Embryonic growth and mobilization of energy and material during incubation in the checkered keelback snake, *Xenochrophis piscator*

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ABSTRACT

We collected 20 checkered keelback snakes (*Xenochrophis piscator*) to study embryonic growth and mobilization of energy and material during incubation. Females laid eggs between late May and late June. The eggs were incubated at 27 °C (±0.3). One egg from each clutch was dissected at five-day intervals starting at oviposition. The mean incubation length at 27 °C was 48.9 days. We identified three phases of embryonic growth or yolk depletion in *X. piscator*. Phase 1, between oviposition and Day 20, was one of minimal transfer of energy and material from yolk to embryo. Phase 2, between Day 20 and Day 39–40, was characterized by increasingly rapid embryonic growth or yolk depletion. Phase 3, between Day 39–40 and hatching, was characterized by reduced embryonic growth or yolk depletion. Approximately 71% of dry mass, 53% of non-polar lipids and 66% of energy were transferred from the egg contents to the hatchling during incubation. Our data confirm that oviposition is not timed to coincide with the onset of rapid embryonic growth in oviparous squamate reptiles. The greater conversion efficiencies of energy and material from egg to hatchling in snakes can be attributed to their lower energetic costs of embryonic development and greater residual yolk sizes.

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1. Introduction

Development of the reptilian embryo involves differentiation and growth, two distinct processes that are integrated but dissociated to some extent during embryogenesis. Early embryonic development is characterized by differentiation (i.e., the origin of tissues and organ systems), whereas late development is characterized by growth (i.e., the increase in the size of the embryo) (Deeming and Ferguson, 1991; Andrews, 2004). Unlike viviparous reptiles that rely upon vitellogenesis and, in various degrees, placentation for embryonic nutrition (Thompson et al., 1999, 2000), oviparous reptiles lay eggs that contain all nutrients necessary for producing a hatchling with some amount of residual yolk (e.g., Wilhoft, 1986; Troyer, 1987; Fischer et al., 1991; Ji and Du, 2001a; Radder et al., 2007). During incubation, embryos use yolk as the source of all organic and most inorganic nutrients and eggshell as the additional source of inorganic nutrients such as calcium (e.g., Packard and Packard, 1984; Shadrix et al., 1994; Thompson et al., 2000; Du et al., 2001; Ji and Du, 2001a; Cai et al., 2007).

Most oviparous squamate reptiles oviposit at later embryonic stages than do turtles, tuataras and crocodilians (Shine, 1983; Andrews and Mathies, 2000). Embryos of these species have completed a substantial proportion of differentiation prior to oviposition but, because differentiation is well advanced before any substantial embryonic growth (Andrews, 2004), the mass of the embryo only represents a very small fraction of the hatchling's mass (e.g., Shadrix et al., 1994; Ji et al., 1997a; Thompson and Stewart, 1997; Ji and Du, 2001a; Du and Ji, 2002; Cai et al., 2007). For these species, the transfer of energy and material from egg to embryo during incubation is nearly equal to the transfer occurring during the whole period of embryonic development (Cai et al., 2007).

Studies of lizards such as the five-lined skink *Eumeces fasciatus* (Shadrix et al., 1994; Thompson and Stewart, 1997), the coral skink *Eumeces anthracinus* (Thompson and Stewart, 1997), the Ordos racerunner *Eremias brenchleyi* (Xu and Wu, 2003) and the northern grass lizard *Takydromus septentrionalis* (Xu et al., 2004) show that embryonic growth is slow until the second quarter of incubation in species where females lay eggs containing embryos at Stage 26–30 in Dufaure and Hubert's (1961) developmental series. Unfortunately, as functions describing embryonic growth and mobilization of energy and material during incubation were not given in these studies, the detailed inverse relationship between embryo and other two egg components (yolk and eggshell) remains unknown in any of these species. Functions established recently for the red-necked keelback snake *Rhabdophis tigrinus lateralis* show three phases of embryonic growth: Phase 1, between oviposition and 36% incubation length, is one of the minimal transfer of energy and material from yolk to embryo; Phase 2, between 36–81% incubation length, is characterized by increasingly rapid embryonic growth; and Phase 3, between 81% incubation length and

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hatching, is characterized by a gradual reduction in embryonic growth (Cai et al., 2007). Might the three phases detected in *R. t. lateralis* be generalisable to reptiles? To answer this question, independent datasets are needed from different snakes and reptilian taxa.

