



**Proximate Constraints on the Evolution of Egg Size, Number, and Total Clutch Mass in Lizards**

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- J. Bacteriol.* **153**, 163 (1983)] with a reporter library and plated on minimal medium [F. Sherman, G. R. Fink, J. B. Hicks, *Laboratory Course Manual for Methods in Yeast Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986)] lacking uracil and tryptophan at a density of ~300 yeast per 10-cm dish. After 3 days of growth at 30°C, the yeast were replica-plated to medium lacking histidine (containing both uracil and tryptophan). After 6 days of growth, the yeast were replica-plated to medium lacking histidine and with or without 1% 5-FOA (PCR, Gainesville, FL). Colonies that grew on 5-FOA-lacking but not 5-FOA-containing plates were picked and the plasmids recovered into bacteria for analysis [C. S. Hofman and F. Winston, *Gene* **57**, 267 (1987)].
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  26. The Pro36-Luc vector contains the firefly luciferase coding region downstream of a minimal prolactin promoter [S. Adler, M. L. Waterman, X. He, M. G. Rosenfeld, *Cell* **52**, 685 (1988)]. The B1 fragment and the B1a oligonucleotide were inserted into the Bam HI site upstream of the promoter. Plasmid p(B1a)<sup>8</sup>-Luc contains eight copies of the B1a oligonucleotide repeated in tandem. The mammalian expression plasmid pCMV has a polylinker downstream of the cytomegalovirus (CMV) promoter. We made pCMV-(NGFI-B) by cutting an NGFI-B cDNA-containing Bluescript (Stratagene) plasmid with Bam HI (which cuts in the polylinker just upstream of the NGFI-B initiation codon) and Mlu I (which cuts the NGFI-B cDNA at nucleotide 1918, just downstream of the termination codon) and then by ligating the product to pCMV cut with Bgl II and Mlu I. We made pCMV-(NGFI-Bt) by cutting a second NGFI-B cDNA-containing Bluescript clone with Aat II [which cuts the NGFI-B cDNA at nucleotide 1353 (amino acid 380, ~80 residues downstream of the zinc fingers)], fusing the product to the downstream Bam HI site in the polylinker, and adding a translation terminator at the Xba I site further downstream. The product was excised from Bluescript with Not I and Nco I (nucleotide 211 of the NGFI-B cDNA), Hind III linkers were added, and the fragment was ligated into the Hind III site of pCMV.
  27. COS-7 cells were grown in Dulbecco's modified Eagles's medium supplemented with 10% fetal bovine serum. Cells were plated 24 hours before transfection at a density of 700,000 cells per 10-cm dish. The cells were transfected by calcium phosphate precipitation [C. Chen and H. Okayama, *Mol. Cell. Biol.* **7**, 2745 (1987)] with a total of 10 µg of plasmid DNA per plate (5 µg of activator plasmid, 5 µg of reporter plasmid). Three days after transfection, the cells were washed twice with ice-cold phosphate-buffered saline and lysed by incubation in 0.7 ml of 50 mM Tris-MES (pH 7.8), 1 mM dithiothreitol, and 1% Triton X-100 for 5 min at room temperature. The lysate was cleared of cellular debris by centrifugation. Luciferase assays were performed on 50 µl of cell lysate as described [J. R. de Wet, K. V. Wood, M. DeLuca, D. R. Helinski, S. Subrami, *Mol. Cell. Biol.* **7**, 725 (1987)] with a Monolight 2010 luminometer (Analytical Bioluminescence Laboratory, San Diego, CA). All values represent the results of at least two different experiments, each performed with different preparation of DNA.
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  30. To screen one mammalian genome equivalent of DNA ( $3 \times 10^9$  bp) for target genes, one would need to plate at least  $6 \times 10^6$  reporter-containing yeast colonies with a modal insert size of 500 bp, a prohibitive number. Yeast ( $\sim 10^7$  bp) and *Drosophila* ( $\sim 10^8$  bp) genomes could be screened completely with  $\sim 10^4$  and  $\sim 10^5$  clones, respectively.
  31. The probability ( $P$ ) of obtaining the observed or a larger number of 7-bp GCN4 sites from each library was calculated with the Poisson distribution,  $P(x) = e^{-m}(m^x/x!)$ ; where  $x$  is the number of sites obtained,  $n$  is the total number of UAS nucleotides selected, and  $m = 2n/4^7$ .
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  33. The GCN4 expression plasmid p164 was obtained from A. Hinnebusch, plasmid p1027 from R. Brent, the Pro36-Luc vector from S. Adler, and the pCMV expression plasmid from M. Roth. We thank T. Ley, S. Adler, and J. Majors for critical reading of the manuscript. Supported by PHS grants NS01018 from the National Institute of Neurological and Communicative Diseases and Stroke and by PO1 CA49712 from the National Cancer Institute and by grant RG1779B2 from the Multiple Sclerosis Foundation.

