

Article

Can snakes use yolk reserves to maximize body size at hatching?

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Handling editor: Zhi-Yun JIA

Received on 29 October 2018; accepted on 12 December 2018

Abstract

We experimentally miniaturized freshly laid eggs of the Chinese cobra *Naja atra* (Elapidae) by removing ~10% and ~20% of original yolk. We tested if yolk-reduced eggs would produce 1) normal-sized hatchlings with invariant yolk-free body mass (and thus invariant linear size) but dramatically reduced or even completely depleted residual yolk, 2) smaller hatchlings with normal-sized residual yolk but reduced yolk-free body mass, or 3) smaller hatchlings of which both yolk-free body mass and residual yolk are proportionally reduced. Yolk quantity affected hatchling linear size (both snout-vent length and tail length) and body mass. However, changes in yolk quantity did not affect incubation length or any hatchling trait examined after accounting for egg mass at laying (for control and sham-manipulated eggs) or after yolk removal (for manipulated eggs). Specifically, yolk-reduced eggs produced hatchlings of which all major components (carcass, residual yolk, and fat bodies) were scaled down proportionally. We show that snakes cannot use yolk reserves to maximize their body size at hatching. Furthermore, our data also suggest that the partitioning of yolk in embryonic snakes is species-specific.

Key words: hatchling phenotype, *Naja atra*, residual yolk, snake, yolk partitioning, yolk removal

One of the central goals of life history studies in invertebrates and non-mammalian vertebrates is to assess maternal investment in offspring via yolk quantity and egg size. It is common and perhaps ubiquitous among these animals that yolk deposited by a mother in individual eggs exceeds the requirements to produce a fully developed young. Therefore, prior to hatching (for oviparous species) or birth (for viviparous species), a fraction of unutilized yolk (i.e., residual yolk) is internalized into the abdominal cavity of the young for later use (Kaplan 1980; Gouiden et al. 1987; Congdon and Gibbons 1989; Kamler et al. 1998; Koláčková et al. 2015). Residual yolk has multiple functions and its size varies within and among taxa, among populations of the same species, among clutches of the same population or family, and even between sexes of the same clutch (Allsteadt and Lang 1995; Ji et al. 2002a; Spencer and Janzen

2014; Koláčková et al. 2015; Wu et al. 2017). One function of residual yolk is to support post-embryonic growth in the same way as yolk does during the embryonic stage, a function that has been reported for a diverse array of ectothermic vertebrates including salamanders (Orr and Maple 1978), lizards (Troyer 1987; Pandav et al. 2006), snakes (Ji et al. 1997, 1999; Ji and Sun 2000; Wu et al. 2017), and turtles (Filoramo and Janzen 1999; Lance and Morafka 2001; Alava et al. 2006; Lee et al. 2007; Van Dyke et al. 2011; Carpentier et al. 2015). However, 1) the coupling mechanism between these 2 stages, and 2) whether stochastic fluctuations in egg size and thus per capita maternal investment alter the partitioning of yolk between these 2 stages of growth remain poorly understood. Previous studies on these aspects have produced mixed conclusions. For instance, a study of the oriental garden lizard *Calotes versicolor*

shows that selection favors the maximization of yolk reserves for post-hatching needs, and the miniaturization of egg size through experimental removal of yolk eggs results in small hatchlings with a normal-sized residual yolk (Radder et al. 2004). Another study on the green iguana (*Iguana iguana*) indicates that the inter-clutch variation in yolk size at hatching is much greater than variation in either body mass or length (Troyer 1983). Overall, this suggests that selection for maximization of body size at hatching and thus post-embryonic growth rate is favored over residual yolk (Troyer 1983).

