Do temperature fluctuations during incubation always play an important role in shaping the phenotype of hatchling reptiles?

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Abstract

Fluctuating temperatures (FTs) influence hatchling phenotypes differently from constant temperatures (CTs) in some reptiles, but not in others. This inconsistency raises a question of whether thermal fluctuations during incubation always play an important role in shaping the phenotype of hatchlings. To answer this question, we incubated eggs of *Naja atra* under one CT (28 °C, CT), two temperature-shift [cold first (CF) and hot first (HF) in which eggs were first incubated at 24 or 32 °C and then at the other, each for 20 days, and finally at 28 °C until hatching], and one FT thermal regimes. Female hatchlings were larger in snout–vent length but smaller in tail length, head size than male hatchlings from the same-sized egg; female hatchlings had more ventral scales than did male hatchlings. The FT and HF treatments resulted in shorter incubation lengths. Tail length was greatest in the CT treatment and smallest in the FT treatment, with the CF and HF treatments in between; head width was greater in the CT treatment than in the other three treatments. Other examined hatchling traits did not differ among treatments. The observed morphological modifications cannot be attributed to the effect of thermal fluctuations but to the effect of temperatures close to the upper and lower viable limits for the species. Our results therefore support the hypothesis that hatchling phenotype is not altered by thermal fluctuation in species with no phenotypic response to incubation temperature within some thresholds.

1. Introduction

An organism's phenotype is determined by the interaction between genetic and environmental factors. Among environmental factors, temperature is the single most important factor affecting many biological processes in organisms. The effects of temperature on developing embryos are more pronounced than those observed in later ontogenetic stages (reviewed in Johnston et al., 1996; Lindström, 1999; Birchard, 2004). Therefore, the embryonic stage provides an excellent time phase to study temperature-induced variation in phenotypic traits. The effect of thermal environments during embryonic development as a proximate source of variation in offspring phenotype has been examined in a wide variety of taxa, and reptiles have proved to be excellent model systems for research in this field. Most studies examining the thermal effects on offspring phenotypic traits in reptiles have been based on oviparous species in which eggs are incubated at constant temperatures (CTs). However, temperatures inside natural nests are rarely constant but vary on a daily and seasonal basis depending on nest depth, microhabitat, locality and year. Thus, the thermal effects demonstrated in CT incubations often do not reflect what truly occurs in nature (reviewed in Ackerman and Lott, 2004; Booth, 2006).

Recent studies have increasingly recognized the importance of simulating natural nest temperatures rather than applying CT regimes (e.g. Overall, 1994; Shine and Harlow, 1996; Shine et al., 1997a, b; Andrews et al., 2000;

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Valenzuela, 2001; Ashmore and Janzen, 2003; Shine, 2004a; Les et al., 2007). These studies generally show that both the mean and the extent of fluctuation in incubation temperatures have important effects on embryogenesis in reptiles, but they do not reach a consistent conclusion regarding the influence of thermal fluctuation on hatchling phenotype. For example, fluctuating temperatures (FTs) influence hatchling size [snout–vent length (SVL), tail length or body mass] differently from CTs with the same mean temperature in some species (Shine and Harlow, 1996; Shine et al., 1997a; Andrews et al., 2000; Webb et al., 2001; Les et al., 2007), but not in others (Chen and Ji, 2002; Ashmore and Janzen, 2003; Chen et al., 2003; Ji et al., 2003, 2007; Lin and Ji, 2004; Hao et al., 2006; Lin et al., 2007). This inconsistency raises a question of whether temperature fluctuations during incubation always play an important role in shaping the phenotype of hatchling reptiles. Species in which fluctuating incubation temperatures can affect offspring phenotypes differently from CT incubation with the same mean temperature, that effect could be due to either thermal fluctuation *per se*, or the fact that fluctuations result in the eggs being exposed to extreme temperatures for brief periods (Shine, 2005). To test between these two possibilities, we need model animals with no phenotypic response to incubation temperature within some thresholds. Species in which incubation temperatures varying within some thresholds do not have differential effects on hatchling phenotype, we hypothesize that hatchling phenotype is not altered by thermal fluctuation.

