

Offspring Sex in a Lizard Depends on Egg Size

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Summary

Current paradigms may substantially underestimate the complexity of reptilian sex determination. In previous work, we have shown that the sex of a hatchling lizard (*Bassiana duperreyi*, Scincidae) does not depend entirely on its genes (XX versus XY sex chromosomes); instead, low nest temperatures can override genotype to produce XX as well as XY males [1–3]. Our experimental studies now add a third mechanism to this list: sex determination via yolk allocation to the egg. Within each clutch, the eggs that produce daughters are larger than those that produce sons. If (and only if) eggs are incubated at low temperatures, removing yolk from a newly laid egg turns the offspring into a male. Adding yolk from a larger (but not smaller) egg turns the recipient egg's offspring into a female. Remarkably, then, offspring sex in this species is the end result of an interaction between three mechanisms: sex chromosomes, nest temperatures, and yolk allocation.

Results and Discussion

The classical view of a fundamental dichotomy between genotypic sex determination (GSD) and environmental sex determination (ESD) [4–7] has been strongly challenged by the discovery that some lizards exhibit multifactorial sex determination [1, 2, 8]. Heteromorphic sex chromosomes determine offspring sex if the eggs are incubated at intermediate temperatures, but are overridden by thermal factors if eggs are incubated under more extreme conditions [1, 8]. Our project was stimulated by a surprising observation: in the montane scincid lizard *Bassiana duperreyi*, large eggs are more likely to produce daughters and small eggs to produce sons [1]. We now report that egg size per se has a direct influence on sex determination in this species and that manipulations of yolk volume change the sex that hatches from the egg. However, this effect is seen only at low incubation temperatures.

We first asked whether the correlation between egg size and offspring sex results from large-egg daughters and small-egg sons within each clutch, versus a trend for some females to specialize in the production of large-egg daughters and others in the production of small-egg sons. Analyses based on 807 eggs from 130 clutches showed that the association between egg size and offspring sex is driven by within-clutch variation, not among-clutch variation. Looking only at offspring from incubation temperatures >20°C (to eliminate thermal effects

on sex ratio), clutches with larger mean egg sizes were not consistently more female biased (range in mean egg mass per clutch 228–460 mg, range in sex ratio 25% to 83% male; correlation $n = 35$ clutches, $r = -0.017$, $p = 0.92$). However, egg size correlated with sex within clutches. Most clutches contained eggs that spanned a wide range of sizes (mean of 20.8% intraclutch range in egg mass at oviposition, maximum 78%, in 130 clutches). Within a clutch, larger eggs generally produced daughters, whereas smaller eggs produced sons (on average, son-producing eggs weighed 4.1% less than daughter-producing eggs in the same clutch; $n = 63$ clutches; paired $t = 3.03$, $df = 11$, $p < 0.015$).

Why did larger eggs produce daughters rather than sons? Because of possible interactions between thermal effects and yolk-allocation effects, we incubated eggs both at cool (16.0°C ± 7.5°C diel range) and warm (22°C ± 7.5°C) temperatures, by using a split-clutch design. Warm-incubated eggs yielded approximately 1:1 sex ratios regardless of our yolk manipulations. Sham controls yielded 45% males, controls were 67% males, and yolk removal yielded 59% males (comparing these three treatments, $\chi^2 = 3.09$, $df = 2$, $p = 0.21$; Figure 1). In all 56 of these cases that we tested using the PCR method, morphological and gonadal sex matched chromosomal sex. Hatching success at this incubation regime was 89% for controls, 92% for sham controls, and 89% for yolk-removed eggs ($\chi^2 = 0.04$, $df = 2$, $p = 0.98$). Thus, yolk allocation did not affect offspring sex when the eggs were incubated under warm conditions.

In striking contrast, removal or addition of yolk to newly laid eggs massively shifted offspring sex ratios when eggs were incubated under cool conditions. After incubation at 16.0°C ± 7.5°C, control eggs yielded 57% male offspring, sham-operated eggs were 61% male, yolk-removed eggs yielded 88% male, and yolk-addition eggs yielded 7% male offspring (Figure 1). Thus, yolk removal generated a strong male bias (versus 50%, $\chi^2 = 47.82$, $df = 1$, $p < 0.001$) and yolk addition generated a strong female bias ($\chi^2 = 21.55$, $df = 1$, $p < 0.001$). Hatching success was 77% overall (79% for controls, 82% for sham removal, 67% for yolk removal, and 94% for yolk addition). The sex-ratio biases at hatching were too high to be attributed to differential sex-specific mortality (even assuming that all unhatched eggs from the yolk-removal and yolk-addition treatments were the “wrong” sex: comparing these two treatments, $\chi^2 = 18.79$, $df = 1$, $p < 0.001$). Genetic sexing methods [2] confirmed that 10% of the control eggs from cool incubation conditions produced discordant (XX) males (4 of 37) but no discordant (XY) females, as expected from previous studies [1, 2]. The genetic assays also showed that yolk manipulation produces higher rates of discordant sex production (up to 68%) that include three discordant females (XY; from yolk addition—see Figure 2) as well as 15 discordant males (XX; from yolk removal). Hence, yolk allocation (as well as temperature) directly affects offspring sex.

