

Embryonic growth and mobilization of energy and material in oviposited eggs of the red-necked keelback snake, *Rhabdophis tigrinus lateralis*

Yao Cai^a, Ting Zhou^a, Xiang Ji^{a,b,*}

^a Jiangsu Key Laboratory for Biodiversity and Biotechnology, College of Life Sciences, Nanjing Normal University, Nanjing 210097, Jiangsu, PR China

^b Hangzhou Key Laboratory for Animal Science and Technology, School of Life Sciences, Hangzhou Normal College, Hangzhou 310036, Zhejiang, PR China

Received 23 August 2006; received in revised form 4 November 2006; accepted 10 November 2006

Available online 5 December 2006

Abstract

We used the red-necked keelback (*Rhabdophis tigrinus lateralis*) as a model animal to study embryonic growth and mobilization of energy and material in oviposited snake eggs. Females ($N=12$) laid eggs between late May and early June. Eggs were incubated at 30 (± 0.3) °C. One egg from each clutch was dissected at five-day intervals starting at oviposition. Incubation length averaged 27.9 days. Three phases of embryonic growth or yolk depletion could be detected in this study. The first phase, between oviposition and Day 10, was one of minimal transfer of energy and material from yolk to embryo. The second phase, between Day 10 and Day 22–23, was characterized by increasingly rapid embryonic growth and yolk depletion. The third phase, between Day 22–23 and hatching, was characterized by a gradual reduction in embryonic growth and yolk depletion. Approximately 73.6% of dry mass, 50.0% of non-polar lipids and 57.8% of energy were transferred from egg to embryo during incubation. Embryos withdrew mineral from the eggshell mainly during the last quarter of incubation. Our data show that oviposition does not coincide with the onset of rapid embryonic growth in oviparous species of squamate reptiles that are positioned midway within the oviparity–viviparity continuum, and that the greater conversion efficiencies of energy and material from egg to hatchling in snakes can be mainly attributed to their lower energetic costs of embryonic development and greater residual yolk sizes.

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Keywords: Reptilia; Colubridae; Red-necked keelback; *Rhabdophis tigrinus lateralis*; Egg; Incubation; Hatchling; Embryonic growth; Conversion efficiency

1. Introduction

Most oviparous squamate reptiles are positioned midway between the two endpoints of the oviparity–viviparity continuum, with females characteristically retaining eggs in utero for about half of the total period of embryonic development (Shine, 1983). Embryos of these species have completed a substantial proportion of differentiation prior to oviposition, but they are an insignificant fraction of egg mass (e.g., Shadrix et al., 1994; Ji et al., 1997a; Ji and Braña, 1999; Ji and Du, 2001; Du and Ji, 2002). Thus, for these species, the transfer of energy and material from egg to embryo during incubation (the post-

ovipositional interval of embryonic development) is approximately equal to the transfer occurring during the whole period of embryonic development (Ji et al., 1997a,b; Ji and Sun, 2000).

Data from studies of lizards such as *Eumeces fasciatus* (Shadrix et al., 1994), *Eremias brenchleyi* (Xu and Wu, 2003) and *Takydromus septentrionalis* (Xu et al., 2004b) show that embryonic growth is slow until the second quarter of incubation in species where the modal embryonic stage at oviposition varies from Stage 26–30 in Dufaure and Hubert's (1961) developmental series. These data therefore provide further evidence that embryonic growth is extremely small in the first half period of embryonic development. Snakes are closely related to lizards, but comparative data are currently unavailable for them. Moreover, as functions describing embryonic growth and mobilization of energy and material from oviposition to hatching were not given in previous studies, the detailed “ebb and flow” relationship between embryo and other two egg

* Corresponding author. Present address: Hangzhou Key Laboratory for Animal Sciences and Technology, School of Life Sciences, Hangzhou Normal College, Hangzhou 310036, Zhejiang, PR China. Tel.: +86 571 28865337; fax: +86 571 28865331.

E-mail address: xji@mail.hz.zj.cn (X. Ji).

components (yolk and eggshell) and instantaneous changes in embryonic mass during the course of incubation remain unclear in oviparous squamate reptiles.