Here, we describe a study incubating eggs of *Naja atra* (Chinese cobra) under four temperature regimes (see below for details) to test our hypothesis. The Chinese cobra is a large-sized (to 1800 mm SVL) and highly venomous elapid snake widely distributed in the southeastern provinces of China, including Taiwan, Hong Kong and Hainan, southwards to northern Vietnam (Wüster et al., 1997; Huang, 1998). Females lay a single clutch of 5–28 pliable-shelled eggs per breeding season stretching from late June to early August (Ji and Wang, 2005). The effects of every viable temperature on incubation length, hatching success and hatchling phenotype have been examined in *N. atra* (Ji and Du, 2001a; Lin, 2005). Incubation temperatures outside the range of 24–32 °C are lethal to developing embryos, whereas temperatures within the range of 26–30 °C do not have differential effects on any examined hatchling trait (Ji and Du, 2001a; Lin, 2005). Therefore, the cobra is ideally suited to testing our hypothesis.

2. Materials and methods

2.1. Egg collection and incubation

Fifteen gravid females (957–1313 mm SVL) were obtained on 22 June 2005 from a private hatchery in Baise (23°91′N, 106°62′E), Guangxi, China. Females were brought to our laboratory at Hangzhou Normal Uni-

versity, where they were housed individually in 60 cm × 60 cm × 50 cm (length × width × height) wire cages placed in a room where temperatures were controlled within the range of 26–30 °C optimal for embryonic development (Ji and Du, 2001a). We checked the cages at least twice daily after the first female laid eggs, thereby ensuring that all eggs were collected within a few hours after oviposition. SVL, tail length and body mass were taken for each postpartum female. Eggs were individually measured (to the nearest 0.01 mm) for length and width with a Mitutoyo digital caliper and weighed (to the nearest 1 mg) on a Mettler balance. A total of 197 eggs, 10–15 from each of the 15 clutches, were divided among the four temperature treatments (see below for details) as equally as possible. The remaining eggs and postpartum females were returned to the hatchery soon after the last female laid her eggs.

Eggs ($N = 197$) used in the present study were individually incubated in covered plastic jars (250 ml) containing 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 being exposed to air inside the jar. Jars were weighed at 5-day intervals, and water was evenly added into substrates when necessary to compensate for evaporative losses and water absorbed by eggs, thereby maintaining a nearly constant substrate water potential.

A total of 149 eggs were assigned to one of the three treatments with the same mean temperature: one constant [28 ± 0.3 °C; hereafter the CT treatment] and two temperature-shift thermal regimes [24–32–28 °C: hereafter the cold first (CF) treatment; 32–24–28 °C: hereafter the hot first (HF) treatment] in which eggs were first incubated at 24 ± 0.3 or 32 ± 0.3 °C and then at the other temperature, each for 20 days [about 1/3 of the mean incubation length (~59 days) at 28 °C; Lin, 2005], and finally at 28 °C until hatching. These temperatures were chosen because the temperature of 28 °C falls within the range of optimal incubation temperatures (26–30 °C) and temperatures below 24 °C and above 32 °C have a lethal effect on embryos in *N. atra* (Ji and Du, 2001a). The temperature-shift sequences in the CF and HF treatments were arranged to equalize the time spent at the three temperatures and to obtain the identical mean temperature in the three treatments. Three Shellab incubators (Sheldon MFG Inc, USA) were used to incubate these eggs; temperature variation inside the incubator was controlled based on recordings from one probe placed in one container with moistened vermiculite similar to those containing eggs. Jars were moved among the shelves in the incubator at 5-day intervals according to a predetermined schedule, thereby minimizing the potential effects of any inconsistencies in temperature within each incubator.

