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Follicle ablation increases offspring size in a lizard with a low clutch frequency

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Abstract. Studies of lizards and snakes have shown that an experimental reduction of offspring number sometimes, but not always, results in an increase in offspring size. We applied the "follicle ablation" technique to an oviparous lacertid lizard (*Eremias argus*) to test the hypothesis that offspring size can be easily altered by manipulating clutch size in species with a low clutch frequency. Our manipulation of clutch size had the effect of inducing variation in egg size in the first post-surgical clutch, with follicle-ablated females producing fewer larger eggs than did controls. Follicle-ablated females produced a second post-surgical clutch as normally as did controls. The proportional amount of resources allocated to reproduction did not shift seasonally in *E. argus*, but females normally switched from producing a larger number of smaller eggs early in the breeding season to a smaller number of larger eggs later in the season. Females used in this study never produce more than two clutches per breeding season. Therefore, our data validate the hypothesis tested. Our data also provide an inference that maximization of reproductive success could be achieved in females of *E. argus* by diverting a larger enough, rather than an extraordinarily high, fraction of the available energy to individual offspring in single reproductive episodes.

Introduction

Maternal fitness depends on offspring size and offspring number that are mutually constrained because of the limited total reproductive investment. The observed relationship between these two life-history variables is the net outcome of complex interactions between numerous factors such as maternal size, food availability, phylogenetic effects, or local environmental factors affecting offspring size and fecundity (Rohr, 2001). Studies on a wide range of animal taxa have shown a positive correlation between offspring size and fitness, often because larger babies 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). However, even in species with strong evidence in support of such a correlation, there is an upper limit to the size of individual offspring, primarily because the selective advantage accrued by increasing effort per offspring can be counterbalanced by the decreased number of offspring produced (Stearns, 1992; Bernardo, 1996; Einum and Flemming, 2000; Agrawal et al., 2001; Roff, 2002). Theoretically, maximization of reproductive success should be achieved in females by producing the greatest number of surviving (not largest) young.

Studies of lizards and snakes by experimental manipulation of clutch size have shown that offspring size may sometimes, but not always, vary in response to variation in offspring number. For example, females undergoing follicle ablation produce fewer larger offspring in the side-blotched lizard *Uta stansburiana* (Sinervo and Licht, 1991a), the common lizard *Lacerta* (*Zootoca*) *vivipara* (Olsson et al., 2002), the king ratsnake *Elaphe carinata* (Ji et al., 2006) and the Chinese cobra *Naja atra* (Ji et al., 2009), because yolk that is normally allocated to a larger clutch, is subsequently channelled into the fewer, remaining follicles. In the northern grass lizard *Takydromus septentrionalis*, however, an experimental reduction of clutch size does not result in enlargement of offspring size, primarily because a given sized female tends to divert a fixed fraction of the available energy to individual offspring in single reproductive bouts

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(Du et al., 2005; Ji and Diong, 2006; Ji et al., 2007). Interestingly, females reproduce more frequently per breeding season in *T. septentrionalis* (up to seven clutches; Ji et al., 2007) than in *U. stansburiana* (up to four clutches; Sinervo and DeNardo, 1996), *L. vivipara* (single clutch for viviparous females; Bauwens and Verheyen, 1987), *E. carinata* (single clutch; Ji et al., 2000) and *N. atra* (single clutch; Ji et al., 2000). This presumably suggests a key difference in egg regulation between *T. septentrionalis* and the other four species and, more importantly, raises a question of whether offspring size is more likely to vary in response to variation in offspring number in species with fewer opportunities to produce an additional clutch per breeding season. To answer this question, one needs to test the hypothesis that offspring size can be easily altered by manipulating clutch size in species with a low clutch frequency.

Here, we describe a study applying the "follicle ablation" technique to the Mongolian racerunner *Eremias argus* to test the hypothesis. This small-sized (up to 70 mm snout-vent length, SVL), oviparous lacertid lizard ranges from the northern parts of China (southward to Jiangsu and westward to Qinghai) to Russia (region of Lake Baikal), Mongolia and Korea (Zhao, 1999). We used lizards from a population in Qinghai (Northwest China) as the model system for two reasons: (1) females never produce more than two clutches per breeding season; and (2) females consistently lay eggs under laboratory conditions (Liu, 2006).