Here, we describe a study where snake eggs were dissected at five-day intervals starting at oviposition to quantify embryonic growth and mobilization of energy and material during incubation. We used the checkered keelback snake *Xenochrophis piscator*, a medium sized (up to 940 mm snout–vent length, SVL) colubrid snake that is widely distributed in the southern part of China (Zhao, 1998), as a model animal for two reasons: (1) the snake is oviparous with negligible embryonic growth prior to oviposition (Ji et al., 2001), and thus the transference of energy and material from egg to hatchling during incubation reflects the overall transfer; and (2) females lay at least 31 eggs/clutch, and the larger clutch size provides an ample opportunity to remove the eggs laid by a single female for dissection at different incubation stages. Our study aims are as follows: (1) to establish mathematical functions describing embryonic growth and mobilization of energy and material during incubation; (2) to examine whether oviposition is timed to coincide with the onset of rapid embryonic growth; and (3) to compare our data with those reported for other oviparous squamate reptiles.

2. Materials and methods

2.1. Animal collection and care

Twenty gravid females (SVL: 658–940 mm; post-oviposition body mass: 97.7–323.5 g) were collected by hand in mid-May 2000 from a population in Yongzhou (26°25′N, 111°36′E), Hunan, central China. Females were placed singly in cloth bags and transported to our laboratory in Hangzhou, where they were housed individually in 600×600×600 mm wire cages. These cages were placed in an indoor animal holding facility where the temperature was controlled within the range from 24 to 28 °C and the fluorescent tubes, which were automatically switched on at 07:00 h (Beijing time), were on a cycle of 14 h light: 10 h dark. Food (rice frogs [*Fejervarya* (*Rana*) *limnocharis*]) was provided *ad libitum*, so that excess food was always available in the cage. We checked the cages at least twice daily for freshly laid eggs as soon as the first female laid eggs, such that eggs were always collected, measured and weighed within 3 h of oviposition. 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. The viability or fertility of freshly laid eggs was judged by the presence of an embryonic disc using a spotlight. Post-oviposition females were individually measured (to the nearest 1 mm) for SVL and tail length, weighed (to the nearest 0.1 g), and then released at their point of capture.