Finally, what causal mechanism links offspring sex to egg size? The link plausibly might be physical, on the basis of amounts of yolk present within the shell; but experiments in which we increased total volume of materials within the egg (by injecting silicone gel) did not change offspring sex

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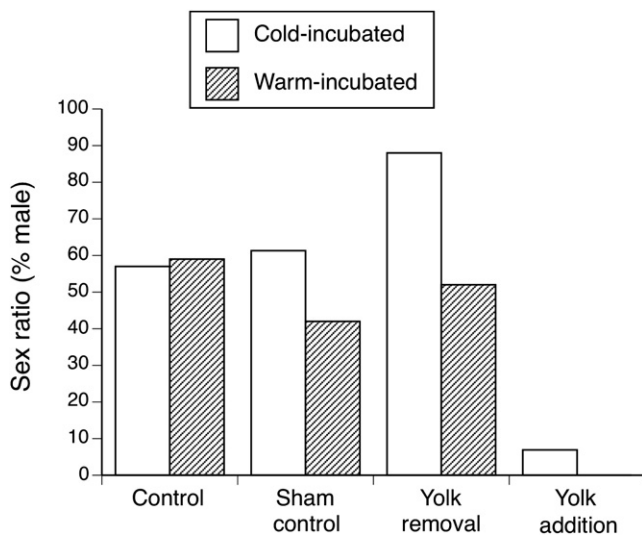


Figure 1. Effects of Egg Size Manipulation on Hatchling Sex

Sex ratio (% male) among hatchling lizards (*Bassiana duperreyi*) from eggs subjected to experimental treatments shortly after they were laid and incubated at either cool temperatures ($16.0^{\circ}\text{C} \pm 7.5^{\circ}\text{C}$; open histograms) or warm temperatures ($22.0^{\circ}\text{C} \pm 7.5^{\circ}\text{C}$; shaded histograms). Some eggs served as unmanipulated controls; some were pierced by a syringe needle to act as sham controls; some had an average of 37 mg of yolk removed through a syringe needle; and the remainder (cool incubation only) had yolk added through a syringe needle. At cool-temperature incubation, the yolk-removal eggs produced mostly male hatchlings (73 M, 10 F) and the yolk-addition eggs produced mostly female hatchlings (2 M, 27 F), whereas the two control groups produced sex ratios close to 50% male (unmanipulated controls 57 M, 43 F; sham-operated controls 46 M, 29 F). At warm incubation temperatures, all three experimental groups produced approximately equal sex ratios. Sham controls yielded 45% males (15 M and 18 F), controls were 67% males (22 M and 11 F), and yolk removal yielded 59% males (24 M and 17 F; comparing these three treatments, $\chi^2 = 6.00$, $df = 2$, $p = 0.19$).

(silicone-injected eggs produced 58% phenotypic males—15 M, 11 F, whereas controls produced 64% males—28 M, 16 F; $\chi^2 = 0.24$, $df = 1$, $p = 0.68$). Instead, the link is physiological: some component of the yolk within larger eggs pushes phenotypic sex toward femaleness. Only 5 of 17 eggs (29%) that received yolk from eggs smaller than themselves produced phenotypic females, compared to 40 of 49 (82%) that received yolk from eggs larger than themselves ($\chi^2 = 15.87$, $df = 1$, $p = 0.0002$).

Remarkably, then, a reproducing female in this lizard population may be able to influence the phenotypic sex of her offspring via three discrete mechanisms: (1) by use of X-bearing versus Y-bearing spermatozoa (not reported in any scincid species to date, but female lacertid lizards can use sperm selectively [9]); (2) by her choice among nest sites offering alternative thermal regimes [10]; and (3) by her allocation of yolk (present study). Although many factors correlate with offspring sex ratios in other vertebrate species, most of these patterns presumably reflect indirect effects mediated via maternal physiology [11, 12]. In contrast, each of the three factors that determine offspring sex in *Bassiana duperreyi* plays direct causal roles. First, incubation at high temperatures generates a match between phenotypic and genetic sex, with heteromorphic sex chromosomes only in males (i.e., XY sex-determining system [1, 2]). Second, incubation at low temperatures can override the chromosomal mechanism to produce XX as well as XY males [1, 2]. Lastly, the present study shows