Here we describe a study in which snake eggs were dissected at five-day intervals after oviposition to quantify embryonic growth and mobilization of energy and material. We used the red-necked keelback (*Rhabdophis tigrinus lateralis*), a small-to-medium sized [up to 850 mm snout-vent length (SVL)] oviparous snake found in most provinces of China (Zhao, 1998), as a model for two reasons. First, the snake is amongst species of oviparous squamate reptiles where embryonic dry mass at oviposition is negligible (Zhao et al., 1997), and thus the transference of energy and material from egg to hatchling during incubation largely reflects the overall transfer. Second, females of *R. t. lateralis* lay at least nine eggs per clutch, and thus provide an ample opportunity to dissect eggs laid by single females at different stages of incubation period. Our study aims are as follows: (1) to present data that can be used to give functions describing embryonic growth and mobilization of energy and material in oviposited snake eggs; (2) to examine whether oviposition coincides with the onset of exponential embryonic growth; and (3) to compare our data with those reported for other oviparous species of squamate reptiles, thereby examining interspecific differences in proportions of energy and material transferred from egg to embryo during incubation.

2. Materials and methods

2.1. Collection and animal care

Twelve gravid females snakes (*R. t. lateralis*) (SVL: 58.0–71.0 cm; post-oviposition body mass: 57.5–124.9 g) were collected in mid-May 2000 from a population in Jiande (29°29' N, 119°16'E), Zhejiang (eastern China), and transported to our laboratory in Hangzhou. Females were housed individually in 60×60×60 cm wire cages placed in a room where the surrounding temperature varied within the range of 26–30 °C on a daily basis, and food [rice frogs (*Rana limnocharis*)] was provided *ad libitum*. We checked the cages at least twice daily since the first female laid eggs, thereby collecting eggs promptly after 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 for SVL and tail length and weighed, and then released to the site where they were originally captured.

2.2. Methods

Viable eggs were either dissected at oviposition or incubated individually in plastic jars (100 ml) with a perforated cover. Jars