2.2. Methods

Viable eggs were either dissected at oviposition or incubated individually in covered plastic jars (100 mL) containing known amounts of vermiculite and distilled water at approximately −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 placed in a Shellab incubator (Sheldon MFG
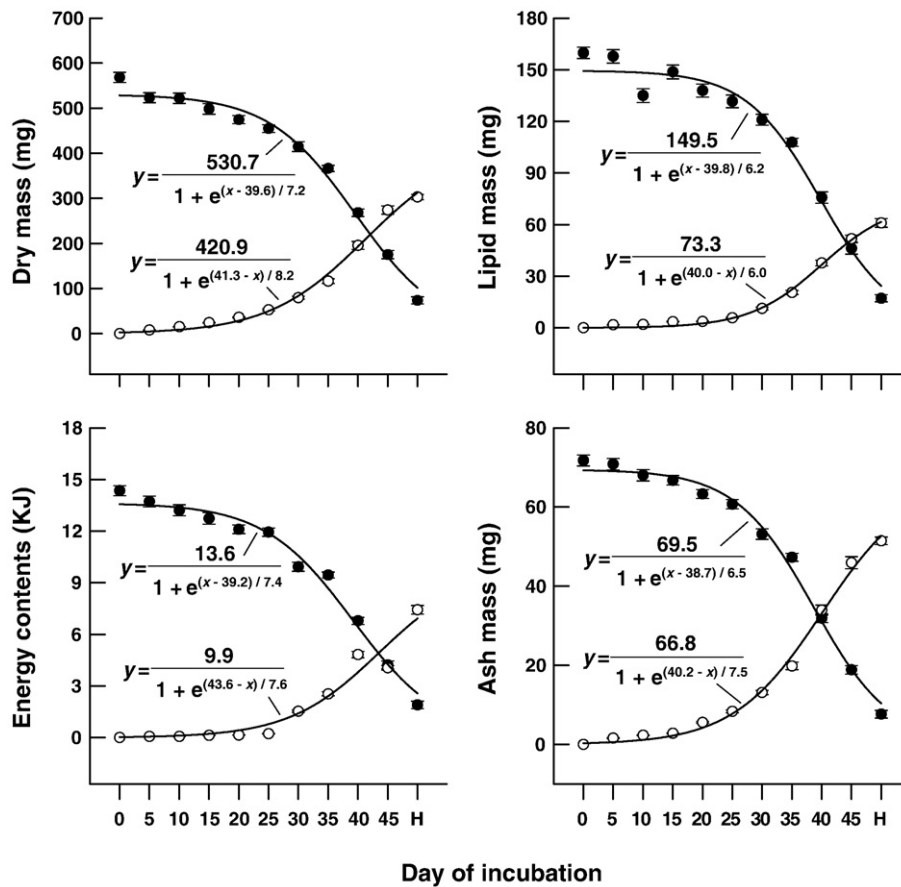

Fig. 1. Adjusted means (±SE) for dry mass, lipid mass, energy content and ash mass of yolks and embryos at different incubation times, with egg mass at oviposition (the covariate) being set at 2400 mg. Solid dots: yolk; open dots: embryo. H: at hatching. Functions and curves describing changes in dry mass, lipid mass, energy content and ash mass of yolks and embryos are given in the figure.

Inc, USA) at 27 °C±0.3. This temperature was chosen because it falls within the range of temperatures optimal for embryonic development in *X. piscator* (Ji et al., 2001). Jars were weighed at five-day intervals, and distilled water was evenly added into substrates when necessary to compensate for evaporative losses and water absorbed by the eggs, thereby maintaining a nearly constant substrate water potential. We moved the jars among shelves daily according to a predetermined schedule to minimize any effects of thermal gradients inside the incubator.

One egg from each of the 20 clutches was dissected at five-day intervals starting at oviposition. Each dissected egg was separated into embryo, yolk and eggshell, and the three egg components were then dried to constant mass in an oven at 65 °C, weighed and preserved frozen for later determination of composition. Hatchlings were collected, measured for SVL and tail length and weighed immediately after they emerged from the egg. One hatchling from each clutch was dissected, and the remaining hatchlings were released to the site where their mothers were collected. Each dissected hatchling was separated into carcass (including fat bodies) and residual yolk, and the two hatchling components as well as the hatched eggshell were also dried to constant mass in an oven at 65 °C, weighed and preserved frozen for later determination of composition.

We extracted non-polar lipids from dried samples in a Soxhlet apparatus for 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 and residual yolk. We determined energy density of dried samples using a WGR-1 adiabatic bomb calorimeter (Changsha Bente Instruments, China). We determined ash content in each sample using a muffle furnace at 700 °C for a minimum of 12 h and weighing the remaining ash.

2.3. Statistical analysis

Prior to all statistical analyses, data were tested for normality using the Kolmogorov–Smirnov test and, for homogeneity of variances, using the Bartlett's test. Percentage data were arc-sine transformed prior to using parametric analyses. We used linear regression analysis, logistic estimation of embryonic growth or yolk depletion (Vleck and Hoyt, 1991), one-way analysis of variance (ANOVA) and one-way analysis of covariance (ANCOVA) with egg mass at oviposition as the covariate to analyze corresponding data. The homogeneity of slopes was checked prior to testing for differences in the adjusted means. Throughout this paper, values are presented as mean±SE, and significance level is set at α=0.05.