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## Proximate Constraints on the Evolution of Egg Size, Number, and Total Clutch Mass in Lizards

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Proximate constraints on egg size, number, and total clutch mass in side-blotched lizards were examined by experimentally reducing average clutch size from 4.6 eggs to one, two, and three eggs. Eggs from experimentally altered clutches were larger than those from controls, reflecting the trade-off between egg size and number. Moreover, the increased frequency of females with oviducally bound eggs or eggs that burst at oviposition suggests that egg size in clutches with very few eggs are at a functional upper size limit. These proximate constraints may also limit evolution of egg size in another group of lizards (*Anolis*) that only produces one-egged clutches.

IMPLICIT IN CONSIDERATIONS OF many traits that covary consistently among phylogenetically related groups of organisms is that these traits are in some fashion constrained and this constraint reflects an underlying mechanistic trade-off among these traits. Indeed, the notion of a trade-off among suites of traits making up the organismal life history has become a paradigm of evolutionary biology (1). For example, the presumed trade-off between

clutch or litter size and offspring size is a fundamental aspect of life history theory (2) and is a pervasive pattern among vertebrates (3). Given functional and energetic limitations on total clutch or litter mass, it seems intuitively obvious that a female producing larger offspring must produce fewer offspring than a female producing a smaller offspring. Moreover, at one end of this continuum, a second type of constraint may impose further limits on adaptive evolution—the diameter of the pelvic girdle has been suggested to be a functional limit to the maximum offspring size that is possible

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at parturition or oviposition. For example, in primates these constraints seem to limit single births to those groups in which neonate head size does not exceed pelvic girdle dimensions (4). Similarly, limitations on adaptive evolution arising from the constraints of the pelvic girdle have been suggested in egg-laying vertebrates such as turtles (5). Despite the central importance of these issues to life history theory, the studies to date have been based on correlational analyses of related groups owing to the difficulty of experimentally manipulating these life history traits, and thus inferences concerning the causes of these patterns in any particular group are elusive (1, 6, 7). We present direct experimental evidence for the existence of these functional constraints in a vertebrate.

Lizards present an opportunity to investigate the causal basis of these constraints in reproduction because results from a comparative analysis can also be experimentally addressed by directly manipulating the ovary. We have developed a method that allows us to experimentally reduce the number of eggs that are ovulated and laid by side-blotched lizards, *Uta stansburiana* (8). *Uta stansburiana* has a reproductive pattern typical of most lizards in that it produces multi-egg clutches (9); this small lizard (average post-oviposition mass of females, 4.0 g) lays an average of 4.6 eggs but can lay up to nine eggs. The reproductive pattern of this species is apparently dichotomous with *Anolis* lizards that are phylogenetically conservative in that only one egg is produced in each clutch (9). Moreover, *Anolis* lizards have a much lower total clutch mass relative to their body size (RCM = total clutch mass/body mass) (9). We present results from one-, two-, and three-egg clutches produced by surgical manipulation and naturally laid clutches (two to nine eggs) from *U. stansburiana*. We also compare our experimentally altered clutches from a small iguanid lizard that lays moderately sized clutches with results from the phylogeneti-

**Table 1.** Frequency of burst eggs or oviducally bound eggs as a function of clutch size for all eggs ( $n = 2512$ ) obtained from clutches ( $n = 538$ ) from female *Uta stansburiana*. The frequency of burst eggs or oviducally bound eggs increases significantly with decreased clutch size (14).

Clutch size	Cases of burst or oviducally bound eggs	Eggs laid ( $n$ )	Burst or oviducally bound eggs (%)
1	4	11	36.4
2	11	152	7.2
3	20	324	6.2
4	19	640	3.0
5	28	780	3.6
6	15	426	3.5
7	2	105	1.9
8	0	56	0.0
9	0	18	0.0

cally conservative *Anolis* lizards. This comparison is facilitated by the close correspondence of body size among lizards from these two groups (9).

