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Incubation and Utilization of Energy and Material during Embryonic Development in Eggs of *Naja naja atra*

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Although it is common in oviparous reptiles that females lay eggs that contain all nutrients for embryonic development, interspecific differences have been found in egg and egg incubation in at least three aspects: conversion of nutrients and/or energy from egg to hatchling (Ewert, 1979; Congdon et al., 1983b; Wilhoft, 1986; Fischer et al., 1991; Ji, 1992), allocation of minerals between yolk and eggshell (Bustard et al., 1969; Jenkins, 1975; Packard and Packard, 1984, 1989; Packard et al., 1984a, b), and embryonic mobilization

pattern of some specific minerals (e.g., calcium; Packard and Packard, 1984, 1986, 1988, 1989; Packard et al., 1984a, b, 1985; Shadrix et al., 1994). Such differences could be related to phylogeny, nest microenvironment, and feeding and foraging strategies of neonates; however, no general conclusions can be drawn presently except that for embryonic mobilization pattern of calcium (Packard and Packard, 1984, 1989; Packard et al., 1984a, b, 1985). Thus, data on egg and hatchling components from more oviparous reptile species are important in answering questions such as why females of some species allocate relatively more material and energy to eggs than those of other species, if the hatchling quality is determined by its feeding ability and food availability of a habitat in which it lives, and what is the exact function of eggshell as a mineral source for a given species.

The cobra *Naja naja atra* is a large, highly venomous snake. Previous work has focused on its morphology, food habits, food assimilation, blood components, visual physiology, and toxicology (see review by Huang and Jin, 1990); however, little information on incubation and reproduction is available other than incidental notes (Hu et al., 1966; Sheng et al., 1988). Herein, we present data on (1) incubation characteristics, (2) egg and hatchling components, (3) energy and material budget during incubation, and (4) sources of calcium and magnesium for embryogenesis.

During mid July 1993, five gravid cobras (snout-vent length: 1035–1142 mm; body mass (excluding the clutch): 376.0–489.3 g) were obtained from a private collector in Beichan, Dinghai, Zhoushan Islands, Zhejiang, eastern China and were transported to our laboratory at Hangzhou Normal College, where they were maintained individually in 800 × 800 × 800 mm wire cages. Food (*Bufo gargarizans* and *Eumeces chinensis*) and water were provided ad libitum. Egg laying started about two weeks after the cobras arrived in the laboratory. We removed eggs from the cages, and measured and weighed them within 6 h of oviposition. We randomly selected two eggs from each clutch, and opened them. Egg contents (yolk plus embryo) of the dissected freshly laid egg were removed, placed in pre-weighed small glass dishes, and weighed to the nearest 0.1 mg. We briefly rinsed shells from the dissected freshly laid eggs, removed surface water using a paper towel, and weighed them to the nearest 0.1 mg. These shells were then frozen for later analysis. All dissected eggs contained a small embryo, which was too small and fragile to be sampled separately, and therefore was included with the yolk.

Eight eggs from two clutches were excluded from experiments, because they either had a less developed eggshell or exhibited reduced viability. Thirty-six eggs, ½-buried on a moistened sand substrate, were incubated in five glass containers (200 × 200 × 200 mm) with pierced covers kept in an incubator at 30 ± 0.3 C. The incubation medium consisted of dry sand to water in a mass ratio of 4:1; water was added periodically to keep the initial water content. Incubation temperature was monitored twice daily using a RC-95 quick-reading electronic thermometer (Shanghai Jinhua Instruments). Within the incubator, positions of the containers were changed at daily intervals to minimize effects of possible temperature gradients. We measured and weighed the incubating eggs to the

nearest 1 mg at weekly intervals before day 42, and at daily intervals thereafter up to one day prior to hatching. At hatching, each hatchling was measured and weighed. Shells from the hatched eggs were reserved in the same way described above for those from the dissected freshly laid eggs. A total of eleven hatchlings (2–3 from each clutch) was sampled for component analyses.

All samples were dried to a constant mass in a ventilated oven at 65 C, cooled to room temperature in a desiccator with silica gel, weighed, and then ground with a mortar and pestle. Non-polar lipids were extracted from samples of egg contents and hatchlings in a Soxhlet apparatus for a minimum of 5.5 h with absolute ether as solvent. The mass of non-polar lipids in each sample was calculated as the difference in sample dry mass before and after extraction.