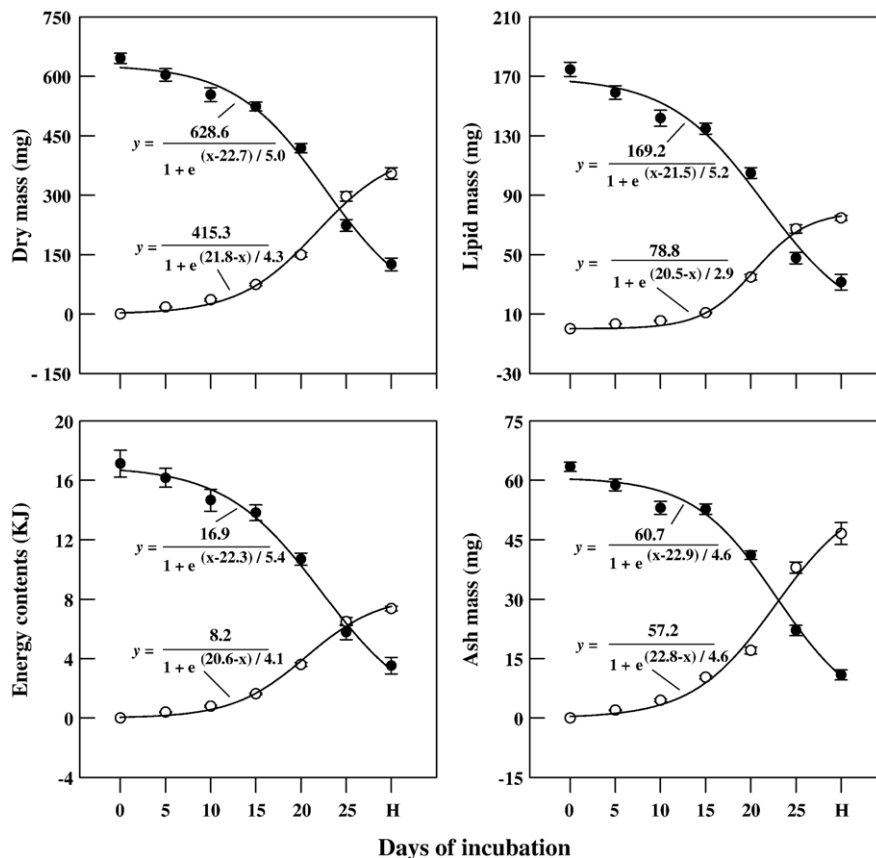


Fig. 1. Adjusted means (and ±SE) for dry mass, lipid mass, energy content and ash mass of yolks and embryos at different stages of incubation, with egg mass at oviposition (the covariate) being set at 2500 mg. Solid dots: yolk; open dots: embryo. H: at hatching. All related functions are given in the figure.

holding eggs contained known amounts of vermiculite and distilled water to produce approximately -220 kPa water potential (Ji and Braña, 1999), and were placed in a Shellab incubator (Sheldon MFG Inc, USA) where the temperature was controlled at $30 (\pm 0.3)$ °C. This temperature was chosen because it falls within the range of temperatures optimal for embryonic development in *R. t. lateralis* (Chen and Ji, 2002). One-third of the egg was buried in the substrate, with the surface near the embryo being exposed to air inside the jar. Jars were weighed every other day and, if necessary, distilled water was mixed into substrates to compensate for evaporative losses and water absorbed by eggs, thereby maintaining the substrate water potential constant. We moved jars among shelves daily according to a predetermined schedule to minimize any effects of thermal gradients inside the incubator.

One egg from each clutch 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 (to the nearest 0.1 mg) 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 hatching 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 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

We used Statistica software package (version 5.0 for PC) to analyze data. All data were tested for normality using Kolmogorov–Smirnov test, and for homogeneity of variance using Bartlett’s test. Percentage data were arc-sine transformed prior to using parametric analyses. We used linear regression analysis, nonlinear estimation, 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 given as mean \pm SE, and significance level is set at $\alpha=0.05$.