Here, we test if stochastic fluctuations in egg size, and thus per capita maternal investment, alter yolk partitioning between the embryonic and post-hatching stages in the Chinese cobra *Naja atra*. We applied a technique of yolk removal that was created for 2 species of sea urchins (*Strongylocentrotus droebachiensis* and *S. purpuratus*; Sinervo and McEdward 1988) and subsequently applied to the western fence lizard *Sceloporus occidentalis*; as the model system (Sinervo 1990), the eastern fence lizard *Sceloporus undulatus* (Storm and Angilletta 2007; Niewiarowski and Angilletta 2008) and the jacky dragon *Amphibolurus muricatus* (Warner and Shine 2007). Specifically, we first miniaturized freshly laid eggs of *N. atra* by surgically removing ~10% and ~20% of original yolk (see below for details) and then tested if yolk-reduced eggs would produce 1) normal-sized hatchlings with invariant yolk-free body mass (and thus invariant linear size) but dramatically reduced or even completely depleted residual yolk (Figure 1A), 2) smaller hatchlings with reduced yolk-free body mass but normal-sized residual yolk (Figure 1B), or 3) smaller hatchlings of which both yolk-free body mass and residual yolk are proportionally reduced (Figure 1C). An earlier study incubating eggs of *N. atra* at multiple constant temperatures within the range of 24°C–32°C shows that the dry mass of residual yolk accounts for 20–33% of the total hatchling dry mass (Ji and Du 2001). Thus, if selection for maximization of body size at hatching is favored over yolk reserves for post-hatching use in this species, we predict that eggs of *N. atra* would have the potential to produce normal-sized hatchlings even undergoing the 20% reduction in yolk quantity.

Materials and Methods

Eggs ($N=165$) used in this study were laid by 11 females collected in late June 2014 from Dinghai (30°02'N, 122°10'E), Zhoushan Islands, East China. Females laid eggs in the laboratory within 2 weeks after capture under the conditions described previously for snakes (Lin et al. 2012). Eggs from individual clutches were assigned among 4 treatments: manipulated (2), sham-manipulated (1) and

control (1). Eighty-eight eggs underwent yolk removal on the day of oviposition, which was accomplished by aspirating yolk with a sterile syringe (#16 needle gauge) inserted into the yolk sac on the side opposite to the embryonic disk. The injection was sealed by a 4 mm × 4 mm sterile adhesive tape. Yolk removal resulted in ~20% (18–26%) mass reduction in 1 group of manipulated eggs ($N=44$), and ~10% (7–12%) mass reduction in the other group ($N=44$). Sham-manipulated eggs ($N=44$) were pierced with a needle but no yolk was removed. Finally, control eggs ($N=33$) were incubated without any kind of manipulation.

Eggs were individually incubated in jars (250 mL) with a substrate of moist vermiculite (~80 mm depth) at a water potential of -220 kPa (Ji and Du 2001). Jars were kept in 2 incubators (Binder Inc., Germany) at 30.0°C. We shuffled the jars within and between incubators every other day to minimize effects of thermal gradients. However, we noticed minor temperature gradients (0.5°C) within incubators. These gradients were measured using Thermochron iButtons (Maxim Integrated Products, USA) that were placed inside jars. We individually weighed jars every 4 days, and added water to the substrate when necessary to compensate for evaporative losses and water absorbed by eggs. Incubation length, defined as the time period between egg laying and pipping, was recorded for each hatched egg (Li et al. 2012; Lu et al. 2012).

Sixty-nine manipulated, 39 sham-manipulated, and 30 control eggs hatched. All hatchlings were collected, weighed, measured for snout-vent length (SVL), and tail length and sexed by manual eversion of hemipenes (Ji and Du 2001) <6 h post-hatching. Eighty-eight hatchlings, 8 (1 male and 1 female from each of the 4 treatments) from each clutch, were frozen at -20°C on the day of hatching. The remaining 50 hatchlings and their respective mothers were released back to the field in mid-September. Frozen hatchlings were later thawed, dissected, and separated into carcass, residual yolk, and fat bodies. The 3 hatchling components were dried in an oven at 60°C for 48 h to obtain dry mass.

We used 2-way ANOVA (for egg mass at laying, incubation length, and hatchling SVL and body mass) or ANCOVA (for other hatchling traits covarying with egg mass) using egg mass at laying (for control and sham-manipulated eggs) or after yolk removal (for manipulated eggs) as a covariate to test if the effects of treatment (manipulated, sham-manipulated, or control), sex and their interaction were significant. Prior to ANCOVAs, we assessed if slopes of the relationship between an examined dependent variable and the covariate were parallel. All analyses were performed with Statistica 8.0 for PC (StatSoft Inc., Tulsa, OK, USA). All values were presented as means ± SE and range. We used a significance level of 0.05.