The remaining 48 eggs [hereafter the FT treatment] were buried 50 cm below the ground surface in a 60 cm × 60 cm × 30 cm soil-constructed chamber in the bush-covered
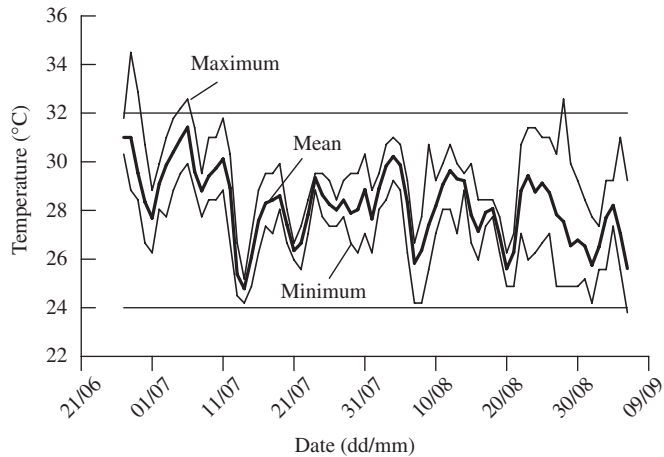
Fig. 1. Temporal variation in daily minimal, mean and maximal temperatures experienced by *N. atra* eggs incubated under fluctuating temperatures. Daily mean temperatures ranged from 24.8 to 31.4 °C with an average of 27.9±0.2 °C, daily minimal temperatures ranged from 22.0 to 30.3 °C with an average of 26.6±0.2 °C, and daily maximal temperatures ranged from 25.2 to 34.2 °C with an average of 29.5±0.2 °C. Exposure of *N. atra* eggs to temperatures higher than 32 °C (the upper horizontal line) or lower than 24 °C (the lower horizontal line) for a prolonged period may dramatically decrease hatchability (Ji and Du, 2001a).

backyard of our laboratory, thereby mimicking thermal conditions in natural nests. The nest depth was chosen because it was about the halfway between the shallowest (~20 cm) and deepest (~80 cm) nests ($N = 8$) located in the field. A Tinytalk datalogger (Gemini Pty, Australia) programmed to record temperature every 1 h was placed in the chamber throughout the incubation period, so that temporal variation in temperatures could be automatically recorded. Temperatures within the chamber varied daily and seasonally (Fig. 1); the maximum magnitude of diel thermal variation, the mean temperature, the lowest temperature and the highest temperature were 8.4, 27.9, 21.8 and 34.5 °C, respectively.

2.2. Measurements and sex determination

Incubation length, measured as the time between oviposition and pipping, was recorded for each egg. Morphological measurements taken for each hatchling included body mass, SVL, tail length, head length, head width, jaw length and the number of ventral scales. Sex was determined by pressing on both sides of the tail base for the presence or absence of hemipenes, and hatchlings with hemipenes were considered as males. All hatchlings were used to evaluate the response to chemical cues following morphological measurements and sex determination. Because physiological and behavioral performances are highly sensitive to variation in body temperature in reptiles, we conducted all trials at the body temperature of 30 °C. This was achieved by placing hatchlings in an incubator at the test temperature for approximately 1 h prior to testing. We presented a cotton-tipped applicator

soaked with cologne water to the lip of hatchlings and recorded tongue flicks for 1 min. Tongue flicking was measured because many reptiles flick their tongues frequently to detect both predators and prey and to gather information about other members of their own species. The behavioral character is therefore a potentially important indicator of fitness. Tongue flicking has been shown to vary with body temperature in reptiles (e.g. Stevenson et al., 1985; Ayers and Shine, 1997; Amo et al., 2004) but, to our knowledge, it is still unknown whether this character varies with incubation temperature.

