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EXPERIMENTAL MANIPULATION OF EGG SIZE AND HATCHLING SIZE IN THE COBRA, *NAJA NAJA ATRA* (ELAPIDAE)

by

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ABSTRACT

We experimentally manipulated eggs of *Naja naja atra* by partially removing yolk (approximately 15% of the total egg wet mass) from the freshly laid eggs to test the effect of such a manipulation on hatchling traits. Hatchlings from treated and control eggs took nearly the same time to complete embryonic development. Experimentally treated eggs produced size-reduced hatchlings. However, when shell excluded egg mass is statistically kept constant miniaturized hatchlings had slightly but significantly larger total dry body mass than did their control sibs. Relatively more yolk remained unutilized at the time of hatching in miniaturized hatchlings. Since no significant difference in body size or carcass size was found between miniaturized and control hatchlings when shell-excluded egg mass is kept constant, the difference in residual yolk dry mass explained much of their difference in dry body mass. The relative difference in residual yolk dry mass also explained much of the difference in lipid mass, energy contents and residual yolk ash mass between miniaturized and control hatchlings.

KEY WORDS: Reptilia, Squamata, Elapidae, *Naja naja atra*, egg manipulation, hatchling, incubation.

INTRODUCTION

In reptiles that lay a varying number of eggs per clutch or per season, egg size is related to offspring number (SMITH & FRETWELL, 1974). Physiologically speaking, clutch size is determined by competition among potential follicles for follicle stimulating hormone (FSH). Follicles receiving more FSH will form a clutch of eggs (see SINERVO, 1994). When clutch size is fixed, reproductive resources available to the mother will determine egg size. Clutch size and/or egg size can be experimentally manipulated at three levels: (1) extraneous provision of FSH to induce more but smaller eggs, (2) partially removing yolked follicles to induce less but larger eggs, and (3) partially removing yolk from the freshly laid eggs to produce artificially miniaturized eggs.

SINERVO & HUEY (1990) miniaturized *Sceloporus occidentalis* eggs and thus hatchlings by partially removing yolk from the freshly laid eggs

and tested interpopulation differences in locomotor performance and morphology of hatchlings. However, they did not give information on the composition of the miniaturized hatchlings, which may be reduced disproportionally and differ from that of control hatchlings. Since artificially miniaturized eggs are not the same as small eggs, it is important to test for differences in composition between hatchlings from treated and control eggs.

In oviparous reptiles, embryos use yolk and eggshell as the sources of nutrients for development and a portion of yolk may remain unutilized at the time of hatching (*e.g.*, EWERT, 1979; PACKARD & PACKARD, 1984; SHADRIX *et al.*, 1994; JI *et al.*, 1997a). Residual yolk reserves can be used for hatchling maintenance and, in some reptiles, also for tissue growth (*e.g.*, KRAEMER & BENNETT, 1981; CONGDON *et al.*, 1983a, b; LONG, 1986; WILHOFT, 1986; FISCHER *et al.*, 1991; JI *et al.*, 1997a, 1999a, b; TUCKER *et al.*, 1998). The function of residual yolk makes the relationship between egg size and hatchling size more complicated than expected because of the inverse relationship between residual yolk size and hatchling size that has been seen in some reptiles (*e.g.*, PACKARD, 1991; JI *et al.*, 1997a). In addition, our experience with incubating reptilian eggs has shown that small hatchlings emerging in a relatively immature condition contain larger residual yolks and leave heavier eggshells with higher levels of inorganic material. Less inorganic material is withdrawn from the eggshell by the embryo.

In this study, we miniaturized *Naja naja atra* eggs to test the effect of experimental manipulation of egg size on hatchling traits. We pay particular attention to possible differences in composition between treated and control hatchlings. We investigate whether miniaturized eggs produce proportionally reduced hatchlings and whether embryos developing in miniaturized and control eggs withdraw the same amounts of inorganic material from the eggshell.

MATERIALS AND METHODS

The cobra *Naja naja atra* (Elapidae) is one of the most conspicuous snake species in southern China. It has received considerable attention because of its economical importance and high abundance in the field. Background data covering a wide range of its biology and ecology are available (HU *et al.*, 1966; SHENG *et al.*, 1988; HUANG & JIN, 1990). In an earlier paper, we reported data on material and energy budgets during embryonic development in eggs incubated at 30°C (JI *et al.*, 1997b).