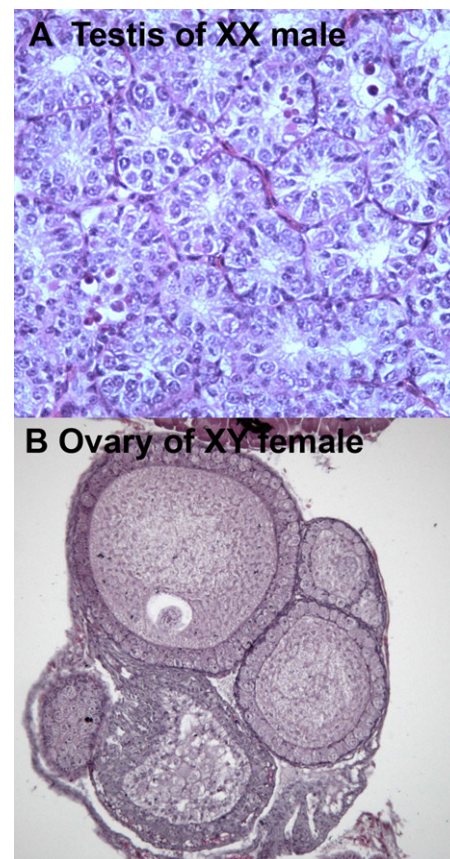


Figure 2. Gonadal Histology of Sex-Reversed Lizards

(A) Cross-section of testis from discordant XX male.

(B) Cross-section of ovary from discordant XY female.

Gonadal sections were taken from male and female *Bassiana duperreyi*, at one year of age, hatched from yolk-manipulated eggs. Chromosomal sex was verified by PCR analysis in both cases. Note (A) the normal testicular development (with distinct seminiferous tubules and spermatogonia) of the XX male and (B) ovarian development of the XY female.

a causal effect of yolk allocation (and thus, egg mass) on offspring sex: if (and only if) eggs are incubated at low temperatures, offspring sex can be changed by adding or removing yolk from the newly laid egg. Previous reports of intraclutch correlations between egg size and offspring sex in vertebrates [13, 14] suggest that *Bassiana duperreyi* may not be unique in this respect, although the physiological mechanisms involved in forging such a link may differ considerably between superficially analogous cases (i.e., biased chromosomal segregation in birds versus yolk-composition effects on phenotypic sex of the offspring in lizards). Differential allocation of yolk among a cohort of simultaneously recruiting follicles is physiologically feasible in many vertebrates [15].

Why did addition of yolk from larger eggs result in female offspring, whereas the same amount of yolk from smaller eggs did not do so? Studies on other lizard species have reported differences in sex-steroid levels between eggs that produce male versus female offspring [16, 17], and exogenous administration of high doses of oestradiol or fadrozole to *Bassiana* eggs massively shift offspring sex ratios [18]. Although a previous study on *Bassiana* [19] reported that levels of most sex steroids in the yolk were not significantly related to egg mass, a reanalysis of the raw data from that study (after In

transformation to reduce variance heterogeneity) suggests that concentrations of dihydrotestosterone (DHT) are higher in smaller eggs (ln DHT versus egg mass: $n = 55$, $r = -0.34$, $p < 0.015$). Hence, allometry of steroid levels offers a potential mechanism for the yolk-allocation effect on offspring sex. Nonetheless, other studies on lizards have concluded that sex differences in yolk steroid levels are minor and probably not biologically significant [20]; thus, the causal physiological basis by which yolk allocation modifies offspring sex in our study species warrants additional study.

More generally, our results suggest that current paradigms substantially underestimate the complexity of sex-determining systems in reptiles. Offspring sex in squamate reptiles may be the end result of multiple factors operating simultaneously (and interactively), given that it is in many invertebrates [21, 22]. Such complexities may evolve in a stepwise fashion. For example, a genetic basis to sex determination in the lineage of lizards containing *Bassiana* may have evolved because of fitness advantages to stable sex ratios close to 50:50 (Fisher's "balance" argument [21]). In severely cold nests close to the upper elevational limits for this species, however, daughters fare less well than sons [23]. Thus, selection has favored the evolution of temperature-dependent sex determination over (and only over) the thermal range at which viability of sons exceeds that of daughters [1]. If nest temperatures average so low that most offspring are born as sons, however, the fitness benefits of producing equal numbers of sons and daughters may override the higher mean viability of sons—and thus, favor a mechanism that moves sex ratios within a clutch back toward 50:50. The yolk-allocation mechanism we have documented does exactly this: by linking offspring sex to egg size, a female can produce fairly equal numbers of sons and daughters over a wide range of incubation temperatures.

