

Nonlinear continuum of egg size-number trade-offs in a snake: is egg-size variation fitness related?

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Abstract The relationship between offspring size and offspring number is crucial to life history evolution. To examine how these two life history variables are coupled and whether an altered balance between them will result in changes in maternal fitness, we manipulated clutch size of the Chinese cobra (*Naja atra*) by using the techniques of hormonal manipulation and follicle ablation. Females receiving exogenous follicle-stimulating hormone produced more but smaller eggs, and females undergoing follicle ablation produced fewer but larger eggs. Neither body size (body mass and snout-vent length) at hatching nor egg mass at oviposition had a role in determining hatchling survival and growth. Female hatchlings were more likely to die in early post-hatching days and grew more slowly than male hatchlings. Our data show that: (1) there is a nonlinear continuum of egg size-number trade-offs in *N. atra* within which there is a single inflexion where the rate at which egg size decreases with increasing clutch size, or clutch size increases with decreasing egg size, is maximized; (2) there is a fixed upper limit to egg size for a given-sized female, and the limit is not determined by her body volume; (3) egg size has no role in determining hatchling survival and

growth; and (4) the extent to which females may enjoy reproductive benefits in a given reproductive episode depends on how well egg size and egg number are balanced.

Keywords *Naja atra* · Chinese cobra · Manipulating clutch size · Offspring survival · Growth

Introduction

Maternal fitness depends on offspring size and offspring number that are mutually constrained by a trade-off resulting from common dependence on the limited reproductive resources (Stearns 1992; Bernardo 1996; Enum and Flemming 2000; Agrawal et al. 2001; Roff 2002). The relationship between offspring size and offspring number is crucial to life history evolution, and has therefore attracted considerable scientific interest over the past decades (Clutton-Brock 1991; Stearns 1992; Williams 1994; Roff 2002). However, how these two variables are coupled and whether an altered balance between them will result in changes in maternal fitness are poorly known in many organisms.

It has been demonstrated in diverse animal taxa that larger offspring have better performance and higher survival (Ferguson and Fox 1984; McGinley et al. 1987; Sargent et al. 1987; Mousseau and Fox 1998; Janzen et al. 2000a, b). Nonetheless, unequivocal evidence in support of a positive relationship between offspring size and fitness is limited. Moreover, in no organism has one seen the evolution of ever-increasing offspring size, primarily due to the fact that the selective advantage accrued by increasing effort per offspring can be counterbalanced by the decreased offspring number and therefore maternal fitness (Stearns 1992; Bernardo 1996; Downhower and Charnov 1998;

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Agrawal et al. 2001; Roff 2002). Several field-based studies suggest that larger offspring size enhances fitness sometimes but not always (Congdon et al. 1999; Warner and Andrews 2002; Husak 2006; Dibattista et al. 2007; Warner and Shine 2007).

The classic offspring size theory predicts that females in a given environment will divide the resources available for reproduction into offspring of an optimal size (Smith and Fretwell 1974). Under this prediction, maximizing maternal fitness will result in a single optimal investment per offspring for any given set of environmental conditions and, as such, changes in total resource acquisition, or in the proportion of reproductive resources allocated to reproduction should not change this optimal investment per offspring and therefore offspring size. However, there is growing recognition that, in many organisms, mothers may adjust the size of their offspring by assessing the environmental circumstance their offspring will encounter according to their own resource availability (Ballinger 1983; Boersma 1997), or offspring size may vary as a function of total reproductive investment or maternal size (Reznick and Bryga 1987; Sargent et al. 1987; Winkler and Wallin 1987; Reznick et al. 1990; Caley et al. 2001).