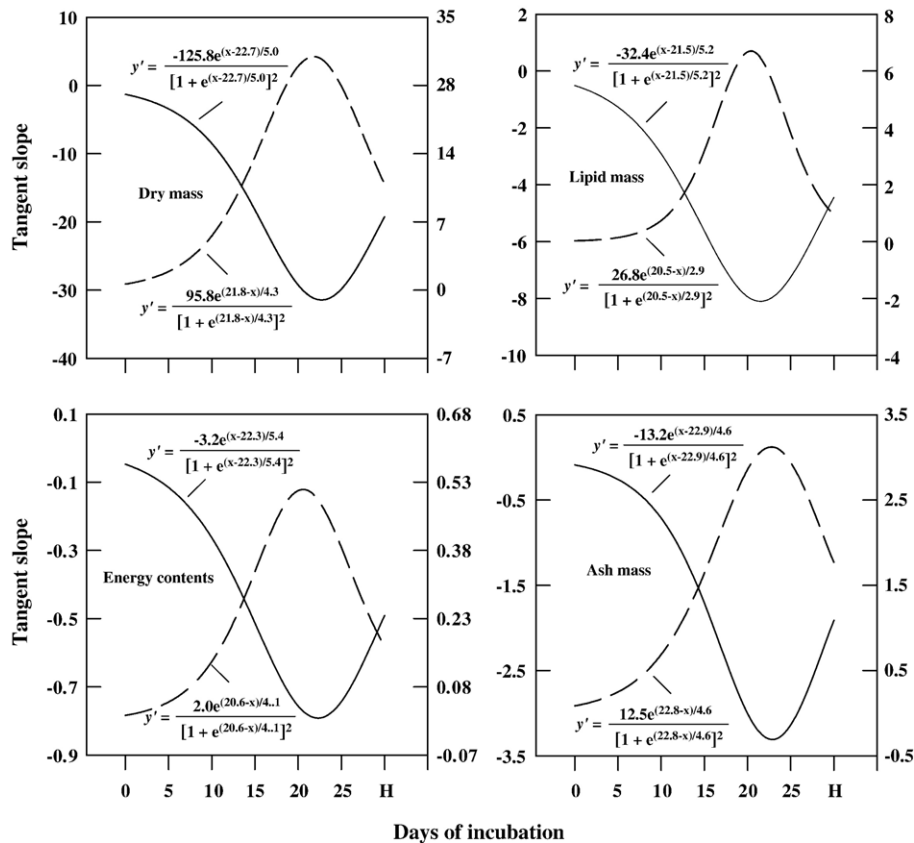


Fig. 2. The derived functions and curves for instantaneous variation in tangent slopes of the corresponding curves in Fig. 1. Solid curves and left axes: yolk; dash curves and right axes: embryo.

3. Results

Females ($N=12$) laid a single clutch of pliable-shelled eggs between late May and early June. Clutch size averaged 15.1 ± 0.9 (range=11–22) eggs, and egg mass averaged 2.56 ± 0.11 (range=1.99–3.05) g. Incubation length, defined as the time elapsed from egg laying to pipping, averaged 27.9 ± 0.4 (range=26.4–28.9) days. Of the 12 embryos identified at oviposition, ten were at Stage 27 and two at Stage 26 in Zehr's (1962) developmental series. Eggs dissected at different stages of incubation did not differ in mean mass at oviposition (ANOVA with day of incubation as the factor: $F_{6, 77}=0.07$, $P=0.999$).

Adjusted means (and SE) for dry mass, lipid mass, energy content and ash mass of embryos and yolks are plotted for each sample day of incubation in Fig. 1. There is a clear-cut “ebb and flow” relationship between embryo and yolk: that is, embryonic growth is tightly associated with yolk depletion, and vice versa (Fig. 1). Functions describing instantaneous changing rates of the functions in Fig. 1 reveal that embryonic growth and yolk depletion are minimal from oviposition to Day 10 and that the five-day interval of the greatest embryonic growth or yolk depletion is between Day 20 and Day 25 (Fig. 2).

Adjusted means (and SE) for dry mass, lipid mass, energy content and ash mass of egg contents (embryo plus yolk for the egg, and carcass plus residual yolk for the hatchling) are plotted for each sample day of incubation in Fig. 3. Functions

describing instantaneous changes in these variables reveal that dry mass, lipid mass and energy content of egg contents all decrease at a progressively greater rate as development proceeds. On the contrary, ash mass of egg contents remains almost unchanged from oviposition to Day 20 but increases dramatically from Day 20 to hatching (Fig. 3).

Adjusted means (and SE) for dry mass and ash mass of eggshells are plotted for each sample day of incubation in Fig. 4. Similar to egg contents, eggshell dry mass generally decreases at a progressively greater rate as development proceeds, whereas eggshell ash mass remained almost unchanged from oviposition to Day 20 but decreases dramatically from Day 20 to hatching (Fig. 4).

Egg contents of the dissected 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 73.6% of dry mass, 50.0% of non-polar lipids and 57.8% of energy were transferred from egg contents to hatchling, with 26.4% of dry mass, 50.0% of non-polar lipids and 42.2% of energy being used for embryogenesis (Table 1).

4. Discussion

At oviposition, embryos of *R. t. lateralis* had completed a substantial proportion of differentiation, but had very small dry