3. Results

Females laid a single clutch of pliable-shelled eggs between late May and late June. Clutch size averaged 56.9±3.1 (range=31–82) eggs, and egg mass averaged 2.40±0.05 (range=2.12–3.16) g. Incubation length, measured as the time between oviposition and pipping, averaged 48.9±0.2 (range=47.3–50.7) days. Embryos identified at oviposition were at Stage 25 in Zehr's (1962) developmental series. Eggs dissected at different stages of incubation did not differ in mean mass measured at oviposition (ANOVA, $F_{10, 209}$=0.28, P=0.986).

Adjusted means (±SE) for dry mass, lipid mass, energy contents and ash mass of embryos and yolks were plotted for each sampling day of incubation in Fig. 1. There was a clear-cut inverse relationship between embryo and yolk; that is, embryonic growth is tightly associated with yolk depletion, and vice versa. Functions describing instantaneous changes in these variables revealed that embryonic growth rate or yolk depletion was less striking between oviposition
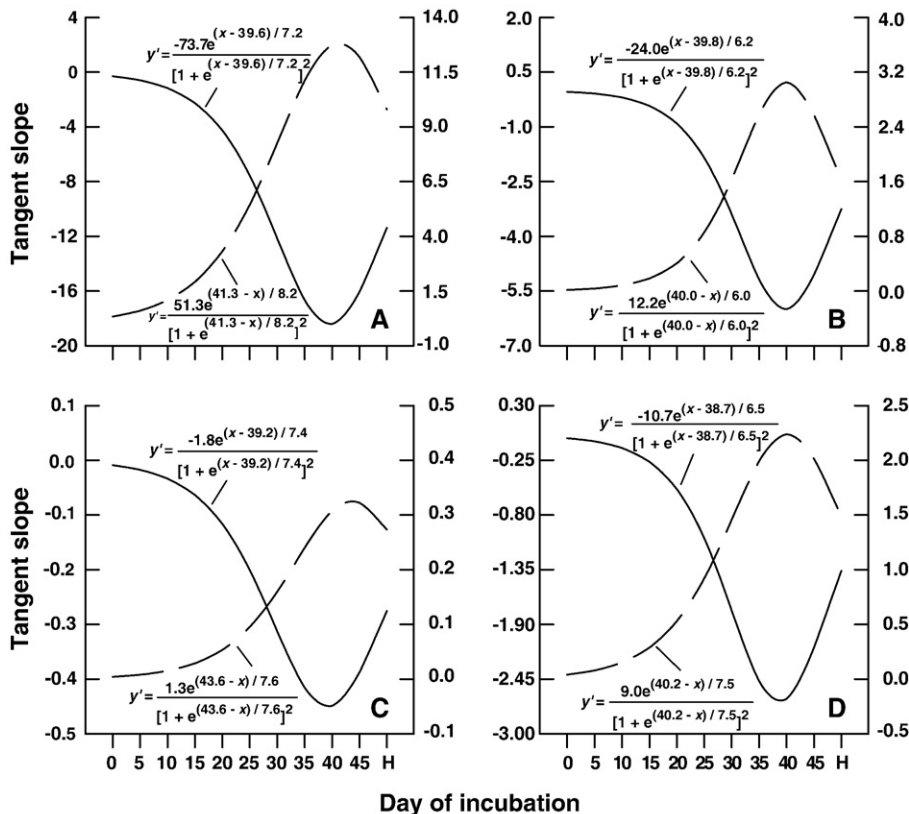

Fig. 2. The derived functions and curves for instantaneous variation in tangent slopes of the corresponding curves in Fig. 1. Solid curves and left axes: yolk; open curves and right axes: embryo. A: dry mass; B: Lipid mass; C: Energy contents; D: Ash mass.

