

The Effects of Incubation Temperature On Hatching Success, Embryonic Use of Energy and Hatchling Morphology in the Stripe-tailed Ratsnake *Elaphe taeniura*

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Abstract.— We incubated eggs of *Elaphe taeniura* at 22, 24, 27, 30 and 32°C to examine the effects of incubation temperature on hatching success, embryonic use of energy and hatchling morphology. Incubation temperature affected incubation length and most hatchling traits examined in this study. Incubation length increased nonlinearly as temperature decreased, with the mean incubation length being 101.7 d at 22°C, 86.0 d at 24°C, 66.3 d at 27°C, 53.9 d at 30°C, and 50.5 d at 32°C. Hatching successes were lower at the two extreme temperatures (41.2% at 32°C, and 50.0% at 22°C) than at the other three moderate temperatures (78.1–79.3%). Hatchlings from the extreme high incubation temperature (32°C) were smaller in body size and wet body mass. High incubation temperatures resulted in production of less developed hatchlings that characteristically had less developed carcasses but contained more unutilized yolks. The proportion of energy transferred from the egg contents to the hatchling was 71.1% at 22°C, 80.2% at 24°C, 81.5% at 27°C, 82.6% at 30°C and 83.9% at 32°C. Taking the lowest hatching success at 32°C and the substantially prolonged incubation lengths at 22°C into account, we conclude that these temperatures are not suitable for embryonic development in *E. taeniura*. Our data confirm the prediction that there are some thresholds over which incubation temperatures can affect hatching success, embryonic use of energy and hatchling morphology.

Keywords.— Reptilia, Colubridae, *Elaphe taeniura*, egg, incubation, temperature, hatchling phenotype.

Introduction

As in other vertebrate and invertebrate taxa, temperature may profoundly influence embryonic development in reptiles. Compared to avian embryos, reptilian embryos can develop under a relatively wide range of temperatures (Birchard, 2004, Booth, 2004). Low temperatures slow embryogenesis but usually have little lethal effect on embryos; high temperatures result in faster embryonic development (and thus, shortened incubation or gestation length) but often increase embryonic abnormality or mortality (e.g. Andrews and Rose, 1994; Andrews et al., 1997; Deeming and Ferguson, 1991; Sexton and Marion, 1974; Shine and Harlow, 1996). Apart from the effects on the rate of embryonic development and embryonic abnormality or mortality, thermal environments experienced by developing embryos also affect a number of phenotypic attributes of the hatchling, including morphology (Du and Ji, 2002; Ji and Du, 2001a, b; Overall, 1994), energy reserves (Du and Ji, 2001), behavior (Burger, 1991; 1998), post-hatching growth (Braña and Ji, 2000; Du and Ji, 2003; Rhen and Lang, 1995), and gender in species with temperature-dependent sex determination (Janzen and Paukstis, 1991). It

has been repeatedly reported for oviparous reptiles that eggs incubated at optimal temperatures not only exhibit high hatching success but also produce good-quality hatchlings.

The range of optimal temperatures for reptilian embryos is often narrow and varies not only among but also within species. For example, the optimal incubation temperatures fall within the range from 24°C to 26°C in *Xenochrophis piscator* (checkered keelback; Ji et al., 2001) and *Deinagkistrodon acutus* (five-paced pit-viper; Lin et al., 2005) but, in *Elaphe carinata* (king ratsnake; Ji and Du, 2001b), *Naja atra* (Chinese cobra; Ji and Du, 2001a), *Rhabdophis tigrinus lateralis* (red-necked keelback; Chen and Ji, 2002), *Dinodon rufozonatum* (red-banded wolf snake; Ji et al., 1999b; Zhang and Ji, 2002), *Ptyas korros* (gray ratsnake; Du and Ji, 2002) and *P. mucosus* (mucous ratsnake; Lin and Ji, 2004), generally within the range from 26°C to 30°C. *Pelodiscus sinensis* (Chinese soft-shelled turtle), however, has a wider range of optimal incubation temperatures, because temperatures exert no important effects on hatching success and hatchling phenotypes within the range from 24°C to 34°C (Du and Ji, 2003; Ji et al., 2003). In *Eumeces chinensis* (Chinese skink), eggs from a lower latitudinal population have a narrower range of optimal incubation

temperatures than do those from a higher latitudinal population, primarily because of more stable thermal environments in the former population (Ji et al., 2002). Overall, previous studies suggest that optimal temperatures for developing embryos differ among reptiles differing in habitat use and/or distributional range.