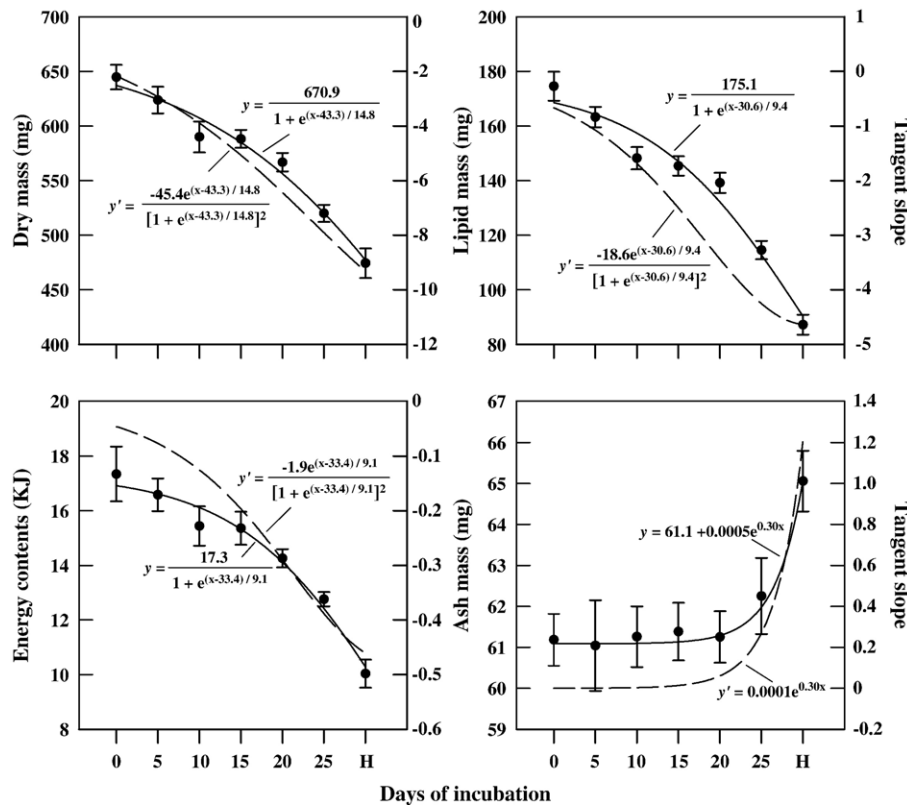


Fig. 3. Adjusted means (and \pm SE) for dry mass, lipid mass, energy content and ash mass of egg contents at different stages of incubation, with egg mass at oviposition being set at 2500 mg. Solid curves: changes in dry mass, lipid mass, energy content and ash mass of egg contents during incubation; dash curves: instantaneous variation in tangent slopes of the solid curves. All related functions are given in the figure.

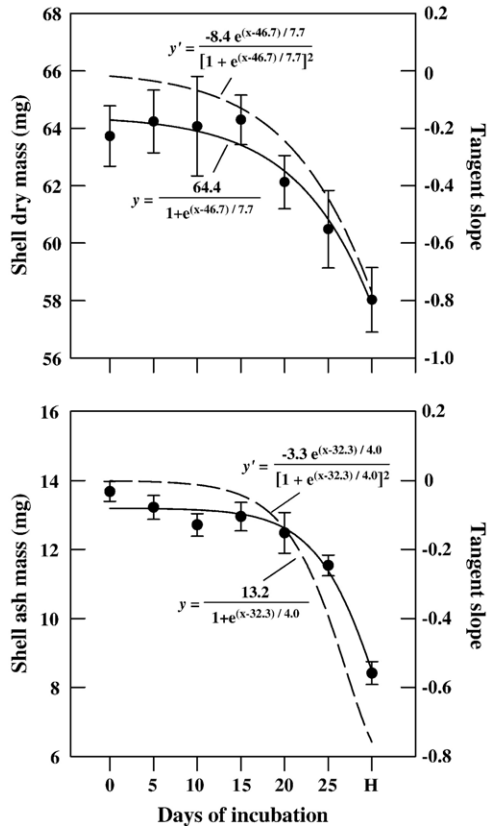


Fig. 4. Adjusted means (and ±SE) for dry mass and ash mass of eggshells at different stages of incubation, with egg mass at oviposition being set at 2500 mg. The dash curves describe instantaneous variation in tangent slopes of the solid curves. All related functions are given in the figure.

mass in comparison to the rest of the egg (Fig. 1). This confirms that *R. t. lateralis* is amongst species of oviparous squamate reptiles where embryonic growth prior to oviposition is negligible (Zhao et al., 1997). Dry mass, lipid mass and energy content of egg contents decreased at ever-increasing rates from oviposition to hatching (Fig. 3), and thus indicate that energetic costs are increasingly greater as embryonic development proceeds. Hatchlings contained more ash than did egg contents at oviposition, whereas eggshells contained less ash at hatching than at oviposition (Table 1). These results indicate that