Studies of squamate reptiles by experimental manipulation of clutch size provide further evidence that offspring size may vary as a response to variation in clutch size or litter size induced by hormonal manipulation or follicle ablation (Sinervo and Licht 1991a, b; Olsson et al. 2002; Ji et al. 2006; but see also Ji and Diong 2006). For example, females undergoing follicle ablation (and thus, decreased clutch size) produce larger offspring in the side-blotched lizard *Uta stansburiana* (Sinervo and Licht 1991b), the common lizard *Lacerta vivipara* (Olsson et al. 2002) and the king ratsnake *Elaphe carinata* (Ji et al. 2006), because yolk, normally allocated to a larger clutch, is subsequently apportioned to the fewer, remaining yolking follicles. The development of techniques for manipulating offspring number has provided a powerful method for examining whether females may adjust the size of their offspring according to variation in offspring number induced by proximate factors. However, as previous studies have not progressed much beyond documenting phenotypic patterns of variation in offspring size, whether offspring-size variation is fitness related and therefore important is still poorly known.

In the present study, we applied the techniques of hormonal manipulation and follicle ablation to the Chinese cobra (*Naja atra*) to examine whether experimental manipulation of clutch size may induce variation in egg size (and thus, hatchling size; Ji and Du 2001) and, if so, how females adjust egg size according to facultative fluctuations in clutch size and therefore reproductive resources available to them, and whether egg-size variation is fitness related. Snakes are particularly suited to such an investigation

partly because, unlike turtles and lizards (Congdon and Gibbons 1987; Sinervo and Licht 1991b), they are free of the pelvic girdle's constraint on offspring size. We used *N. atra* as the model organism mainly for two reasons: (1) egg size varies considerably among females differing in body size, or clutch size, and a phenotypic trade-off between egg size and number exists under both natural and laboratory resource conditions; and (2) females consistently lay eggs in laboratory enclosures (Ji and Wang 2005).

Materials and methods

Study species

The Chinese cobra (*Naja atra*) is a large-sized elapid snake that is widely distributed in the southeastern provinces of China, including Taiwan, Hong Kong and Hainan, southwards to northern Vietnam (Wüster et al. 1997). Life history traits such as the minimum size at maturity, timing of oviposition, clutch size, egg size and clutch mass differ among geographically separated populations (Ji and Wang 2005). In Zhoushan Islands (eastern China) where is the northern limit of the species' distributional range, females larger than 88 cm snout-vent length (SVL) can lay a single clutch of from eight to 28 eggs per breeding season stretching from July to August (Ji and Wang 2005). Larger females in general produce more and larger eggs than do smaller ones; at the same time, an increase in clutch size tends to be counterbalanced by egg size, with eggs from larger clutches being smaller than those of smaller clutches (Ji and Wang 2005).

Experimental manipulation of clutch size

In early April 2003, we employed local people to collect 142 females (91–126 cm SVL) and 18 males (110–135 cm SVL) from a previously studied population in Dinghai (30°02'N, 122°10'E), Zhoushan Islands, and maintained them together in a 24 m × 14 m × 2.5 m (length × width × height) outdoor enclosure built at Hangzhou Normal University. Females were marked via unique combinations of clipped ventral scales for future identification. Food [common toad (*Bufo gargarizans*) and Chinese skink (*Eumeces chinensis*)] and water were provided ad libitum.

In mid-April, 50 females at their earliest stages of vitellogenesis were assigned equally to two groups for follicle stimulating hormone (FSH) treatment, one (the control group) of which received 0.2 ml saline injected subcutaneously under the dorsal skin every other day for 20 days. Females in the other group (the experimental group) received 0.2 ml porcine FSH (product no. F-2293; Sigma)

dissolved in saline to the dosage of 10 units/kg (i.e., 1 unit/kg every other day) at the same interval. FSH increases clutch size by stimulating the growth of additional follicles that might otherwise undergo atresia (Sinervo and Licht 1991a; Sinervo 1999).

In mid-May, females with small-sized yolking follicles (~20 mm length) were assigned to one of the five (three follicle-ablated, one sham-ablated and one control) treatments. Two ($n = 25$), four ($n = 25$) or six ($n = 15$) yolking follicles in the follicle-ablated females were ablated by removing ~0.5 ml yolk from each ablated follicle with sterile syringes. Fifteen sham-ablated females underwent the same protocol without any yolk extraction.