We obtained eggs laid in captivity by females from May through August 1990 (10). To produce one-, two-, and three-egg clutches we surgically exposed the ovaries of early- to mid-stage vitellogenic females and removed yolk from some of these follicles (yolkectomy) which effectively terminates their growth (8, 11). Females undergoing the yolkectomy surgery ( $n = 73$ ) typically produce larger eggs than control females; yolk normally allocated to a larger clutch is now distributed among the fewer yolking follicles (one to three) that were left intact. We compared clutches from sham-manipulated ( $n = 22$ ) females with those from unmanipulated females ( $n = 90$ ). All females underwent the major period of vitellogenesis in the laboratory. We also obtained a large sample of eggs ( $n = 352$ ) from females that were taken from nature with oviducal eggs (that is, all vitellogenesis had taken place in nature).

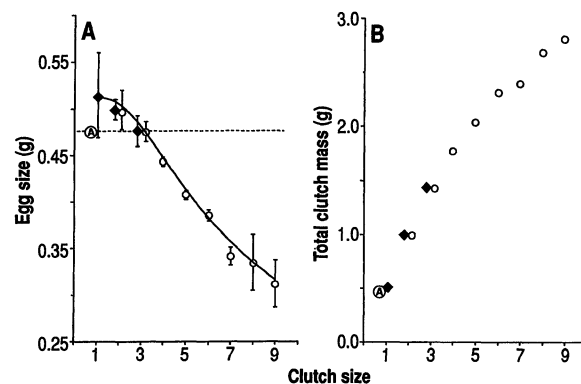
Of the large number of clutches ( $n = 464$ ) obtained from females that were unmanipu-

lated (either maintained in the laboratory or taken from females yolking eggs in nature) and sham-manipulated females, a small fraction laid two- (2.6%) and three-egg (13.8%) clutches naturally (none laid one-egg clutches) that we could compare directly with females laying experimentally altered clutches of one, two, and three eggs. For example, the size of eggs from clutches of three or fewer eggs produced by natural or experimental means was the same (Fig. 1A) (12). Thus, our surgical procedures had no apparent effect on the size of the eggs (13) except to make them larger than eggs from females laying more than three eggs in a fashion that reflects the egg size and egg number trade-off (8). The curve relating egg size and egg number is markedly nonlinear with a sigmoidal shape (Fig. 1A) suggesting that a functional ceiling in egg size has been reached in the one-, two-, and three-egg clutches. Indeed the number of eggs that became oviducally bound or eggs that burst at oviposition increased with decreased clutch size in *U. stansburiana* and this effect becomes most pronounced in clutches of three and fewer eggs (Table 1) (14, 15). It is likely that these post-ovulation proximate constraints on egg size are mediated by the morphological constraint of the pelvic girdle as has been suggested in other vertebrates (4, 5). Because of the sigmoidal shape of the curve relating egg size and clutch size (15), total clutch mass also decreases dramatically with clutch size, particularly in clutches with three and fewer eggs (Fig. 1B).

On the basis of our results for *U. stansburiana*, it is clear why there are no females laying one-egg clutches and why few lay two-egg clutches. Whereas instances of burst eggs only result in the death of the embryo, cases of oviducally bound eggs typically kill both the female along with any or all eggs that are oviducally bound. Each of these events reflects selection against a female with that reproductive pattern and would presumably also occur in natural populations. It is noteworthy that *Anolis* lizards (16) that exclusively lay one-egg clutches (Fig. 1A) lay eggs (0.48 g) that are slightly smaller than the egg size of *U. stansburiana* from one-, two-, and three-egg clutches—those clutch and egg sizes with which *U. stansburiana* experienced the greatest difficulty (Fig. 1A). Thus, our elucidation of the proximate constraints (17) on maximum egg size in *U. stansburiana* based on experimentally altered clutches suggest that the evolution of egg size in *Anolis* lizards may have been similarly constrained.