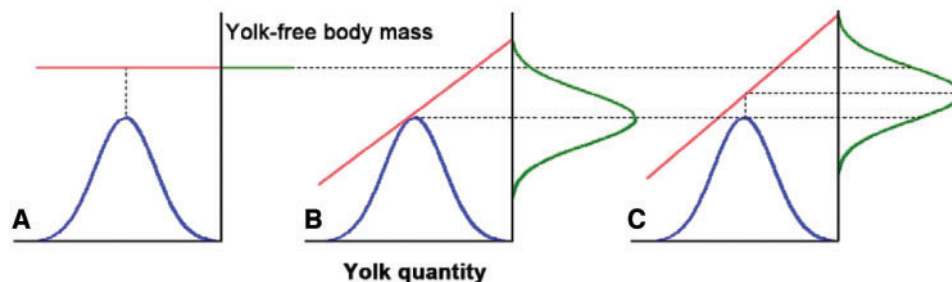


Figure 1. Hypothetical relationships between yolk-free body mass (green line or curves) at hatching and yolk quantity (blue curve) in yolk-reduced (miniaturized) eggs that produce 1) normal-sized hatchlings (unchanged yolk-free body mass) with no or dramatically reduced residual yolk (A), 2) smaller hatchlings with normal-sized residual yolk (B), or 3) smaller hatchlings of which yolk-free body mass and residual yolk both are reduced (C). Green line or curves show yolk-free body mass at hatching; blue curve shows yolk quantity; red line shows the relationship between yolk-free body mass at hatching and the quantity of yolk used to support somatic tissue growth during the embryonic stage.

Table 1. Descriptive statistics, expressed as mean \pm SE and range, for size, mass and composition of hatchlings derived from manipulated (miniaturized), sham-manipulated and control eggs

	Control		Sham-manipulated		Miniaturized by 10%		Miniaturized by 20%	
	Females	Males	Females	Males	Females	Males	Females	Males
Initial egg mass	17.4 \pm 1.1	17.8 \pm 1.0	17.5 \pm 1.0	17.6 \pm 1.0	18.0 \pm 0.9	16.6 \pm 0.7	18.0 \pm 0.9	17.2 \pm 1.0
	11.9 – 22.8	12.2 – 23.3	12.1 – 23.2	12.6 – 23.5	13.3 – 22.2	12.4 – 21.0	12.2 – 23.1	12.1 – 22.5
Miniaturized egg mass	–	–	–	–	16.2 \pm 0.7	14.8 \pm 0.6	14.4 \pm 0.7	13.9 \pm 0.8
					12.0 – 19.5	11.1 – 19.0	10.0 – 18.4	9.6 – 18.5
Snout-vent length	26.1 \pm 0.6	26.3 \pm 0.4	26.4 \pm 0.5	26.4 \pm 0.5	26.0 \pm 0.3	24.8 \pm 0.6	24.9 \pm 0.3	24.1 \pm 0.4
	21.5 – 29.3	24.8 – 28.8	22.9 – 28.5	23.8 – 29.8	24.4 – 27.4	20.8 – 28.2	23.6 – 26.7	21.4 – 25.8
Tail length	4.6 \pm 0.1	4.8 \pm 0.1	4.5 \pm 0.1	4.9 \pm 0.1	4.3 \pm 0.1	4.6 \pm 0.1	4.2 \pm 0.1	4.4 \pm 0.1
	3.9 – 5.3	4.6 – 5.2	4.0 – 5.0	4.5 – 5.6	3.8 – 5.0	4.0 – 5.4	3.7 – 4.7	4.1 – 4.8
Wet body mass	13.1 \pm 0.8	13.7 \pm 0.7	13.7 \pm 0.7	14.0 \pm 0.8	13.3 \pm 0.7	11.4 \pm 0.8	11.6 \pm 0.6	11.3 \pm 0.6
	9.7 – 17.3	10.5 – 17.2	10.6 – 17.6	10.7 – 18.5	9.2 – 16.0	6.2 – 14.8	9.0 – 14.7	8.0 – 14.9
Dry body mass	3.42 \pm 0.21	3.38 \pm 0.21	3.43 \pm 0.19	3.48 \pm 0.17	3.28 \pm 0.21	2.78 \pm 0.20	2.92 \pm 0.17	2.86 \pm 0.18
	2.44 – 4.31	2.40 – 4.63	2.68 – 4.38	2.52 – 4.15	2.05 – 4.20	1.72 – 3.87	2.14 – 3.73	1.98 – 3.95
Carcass dry mass	1.87 \pm 0.14	1.94 \pm 0.11	1.89 \pm 0.10	2.03 \pm 0.10	1.79 \pm 0.08	1.65 \pm 0.11	1.63 \pm 0.08	1.59 \pm 0.08
	1.09 – 2.45	1.48 – 2.44	1.37 – 2.53	1.41 – 2.50	1.37 – 2.11	1.06 – 2.05	1.30 – 2.09	1.19 – 2.07
Residual yolk dry mass	1.04 \pm 0.09	0.94 \pm 0.08	1.00 \pm 0.08	0.91 \pm 0.05	1.00 \pm 0.12	0.68 \pm 0.08	0.84 \pm 0.09	0.83 \pm 0.09
	0.66 – 1.55	0.54 – 1.51	0.65 – 1.51	0.70 – 1.18	0.35 – 1.58	0.35 – 1.24	0.48 – 1.50	0.37 – 1.23
Fat body dry mass	0.52 \pm 0.05	0.50 \pm 0.04	0.54 \pm 0.04	0.54 \pm 0.03	0.50 \pm 0.03	0.46 \pm 0.04	0.45 \pm 0.03	0.44 \pm 0.03
	0.31 – 0.76	0.34 – 0.70	0.32 – 0.76	0.38 – 0.72	0.30 – 0.66	0.21 – 0.64	0.35 – 0.63	0.33 – 0.66