In early July 1998, we obtained 43 gravid cobras (snout-vent length: 84.0-120.5 cm; postpartum body mass: 126.5-592.0 g) from dealers in

Yiwu and Jiande, Zhejiang, eastern China. The cobras were brought to our laboratory at Hangzhou Normal College, where they were randomly assigned 1-2 to a 50 × 45 × 35 (length × width × height) cm wire cage placed in an air-conditioned room at 28-30°C. Food (toads and skinks) and water were provided ad libitum. The animals began to lay eggs a few days after their arrival. Cages were checked for a minimum of six times daily for the presence of eggs so that eggs could be collected, weighed and measured promptly. The majority of eggs were used to test the effects of hydric and thermal environments on incubating eggs and hatchling characteristics, and data will be reported elsewhere. We randomly selected 1 to 3 freshly laid eggs from each of the clutches with more than 8 eggs and removed part of yolk using a sterilized syringe. We also randomly selected 1 to 2 eggs from each clutch as a control (unmanipulated) sample.

Eggs were incubated at 30 (± 0.3)°C in plastic containers (25 × 18 × 7 cm) with pierced covers. The containers contained known amounts of vermiculite and distilled water (3 g water/1 g dry vermiculite) resulting in approximately 0 kPa water potential. Eggs were 1/3-buried in the substrate, with the surface near the embryo being exposed to air inside the container. We moved containers among shelves in the incubator daily according to a predetermined schedule to minimize any effects of thermal gradients inside the incubator. The temperature in close proximity to the eggs was monitored twice daily using a digital thermometer. Containers were weighed daily and, if necessary, distilled water was mixed evenly into substrates to compensate for small evaporative losses and water absorbed by eggs, thereby maintaining the water potential of the substrate constant. Duration of incubation was defined as the time elapsed from egg laying to hatchling emergence.

A total of 85 hatchlings were sexed, measured (snout-vent length and tail length), weighed, and then killed by freezing. The hatchlings were later thawed and separated into carcass, yolk sac (residual yolk) and fat bodies. The three components were dried to constant mass at 65°C, weighed, and preserved for later determination of composition.

We extracted non-polar lipids from dried samples of carcass and residual yolk in a Soxhlet apparatus for a minimum of 5.5 h using absolute ether as solvent. The amount of lipids in a sample was determined by subtracting the lipid-free dry mass from the total sample dry mass. We determined energy contents of the three components of hatchling using a GR-3500 adiabatic bomb calorimeter (Changsha Instruments, China). Inorganic contents of eggshell, carcass and residual yolk were determined by burning samples in a muffle furnace at 700°C for a minimum of 12 h and weighing the remaining ash.

A preliminary analysis showed that there were no between-sex differences in hatchling traits except for shorter tails in females, so we pooled

data other than those on the tail length for both sexes. To avoid pseudo-duplication, we blocked data by the clutch and then tested for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Bartlett's test). LN transformations were performed when necessary to achieve the conditions for using parametric tests. We used regression statistics, one-way and two-way analysis of variance (ANOVA) and one-way analysis of covariance (ANCOVA) to analyze data. For miniaturized eggs, we considered post-treatment egg mass as their mass. In all ANCOVA models, we used shell-excluded egg mass (egg mass — shell dry mass at the time of hatching) as the covariate. Throughout this paper, values are presented as mean $\pm$ 1 standard error, and significance level is set at $\alpha = 0.05$.

RESULTS

Clutch size and egg mass in our sample averaged 11.1 $\pm$ 0.4 g (range 6-17, $N = 43$) and 16.2 $\pm$ 0.5 g (range 11.9-29.1, $N = 43$), respectively. Both clutch size ($r^2 = 0.37$, $F_{1,41} = 24.806$, $P < 0.0001$) and clutch mass ($r^2 = 0.53$, $F_{1,41} = 46.591$, $P < 0.0001$) were positively correlated with female SVL.

As expected there was no significant difference in the average pre-treatment mass between miniaturized and control eggs (Table 1, $F_{1,70} = 1.941$, $P > 0.05$). The average mass of miniaturized eggs was approximately 85% of the average pre-treatment mass, and the difference was significant (Table 1, $F_{1,50} = 10.246$, $P < 0.01$). The overall sex ratio of hatchlings (females/males = 42/43) was almost 1 : 1. There was no significant difference in the post-treatment mass between miniaturized eggs producing male and female hatchlings, respectively (Table 1, $F_{1,24} = 0.048$, $P > 0.05$). A two-way ANOVA did not show any difference in incubation length between sexes or between treatments ($F_{1,68} = 0.365$, $P > 0.05$; $F_{1,68} = 1.413$, $P > 0.05$; Table 1). When pooling data for both sexes, we again found that the incubation length was independent of treatment ($F_{1,23} = 2.049$, $P > 0.05$) but there was a significant inter-individual variation in incubation length ($F_{23,23} = 10.246$, $P < 0.001$).