2.3. Data analyses

We used Statistica software package (Version 5.0 for PC) to analyze the data. Data on hatchlings of the same sex were blocked by the clutch to avoid pseudo-replication. We used *G* test to examine whether hatching success, the sex ratio of hatchlings and the number of deformed hatchlings differed among the four treatments. We used a multivariate analysis of covariance (MANCOVA, with egg mass at oviposition as the covariate) to examine the effects of the same factors (sex and incubation treatment) and their interaction on hatchling morphological traits other than the number of ventral scales. Prior to this analysis, the assumption of homogeneity of variances was tested at univariate (Bartlett's test) and multivariate (Box's *M* test) levels. We used a two-way analysis of variance (ANOVA, with incubation treatment and sex as the factors) to analyze the number of ventral scales and the number of tongue flicks per minute because these two traits were independent of egg mass in all sex × incubation treatment combinations. No data required transformation to meet the assumptions for parametric analyses when using such analyses. Values are presented as mean ± SE, and the significance level is set at $\alpha = 0.05$.

3. Results

Females ($N = 15$) laid eggs between 26 June and 7 July. Clutch size, varying from 11 to 22 eggs, was positively correlated with maternal SVL ($r^2 = 0.67$, $F_{1, 13} = 25.92$, $P < 0.001$), whereas mean egg mass of clutches, varying from 12.1 to 22.7 g, was not ($r^2 = 0.08$, $F_{1, 13} = 1.12$, $P = 0.309$).

Hatching success, the sex ratio of hatchlings and the number of deformed hatchlings (either tail or trunk malformation) did not differ among the four treatments (*G* test; all $P > 0.95$) (Table 1). Incubation length was independent of eggs mass (simple linear regression; all $P > 0.186$), and did not differ between the sexes within each treatment (one-way ANOVA; all $P > 0.173$). Incubation length differed among the four treatments (one-way ANOVA; $F_{3, 116} = 27.37$, $P < 0.0001$), with the FT and HF treatments resulting in shorter incubation lengths (Table 1).

Table 1
Effects of incubation thermal environments on duration of incubation, hatching success, and sex ratio and abnormality of hatchlings

Thermal treatments	Incubated eggs	Duration of incubation[*] (d)	Hatching success (%)	Sex ratio (♀♀/♂♂)	Abnormality (%)
Fluctuating temperature	48	$56.4^b \pm 0.3$ (54.0−59.8)	89.6 (43/48)	21/22	4.2 (2/48)
Constant temperature	49	$59.8^a \pm 0.3$ (54.8−64.4)	87.8 (43/49)	20/23	2.0 (1/49)
Cold first	50	$59.9^a \pm 0.4$ (54.8−63.7)	90.0 (45/50)	21/24	2.0 (1/50)
Hot first	50	$57.5^b \pm 0.4$ (53.2−61.2)	94.0 (47/50)	22/25	4.0 (2/50)

Data on duration of incubation are expressed as mean ± SE (range). Means with different superscripts differ significantly (Tukey's *post-hoc* comparison, $\alpha = 0.05$).
[*]Data from the same clutch are blocked.