The stripe-tailed ratsnake *Elaphe taeniura* is a large sized (to 1800 mm SVL [snout-vent length]) oviparous colubrid snake that ranges from the central and southern provinces of China to Korea, Burma, Laos, Vietnam and India (Zhao, 1993). Wild population of this snake have declined dramatically due to habitat loss and over-harvesting over the past two decades (Zhao, 1998). Fecundity, reproductive output and embryonic mobilization of energy and material during incubation have been reported for snakes from Zhoushan Islands (Ji et al., 1999a; 2000). Because eggs were never incubated at multiple temperatures, the range of incubation temperatures optimal for developing embryos of *E. taeniura* remains unknown. To fill this gap, we incubated eggs produced by females from a southern population (Guangxi, China) at five constant temperatures ranging from 22°C to 32°C. Specifically, our aims are to (1) examine the effects of incubation temperatures on hatching success, embryonic use of energy and hatchling morphology, and (2) determine the range of optimal temperatures for embryos of *E. taeniura*.

Materials and Methods

We obtained 13 gravid *E. taeniura* (SVL: 1220–1690 mm; postoviposition body mass: 277.9–755.0 g) in June 1998 from a private hatchery in Guilin (Guangxi, southern China), and brought them to our laboratory in Hangzhou, where they were maintained in a 2000 x 800 x 800 (length x width x height) mm wire cage placed in a room inside which air temperatures were never higher than 30°C. Food (eggs of *Coturnix coturnix*) and water were provided *ad libitum*. The snakes laid eggs between

23 June and 3 July (clutch size: 8.8 ± 0.4 , range: 7–11). We collected the eggs within a few hours after being laid. Each egg was 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. One freshly laid egg from each clutch was dissected to determine the composition of eggs. Egg contents (yolk plus embryo) were placed in pre-weighed glass dishes, and weighed. Shells were briefly rinsed, dried by blotting with paper towels and weighed. Egg contents and shells were weighed again after oven drying to constant mass at 65°C. The remaining eggs were incubated systematically at five constant temperatures (22, 24, 27, 30, and 32 [± 0.3]°C); such that eggs from single clutches were distributed almost equally among the five temperature treatments.

Eggs were individually incubated in covered plastic jars containing known amounts of vermiculite and distilled water at approximately -12 kPa water potential (vermiculite: water = 1:2). 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 equally assigned to five incubators (Guangzhou medical instrument, China), with incubation temperatures set at 22, 24, 27, 30, and 32 (± 0.3)°C, respectively. 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. 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.

The duration of incubation, measured as the number of days to pipping, was recorded for each egg. Hatchlings were collected, measured (for SVL and tail length), and weighed a few hours after hatching, and 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

Table 1. The effect of temperature on incubation duration, hatching success, and sex ratio in *Elaphe taeniura*. Data on incubation duration are expressed as mean \pm 1 SE.

| Temperature (°C) | Duration of incubation (d) | Hatching success (%) | Sex ratio (♀♀/♂♂) |
|------------------|----------------------------|----------------------|----------------------|
| 22 | 101.7 \pm 1.3 | 50.0 (6/12) | 2/4 |
| 24 | 86.0 \pm 0.6 | 78.1 (25/32) | 12/13 |
| 27 | 66.3 \pm 0.7 | 79.2 (19/24) | 13/6 |
| 30 | 53.9 \pm 0.4 | 79.3 (23/29) | 11/12 |
| 32 | 50.5 \pm 0.5 | 41.2 (7/17) | 2/5 |