Miniaturized eggs produced size-reduced hatchlings of which the three components are all reduced (Table 1, ANOVA, $P < 0.001$ for all traits). However, when controlling for variation in shelled-excluded egg mass, dry body mass, lipid mass and energy contents of the hatchling, residual yolk dry mass and residual yolk ash mass were significantly larger in miniaturized hatchlings, whereas other hatchling traits did not vary between miniaturized and control individuals (Table 2). At the time of hatching, shells were heavier in miniaturized eggs, whereas ash remaining

TABLE 1

Mass of miniaturized and control eggs of *Naja naja atra*, duration of incubation at 30° C and size, mass and major components of newly emerged hatchlings. Data are reported as direct values in mean ± 1 standard error. Mass is reported in grams, length in centimeters, and incubation length in days.

	Control eggs		Miniaturized eggs	
	Male $N = 22$	Female $N = 24$	Male $N = 13$	Female $N = 13$
Egg mass				
Original mass	16.1 ± 0.6	16.1 ± 0.6	15.1 ± 0.8	15.3 ± 0.7
Manipulated mass	–	–	12.8 ± 0.6	13.1 ± 0.7
Shell-excluded mass	15.8 ± 0.5	15.9 ± 0.6	12.5 ± 0.6	12.8 ± 0.7
Duration of incubation	45.9 ± 0.4	46.3 ± 0.4	45.6 ± 0.4	45.7 ± 0.3
Hatchling size and mass				
Snout-vent length	27.0 ± 0.3	27.1 ± 0.2	25.6 ± 0.4	25.9 ± 0.3
Tail length	5.02 ± 0.09	4.62 ± 0.05	4.83 ± 0.07	4.33 ± 0.06
Wet body mass	12.5 ± 0.5	12.7 ± 0.5	10.3 ± 0.6	10.4 ± 0.6
Dry body mass	3.00 ± 0.16	3.12 ± 0.19	2.52 ± 0.20	2.44 ± 0.17
Carcass dry mass	1.73 ± 0.05	1.75 ± 0.06	1.46 ± 0.07	1.46 ± 0.06
Yolk sac dry mass	0.79 ± 0.10	0.87 ± 0.12	0.58 ± 0.12	0.58 ± 0.10
Fatbody dry mass	0.48 ± 0.03	0.50 ± 0.03	0.46 ± 0.06	0.40 ± 0.03

in the shell did not vary between miniaturized and control eggs when controlling for variation in shelled-excluded egg mass (Table 2).

DISCUSSION

Dried eggshells at the time of hatching account for approximately 2.2% of the total egg mass in miniaturized eggs and 1.8% of the total egg mass in control eggs. Due to this difference, original egg mass cannot be used as a covariate, because the reduced mass of miniaturized eggs results in a relative increase in shell mass (Table 2). Using shell-excluded egg mass as the covariate avoids this problem, but it can complicate our analysis if there is any difference in shell mass between miniaturized and control eggs. Fortunately, an ANCOVA using the pre-treatment egg mass as the covariate does not show any difference in shell dry mass between miniaturized and control eggs at the time of hatching ($F_{1,69} = 0.005, P > 0.05$). Therefore, shell-excluded egg mass is a more reliable covariate, and proved to be the most robust covariate in all of our ANCOVA models.

Experimentally miniaturized *N. n. atra* eggs produced size-reduced hatchlings of which, however, the composition seems not to be reduced