Materials and methods

Animal collection and care

Adult lizards (>51 mm SVL) were collected by noose or by hand in early May 2006 from a population in Gonghe (36°03′N, 101°13′E; altitude ~2300 m ASL), Qinghai. Lizards were transported to our laboratory in Hangzhou, where they were sexed and marked by painting. Between 10 and 12 lizards, 5-6 of each sex, were housed together in each 900 mm × 650 mm × 600 mm (length × width × height) communal cage, in an indoor animal holding facility. Communal cages contained a substrate of sand (~50 mm depth), with rocks and pieces of clay tiles provided as shelter and basking sites. Thermoregulatory opportunities were provided during daylight hours by a 100-W incandescent lamp suspended over one end of the cage; overnight temperatures followed indoor ambient temperatures (20-28°C). Food [mealworms (larvae of *Tenebrio molitor*) and house crickets (*Achetus domesticus*)] dusted with multivitamins and minerals and water were provided daily, so that excess food was always available in the communal cages. Females with shelled oviductal eggs were removed from the communal cages, and housed individually in 200 mm × 150 mm × 200 mm egg-laying cages with 4 cm depth moist soil and a 20-W spotlight mounted in each cage to allow thermoregulation.

Eggs were collected, measured (to the nearest 0.1 mm) for length and width with a Mitutoyo digital caliper and weighed (to the nearest 1 mg) on a Mettler balance within 2 hours after oviposition, thereby minimizing any uncertainty about the egg mass due to loss or gain of water (Hao et al., 2006). Post-oviposition females were measured for SVL and weighed before they were returned to the communal cages, where they remained until they again carried shelled oviductal eggs, at which time they were once again transferred to the egg-laying cages.

Follicle ablation

We palpated females between 12-27 May to determine their reproductive conditions, and assigned a total of 60 females at early stages of vitellogenesis to follicle-ablated ($N = 30$), sham-ablated ($N = 15$) and control ($N = 15$) groups. Prior to surgery, we anaesthetized females with ether. We taped the anaesthetized female to a sterile board and prepared for aseptic surgery by cleaning the abdominal area with 75% alcohol. Thereafter, a 5-7 mm incision was made 2-3 mm to the left of the mid-vent line with a sterile scalpel. We lifted the left-sided ovary of the follicle-ablated female out of the incision with a pair of forceps, counted and measured all yolking follicles, and then ablated them with sterile syringes. Between one and two yolking follicles, measuring 2.5-4.0 mm diameter, were ablated. The incision was closed using a surgical suture and cleaned daily with 75% alcohol. After a 5-day recovery period, females were moved back into the communal cages. Females in the sham-ablated group underwent the same protocol without any follicle ablation.

Data analysis

Females producing abnormal clutches with various numbers of eggs containing condensed yolk or infertile eggs were excluded from analyses. Prior to using parametric tests, all data were tested for homogeneity of variances using the Bartlett's test, and for normality using the Kolmogorov-Smirnov test. We used linear regression analysis, one- and two-way ANOVA and one- and two-way ANCOVA to analyze the corresponding data suitable for parametric tests. Prior to testing differences in adjusted means, the homogeneity of slopes was checked. We used the G-test to examine whether females of different treatments differed in the number of individuals producing a second clutch. Values are presented as mean ± standard error, and the significance level is set at $\alpha = 0.05$.

Results

Females producing abnormal clutches were found in each treatment. Twenty-seven follicle-ablated females produced a post-surgical clutch (their first clutch since start of laying) between 27 May and 24 June, and 14 of them produced a second clutch between 14 June and 3 July. Fourteen sham-ablated females produced a post-surgical clutch between 24 May and 14 June, and 7 of them produced a second clutch between 18 June and 5 July. Thirteen control females produced a first clutch between 23 May and 14 June, and 5 of them produced a second clutch between 18-29 June. Follicle-ablated, sham-ablated and control females did not differ from each other in the number of individuals producing a second clutch ($G = 0.25$, $df = 2$, $P > 0.850$).