Females of different treatments were maintained together in the enclosure described above, and those with shelled oviductal eggs were individually housed in 50 cm × 45 cm × 35 cm wire cages placed in an indoor animal holding facility where the temperature was controlled within the range 26–30°C, optimal for embryonic development. Eggs were collected, measured and weighed within a few hours after oviposition, thereby avoiding any uncertainty of the initial egg mass due to loss or gain of water (Ji and Du 2001); SVL, tail length and body mass were taken for each post-oviposition female. Seven females died during the course of the experiment, and 15 females either deposited eggs in the enclosure (and thus, eggs could not be allocated accurately to the mother) or produced abnormal clutches with various numbers of eggs containing condensed yolk for unknown reasons; these females, including one FSH-injected, five two-follicle ablated, two four-ablated, four six-ablated and ten control individuals, were found in each treatment, and were excluded from analysis.

To judge whether clutches from captive females resembled those from wild-caught females, we collected an additional sample of 20 gravid females (96–125 cm SVL) from the same population in early July 2003. These females were individually housed in egg-laying cages described above until they laid eggs between 12 and 20 July.

Egg incubation and hatchling husbandry

Fifty-four eggs, two to three from each of the 20 clutches from wild-caught females, were individually incubated in covered plastic jars (250 ml) with known amounts of vermiculite and water at about –220 kPa water potential (1 g dried vermiculite/1 g water; Ji and Du 2001). One-third of the egg was buried lengthwise in the substrate, with the surface near the embryo exposed to air inside the jar. Jars were placed in a 4 m × 3 m artificial atmospheric phenomena simulator (AAPS) room where temperatures varied from 26 to 30°C optimal for embryonic development (Ji and Du 2001; Lin et al. 2008). Jars were weighed at 5-day intervals,

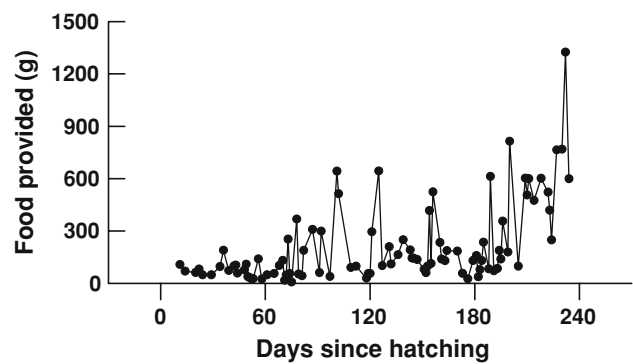


Fig. 1 Food supplied in the first 240-day period. *Solid dots* represent the time and amount of food provided

and water was added to substrates when necessary to compensate for evaporative losses and water absorbed by eggs. Incubation length was defined as the time between egg laying and pipping.

After the eggs had hatched, each hatchling was measured [SVL and tail length (TL)], weighed (initial body mass) and sexed by manual eversion of hemipenes on males (Ji and Du 2001). Each hatchling was marked with a 10 mm × 1.5 mm (length × diameter) implantable PIT tag for future identification. All hatchlings were housed in a 10 m × 6 m greenhouse for 240 days. The greenhouse contained soil substrate, with rocks, shrub stumps, wooden blocks and pieces of clay tiles provided as shelter for the hatchlings. Air temperatures in the greenhouse varied between 20 and 30°C, and hatchlings could thermoregulate behaviorally when heat from the sun was available. Food [neonatal rats (*Rattus norvegicus* and *Mus musculus*) and Chinese weatherfish (*Misgurnus anguillicaudatus*)] was provided in an irregular way to simulate natural variation in food availability (Fig. 1), and water was always available. The hatchlings were measured (SVL and TL) and weighed at 60-day intervals to obtain growth data in the first 240-day period.

Statistical analyses

All statistical analyses were performed with the Statistica software package (version 5.0 for PC). Data on hatchlings of the same sex were blocked by clutch to avoid pseudo-replication. Prior to analyses, data were tested for normality using the Kolmogorov–Smirnov test and for homogeneity of variances using the Bartlett’s test. We used linear regression analysis, one-way ANOVA, one-way analysis of covariance (ANCOVA) (to correct for female SVL and test for homogeneity of slopes), repeated-measures ANOVA and Tukey’s post hoc tests to analyze the corresponding data. All values are presented as mean ± SE, and the significance level is set at $\alpha = 0.05$.