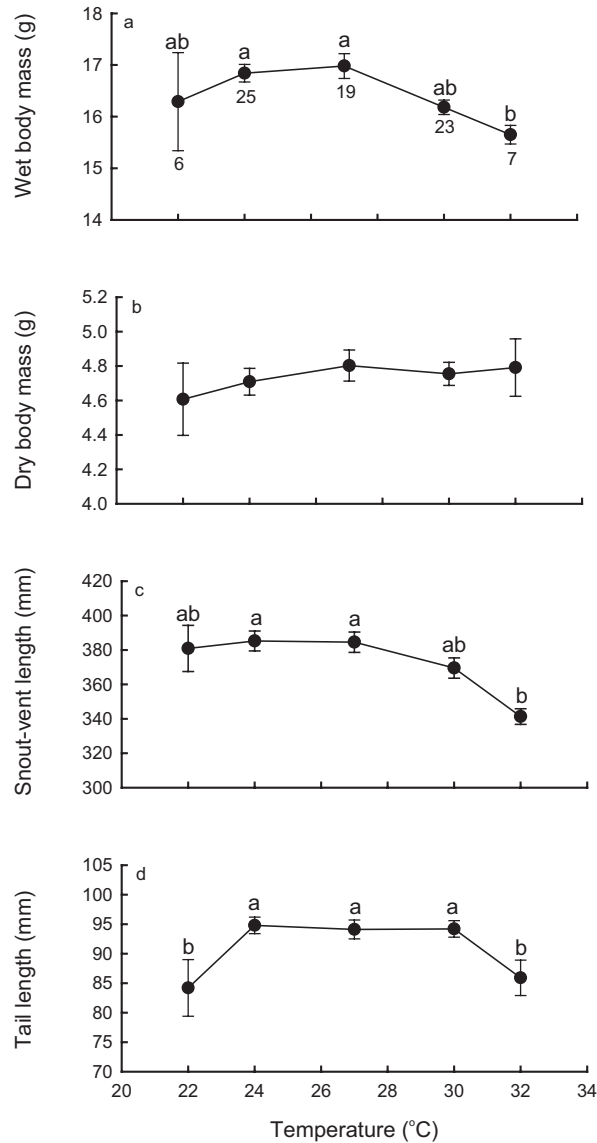


Figure 1. Adjusted means (± 1 standard error) for body mass, SVL and tail length of hatchlings from different incubation temperatures, with initial egg mass being set at 23.5 g. Adjusted means with different letters differ significantly. Numbers under the error bars in the upper plot are sample sizes, and are applicable to the other two plots.

three components of the hatchling were dried in an oven (65°C) to constant mass, weighed 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 of egg contents and hatchlings in a Soxhlet apparatus for a minimum of 5.5 h using absolute ether as solvent. The amount of lipids in each sample was determined by sub-

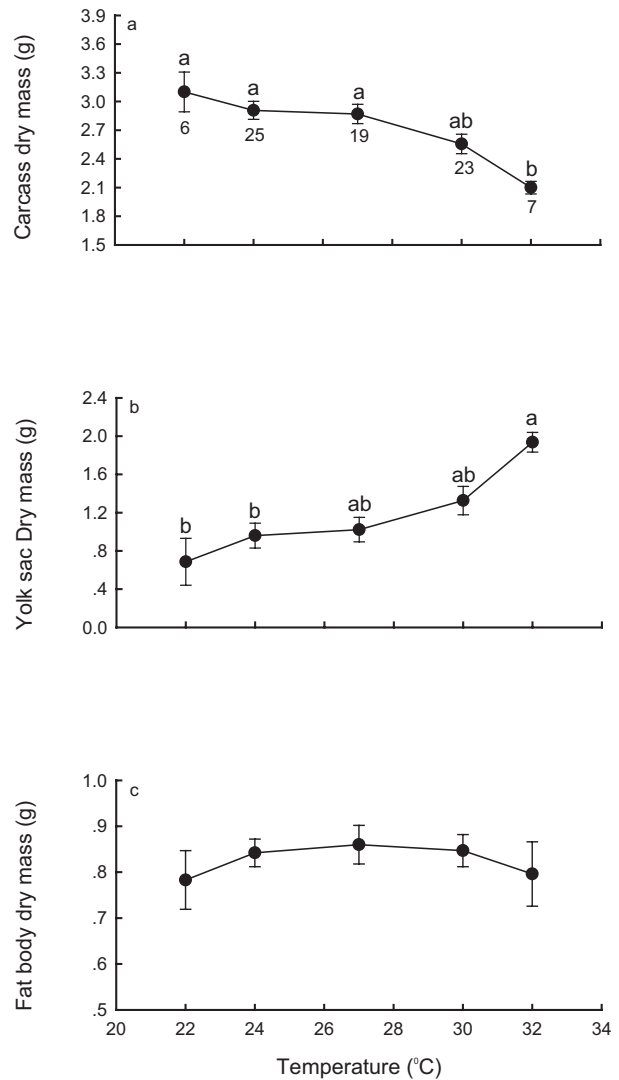


Figure 2. Carcass, yolk sac and fat bodies of hatchling *Elaphe taeniura* incubated at different temperatures. Graphs show adjusted mean (\pm standard error), with initial egg mass as the covariate. Adjusted means with different letters above the error bars differ significantly. Numbers under the error bars in the upper graph are sample sizes, and apply to all graphs within this figure.

tracting the lipid-free dry mass from the total sample dry mass. We determined energy density of each dried sample using a GR-3500 adiabatic bomb calorimeter (Changsha Instruments, China).