Table 1
Composition of freshly laid and hatched eggs of *R. t. lateralis*

	Freshly laid egg	Hatched egg	<i>F</i> _{1, 21}
	Egg contents	Hatchling	
Wet mass (g)	2.49±0.12	2.08±0.09	8.47**
Dry mass (mg)	644.6±11.2	474.3±13.6	89.27***
Lipid mass (mg)	174.5±5.3	87.2±3.7	175.60***
Energy content (KJ)	17.3±1.0	10.0±0.5	42.48***
Ash mass (mg)	61.2±0.6	65.1±0.7	15.51***
	Eggshell		
Dry mass (mg)	62.9±1.4	58.0±1.1	7.64*
Ash mass (mg)	13.9±0.3	8.4±0.3	159.98***

Data are expressed as adjusted mean±SE, with egg mass at oviposition (set at 2500 mg) as the covariate. *N*=12 (clutches). *F* values of ANCOVA are given in the table, and symbols immediately after *F* values represent significance level: **P*<0.05, ***P*<0.01, and ****P*<0.001.

embryos of *R. t. lateralis*, like other oviparous reptiles (e.g., Packard and Packard, 1989; Shadrix et al., 1994; Ji et al., 1997a; Ji and Du, 2001; Zheng et al., 2006), use eggshell as a secondary source of minerals for development. Mineral uptake from the eggshell occurred mainly during the last quarter of embryonic development in *R. t. lateralis* (Figs. 3 and 4). This pattern of mineral uptake is similar to that reported for *E. fasciatus* (Shadrix et al., 1994), but whether the pattern is generalisable to oviparous reptiles is unclear because of the lack of comparative data.

Three major phases of embryonic growth during incubation can be identified in *R. t. lateralis* (Fig. 2). The first phase, between oviposition and Day 10, is one of minimal transfer of energy and material from yolk to embryo. Embryonic growth is relatively slow during this phase. Thus, as in *E. fasciatus* (Shadrix et al., 1994), *E. brenchleyi* (Xu and Wu, 2003) and *T. septentrionalis* (Xu et al., 2004b), oviposition does not coincide with the onset of rapid embryonic growth in *R. t. lateralis*. The second phase, between Day 10 and Day 22–23, is characterized by increasingly rapid embryonic growth and mobilization of energy and material. The most notable feature of this phase is that the greatest embryonic growth and yolk depletion occur at

Table 2

A comparison of the conversion efficiencies of dry mass, non-polar lipids and energy between *Rhabdophis tigrinus lateralis* and other species of oviparous squamate reptiles where embryonic dry mass is almost negligible as a contributor to overall egg mass at oviposition

	Hatching dry mass (mg)	Conversion efficiency (%)			Data resources
		Dry material	Non-polar lipids	Energy	
Lizards					
<i>Calotes versicolor</i>	108	61	29	51	Ji et al., 2002
<i>Eremias brenchleyi</i>	100	67	33	60	Xu and Wu, 2003
<i>Eumeces chinensis</i>	203	68	45	64	Ji et al., 1996
<i>Eumeces elegans</i>	82	58	40	53	Xu et al., 2004a
<i>Morethia adelaidensis</i>	46	66	–	58	Thompson and Russell, 1999
<i>Morethia boulengeri</i>	36	74	–	58	Thompson and Russell, 1999
<i>Podarcis muralias</i>	71	75	46	61	Ji and Braña, 1999
<i>Takydromus septentrionalis</i>	74	70	37	53	Xu et al., 2004b
Snakes					
<i>Dinodon rufozanatum</i>	1800	81	70	79	Ji et al., 1999b
<i>Elaphe carinata</i>	6820	81	64	72	Ji et al., 1997a
<i>Elaphe taeniura</i>	5610	84	75	81	Ji et al., 1999a
<i>Naja naja atra</i>	3500	74	64	69	Ji et al., 1997b
<i>Ptyas korros</i>	1590	77	54	69	Ji and Sun, 2000
<i>Rhabdophis tigrinus lateralis</i>	474	74	50	58	This study
<i>Xenochrophis piscator</i>	354	75	52	66	Ji et al., 2001
<i>Zaocys dhumnades</i>	2010	76	63	70	Ji, unpubl. data

the end of the phase (Fig. 2). The third phase, between Day 22–23 and hatching, is characterized by a gradual reduction in embryonic growth and yolk mobilization. Snake embryos do not accumulate minerals withdrawn from the eggshell in the yolk (Packard et al., 1984; Packard and Packard, 1988; Ji et al., 1997a,b). Thus, the gain in ash mass of egg contents during this phase signifies that this is a time for embryos of *R. t. lateralis* to undergo significant skeletal ossification.