Results

The saline-injected, sham-ablated and control females in the latter experiment did not differ from each other in any examined trait [ANOVA (for SVL) or ANCOVA (for the other traits); all $P > 0.05$]. We therefore pooled data for these females and considered them together as controls. Twenty-four FSH-injected, 20 two-follicle ablated, 23 four-follicle ablated, 11 six-follicle ablated and 42 control females laid clutches between 8 and 25 July. Females of different treatments did not differ from each other in SVL (ANOVA; $F_{4,115} = 1.61$, $P = 0.175$). Clutch size, clutch mass, clutch mean egg mass (hereafter “egg mass”) and post-oviposition body mass were all positively correlated with female SVL (all $P < 0.05$). ANCOVAs with SVL as the covariate revealed that females of different treatments differed in clutch size (elevation, $F_{4,114} = 57.55$, $P < 0.0001$; slope, $F_{4,110} = 0.80$, $P = 0.528$), egg mass (elevation, $F_{4,114} = 42.89$, $P < 0.0001$; slope, $F_{4,110} = 0.76$, $P = 0.552$) and clutch mass (elevation, $F_{4,114} = 3.30$, $P = 0.013$; slope, $F_{4,110} = 1.01$, $P = 0.407$) but not in post-oviposition mass (elevation, $F_{4,114} = 1.86$, $P = 0.123$; slope, $F_{4,110} = 1.16$, $P = 0.332$).

Figure 2 shows adjusted means (+SE) for clutch size, egg mass and clutch mass of females of which body sizes were controlled at 106 cm SVL (the overall mean SVL of females). The females receiving exogenous FSH produced more but smaller eggs than the control females, whereas the females undergoing follicle ablation produced fewer but larger eggs than the control females. The FSH-injected females and the females undergoing two-follicle ablation did not differ from the control females in clutch mass, whereas the females undergoing four- and six-follicle ablation apparently reduced their total reproductive investment and therefore clutch mass.

The function (the solid curve) depicting the relationship between egg size and egg number reveals that egg size non-linearly increases with decreasing clutch size, with the tangent slope of the function for the six-follicle ablation treatment being -0.09 (Fig. 3). The function (the dashed curve) depicting instantaneous variation in tangent slopes has a single inflexion where the \log_e -transformed value of clutch size (C_1) is 2.75 (i.e., 15.6 eggs) for the female of which SVL is set at 106 cm (Fig. 3). Within the range of clutch sizes involved, egg size increases at decreasing rates at the change-over from C_1 to C_{1-i} , but at increasing rates at the change-over from C_{1+i} to C_1 (Fig. 3). Taken together, these two functions or curves show that: (1) there is a non-linear continuum of egg size-number trade-offs in *N. atra* within which there is a single point where the rate at which egg size decreases with increasing clutch size, or clutch size increases with decreasing egg size, is maximized; and (2) there is a fixed upper limit to egg size for a given-sized female, and the limit is not determined by her body volume.