TABLE 2

A comparison of traits of hatchlings from miniaturized and control eggs of *Naja naja atra*. For miniturized hatchlings adjusted means with shell-excluded egg mass as covariate are given. Data are reported as adjusted means ± 1 standard error. Mass is reported in grams, length in centimeters, and energy contents in kilojoules. F values correspond to single models of one-way ANCOVA. Symbols immediately after F values represent significance levels: NS $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Variable	Treatment		F
	Control hatchlings	Miniaturized hatchlings	
Snout-vent length	26.8 ± 0.1	26.5 ± 0.2	2.026^{ns}
Tail length	4.97 ± 0.08 (M)	5.00 ± 0.07 (M)	0.053^{ns}
	4.58 ± 0.04 (F)	4.44 ± 0.05 (F)	3.716^{ns}
Wet body mass	11.9 ± 0.1	12.2 ± 0.1	1.357^{ns}
Dry body mass	2.85 ± 0.06	3.09 ± 0.07	5.012^{*}
Carcass dry mass	1.67 ± 0.03	1.64 ± 0.02	0.439^{ns}
Yolk sac dry mass	0.71 ± 0.05	0.92 ± 0.05	6.636^{*}
Fatbody dry mass	0.46 ± 0.02	0.51 ± 0.03	1.965^{ns}
Hatchling lipid mass	0.79 ± 0.02	0.90 ± 0.03	5.243^{*}
Hatchling energy contents	68.4 ± 1.5	74.6 ± 2.0	4.329^{*}
Hatchling ash mass	0.294 ± 0.005	0.308 ± 0.005	2.901^{ns}
Carcass lipid mass	0.124 ± 0.003	0.131 ± 0.003	0.989^{ns}
Carcass ash mass	0.250 ± 0.004	0.248 ± 0.003	0.141^{ns}
Yolk lipid mass	0.203 ± 0.011	0.253 ± 0.013	7.370^{**}
Yolk sac ash mass	0.044 ± 0.003	0.061 ± 0.004	8.263^{**}
Shell dry mass	0.277 ± 0.004	0.307 ± 0.004	25.751^{***}
Shell ash mass	0.058 ± 0.002	0.063 ± 0.002	1.079^{ns}

proportionally. Although body dimension (snout-vent length and tail length), carcass dry mass and fatbody dry mass did not vary between miniaturized and control hatchlings, relatively more yolk remained at the time of hatching in miniaturized hatchlings for some unknown reason. Since the adjusted mean of dry body mass includes residual yolk dry mass (Table 2), the difference in residual yolk dry mass (0.21 g) explains much of the difference in body dry mass (0.24 g) between miniaturized and control hatchlings. Because the maximum energy density usually occurs in the samples with the highest level of lipids (JI, 1992) and miniaturized *N. n. atra* hatchlings contain a relatively larger lipid mass, it is not surprising that miniaturized hatchlings have higher energy contents than do control hatchlings.

Eggshell has been known to decrease in mass during incubation, mainly because developing embryos withdraw a portion of minerals for development from the source (*e.g.*, PACKARD & PACKARD, 1984, 1986, 1989; PACKARD *et al.*, 1984a, b; SHADRIX *et al.*, 1994; JI *et al.*, 1996,

1997a, b). In *N. n. atra*, the eggshell has lost approximately 14% dry mass and 29% ash by the end of incubation (JI *et al.*, 1997b). The decrease in ash mass during incubation is more pronounced than the decrease in shell dry mass, but both are positively correlated with freshly laid egg mass (JI *et al.*, 1997b). The same phenomenon has also been found in other species of snakes studied, *e.g.*, *Elaphe carinata* (JI *et al.*, 1997a), *Rhabdophis tigrinus lateralis* (ZHAO *et al.*, 1997) and *Elaphe taeniura* (JI *et al.*, 1999a). From this positive correlation one would expect that miniaturized *N. n. atra* eggs shells contain proportionaly less ash than control eggs. However, miniaturizing egg size does not result in a noticeably decreased embryonic mobilization of ash from the shell, as indicated by the lack of the difference in relative shell ash mass between miniaturized and control eggs at the time of hatching (Table 2). This presumably suggests that embryos developing within miniaturized eggs mobilize extra ash from the shell. Since inorganic material, both from yolk and eggshell, can be incorporated into the carcass, an increased embryonic mobilization of ash from the egg shell might be important for early growth of miniaturized hatchlings. However, miniaturized hatchlings did not contain proportionally higher amounts of ash than did control hatchlings (Table 2). The greater ash mass in the yolk (1.4 × control) is largely explained by the relatively higher amount of residual yolk (1.3 × control) present in miniaturized hatchlings (Table 2).

In summary, the most striking feature of this study is that miniaturized *N. n. atra* eggs produce size-reduced hatchlings of which the composition is not reduced proportionally, with significantly more yolk remaining at the time of hatching. Since no significant differences in relative fat body size and carcass size have been found between miniaturized and control hatchlings when controlling for shell-excluded egg mass, the difference in residual yolk dry mass explains much of the difference in dry body mass. The difference in residual yolk dry mass between miniaturized and control hatchlings also explains much of the relative differences in lipid mass, energy contents and residual yolk ash mass. However, to what extent these differences influencing hatchling performance remains unclear. Thus, in future studies, it is could be interesting to test the possible difference in performance between miniaturized and control *N. n. atra* hatchlings.

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