All mass units are in g, and length units in cm.

Results

Table 1 shows descriptive statistics for size, mass, and composition of hatchlings from manipulated, sham-manipulated, and control eggs. Eggs assigned to the 4 treatments did not differ from each other in mean mass at laying ($F_{3,80} = 0.05$, $P = 0.983$) or mean incubation length ($F_{3,80} = 1.39$, $P = 0.251$). We found that eggs producing male and female hatchlings did not differ in mean mass ($F_{1,80} = 0.40$, $P = 0.529$) or mean incubation length ($F_{1,80} = 0.03$, $P = 0.859$). We determined that the sex \times treatment interaction was not a significant source of variation in egg mass at laying ($F_{3,80} = 0.41$, $P = 0.745$) or incubation length ($F_{3,80} = 0.80$, $P = 0.497$). Data pooled for the 4 treatments and sexes showed that incubation lengths ranged from 43.4 to 49.9 days, with a mean of 47.0 days.

None of the examined hatchling traits differed among the 4 treatments after accounting for egg mass at laying (for control and sham-manipulated eggs) or after yolk removal (for manipulated eggs) (all $P > 0.240$). The tail length was the only trait that differed between male and female hatchlings, with males having a longer tail than females ($F_{1,79} = 31.46$, $P < 0.0001$). The sex \times treatment interaction was not a significant source of variation in any examined hatchling trait (all $P > 0.372$). The 10% reduction in egg mass resulted in production of hatchlings that were about 3% shorter in SVL, 8% lighter in wet mass and 10% lighter in dry mass. The 20% reduction in egg mass resulted in hatchlings that were about 7% shorter in SVL, 17% lighter in wet mass, and 16% lighter in dry mass.

Discussion

We found that yolk-reduced eggs produced smaller hatchlings that were shorter and lighter than controls. None of the examined hatchling traits differed among the 4 treatments (manipulated [2], sham-manipulated [1] and control [1]) after accounting for yolk quantity at laying (for controls) or after yolk removal (for the manipulated treatments). Therefore, our results are consistent with the hypothesis

that yolk-reduced eggs produce smaller hatchlings of which both yolk-free body mass and residual yolk are proportionally reduced (Figure 1C).

The proportions of yolk allocated to produce either larger hatchlings with smaller yolk reserves or smaller hatchlings with larger amounts of residual yolk vary considerably among studied vertebrate species and even between the sexes of the same clutch (Allsteadt and Lang 1995; Nagle et al. 2003; Gao et al. 2010; Spencer and Janzen 2014; Koláčková et al. 2015). There is also evidence that yolk partitioning in embryonic snakes is phylogenetically related or species-specific (Wu et al. 2017). For instance, the relative size of residual yolk is far smaller in the short-tailed pit viper *Gloydius brevicaudus* (~3% of the body dry mass; Gao et al. 2010) than in the 5-paced pit viper *Deinagkistrodon acutus* (~33% of the body dry mass; Lin et al. 2005). While larger residual yolks provide sustenance for longer periods and perhaps support early growth better, larger hatchlings with smaller yolk reserves but more developed bodies are favored when resources are abundant or selection for high performance is strong (Booth 2000; Ji and Sun 2000; Radder et al. 2004, 2007; Van Dyke et al. 2011; Pezaro et al. 2013). Natural selection for a given relative size of residual yolk is influenced by the feeding ability of newborn offspring or, probably, the period when they have a negative energy balance (Lance and Morafka 2001; Pezaro et al. 2013; Spencer and Janzen 2014). Here, we found that body size (both SVL and tail length) and mass were the only traits affected by experimental miniaturization of egg size. All other examined hatchling traits including yolk-free dry body mass (carcass dry mass + fat body dry mass) and residual yolk dry mass were not affected by the manipulations of egg size after accounting for yolk quantity. Below, we summarize the main consequences of our findings on yolk partitioning strategies in *N. atra*.