All data were tested for normality using Kolmogorov-Smirnov test and for homogeneity of variance using Bartlett's test. Parametric analyses were used to analyze data when the assumptions for these analyses were met; otherwise, nonparametric analyses were used. Values are presented as mean ± 1 standard error, and the significance level is set at $\alpha = 0.05$.

Table 2. Lipids and energy in egg contents and shell dry mass of hatched eggs and freshly laid eggs in *Elaphe taeniura*. Data are expressed as adjusted mean \pm 1 SE, with initial egg mass as the covariate.

| | Egg contents (n = 11) | Hatchlings | | | | |
|--------------------|--------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| | | 22°C (n = 6) | 24°C (n = 25) | 27°C (n = 19) | 30°C (n = 23) | 32°C (n = 7) |
| Lipid mass (g) | 1.956 \pm 0.079 | 1.082 \pm 0.047 | 1.269 \pm 0.039 | 1.316 \pm 0.048 | 1.362 \pm 0.046 | 1.387 \pm 0.101 |
| Energy (kJ) | 143.9 \pm 4.3 | 102.3 \pm 3.9 | 115.4 \pm 2.5 | 117.3 \pm 3.1 | 118.9 \pm 2.6 | 120.8 \pm 4.9 |
| Shell dry mass (g) | 1.555 \pm 0.031 | 1.401 \pm 0.075 | 1.411 \pm 0.011 | 1.408 \pm 0.029 | 1.380 \pm 0.029 | 1.397 \pm 0.030 |

Results

The mean values for incubation length varied considerably among the five temperature treatments (ANOVA; $F_{4,75} = 687.2$, $p < 0.0001$). Incubation length decreased as incubation temperature increased, but not in a linear pattern. The mean incubation length was shortened by 3.5 d from 30°C to 32°C, 12.4 d from 27°C to 30°C, 19.7 d from 24°C to 27°C, and 15.7 d from 22°C to 24°C (Table 1). Incubation temperature affected hatching success (G-test, $G = 11.51$, $df = 4$, $p < 0.05$), but not the sexual phenotype of hatchlings ($G = 4.73$, $df = 4$, $p > 0.25$). Hatching successes were apparently lower at the two extreme temperatures (22°C and 32°C) than at the other three moderate temperatures (24, 27, and 30°C) (Table 1).

There were no between-sex differences in all examined hatchling variables (all $p > 0.05$), so we pooled data for both sexes. All examined hatchling variables, except yolk sac, were positively correlated with initial egg mass). ANCOVAs with initial egg mass as the covariate showed that incubation temperatures significantly affected wet body mass ($F_{4,74} = 3.67$, $p < 0.01$; Fig. 1a), SVL ($F_{4,74} = 4.21$, $p < 0.01$; Fig. 1c) and tail length ($F_{4,74} = 4.31$, $p < 0.01$; Fig. 1d) of hatchlings, but not hatchling dry body mass ($F_{4,74} = 0.38$, $p > 0.05$; Fig. 1b). Mean values for wet body mass, SVL and tail length were all smaller in hatchlings incubated at 32°C than in hatchlings incubated at the other four temperatures (Fig. 1a, c, d). Hatchlings from different incubation temperatures differed in carcass mass (ANCOVA with the initial egg mass as the covariate: $F_{4,74} = 6.36$, $p < 0.001$; Fig. 2a) and yolk sac dry mass (ANOVA: $F_{4,75} = 4.79$, $p < 0.01$; Fig. 2b), but not fatbody dry mass ($F_{4,74} = 0.37$, $p > 0.05$; Fig. 2c). Hatchlings from 32°C had lighter carcass dry mass but larger residual yolk sac, whereas hatchlings from 22°C had heavier carcass but smaller residual yolk sac (Fig. 2a, b).

Energy contents ($F_{4,74} = 2.78$, $p < 0.05$) and non-polar lipids ($F_{4,74} = 2.80$, $p < 0.05$) differed among hatchlings from different incubation temperatures, with hatchlings incubated at 22°C containing lower quantities of energy and non-polar lipid than did those incubated at

other four temperatures (Table 2). Conversion efficiency of energy during incubation at 22°C (71.1%) was thus lower than those at 32, 30, 27, and 24°C (83.9%, 82.6%, 81.5%, and 80.2%). Similarly, conversion efficiency of lipid at 22°C (55.3%) was lower than those at 32, 30, 27, and 24°C (70.9%, 69.%, 67.3% and 64.9%). Additionally, shells of hatched eggs were significantly lighter than shells of freshly laid eggs ($F_{5,84} = 3.19$, $p < 0.05$; Table 2).