Ash and energy density of egg contents and hatchling were determined using a JR-2800 adiabatic bomb calorimeter (Changsha Instruments). Titrations were performed on the residue after calorimetry to correct for nitrogenous wastes. Further corrections were performed for fuse wire burning. Samples of eggshells were burned in a muffle furnace at 550 C for 24 h to determine ash mass.

Total nitrogen of the samples of egg contents and hatchling was determined using a micro-Kjedahl procedure. Total protein was estimated by multiplying total nitrogen content by 6.25 (Card and Nesheim, 1966).

Samples of egg contents and hatchling for calcium and magnesium determinations were weighed out into glass tubes and digested completely in hot concentrated nitric acid. Digestates were brought to volume in volumetric glassware and stored in a refrigerator until analysis for calcium and magnesium. Concentrations of the two elements in digestates were determined using a WFX-1B model atomic absorption spectrometer (Beijing 2nd Optical Instruments). We did not determine individually the amounts of calcium and magnesium in eggshells. To check if there were any differences in calcium and magnesium contents between shells from freshly laid and hatched eggs, we took an equal amount of sample from each shell, pooled separately the samples from freshly laid and hatched eggs, and treated them as two different samples.

Because all components were highly correlated with total egg wet mass at oviposition, we compared all means using analysis of covariance (ANCOVA) with total egg wet mass at oviposition as the covariate. The homogeneity of slopes was checked prior to testing for differences in the adjusted means. Descriptive statistics are presented as mean ± 1SE.

Clutch size averaged 10.8 ± 1.4 (range = 9–16, $N = 5$). Descriptive statistics of size and mass of *N. n. atra* eggs (including abnormal ones) and hatchlings are shown in Table 1. Eggs increased in wet mass during incubation and, one day prior to hatching, weighed $114 \pm 1.2\%$ (range = 105.9–126.7, $N = 29$) of total egg wet mass at oviposition. Hatching success was 80.6% (29/36). The incubation duration averaged 50.7 ± 0.1 (range = 49.5–51.5, $N = 29$) days.

Data on egg and hatchling components are shown in Table 2. Egg contents averaged 75.5% water by mass. Egg contents averaged 92.8% organic material, 7.2% ash, 31.5% non-polar lipids, 49.9% protein, 1.14%

TABLE 1. Descriptive statistics of size and mass of *Naja naja atra* eggs and hatchlings from five clutches.

	N	Mean	Mini- mum	Maxi- mum	1 SE
Egg measurements					
Egg length (mm)	54	49.4	38.1	65.5	1.0
Egg width (mm)	54	26.2	22.9	27.7	0.2
Egg wet mass (g)	54	20.0	12.9	26.5	0.5
Hatchling measurements					
Snout-vent length (mm)	29	286.1	251.9	308.3	2.2
Tail length (mm)	29	46.1	40.3	51.3	1.1
Hatchling wet mass (g)	29	15.3	10.9	18.9	0.4

calcium, and 0.426% magnesium by dry mass (Table 2). Shells from the dissected freshly laid eggs averaged 8.3% of the total egg dry mass, and 79.3% organic material and 20.7% ash by dry mass (Table 2). Shells from the dissected freshly eggs had a noticeably higher level of calcium (9.73%) but a lower level of magnesium (0.643%) than did shells from the hatched eggs (calcium: 5.69%; magnesium: 0.827%).

Hatchlings averaged 75.9% water by mass. Hatchlings averaged 90.2% organic material, 9.8% ash, 27.7% non-polar lipids, 47.8% protein, 1.80% calcium, and 0.582% magnesium by dry mass (Table 2). Shells from hatched eggs averaged 82.9% organic material and 17.1% ash by dry mass (Table 2).

Hatchlings contained lower quantities of total dry mass, organic mass, non-polar lipids, protein, and energy but a higher quantity of calcium than did egg contents. There were no significant differences in quantities of ash mass and magnesium between egg contents and hatchling (Table 2). Shell from hatched eggs contained lower quantities of total dry mass, organic mass, and ash mass than did shells from freshly laid eggs (Table 2).

During incubation, approximately 25.5% dry mass, 35.6% non-polar lipids, 29.7% protein, and 30.6% energy in egg contents of the freshly laid egg were utilized for embryogenesis; the remaining 74.5% dry mass, 64.4% non-polar lipids, 70.3% protein, and 69.4% energy were stored in the hatchlings (Table 2). Fully developed hatchlings could obtain all magnesium from the yolk, but withdrew 13.6% of their total calcium requirements from sources other than yolk (Table 2).

