

The sex ratio of hatchlings differed significantly between temperature treatments, but we do not think this is because there is a temperature-dependent sex determination mechanism in *T. septentrionalis* for two reasons: (1) the lizard has heteromorphic sex chromosomes (Guo and Dong, '89); and (2) only hatchlings derived from eggs incubated at 33°C were highly biased to males (Table 1). Most probably, this bias of sex ratio results from the between-sex difference in thermal tolerance of embryos (Elphick and Shine, '99).

For any given species, there is a range of viable incubation temperatures within which embryos can not only develop successfully but also produce healthy hatchlings. The upper and lower limits of viable incubation temperatures can be determined by evaluating IND, survival of embryo and hatchling traits (Ji and Du, 2001a,b; Du et al., 2003). In this study, hatching success was highest at 27°C but decreased dramatically at temperatures higher than 30°C. Moreover, hatchlings from temperatures higher than 30°C were small in size and had poor performance in the racetrack. Accordingly, the temperature of 30°C is close to the upper limit of viable incubation temperatures in this species.

By now, empirical evidence for the influence of fluctuating temperature on egg incubation is limited to very few species of reptiles (Overall, '94; Shine and Harlow, '96; Shine et al., '97; Doody, '99; Andrews et al., 2000; Ashmore and Janzen, 2003; Shine et al., 2003). Thus, no general conclusion about the ecological consequence of this temperature treatment can be

drawn at this time. In this study, the mean IND of eggs incubated at the fluctuating temperatures (mean = 24.2°C) is longer than that of eggs incubated at a temperature of 24°C. This result is consistent with that reported for *Bassiana duperreyi* (Shine et al., '97) and *Apalone mutica* (Ashmore and Janzen, 2003), whose eggs are incubated at fluctuating temperatures. In contrast, increasing temperature fluctuation reduces (Overall, '94; Shine and Harlow, '96) or does not affect (Andrews et al., 2000) IND in some other squamate reptiles. Increasing variances of temperatures result in enhanced locomotor performance in hatchling *T. septentrionalis* (Fig. 4). This result has been reported for some reptilian species (Shine and Harlow, '96; Ashmore and Janzen, 2003), but not for others (Shine et al., '97; Doody, '99).

We are quite confident that thermal environments in our outdoor incubator could be similar to those of natural nests in *T. septentrionalis* for three reasons. Firstly, *T. septentrionalis* is locally abundant in Hangzhou where our experiments were conducted. Secondly, our outdoor incubation experiment (from mid-April to the end of July) covered nearly the whole breeding season of *T. septentrionalis* (Wang, '66). Finally, the surrounding of our outdoor incubator was so elaborately established that it is almost the same as the natural habitat used by the lizard. Therefore, we believe that the results from the outdoor incubation are ecologically meaningful. Results from our outdoor incubation indicate that it is the variance rather than the mean of fluctuating temperatures that exerts more significant effects on hatchling traits in *T. septentrionalis* (Table 3, Fig. 4). The small variation in mean temperatures (23.4–27.2°C) explains why the mean of fluctuating temperature in this study has little effect on the examined traits. Hatchlings derived from constant temperatures of 24°C and 27°C did not differ in all examined hatchling traits (Figs. 2 and 3; Table 2). This result provides further evidence that mean temperatures ranging from 24°C to 27°C do not affect hatchling traits. Taken together, the variances of fluctuating temperatures may be ecologically more meaningful than the means of fluctuating temperatures, especially given that constant temperature rarely occurs in the natural nest.

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