Naja atra is not one of the species where the advantages of maximizing body size prior to hatching is favored over the advantages of maximizing residual yolk (see *I. iguana*; Troyer 1983). In these cases, yolk-reduced eggs are more likely to produce hatchlings with unchanged yolk-free body mass (Troyer 1983). *Naja atra* is also not

one of the species where selection for maximization of yolk reserves at hatching is favored over body size (see *C. versicolor*; Radder et al. 2004). In these cases, yolk-reduced eggs are more likely to produce small hatchlings with normal-sized residual yolk (Radder et al. 2004). Instead, *N. atra* is a species where yolk-reduced eggs produce smaller hatchlings with reduced yolk-free body mass and residual yolk. Specifically, yolk-reduced *N. atra* eggs produce hatchlings with all major body components (carcass, fat bodies and residual yolk) scaled down proportionally. This finding suggests that Chinese cobras cannot actively partition more yolk reserves to maximize body size at hatching.

Reproductive females of *N. atra* can adjust egg size in response to fluctuations in resource availability (Ji and Wang 2005) or clutch size (Ji et al. 2009). Based on our findings, the following prediction can be extrapolated to wild populations of *N. atra*. Changes in egg size may influence hatchling linear size and mass, but not proportional amounts of major hatchling components (including yolk reserves for post-hatching utilization). In fact, snakes cannot control the use or sequestration of yolk, although the residual yolk has a functional role in buffering the embryos from unpredictable environments and its quantity is affected by the environmental conditions, extremely hot and/or dry environments in particular, experienced during embryonic development (Lee et al. 2007; Van Dyke et al. 2011; Pezaro et al. 2013; Wu et al. 2017). For example, high incubation or gestation (for viviparous species) temperatures often result in the production of smaller offspring that characteristically have less developed bodies and contain more unutilized yolk in a diverse array of reptilian species (Packard et al. 1988; Booth and Astill 2001; Gao et al. 2010; Warner et al. 2012). This is also the case in *N. atra* (Ji and Du 2001; Lin et al. 2008). For a given snake species, the size of residual yolk is variable but such a variability does not have anything to do with yolk partitioning strategies.

Our study also shows that yolk removal does not affect the rate of embryonic development, substantiating an earlier conclusion that egg size is not a determinant of incubation length in *N. atra* (Ji and Du 2001; Lin et al. 2008; Ji et al. 2009). Overall, this has been showed in *S. undulates* (Warner and Andrews 2002; Storm and Angilletta 2007), the green anole lizard *Anolis carolinensis* (Goodman 2010), and the Mexican axolotl *Ambystoma mexicanum* (Brain and Landberg 2017). However, our findings are inconsistent with studies of *S. occidentalis* (Sinervo 1990), *C. versicolor* (Radder et al. 2004; but see also Ji et al. 2002b) and different salamander species (e.g., *Ambystoma barbouri* and *A. maculatum*; Landberg 2014, 2015) where yolk removal actually induce fast embryonic development and thus early hatching.

Acknowledgment

The work was supported by grants from the National Natural Science Foundation of China to X.J. (31272294) and Y.-F.Q. (31200283) and the Natural Science Foundation of Zhejiang Province to L.-H.L. (LY17C030003). We thank Ling Zhang for assistance in the laboratory. We thank Daniel Portik and Cristian Román for English editing.

Authors' Contributions

Y.-F.Q. and X.J. conceived and designed the experiments. X.J. supervised the study. Y.-F.Q., S.-Z.Z., X.-F.J., and L.-H.L. performed the experiments. Y.-F.Q. and X.J. analyzed the data. Y.-F.Q. and X.J. wrote the manuscript. All authors reviewed and contributed to editing of the manuscript and approved its final publication.

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