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

Variable	Sex	Thermal treatments			
		FT	CT	CF	HF
Egg mass (g)	♀♀	19.2±0.6 14.6−22.2	17.8±0.7 13.8−22.3	18.2±0.6 14.8−22.1	17.5±0.7 12.8−21.7
	♂♂	16.9±0.7 11.6−24.1	18.5±0.7 14.7−23.6	18.0±0.7 12.5−23.0	18.7±0.7 14.8−22.3
Snout–vent length (cm)	♀♀	28.4±0.4 25.6−30.4	28.3±0.3 26.3−30.3	28.3±0.3 26.2−29.6	28.1±0.3 26.0−30.1
	♂♂	27.5±0.3 24.8−29.0	28.3±0.3 26.3−30.4	27.4±0.3 24.8−28.9	27.3±0.3 25.3−28.9
Tail length (cm)	♀♀	4.8±0.1 4.0−5.2	4.7±0.06 4.4−5.4	4.6±0.1 4.0−5.2	4.8±0.05 4.5−5.2
	♂♂	5.1±0.1 4.5−5.6	5.3±0.1 4.7−5.9	5.0±0.1 4.6−5.7	5.1±0.1 4.5−5.6
Body mass (g)	♀♀	15.0±0.6 9.0−17.5	14.2±0.5 11.1−17.3	14.5±0.4 12.4−17.3	14.1±0.5 10.2−17.1
	♂♂	13.9±0.5 10.5−18.1	15.0±0.5 12.0−17.5	14.2±0.6 9.3−17.5	14.5±0.5 11.7−17.1
Head length (mm)	♀♀	15.4±0.1 14.6−16.1	15.6±0.1 14.8−16.5	15.6±0.1 14.9−16.3	15.6±0.1 15.1−16.3
	♂♂	15.7±0.1 14.9−16.7	16.0±0.1 14.9−16.5	15.7±0.1 14.9−16.4	15.8±0.1 15.0−16.9
Head width (mm)	♀♀	7.3±0.07 6.9−7.7	7.4±0.08 7.0−7.9	7.2±0.08 6.8−7.4	7.2±0.07 6.7−7.6
	♂♂	7.4±0.07 7.1−8.1	7.6±0.08 7.0−8.2	7.4±0.08 6.9−7.9	7.4±0.06 7.1−7.8
Jaw length (mm)	♀♀	11.3±0.1 10.5−12.0	11.4±0.2 9.5−13.0	11.4±0.1 10.6−12.4	11.3±0.1 10.8−11.9
	♂♂	11.4±0.2 10.8−13.4	11.7±0.1 11.2−12.4	11.4±0.1 10.6−11.8	11.4±0.1 10.5−12.5
Ventral scales	♀♀	172.8±1.1 162−178	173.5±0.7 170−178	173.6±0.8 169−179	173.8±1.0 167−182
	♂♂	169.8±1.4 161−181	168.3±0.9 162−175	168.0±0.5 163−170	167.8±1.3 155−177

Data are expressed as mean ± SE and range. FT: fluctuating-temperature treatment; CT: constant-temperature treatment; CF: cold first treatment; HF: hot first treatment.

The variance of temperatures experienced by eggs during the whole incubation period in the FT treatment, varying from 2.5 to 3.8 (mean = 2.8), was not a significant predictor of all examined hatchling traits in both sexes (simple linear regression; all $P > 0.128$). Egg mass was not a significant predictor of the number of ventral scales (simple linear regression; $P > 0.230$ in all the four treatments), but it explained a substantial amount of variation in other examined hatchling morphological phenotypes (simple linear regression; $P < 0.001$ in all the four treatments).
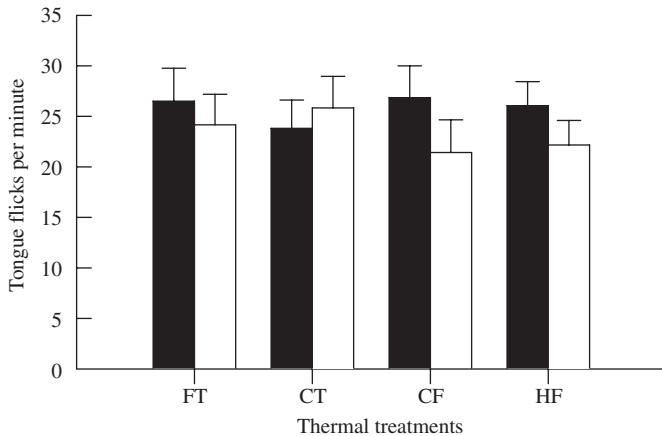
Fig. 2. Mean values (+SE) for the number of tongue flicks per minute of hatchlings derived from eggs incubated under different temperature regimes. Black bars: females; open bars: males. FT: fluctuating-temperature treatment; CT: constant-temperature treatment; CF: cold first treatment; HF: hot first treatment.