Our results indicate that egg size, egg number, total clutch mass, and relative clutch mass are physiologically coupled life history traits (18). Given the generality of

**Fig. 1.** (A) Relation between clutch size and egg size (mean  $\pm$  SE) for experimental ( $\blacklozenge$ ) and naturally laid eggs ( $\circ$ ) obtained from female *Uta stansburiana* (eggs from laboratory-maintained females that were sham-manipulated or unmanipulated and eggs from females taken from nature have been pooled). The effects of post-oviposition body size on egg size has been removed by ANCOVA of ln-transformed variables (15). Dashed horizontal line indicates the approximate threshold of egg sizes above which we observed a large increase in the frequency of burst or oviducally bound eggs (that is,  $\leq 3$  eggs or  $\geq 0.475$  g). (B) The relation between total clutch mass and clutch size calculated by multiplying egg size by clutch size. The egg size and total clutch mass for *Anolis* lizards that only produce one-egg clutches is provided ( $\circ$ ) for comparison (16).



the trade-off between egg size and egg number (3), and the relatively conserved aspects of hormonal regulation of reproduction among vertebrates (19), our results are likely to be generalizable to other vertebrate life histories. Within the class reptilia, the potential constraints of the pelvic girdle on maximum offspring size may be taxon-specific. For example, these constraints may be more stringent in groups like turtles (5) and lizards because of the pelvic girdle's central role in tetrapod locomotion. Snakes, the closest relative of lizards, may be free of this additional locomotor constraint, but comparative data for egg size in snakes is currently unavailable for testing this hypothesis.

It is clear that the dichotomy between lizards that produce one-egged as opposed to multi-egged clutches as reflected in the comparative literature (9, 20, 21) is actually an artifact of the highly nonlinear nature of the egg size and egg number trade-off. Because no *Anolis* lizard is known to ovulate or lay more than one egg at a time and other lizards typically lay clutches of two or more eggs, there was previously no direct way to compare these life history strategies. In light of our experimental observations, it is clear that *Anolis* and *Uta* are not that different in terms of the factors governing egg size and total clutch mass (Fig. 1). As *Anolis* lizards evolved a fixed, one-egged strategy from the life history strategy of multi-egged clutches more typical of other lizards, and presumably the primitive condition, relatively small changes would have been required in the mechanisms underlying control of reproduction. Clutch size, egg size, total clutch mass, and relative clutch mass would have evolved in a concordant fashion owing to the physiological and hormonal coupling between these traits. Moreover, if the proximate constraints identified in *U. stansburiana* were also acting as ultimate constraints, then even with chronic selection for increased egg size in one-egged species such as *Anolis* spp., no further evolution of egg size would occur. Our experimental methodology of egg enlargement in combination with complementary manipulations involving egg miniaturization (7, 8) could be used to gain insights into the presumed selective pressures (7, 9, 20, 22) and constraints that ultimately served to differentiate the reproductive strategies of lizards in natural populations.