Sham-ablated and control females did not differ in any reproductive trait listed in table 1 [one-way ANCOVA with SVL as the covariate (for post-oviposition body mass and clutch mass) or one-way ANOVA (for the other traits); all $P > 0.144$]. We therefore pooled data for these females and considered them together as controls (table 1). The mean clutch mass was greater in control than in follicle-ablated females producing the first clutch (one-way ANCOVA with SVL as the covariate; $F_{1,76} = 32.15$, $P < 0.0001$), but it did not differ between controls producing the first clutch and females producing the second clutch (one-way ANCOVA; $F_{1,50} = 1.18$, $P = 0.283$).

Two-way ANOVA revealed that follicle-ablated and control females did not differ in SVL ($F_{1,76} = 0.03$, $P = 0.870$), nor did females producing the first and second clutches differ in the trait ($F_{1,76} = 0.34$, $P = 0.562$). Two-way ANCOVA with SVL as the covariate revealed that follicle-ablated and control females did not differ in post-oviposition body mass ($F_{1,75} = 0.86$, $P = 0.357$), nor did females producing the first and second clutches differ in the trait ($F_{1,75} = 0.76$, $P = 0.387$). The interaction between treatment (follicle-ablated females versus controls) and clutch order (first versus second clutches) was not a significant source of variation in these two traits (both $P > 0.417$).

Follicle-ablated females produced fewer but larger eggs in the first clutch than did control females (one-way ANOVA; clutch size-$F_{1,52} = 67.87$, $P < 0.0001$; egg mass-$F_{1,52} = 15.73$, $P < 0.0003$). Their original clutch sizes (3.9 ± 0.1, range = 3-5), after adding the ablated yolking follicles, did not differ from those of controls (one-way ANOVA; $F_{1,52} = 1.05$, $P = 0.311$). Second post-surgical clutches (the sec-

Table 1. Descriptive statistics for reproductive traits of follicle-ablated and control females of *Eremias argus*. Data are expressed as mean ± standard error and range.

	Follicle-ablated females		Control females	
	First clutch	Second clutch	First clutch	Second clutch
N	27	14	27	12
Snout-vent length (mm)	58.5 ± 0.5	59.0 ± 0.7	58.5 ± 0.5	58.8 ± 0.6
	51.8-63.8	52.9-63.1	51.8-62.4	54.4-62.3
Post-oviposition body mass (g)	3.7 ± 0.1	3.8 ± 0.1	3.7 ± 0.1	3.6 ± 0.1
	2.9-4.7	3.2-4.9	2.4-5.7	2.9-4.7
Clutch size	2.2 ± 0.1	2.9 ± 0.1	3.7 ± 0.1	3.1 ± 0.2
	1-3	2-3	2-5	2-4
Clutch mass (g)	1.14 ± 0.07	1.52 ± 0.08	1.62 ± 0.06	1.58 ± 0.09
	0.48-1.92	0.94-2.04	0.95-2.43	1.01-2.08
Egg mass (g)	0.51 ± 0.02	0.53 ± 0.02	0.44 ± 0.01	0.52 ± 0.02
	0.37-0.67	0.43-0.68	0.36-0.53	0.44-0.60
Egg length (mm)	14.9 ± 0.3	14.7 ± 0.2	13.5 ± 0.2	14.7 ± 0.4
	12.4-19.3	13.5-16.5	12.1-15.1	13.0-18.2
Egg width (mm)	8.2 ± 0.1	8.2 ± 0.1	7.7 ± 0.1	8.3 ± 0.1
	7.2-9.4	7.7-8.9	6.6-9.0	7.9-8.9

ond clutch since start of laying) from follicle-ablated females did not differ from those of controls (one-way ANOVA; clutch size-$F_{1,24} = 1.20$, $P = 0.285$; clutch mass-$F_{1,24} = 0.20$, $P = 0.660$; egg mass-$F_{1,24} = 0.50$, $P = 0.488$).