The number of ventral scales was affected by sex ($F_{1, 112} = 49.58$, $P < 0.0001$) but not by incubation treatment ($F_{3, 112} = 0.13$, $P = 0.944$), and the interaction between sex and incubation treatment was not a significant source of variation in the trait ($F_{3, 112} = 0.92$, $P = 0.432$). Other examined hatchling morphological traits were affected by both sex (Wilks' Lambda = 0.413, df = 6, 105, $P < 0.0001$) and incubation treatment (Wilks' Lambda = 0.701, df = 18, 297, $P < 0.004$), but not by the interaction between sex and incubation treatment (Wilks' Lambda = 0.893, df = 18, 297, $P = 0.834$). Female hatchlings were larger in SVL but smaller in tail length, head length and head width than male hatchlings from the same-sized egg; female hatchlings had more ventral scales than did male hatchlings (Tukey's *post-hoc* test, all $P < 0.001$; Table 2). Tail length was greatest in the CT treatment and smallest in the FT treatment, with the CF and HF treatments in between; head width was greater in the CT treatment than in the other three treatments (Tukey's *post-hoc* test, all $P < 0.05$; Table 2).

Neither sex ($F_{1, 112} = 1.33$, $P = 0.251$) nor incubation treatment ($F_{3, 112} = 0.08$, $P = 0.971$) affected the number of tongue flicks per minute (Fig. 2). The interaction between sex and incubation treatment was not a significant source of variation in this behavioral phenotype ($F_{3, 112} = 0.59$, $P = 0.621$).

4. Discussion

Incubation temperature affects a number of phenotypic traits of hatchling reptiles (reviewed in Deeming and Ferguson, 1991; Birchard, 2004; Deeming, 2004; Valenzuela, 2004; Booth, 2006). The most clear pattern in this field is that incubation at higher temperatures results in shorter incubation lengths and a lesser amount of yolk converted to somatic tissue such that hatchlings incubated at higher temperatures tend to have smaller carcasses (and thus,

smaller body dimensions) but larger residual yolks (e.g. Webb et al., 1987; Phillips and Packard, 1994; Allsteadt and Lang, 1995; Ji and Braña, 1999; Ji et al., 2002; Hao et al., 2006). Studies using CT incubation usually show the existence of lower and upper thresholds over which the temperature effects are important and affect several aspects of a hatchling's morphology and performance. For example, hatchlings incubated at temperatures within the range of 24–30 °C do not differ in any examined morphological trait in *Eumeces chinensis* (Chinese skink; Ji and Zhang, 2001), *Calotes versicolor* (oriental garden lizard; Ji et al., 2002; but see also Radder et al., 2002), *Elaphe carinata* (king ratsnake; Ji and Du, 2001b) and *Bungarus multicintus* (multi-banded krait; Ji et al., 2007). The range of temperatures exerting no differential effects on hatchling phenotypes varies across species, being broadly correlated with the mean temperature of a species' natural incubation environment (e.g. Lin and Ji, 1998; Du and Ji, 2003; Booth, 2004; Lin et al., 2005).

There have been some studies applying natural or FT incubation (e.g. Shine and Harlow, 1996; Shine et al., 2004a, b, 1997a, b; Andrews et al., 2000; Webb et al., 2001; Ashmore and Janzen, 2003; Du and Ji, 2006; Ji et al., 2007; Les et al., 2007). In general, these studies show that FTs influence hatchling traits differently from CTs even when the means were the same between treatments. Nonetheless, whether the observed effects result from thermal fluctuation, temperature extremes or both is largely unknown due to the relative paucity of studies examining the ways that specific attributes of incubation thermal regimes affect hatchling phenotypes.

In the present study, tail length and head width differed among the four treatments, whereas other examined hatchling traits did not (Table 2, Fig. 2). It is worth noting that eggs in the FT, CF and HF treatments experienced temperatures close to the upper and lower limits of viable range (slightly wider than the range of 24–32 °C; Ji and Du, 2001a) for *N. atra*. Hatchlings in these three treatments had relatively shorter tails as compared with those in the CT treatment (Table 2). This morphological modification is highly consistent with the finding from an earlier study of *N. atra* where eggs incubated at temperatures lower than 26 °C or higher than 30 °C produced hatchlings with shorter tails as compared with those incubated at moderate temperatures (Ji and Du, 2001a). This consistency is unlikely to come upon by chance but implies that the mean rather than the fluctuation of incubation temperatures has the main influence in determining the tail size of hatchling *N. atra*. The mean value for head width was greater in the CT treatment than in the other three treatments. This result presumably suggests that incubation at temperatures over some thresholds may modify the head shape (the ratio of head width to length) of hatchling *N. atra*, but it does not provide any evidence showing the importance of thermal fluctuation *per se* in determining this hatchling phenotype. Therefore, taken together, our data show that thermal fluctuations during incubation do