Table 1
Composition of freshly laid and hatched eggs of *Xenochrophis piscator*

	Freshly laid egg	Hatched egg	$F_{1,\ 37}$
	Egg contents	Hatchling	
Wet mass (g)	2.29±0.01	1.77±0.04	148.38***
Dry mass (mg)	604.4±13.5	431.8±12.5	124.54***
Lipid mass (mg)	160.0±3.3	84.8±5.8	120.72***
Energy contents (KJ)	14.4±0.3	9.5±0.3	113.74***
Ash mass (mg)	68.7±1.6	72.1±1.3	2.55[NS]
	Eggshell	Eggshell	
Dry mass (mg)	56.1±1.2	48.7±1.0	21.00***
Ash mass (mg)	13.6±0.8	10.0±0.4	14.20**

Data are expressed as adjusted mean±SE, with egg mass at oviposition (set at 2400 mg) as the covariate. $N=20$ (clutches). F values of ANCOVA are given in the table, and symbols immediately after F values represent significance level.
[NS]$P<0.05$.
**$P<0.01$.
***$P<0.001$.

and Day 20 (41% incubation length) and that the 10 day interval between Day 35 and 45 (between 72 and 92% incubation length) represented the greatest embryonic growth or yolk depletion (Fig. 2).

Egg contents of the freshly laid eggs contained higher quantities of wet material, dry material, non-polar lipids and energy but less ash than did hatchlings (Table 1). Eggshells contained higher quantities of dry material and ash at oviposition than at hatching (Table 1). During incubation, approximately 71% of dry mass, 53% of non-polar lipids and 66% of energy were transferred from egg contents to hatchling, with 29% of dry mass, 47% of non-polar lipids and 34% of energy used for embryogenesis (Table 1).

4. Discussion

Consistent with the results reported for other oviparous snakes that oviposit at Zehr stage 23–27 (Ji et al., 1999a,b; Ji and Du, 2001a,b; Du and Ji, 2002; Lin and Ji, 2004; Cai et al., 2007; Du et al., 2007), embryos of *X. piscator*, though having completed a substantial proportion of differentiation at oviposition, had negligible dry mass in comparison to the rest of the egg (Fig. 1). Accordingly, we conclude that *X. piscator* is amongst species where the yolk-to-embryo transfer of material and energy during post-ovipositional embryogenesis is nearly equal to the transference overall.

Hatchlings contained more ash than did egg contents at oviposition, whereas eggshells contained less ash at hatching than at oviposition (Table 1). Thus, embryos of *X. piscator*, like other oviparous reptiles, use eggshell as a secondary source of minerals (ash) for development. Fully developed embryos of *X. piscator* should withdraw approximately 4.8% of their total mineral requirements from the eggshell (Table 1). This proportion is smaller than the values reported for the king ratsnake *Elaphe carinata* (11.9%; Ji et al., 1997a), the Chinese beauty snake *E. taeniura* (16.9%; Ji et al., 1999a), the Chinese ratsnake *Zaocys dhumnades* (9.3%; Du et al., 2007) and *R. t. lateralis* (6.0%; Cai et al. 2007), but greater than the values reported for the Chinese cobra *Naja atra* (1.3%; Ji et al., 1997b) and the Indochinese ratsnake *Ptyas korros* (3.4%; Du and Ji, 2002). This series of comparisons suggests that the proportional amount of minerals withdrawn by the embryo from the eggshell varies considerably among species of snakes. Such differences presumably reflect the interspecific differences in eggshell structure and/or allocation of minerals between eggshell and yolk.

The conversion efficiencies (proportions) of dry materials (74–84%), lipids (50–75%) and energy (58–79%) transferred from egg to hatchling (including the internalized yolk) summarized by Cai et al. (2007) for eight species of oviparous snakes (including one elapid and seven colubrid snakes) range from 50–84%. In 15 species of oviparous lizards (including one phrynosomatid, one gekkonid, two agamid,