Although a small embryo was present in the freshly laid egg, its mass, relative to the whole egg mass, was negligible. Thus, we can reasonably consider the transference of energy and material from egg to hatchling during incubation as an approximation to the transference overall. This makes it possible to compare our data with those for other oviparous reptiles whose freshly laid eggs also contain a very small embryo.

Dry mass conversion from egg to hatchling in *N. n. atra* (74.5%) was similar to the values reported for the chicken turtle (72%; Congdon et al., 1983a) and the painted turtle (72%; Ewert, 1979), but lower than the value reported for the American alligator (79%; Fischer et al., 1991). The proportion of non-polar lipids transferred from egg to hatchling in *N. n. atra* (64.4%) was lower than the value reported for the American alligator (74.3%; Fischer et al., 1991), but higher than the values reported for some turtles and lizards (50–60%; Congdon et al., 1983b; Wilhoft, 1986; Ji, 1992). Energy conversion efficiency during incubation in *N. n. atra* (69.4%) was greater than the value reported for the snapping turtle (60%; Wilhoft, 1986).

Evidently, the above comparisons show interspecific differences in conversions of material and energy from egg to hatchling. However, explanations for these differences are unknown at this time, mainly because the data cited above were obviously from species whose eggs were incubated under very different conditions. Previous studies showed the influence of incubation conditions on egg survival and hatchling parameters (Snell and Tracy, 1985; Gutzke and Packard, 1987;

TABLE 2. Components and F values of the ANCOVA for 10 freshly laid eggs and 11 hatchling *Naja naja atra*. Data are expressed as adjusted mean \pm 1 SE with total egg wet mass at oviposition as covariate. Symbols immediately after F values represent significant levels: NS $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

	Freshly laid egg	Hatched egg	F
	Egg contents	Total hatchling	
Wet mass (g)	18.9 \pm 0.3	14.7 \pm 0.3	51.74***
Dry mass (g)	4.7 \pm 0.2	3.5 \pm 0.1	34.81***
Water (g)	14.3 \pm 0.2	11.2 \pm 0.2	79.80***
Organic mass (g)	4.39 \pm 0.17	3.13 \pm 0.10	38.34***
Ash mass (g)	338.3 \pm 9.0	342.8 \pm 0.8	0.08 ^{NS}
Non-polar lipid (g)	1.49 \pm 0.06	0.96 \pm 0.04	50.73***
Protein (g)	2.36 \pm 0.09	1.66 \pm 0.06	37.47***
Calcium (mg)	53.9 \pm 1.5	62.4 \pm 2.8	5.73*
Magnesium (mg)	20.2 \pm 0.6	19.3 \pm 0.9	0.57 ^{NS}
Energy (Kcal)	28.8 \pm 0.2	20.0 \pm 0.6	41.39***
	Eggshell	Eggshell	
Dry mass (mg)	425.7 \pm 7.4	367.3 \pm 6.3	33.31***
Organic mass (mg)	337.7 \pm 6.4	304.5 \pm 5.4	14.45**
Ash mass (mg)	88.0 \pm 1.4	62.9 \pm 1.1	176.93***

Packard, 1991; Vleck, 1991; Dmi'el et al., 1993), so interspecific comparisons might be less informative except those between species whose eggs are incubated under similar conditions. Our experience with incubating eggs of different squamate species is that the values of material and energy budget during incubation are strongly influenced by the level of reproductive investment in the egg, costs of embryonic development, and the proportional amounts of posthatching yolk and fat bodies in the hatchling (Ji et al., 1997a, 1997b).

Similar to other squamates (Packard et al., 1984a, 1985; Packard and Packard, 1988), turtles (Packard et al., 1984b; Packard and Packard, 1986), and the American alligator (Packard and Packard, 1989), *Naja naja atra* embryos should withdraw a noticeable amount of calcium from the eggshell. The result that eggshells decreased in mass and calcium content during incubation supported this interpretation. The level of calcium withdrawn by *N. n. atra* (13.6%) embryos from sources other than yolk was much lower than the values reported for crocodylians and turtles (50–80%; Bustard et al., 1969; Jenkins, 1975; Packard and Packard, 1984, 1989); it was also lower than the values reported for the colubrid snake *Coluber constrictor* (20%; Packard et al., 1984a) and the skink *Eumeces fasciatus* (39%; Shadrix et al., 1994). These differences presumably reflect the interspecific differences in eggshell structure and allocation of minerals between yolk and eggshell.