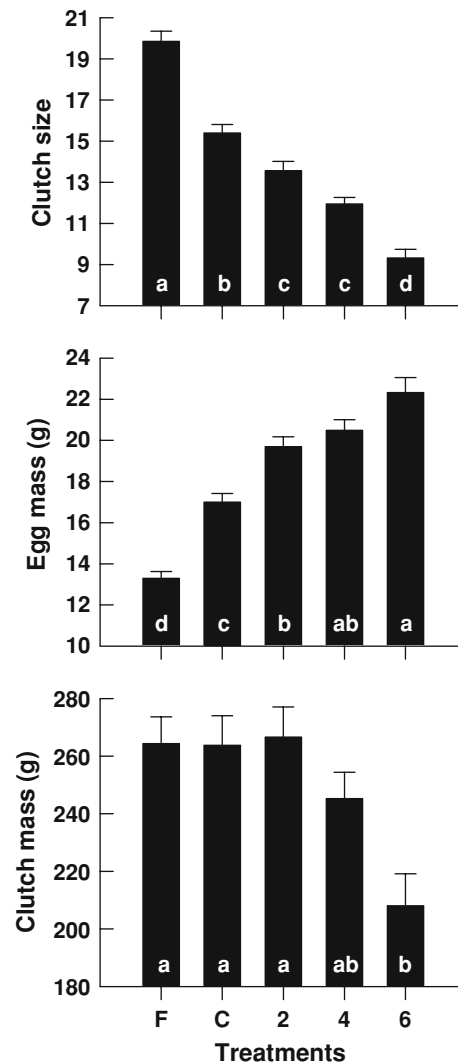


Fig. 2 Adjusted means (+SE) for clutch size, egg mass and clutch mass of females undergoing different treatments, with female snout-vent length (SVL) set at 106 cm. Adjusted means with different letters differ significantly (Tukey's post hoc test, $\alpha = 0.05$; $a > b > c > d$). F Follicle-stimulating hormone-injected females ($n = 24$), C control females ($n = 42$), 2 two-follicle ablated females ($n = 20$), 4 four-follicle ablated females ($n = 23$), 6 six-follicle ablated females ($n = 11$)

The control females did not differ from the wild-caught females in clutch size, egg mass and post-oviposition body mass (ANCOVA; all $P > 0.104$). Data pooled for these females showed that clutch size ($F_{1,60} = 68.66$, $P < 0.0001$) and egg mass ($F_{1,60} = 11.84$, $P < 0.002$) were both positively correlated with female SVL (Fig. 4).

A total of 48 hatchlings (21 males and 27 females) were produced in the AAPS room (Table 1). Female hatchlings were more likely to die in early post-hatching days (Table 1). Of the ten dead hatchlings, three (females) died in the first 60-day period, six (females) died during the period of day 60–120, and one (male) died at 226 days of age. Nine of them died in competition with others for food,

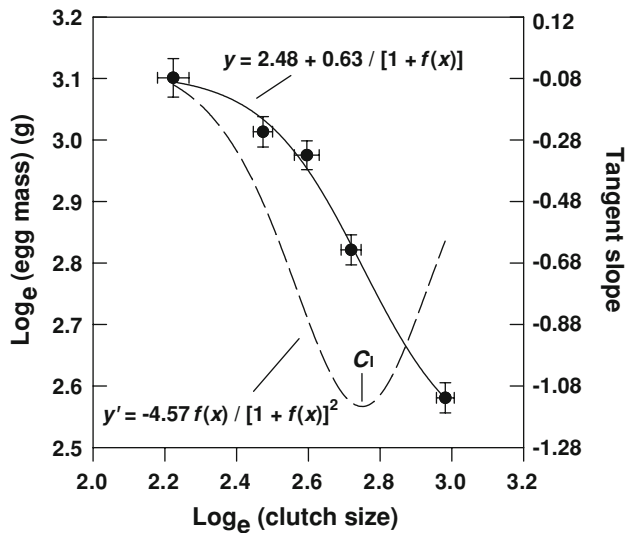


Fig. 3 The relationship between egg size and egg number. Data are expressed as mean \pm SE. The *solid curve* is generated from a fit of the standard curve on size-specific \log_e -transformed data of egg size and clutch size, with female SVL set at 106 cm. The *dashed curve* describes instantaneous variation in tangent slopes of the *solid curve*. $f(x) = 10^{3.15(x-2.75)}$. C_1 Clutch size at the inflexion