three lacertid and eight sincid lizards) studied to date, 41–85% of dry material, 29–46% of lipids and 51–64% of energy are transferred from egg to hatchling (Thompson et al., 2000; Cai et al., 2007). Our study of *X. piscator* adds one smallest [dry material (71%)] and two smaller-than-average conversion efficiencies [lipids (53%) and energy (66%)] to snakes. Reanalyzing data on nine snake (including *X. piscator*) and 15 lizard species, we find that the conversion efficiencies of dry mass ($F_{1,\ 22}=6.21$, $P=0.021$), lipids ($F_{1,\ 14}=30.64$, $P<0.0001$) and energy ($F_{1,\ 15}=19.58$, $P<0.0005$) are on average smaller in lizards than in snakes. Available data allow us to draw two general conclusions: (1) the conversion efficiencies differ among species, especially between lizards and snakes; and (2) the proportional amounts of dry material (68% vs. 77% mean values), lipids (39% vs. 61% mean values) and energy (57% vs. 70% mean values) transferred from egg to hatchling were on average smaller in lizards than in snakes.

Given that snakes are closely related to lizards, why are the conversion efficiencies on average greater in snakes than in lizards? The answer presumably lies in the phylogenetic differences in residual yolk size between snakes and lizards. It is widespread among oviparous reptiles that nutrients stored in the freshly laid egg exceed the material and energy requirements for producing a complete hatchling, and thus a portion of yolk (i.e., residual yolk) remains unused at hatching (e.g., Kraemer and Bennett, 1981; Troyer, 1987; Fischer et al., 1991; Ji et al., 1997a). Residual yolk is used by hatchling reptiles for maintenance and early growth, and has been shown to enhance their survivorship (e.g., Troyer, 1987; Congdon and Gibbons, 1989; Fischer et al., 1991; Radder et al., 2007). Hatchling snakes (including *X. piscator*) generally contain more residual yolk than do hatchling lizards (11–26% vs. Nil-12% of the total hatchling dry mass; Ji et al., 2001; Cai et al., 2007). The conversion efficiencies are greater in species where more yolk remains unused at hatching (Ji et al., 2002). Therefore, the greater residual yolk size provides a reasonable explanation for the greater conversions efficiencies in snakes.

Based on the derived mathematical functions and curves in Fig. 2, we can arbitrarily identify three phases of embryonic growth and mobilization of energy and material in *X. piscator*. The first phase, between oviposition and Day 20 (~41% incubation length), represents one of minimal transfer of energy and material from yolk to embryo. Embryonic growth is slow during this phase. We therefore conclude that, as in the bearded dragon *Amphibolurus* (*Pogona*) *barbata* (Packard et al., 1985), the five-lined skink *E. fasciatus* (Shadrix et al., 1994; Thompson and Stewart, 1997), the coral skink *E. anthracinus* (Thompson and Stewart, 1997), the Ordos racerunner *E. brenchleyi* (Xu and Wu, 2003), the northern grass lizard *T. septentrionalis* (Xu et al., 2004) and the red-necked keelback *R. t. lateralis* (Cai et al., 2007), oviposition does not coincide with the onset of rapid embryonic growth in *X. piscator*. The second phase, between Day 20 and Day 39–40 (~81% incubation length), is one of increasingly rapid embryonic growth and mobilization of energy and material, with the greatest embryonic growth or yolk depletion occurring at the end of the phase. The third phase, between Day 39–40 and hatching, is characterized by a gradual reduction in embryonic growth and yolk mobilization. The first phase ends at a later incubation stage in *X. piscator* than in *R. t. lateralis* (~36%; Cai et al., 2007), presumably because females oviposit at an earlier embryonic stage in the former (Stage 25) than in the later (Stage 26–27; Cai et al., 2007) species. The two species differ in incubation length even when their eggs are incubated under identical thermal conditions. For example, the mean incubation length at 27 °C is 48.9 days in *X. piscator*, and 32.7 days in *R. t. lateralis* (Chen and Ji, 2002). Interestingly, however, the second phase identified in this study ends at an incubation stage (~81%) surprisingly consistent with that reported for *R. t. lateralis* (~81%; Cai et al., 2007). This consistency together with the similarity of the three phases between the two snake species provides an inference that, at least in some species of snakes, there is a common pattern of embryonic growth and mobilization of energy and material during embryogenesis.

Acknowledgements

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