Discussion

Our manipulation of clutch size via follicle ablation had the effect of inducing variation in egg size in the first but not the second post-surgical clutch. Follicle-ablated females produced a second clutch as normally as did control ones, suggesting that follicle ablation did not influence clutches subsequent to the post-surgical one. Consistent with the results reported for *U. stansburiana* (Sinervo and Licht, 1991a), *L. vivipara* (Olsson et al., 2002), *E. carinata* (Ji et al., 2006) and *N. atra* (Ji et al., 2009), females of *E. argus* undergoing follicle ablation produced larger eggs. Therefore, our data support the hypothesis that offspring size can be easily altered by manipulating clutch size in species with a relatively low clutch frequency.

Control females producing the first clutch did not differ from those producing the second clutch in clutch mass. This result, together with the observation that females producing the first and second clutches did not differ in post-oviposition body mass, provides an inference that the proportional amount of resources allocated to reproduction does not shift seasonally in *E. argus* under laboratory conditions. However, females did switch from producing a larger number of smaller eggs early in the breeding season to a smaller number of larger eggs later in the season (table 1). Seasonal shifts in clutch characteristics (clutch size, clutch mass, or egg mass) has been reported for several lizard species (e.g. Ferguson and Snell, 1986; James and Whitford, 1994; Ji and Braña, 2000; Ji et al., 2007). Dividing accessible resources into smaller amounts for each egg allows females to increase the number of eggs produced, and hatchlings from eggs produced early in the breeding season have a longer growth period prior to the onset of the first winter. Therefore, laying more but smaller eggs early in the breeding season could be a reproductive tactic in *E. argus* that improves female reproductive success.

In squamate reptiles, a female's somatic condition (indicative of energy reserves) determines the number of oocytes to be incorporated in the clutch, and the energy available for vitellogenesis until the time of ovulation determines final egg size (Ballinger, 1983; Doughty and Shine, 1997; Ji and Wang, 2005). In our study, females were maintained under the laboratory conditions without any food limitation and, thus, egg size was unlikely to be constrained by energy availability. Moreover, total reproductive allocation was also unlikely to be constrained by the amount of space available to hold eggs within a follicle-ablated female's abdomen because of the reduced clutch mass and therefore clutch volume (table 1).

So, why did females undergoing follicle ablation not produce as large eggs as they could to fill the available abdomen space to a large extent? One plausible explanation is that, as in other small-bodied lizards and turtles (Congdon and Gibbons, 1987; Long and Rose, 1989; Sinervo and Licht, 1991b; Kratochvil and Frynta, 2006), the maximum egg size is constrained by the pelvic girdle dimension in *E. argus*. Compared to egg length, egg width is more likely to be constrained by the pelvic girdle dimension in groups like lizards and turtles (Rollinson and Brooks, 2008). In our study, follicle-ablated and control females producing the first clutch and those producing the second clutch did not differ from each other in the ratio of egg width over egg length (one-way ANOVA; $F_{2,77} = 2.34$, $P = 0.103$). This presumably suggests that, inconsistent with the results reported for *U. stansburiana* (Sinervo and Licht, 1991b) and *E. carinata* (Ji et al., 2006), females of *E. argus* cannot increase egg mass by means of an increase in egg length when egg width is limited. Another plausible explanation is that, as in *T. septentri-*

onalis (Ji and Diong, 2006), *E. carinata* (Ji et al., 2006) and *N. atra* (Ji et al., 2009), there is a space-independent upper limit to reproductive investment per offspring within each particular clutch in *E. argus*. As high expenditure in current reproduction can reduce "residual reproductive value" by decreasing survival, growth, or the amount of energy available for future reproduction (Schwarzkopf, 1994), we may therefore anticipate that maximization of reproductive success could be achieved in *E. argus* by diverting a large enough, rather than an extraordinarily high, fraction of the available energy to individual offspring in single reproductive episodes.

In summary, our data show that follicle ablation results in enlargement of egg size (and thus, hatchling size; Hao et al., 2006) in *E. argus*, and validate the hypothesis that offspring size can be easily altered by manipulating clutch size in lizards with a low clutch frequency. Females of *E. argus* normally switch from producing a larger number of smaller eggs early in the breeding season to a smaller number of larger eggs later in the season. Females do not produce as large eggs as they could to fill the available abdomen space when undergoing follicle ablation.

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