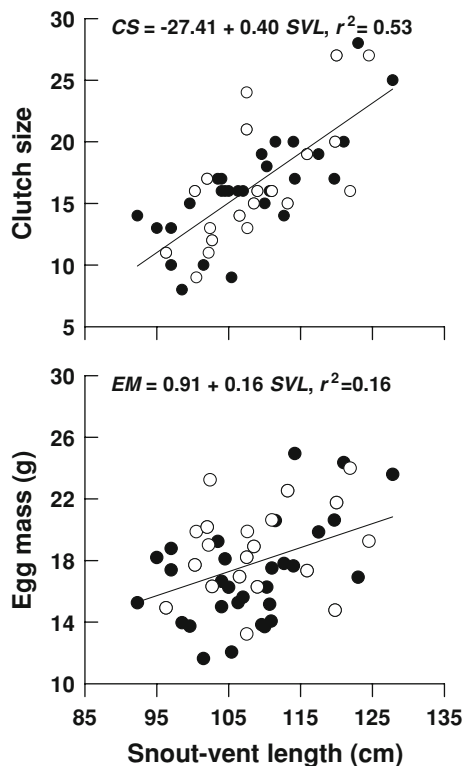


Fig. 4 Linear regressions of clutch size and egg mass on female SVL. *Solid dots* represent clutches prepared in captivity, and *open dots* represent clutches prepared in the wild. The regression equations and coefficients are given in the figure

and one (female) died from some unknown cause. Male and female hatchlings (ANOVA; $F_{1,46} = 0.99$, $P = 0.326$) did not differ in incubation length, nor did dead and live

Table 1 Descriptive statistics for egg mass at oviposition, incubation length and hatchling size at hatching

	Males		Females	
	Live	Dead ^a	Live	Dead ^a
<i>n</i>	20	1	18	9
Egg mass (g)	19.6 \pm 0.5 16.2–23.7	19.3	19.6 \pm 0.6 14.8–24.8	18.6 \pm 0.7 16.3–21.9
Incubation length (days)	60.2 \pm 1.0 54–68	67	58.4 \pm 1.2 53–69	60.1 \pm 2.1 53–69
Body mass (g)	15.2 \pm 0.4 12.6–18.0	15.3	14.9 \pm 0.5 10.8–18.4	14.2 \pm 0.5 12.3–17.0
Snout-vent length (mm)	282.4 \pm 1.9 266–300	278	277.3 \pm 2.9 243–294	273.8 \pm 4.2 247–284

Eggs were laid by wild-caught females and incubated at temperatures varying from 26 to 30°C

^a Of the ten dead hatchlings, three females died in the first 60-day period, six females died during the period of day 60–120, and one male died at 226 days of age

hatchlings (ANOVA; $F_{1,46} = 0.64$, $P = 0.428$). Mean values for body size at hatching (ANOVA; mass, $F_{1,46} = 0.87$, $P = 0.356$; SVL, $F_{1,46} = 3.67$, $P = 0.061$) and egg mass at oviposition (ANOVA; $F_{1,46} = 0.22$, $P = 0.642$) did not differ between male and female hatchlings, nor between dead and live hatchlings (ANOVA; mass, $F_{1,46} = 1.37$, $P = 0.248$; SVL, $F_{1,46} = 2.20$, $P = 0.145$; egg mass, $F_{1,46} = 1.27$, $P = 0.265$). An ANCOVA with SVL as the covariate revealed that, at hatching, dead and live hatchlings did not differ in body mass (elevation, $F_{1,45} = 0.006$, $P = 0.941$; slope, $F_{1,44} = 0.59$, $P = 0.448$) and therefore body condition.

Post-hatching growth was evident in both sexes (Fig. 5), with males growing more rapidly than did females (Table 2). Mass gain in the first 240-day period was unrelated to body size at hatching or egg mass in both sexes (all $P > 0.355$).

Discussion

Our manipulation of clutch size had the desired effect of inducing variation in egg size: females receiving exogenous FSH produced more but smaller eggs, and females undergoing follicle ablation produced fewer but larger eggs (Fig. 2). These results together with the observation that the control females resembled the wild-caught females in clutch information suggest that *N. atra* is among reptilian species where females may adjust offspring size according to facultative fluctuations in clutch or litter size (Sinervo and Licht 1991a, Sinervo and Licht 1991b; Olsson et al. 2002; Ji et al. 2006). Post-oviposition body mass did not differ among treatments, presumably because females did not