not have the main influence in shaping the phenotype of hatchling *N. atra*. This result is largely consistent with the findings from our earlier studies of other reptiles such as *Rhabdophis tigrinus lateralis* (red-necked keelback; Chen and Ji, 2002), *E. chinensis* (Chen et al., 2003), *Pelodiscus sinensis* (Chinese soft-shelled turtle; Du and Ji, 2003; Ji et al., 2003) and *Eremias argus* (Mongolian racerunner; Hao et al., 2006). In these species, thermal fluctuations during incubation do not play an important role in modifying hatchling trait as long as eggs are not exposed to extreme temperatures for long periods of time. In *B. multicintus*, however, fluctuating incubation temperatures do not influence hatchling size and morphology any differentially from CTs with the same mean, but have a positive effect on locomotor performance of hatchlings (Ji et al., 2007). It seems that phenotypic responses to thermal fluctuations during incubation differ among species, and that some hatchling traits are likely more sensitive to incubation thermal environments than others.

Intuitively, eggs incubated at FTs should take a longer time to hatch than those at CTs with the same mean, because incubation length increases at an ever-increasing rate as temperature decreases in reptiles (Deeming and Ferguson, 1991). Interestingly, however, fluctuating (or variable) temperatures result in longer incubation periods relative to constant (or stable) temperatures in some species (Shine, 2004b; Hao et al., 2006; Braña and Ji, 2007; Les et al., 2007), but not in others (Andrews et al., 2000; Du and Ji, 2003, 2006; Shine, 2004a; Ji et al., 2007; Lin et al., 2007). In *N. atra*, incubation length decreases by approximately 33 days as temperature increases from 24 to 28 °C, and by approximately 17 days as temperature increases from 28 to 32 °C (Ji and Du, 2001a). However, in opposition to what is expected, the FT treatment resulted in shorter incubation periods relative to the CT treatment with nearly the same mean temperature (Table 1). This result provides an inference that, as in *Bassiana duperreyi* (three-lined skink; Shine, 2004a), incubation at stable temperatures may lead to delayed hatching in *N. atra*.

Another unexpected observation is that incubation length differed between the HF and CT treatments but not between the CF and CT treatments (Table 1). Given that the HF and CF treatments were designed to have the identical mean temperature as the CT treatment but differ in the time sequence of different temperatures applied, the shorter incubation length in the HF treatment therefore suggests that temperatures affect developmental rates in different ways depending upon the phase of incubation in which they were applied. In reptiles as well as in other animal taxa, growth and development are processes to some extent dissociated during the embryogenesis, with early development being characterized by tissue differentiation and organogenesis, and late development by rapid growth in size (Deeming and Ferguson, 1991; Andrews and Mathies, 2000; Andrews, 2004; Cai et al., 2007). Thus, for future studies, it could be important to design more sophisticated experiments that allow examination of the

effects of thermal environments at different stages of embryonic development, so that the effects of seasonal shifts in incubation temperature on hatching success, incubation length and hatchling phenotype can be experimentally evaluated.

Taken together, our results show that thermal fluctuations during incubation play an important role in influencing incubation length but not the phenotype of hatchlings in *N. atra*. The observed morphological modifications (tail length and head width) cannot be attributed to the effect of thermal fluctuations but to the effect of temperatures close to the upper and lower limits of the viable range for the species. Overall, our results support the hypothesis that hatchling phenotype is not altered by thermal fluctuation in species with no phenotypic response to incubation temperature within some thresholds.

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