Discussion

As in numerous other reptiles, thermal environments experienced by developing embryos affect hatching success, incubation length, embryonic expenditure of energy, and linear dimensions (SVL and tail length) and body composition of hatchlings in *Elaphe taeniura*. Our results provide support for the previous conclusion that reptilian embryos developing at relatively low or moderate temperatures produce well developed and thus, larger hatchlings (e.g. Deeming and Ferguson, 1991; Du and Ji, 2002; Ji and Du, 2001a, b). The larger hatchling size has an association with the greater carcass dry mass (Chen and Ji, 2002; Du and Ji, 2002; Ji et al., 1997; Ji and Sun, 2000). Data from the current study proved that this conclusion is also true in *E. taeniura* (Fig. 1; Fig. 2). The finding that more yolks remain unutilized at hatching when eggs are incubated at high temperatures has been reported for nearly all reptiles studied to-date (e.g., Beuchat, 1988; Du and Ji, 2002; Ji and Du, 2001a, b; Lin et al., 2005; Phillips et al., 1990; Phillips and Packard, 1994). In *E. carinata* (Ji et al., 1997), *E. taeniura* (Ji et al., 1999a), *D. rufozonatum* (Ji et al., 1999b) and *P. korros* (Ji and Sun, 2000), hatchlings exhibit a substantial increase in size (SVL) during the first post-hatching days due to the transfer of resources in the residual yolk into carcass.

The proportion of lipids transferred from the egg contents into the hatchling was noticeably lower than those of energy. Given that mass-specific energy density is much higher in lipids than in proteins and carbohydrates, this result provides evidence that lipids are the main source of energy for embryonic development.

Hatchling size and mass were primarily determined by the embryonic expenditure of energy during incubation after removing the influence of variation in initial egg mass (Du and Ji, 2001, 2003; Ji and Du, 2001a, b). Given that the total energy invested in an egg is fixed, any increase in embryonic expenditure of energy during incubation may inevitably result in production of small sized or lighter hatchlings. Incubating eggs at low temperatures or high temperatures may increase energy expenditure for embryonic development due to the increased incubation length and/or embryonic metabolism (Booth, 1998; Booth and Astill, 2001; Packard and Packard, 1988). Consequently, eggs incubated at moderate temperatures usually produce larger and heavier hatchlings than did those at low or high temperatures.

A prolonged exposure of eggs of *E. taeniura* to temperatures lower than 24°C or higher than 30°C may have a detrimental effect on embryonic development, as indicated by the fact that hatching success decreases dramatically at temperatures outside this temperature range (Table 1). The mean incubation length at 30°C is 53.9 days, approximately 3.4 days longer than that at 32°C, so the ecological disadvantage of the increased incubation length (and thus, decreased growth period prior to the onset of the first winter) due to a decrease in incubation temperature from 32°C to 30°C is less pronounced. Considering that less developed hatchlings are produced at 32°C and that hatching success is low at this temperature, we conclude that the temperature of 32°C is outside the range of optimal temperatures for incubating eggs of *E. taeniura*. Eggs incubated at 24°C exhibit high hatching success and produce well developed hatchlings. However, the majority of hatchlings from eggs incubated at 24°C appear between late September and mid-October, so the growth prior to the onset (late November) of the first winter for these hatchlings is about 1.5–2 months. As incubation length increasingly increases as temperature decreases in *E. taeniura* (Table 1), we expect that the disadvantage of incubating eggs of *E. taeniura* at temperatures lower than 24°C can be very pronounced due to the increasingly prolonged incubation length. For eggs of reptiles incubated under natural conditions, the prolonged incubation length at low temperatures also increases exposure of eggs to the effects of adverse biotic (increased microbial contamination) and abiotic factors (extreme thermal and hydric conditions) in the incubation environment of the eggs, which potentially reduces hatching success. Thus, the temperature of 24°C is suitable but not optimal for incubating eggs of *E. taeniura*. Taking the energy expenditure during incubation, the rate of embryonic development and hatchling phenotypes into account, we consider that temperatures around 27°C are optimal for incubating eggs of *E. taeniura*.

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