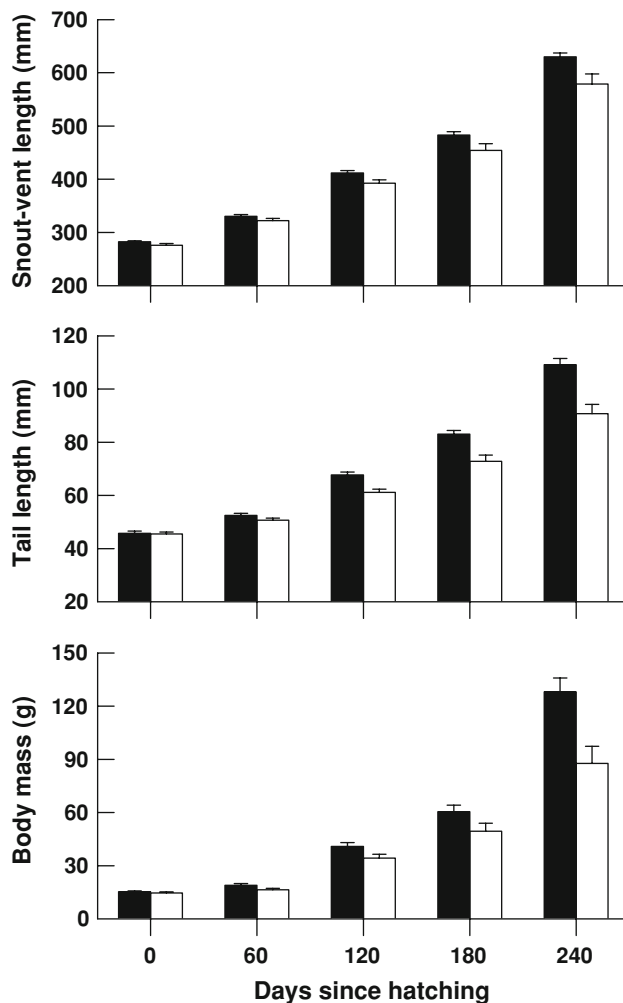


Fig. 5 Mean values (+SE) for hatchling growth in the first 240-day period. *Solid bars* represent males, and *open bars* represent females. Comparisons at each time step are provided in Table 2

accumulate energy but diverted most of their current surplus energy to production of eggs during the breeding season, or because they were not energy limited and ate in sufficient quantity to maximize energy stores and production of eggs.

There are three interesting findings in this study: (1) there is a nonlinear continuum of egg size-number trade-offs in *N. atra* (Fig. 3); (2) hatchling survival and growth, two highly fitness-related traits, are both unrelated to egg size in the species; and (3) female hatchlings are more likely to die in early post-hatching days and grow more slowly than male hatchlings (Fig. 5). The first two findings provide the following three inferences regarding egg size-number strategies in *N. atra*.

Firstly, there is a fixed upper limit to egg size for a given-sized female. The upper limit to reproductive investment (or reproductive output) is set by the body volume in the Zhoushan population (Ji and Wang 2005). With the six-follicle ablation, the mean egg mass increased from 17.0 to 22.3 g but the mean clutch mass dropped from 263.8 to 208.0 g for females of which body sizes were controlled at 106 cm SVL. This suggests that females producing the largest eggs do not completely fill the available body volume and that the potential constraints of body volume on total reproductive investment as well as egg size can be mitigated in females producing smaller clutches. The captive females had free access to food and, as such, both clutch mass and egg mass were unlikely to be constrained by food availability. Therefore, the result that six-follicle ablated females did not produce even larger eggs to fill the available body volume suggests that, as in *E. carinata* (Ji et al. 2006), there is a fixed upper limit to egg size for a given-sized female in *N. atra*, and the limit is not determined by her body volume. The mean egg mass in the six-follicle ablation treatment is very close to this limit, because the tangent slope (-0.09) of the function depicting the relationship between egg size and number for this treatment is nearly equal to zero at which eggs are theoretically maximized for size (Fig. 3).