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- Lizards were taken from three locales in the inner Coast Range of California. Female and male *U. stansburiana* were collected during early April 1990 from three study populations in the Coast Range of California located along Corral Hollow Road (Contra San Joaquin County, CA), Del Puerto Canyon Road (Stanislaus County, CA), and near Los Banos Creek along Billy Wright Road (Merced County, CA). Details of husbandry and egg oviposition conditions can be found in Sinervo and Licht (8). Because of the low-frequency of eight- ( $n = 3$ ) and nine-egged ( $n = 1$ ) clutches in 1990, we used data on eight- ( $n = 5$ ) and nine-egged clutches ( $n = 1$ ) from 1989 (8) to increase their frequency in the statistical analyses.
- Animals were anesthetized using Metaflane (10 to 15 min) before surgery.
- We could detect no significant difference in egg mass between natural ( $n = 12$  for two-egged clutches and  $n = 64$  for three-egged clutches) and experimental clutches ( $n = 5$  for one-egged clutches,  $n = 42$  for two-egged clutches, and  $n = 24$  for three-egged clutches) with three or fewer eggs [ $F(1,142) = 1.63$ ,  $P > 0.20$ ] by analysis of covariance (ANCOVA) and neither of the two covariates, number of eggs per clutch [ $F(1,142) = 1.23$ ,  $P > 0.27$ ] or ln-transformed post-oviposition mass of the female as covariates [ $F(1,142) = 3.63$ ,  $P > 0.06$ ] was significant.
- As has been found to be the case in other experiments (8), the average egg sizes of sham-manipulated ( $n = 22$ ) and unmanipulated females that underwent the major period of vitellogenesis in the laboratory ( $n = 90$ ) was not significantly different [ $F(1,110) = 2.95$ ,  $P > 0.09$ ] [ANCOVA: covariate for number of eggs per clutch was significant,  $F(1,110) = 51.82$ ,  $P < 0.001$ , as was ln-transformed post-oviposition mass of the female,  $F(1,110) = 25.34$ ,  $P < 0.001$ ].
- Of the total number of eggs that either burst or became egg-bound ( $n = 99$ , Table 1), most of the eggs burst during oviposition ( $n = 86$ ). Those eggs that became egg-bound in the oviduct ( $n = 13$ ) occurred in the following frequencies:  $n = 1$  for one-egged,  $n = 9$  for two-egged,  $n = 2$  for three-egged, and  $n = 1$  for four-egged clutches. The increase in the frequency of burst eggs or oviductally bound eggs (pooled) with decreased clutch size was significant ( $P < 0.05$ ) based on results from logistic regression analysis of the fate of individual eggs [iteratively weighted least squares analysis, L. Wilkinson, "SYSTAT: The system for statistics" (SYSTAT, Evanston, IL, 1987)]. Parameter estimates (with standard errors) for the logistic regression are as follows:  $B_0 = -1.651 \pm 0.367$  and  $B_1 = -0.339 \pm 0.076$ . The relation was also statistically significant when the data for one-egged clutches were removed from the analysis.
- The sigmoidal shape of the curve is the result of two countervailing effects on egg size. At the largest and intermediate clutch sizes, the curve is governed by the inverse relation between egg and clutch size. However, owing to the functional limits on egg size (14) and underlying regulation of egg size (8), this inverse relation levels off at the smallest clutch sizes. Indeed, it is likely that those eggs that burst or became egg-bound at the smallest clutch sizes were actually larger than the sample of eggs that were laid without bursting. Thus, the sigmoidal curve based on our experimental data reflects any selection against large eggs owing to functional limitations. Because of the nonlinear relation between clutch size and egg size over the full range of clutch sizes (one to nine eggs), we used clutch size as a factor [ $F(8,528) = 31.77$ ,  $P < 0.001$ ] and ln-transformed post-oviposition mass of the female as a covariate [ $F(1,528) = 64.23$ ,  $P < 0.001$ ] in our analysis of ln-transformed egg mass. Heterogeneity of slopes among clutch sizes was not statistically significant ( $P > 0.24$ ), thus, the pooled allometric slope relating ln-transformed egg mass and ln-transformed post-oviposition mass of the female was 0.291. The means and standard errors for egg size in Fig. 1A were based on the analysis of ln-transformed data. Data reflects egg mass at each clutch size for an average-sized female *U. stansburiana* weighing 4.05 g. A descriptive curve was fit to the size-corrected data by nonlinear regression. Parameter values (with linearized estimates of the standard error) for the sigmoidal curve relating egg mass and egg number, egg mass =  $a \times \exp[-b \times \exp(-c \times 1/\text{eggs})]$  are as follows:  $a = 0.509 \pm 0.010$ ,  $b = 1.323 \pm 0.206$ , and  $c = 9.119 \pm 1.154$ .
- The average egg size, 0.48 g, for a 4.05-g *Anolis* lizard (that is, average size of *Uta* in our study) was calculated based on tabulated data and published regressions presented by Andrews and Rand (9) that relates egg size with various measures of body size.
- The functional limits on egg size as imposed by the diameter of the pelvic girdle are ameliorated to a certain extent in lizards because as eggs become large, either by experimental manipulation (8) or through evolution [Y. L. Werner, *Israel J. Zool.* 35, 199 (1989)], egg length increases dramatically relative to egg width.
- These correlations between egg size, egg number, and total clutch mass are explicable in terms of the known mechanisms governing reptilian reproduction, and these mechanisms suggest a mechanistic model whereby this suite of life history traits could be coupled physiologically. Clutch size in lizards is determined by the levels of gonadotropin during the earliest stages of vitellogenesis (8). Once clutch size is determined, the egg size of the resultant clutch is also determined. Thus, the trade-off between egg size and egg number results from an underlying physiological and hormonal coupling between these two traits (8). We hypothesize that the additional coupling between egg and clutch size and total clutch mass would arise if the levels of vitellogenin produced by the liver in response to estrogen [W. E. Hahn, *Comp. Biochem. Physiol.*, 23A, 83 (1967); J. F. Gerstle and I. P. Callard, *Comp. Biochem. Physiol.* 42A, 791 (1972); Z. Yaron and L. Widzer, *Comp. Biochem. Physiol.* 60A, 279 (1978); J. Gavaud, *Gen. Comp. Endocrinol.* 63, 11 (1986)] is also quantitatively related to the number of yolk follicles and their estrogen production.
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