As in the American alligator (Packard and Packard, 1989), *Naja naja atra* embryos can obtain all magnesium from the yolk. Although we do not know if there is a precise cation replacement mechanism, we do not have any reasons to think the relative increase in magnesium level in hatched eggshells not to be partly the result of selective depletion of calcium by embryos from the eggshell. This might also provide an additional evidence that demonstrates that it is not necessary for *N. n. atra* embryos to withdraw magnesium from the eggshell.

In this study, we only employed one temperature-humidity regime to incubate *N. n. atra* eggs. This approach does not furnish information on variability in embryonic development condition at hatching and energy reserve in the hatchling. Thus, more extensive and sophisticatedly designed studies in this field could be very helpful in our understanding of storage and utilization pattern of energy and material in eggs of oviparous reptiles.

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Skeletochronology of *Bufo raddei* from the Gobi Desert

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The Mongolian toad (*Bufo raddei*) is one of the most widespread amphibians in Central Asia. Its distribution extends from the Lake Baikal region in the north to the Huang He River valley in the south, and from

Xinjiang and south-western Mongolia in the west to Primorye and Korea in the east. Mongolian toad populations from arid zones live at an ecological extreme for amphibians. Winter temperatures below –20 C and summer temperatures of +40 C (air) and +70 C (soil) are normal in the Gobi Desert. The population biology of the Mongolian toad is unknown in the Gobi (Wang and Shi, 1958; Ye, 1965; Piechocki and Peters, 1966; Borkin and Kuzmin, 1988; Zhao and Adler, 1993). Therefore, studies of its biology in such an extreme climate may be important in understanding adaptations of amphibians to arid conditions.

Specimens were collected in Mongolia from June through August 1991 and 1993 in the Valley of the Gobi Lakes, Bayankhongor Aimag (along the shores of the Tuin-Gol River and the weakly saline lakes Orog-Nuur and Boontsagaan-Nuur) and at Lake Ugi-Nuur in Arkhangai Aimag (Fig. 1). The first three locations were grassland oases in semi-desert habitat whereas the fourth location was in grassland steppe. We measured snout-vent lengths (SVL, mm) with calipers and rulers and body mass (mg) with balances. After field measurements were taken, most of the animals were released where captured. Tadpole developmental stages were determined using the stages in Gosner (1960).

Hip bones of newly metamorphosed toadlets (N = 24) were fixed in 5% neutral formalin and examined using skeletochronological techniques. The age of postmetamorphic toads was determined by the number of annual rings (lines of arrested growth—LAGs) in transverse sections of the third phalanx of the third toe of the right hindleg (Smirina, 1972; Hemelaar and Van Gelder, 1980; Hemelaar, 1981). Diameters of annual rings and of marrow cavity were measured under the microscope; these data were used for calculation of relative growth of the bone. To avoid age estimation errors resulting from growth, we analyzed annual rings only in samples collected over a period of 2–4 consecutive days. We examined 583 specimens of which it was impossible to determine the age of 54 individuals. We measured SVL and determined sex in an additional 85 toads that were not examined for age. Metric measurements were compared to the numbers of LAGs using Pearson and Spearman rank correlations. Usual t-tests were used to compare means. The program PC - Statistica for Windows, release 4.3, was used for statistical calculations.

Toad tadpoles (total length 18–41 mm, stage 32–41) were found in mid-July in small wetlands, springs, and fishless stream pools. They were absent in the lakes, probably because of the saline water. Metamorphosing larvae (stages 42–45) and newly metamorphosed toadlets (stage 46, SVL = 13–23 mm) were found in sympatry with older toads. Adults regularly were found in water on hot sunny days.

We did not find LAGs in the newly metamorphosed toadlets (SVL \pm SE = 17.4 \pm 0.32 mm). Specimens of SVL 20–47 mm (on average 26.3 \pm 1.3–41.3 \pm 1.3 mm depending upon locality) collected at the same time had one LAG. They were significantly longer than newly metamorphosed toadlets ($t = 8.593$, $df = 29$, $P < 0.001$). These larger tadpoles probably had overwintered once.

Although we have no long-term mark-recapture data that permit us to make firm conclusions on the