Secondly, within the continuum there is an inflexion where the rate at which egg size decreases with increasing clutch size, or clutch size increases with decreasing egg size, is maximized (Fig. 3). As hatchling survival and growth were both unrelated to body size (SVL and body mass) at hatching or egg mass at oviposition, egg size has

Table 2 Results of repeated-measures ANOVA for snout-vent length, tail length and body mass of hatchling *Naja atra*, with age as the within-subject factor and sex as the between-subject factor

	Sex	Age	Interaction
Snout-vent length	$F_{1,23} = 8.38, P < 0.009$ F < M	$F_{4,92} = 561.25, P < 0.0001$ D_0 e, D_{60} d, D_{120} c, D_{180} b, D_{240} a	$F_{4,92} = 2.85, P = 0.028$
Tail length	$F_{1,23} = 22.47, P < 0.0001$ F < M	$F_{4,92} = 394.45, P < 0.0001$ D_0 e, D_{60} d, D_{120} c, D_{180} b, D_{240} a	$F_{4,92} = 11.03, P < 0.0001$
Body mass	$F_{1,23} = 9.73, P < 0.005$ F < M	$F_{4,92} = 177.36, P < 0.0001$ D_0 d, D_{60} d, D_{120} c, D_{180} b, D_{240} a	$F_{4,92} = 8.12, P < 0.0001$

Means with *different letters* differ significantly (Tukey's post hoc test, $\alpha = 0.05$; a > b > c > d > e). F Females, M males, D_0 0 days of age, D_{60} 60 days of age, D_{120} 120 days of age, D_{180} 180 days of age, D_{240} 240 days of age

therefore no role in determining hatchling survival and growth. Considering that maternal fitness depends on the number of surviving young, we conclude that clutch size rather than egg size plays a key role in determining maternal fitness in *N. atra*. The potential gain of maternal fitness due to the decreased egg size becomes increasingly more pronounced in females producing clutches smaller than C_1 because egg size decreases at increasing rates when females shift clutch size from C_{1-i} to C_1 . On the contrary, the potential gain of maternal fitness due to the decreased egg size becomes increasingly less pronounced in females producing clutches larger than C_1 because egg size decreases at decreasing rates when females shift clutch size from C_1 to C_{1+i} (Fig. 3). Reproduction involves benefits and costs, whereby increased expenditure into current reproduction can reduce future reproductive success. Therefore, the pattern of egg size-number trade-offs presumably suggests that maximization of reproductive benefits could be achieved in female *N. atra* by shifting clutch size towards a convergent point (the inflexion per se) where maternal fitness and costs associated with current reproduction are well balanced.

Thirdly, C_1 is much closer to the mean clutch size recorded in wild-caught females of the same size (106 cm SVL). Females collected in July 2003 did not differ from those preparing clutches in nature during 1998–1999 ($n = 34$; Ji and Wang 2005) in clutch size (ANCOVA; $F_{1,51} = 0.03$, $P = 0.874$). Pooling data for these females, we develop a linear regression model ($CS = -24.04 + 0.37 SVL$) that predicts a clutch size of 15.2 eggs from 106 cm SVL, which is surprisingly consistent with C_1 (15.6 eggs). This consistency of clutch size suggests that females in the wild tend to shift clutch size towards a size-specific convergent point by producing an intermediate number of eggs.

We are currently unable to explain why females are more likely to die in early post-hatching days and grow more slowly than males. However, the lack of differences in the mean initial body or egg mass between dead and alive hatchlings and between male and female hatchlings provides further evidence that egg-size variation has no role in influencing hatchling survival and growth in *N. atra*.

In summary, our data show that follicle ablation may result in enlargement of egg size and that exogenous provision of FSH may result in a decrement of egg size in *N. atra*, and confirm that eggs of this species may vary as a result of facultative fluctuations in clutch size (Ji and Wang 2005). There is a fixed upper limit to egg size for a given-sized female *N. atra*, and the limit is not determined by her body volume. Egg size has no role in determining hatchling survival and growth and, in a given reproductive episode, the extent to which females may enjoy reproductive benefits depends on how well egg size and egg number are balanced.

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