Table 2 summarizes the proportions (conversion efficiencies) of dry material, non-polar lipids and energy transferred from egg contents of the freshly laid egg to hatchling recorded in 16 oviparous species of squamate reptiles. The efficiencies in the table differ among species, especially between lizards and snakes. These efficiencies provide information that allows us to draw two conclusions: (1) smaller quantities of dry material ($F_{1, 14}=17.76$, $P<0.001$; 67.4 vs. 77.8% mean values), non-polar lipids ($F_{1, 12}=28.63$, $P<0.0002$; 38.3 vs. 61.5% mean values) and energy ($F_{1, 14}=19.30$, $P<0.001$; 57.3 vs. 70.5% mean values) are transferred from egg to hatchling in lizards than in snakes; and (2) hatchling dry mass, a measure directly related to reproductive investment in single offspring, is not a determinant of the conversion efficiencies in either lizards or snakes (linear regression analyses with hatchling dry mass as the independent variable; all $P>0.063$).

Of the above three conclusions, the second one is particularly interesting, because it presumably reflects the phylogenetic differences in energetic cost of embryonic development and residual yolk size between lizards and snakes. The energetic cost of embryonic development in snakes is the lowest amongst reptiles while that of lizards is among the highest (Dmi'el, 1970; Black et al., 1984; Whitehead, 1987; Booth and Thompson, 1991; Vleck and Hoyt, 1991). Thus, the lower energetic cost of embryonic development provides one explanation for the observed greater conversions efficiencies in snakes. It is common in oviparous reptiles that the nutrient provision in eggs exceed the needs for producing a complete hatchling, and thus a portion of yolk (i.e., residual yolk) may remain unused at hatching (e.g., Kraemer and Bennett, 1981; Troyer, 1983; Fischer et al., 1991; Ji et al., 1997a). Residual yolk can be used by hatchling reptiles for maintenance metabolism and/or tissue growth, and may therefore enhance their survivorship (e.g., Troyer, 1987; Congdon and Gibbons, 1989; Fischer et al., 1991; Ji et al., 1997a,b). Interestingly, hatchling snakes (of the total hatchling dry mass) generally contain more residual yolk than do hatchling lizards (11–25% vs. 0–12% of the total hatchling dry mass; see references in Table 2). The conversion efficiencies should be greater in species where female provision eggs with more yolk. Thus, the greater residual yolk size provides another explanation for the greater conversions efficiencies in snakes.

In summary, our data show that there are three phases of embryonic growth or yolk depletion in *R. t. lateralis*, and confirm that oviposition does not coincides with the onset of exponential embryonic growth in oviparous squamate reptiles that are positioned midway within the oviparity–viviparity continuum. Embryos of *R. t. lateralis* use eggshell as a secondary source of minerals for development, and withdraw minerals from the eggshell mainly during the last quarter of

incubation. The conversion efficiencies of energy and material from egg to hatchling are greater in snakes than in lizards, primarily because energetic cost of embryonic development are lower but residual yolk sizes are greater in snakes.

Acknowledgements

We thank Hui-Li Chen, Lai-Gao Luo, Chao-Hua Zhang and Ling Zhang for their assistance in the laboratory. The experiment complied with the current laws on animal welfare and research in China, was conducted under the authority of the Zhejiang Provincial Bureau of Forestry. This work was supported by grants from the Zhejiang Provincial Natural Science Foundation (Research Project Grant RC97019) and the Zhejiang Provincial Government for the Key Discipline of Zoology to XJ.

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