This stepwise model of accumulation of multiple complementary sex-determining systems should be amenable to historical (phylogenetic) analysis; for example, it predicts lability in sex-determining systems even among conspecific populations exposed to differing selective regimes. Future work also could usefully examine the selective forces involved: for example, linking offspring sex to egg size may enhance fitness because of sex differences in optimal body sizes at hatching (as documented in other species of montane lizards sympatric with *B. duperreyi* [24]). Regardless of the adaptive significance of multifactorial sex determination, our study shows that the sex of a neonatal lizard is the end result of a complex set of factors, including direct maternal control over offspring sex by means of yolk allocation. We doubt that *Bassiana duperreyi* is unique in this respect: the magnitude of such effects (and hence the ease of their discovery) undoubtedly depends on the incubation temperatures used in experiments and on the magnitude of variance in egg size within clutches. Thus, effects of egg size on sex determination might be common (especially in species with TSD) but not readily detectable under the experimental protocols used in previous work.

Experimental Procedures

Collection and Husbandry

Adult female lizards (*Bassiana duperreyi*, Scincidae) from the Brindabella Range (148°50'E, 35°21'S) of southeastern Australia produce a single clutch of 3–11 eggs each year [1, 25]. We collected females ~1 week prior to laying (range 4–12 days), allowed them to oviposit in captivity at the University of Sydney, and incubated their eggs on moist vermiculite (water potential: -200 kPa) at a diel cycle of $16.0^\circ\text{C} \pm 7.5^\circ\text{C}$ (cool) or $22^\circ\text{C} \pm 7.5^\circ\text{C}$ (warm).

These incubation regimes mimic those in natural nests at high versus low elevations [1].

Experimental Manipulation of Newly Laid Eggs

Less than 12 hr after eggs were laid, we changed egg size by inserting a small needle (24 gauge) into the egg at a point as far as possible from the embryo (as visualized by candling the egg) and either removing yolk, or adding yolk that had previously been removed from another egg. Using a split-clutch design, we divided the eggs into four treatment groups: (1) control; (2) yolk removal (such that eggs were reduced to the mass of the smallest egg within their clutch of origin; amount of yolk removed averaged 37.0 ± 11.09 mg [SEM] per egg); (3) yolk addition (26.0 ± 1.56 mg of the previously removed yolk was added to another egg in the same clutch); and (4) sham-yolk removal (needle penetrating the egg, but <5 mg of yolk removed). Because of a scarcity of eggs, we omitted the "yolk addition" treatment for warm-incubated eggs. Thus for warm incubation, eggs from each clutch were divided into three groups only: control, yolk removal, and sham-yolk removal.

To investigate whether the size of the donor egg affected the sex of offspring emerging from the recipient egg, we added 10–30 mg of yolk to 66 small eggs (one from each clutch); the donor egg was larger than the recipient egg in some of these cases and smaller in others. To see whether the yolk-addition effect on offspring sex might be due simply to the additional volume of materials inside the egg, we also added silicone gel (Factor II, AZ, USA; used in cosmetic implantation) rather than yolk. For this purpose, we increased the sizes of 39 small eggs (each from different clutches) by adding 39.0 ± 7.0 mg of silicone gel. These manipulated eggs as well as 54 control (needle-pierced only) eggs were incubated at cool temperatures. The eggs used in these experiments (effects of donor-egg size and of silicon-gel addition) were run on a different set of clutches than those on which we conducted the initial work (on effects of yolk removal and addition).

Determination of Genotypic and Phenotypic Offspring Sex

We assessed offspring sex by eversion of the hemipenes in males [26] and verified this by histological examination of gonads at 10 weeks of age ($n = 12$ hatchlings). To identify chromosomal sex of hatchlings ($n = 137$), one of us (AQ) applied PCR-based tests for the Y chromosome sequence of *B. duperreyi* [2] on the basis of genomic DNA extracted from tail-tip tissue. For each individual, we performed two separate PCRs to identify genetic sex: the first PCR amplified a 185 bp Y chromosome fragment (males only), and the second PCR amplified a 92 bp fragment (males only), nested within the 185 bp fragment. The sex of the discordant animals hatched from the yolk-manipulated eggs (i.e., XX males, XY females; identified by molecular sexing methods) was reconfirmed at 1 year of age by gonadal histology of five randomly selected individuals. The specimens used for genotypic sexing in this paper drew from the sample of 78 animals sexed for the identification, characterization, and testing of the sex marker [3] and the 137 animals used to demonstrate sex reversal in this species [2], plus an additional 47 specimens.

Statistical Analyses

We used linear correlation analysis to compare clutch sex ratios to mean egg sizes and paired *t* tests to compare mean masses of eggs producing sons versus daughters within each clutch. Data on the effects of incubation temperature and yolk manipulation on offspring sex were analyzed by contingency-table tests.

Acknowledgments

The junior authors thank the senior author for the inspiration and friendship he gave so generously during his tragically brief life. Thanks also to Melanie Elphick and Betsy Roznik for laboratory assistance. This project was supported by the Australian Research Council.

Received: March 18, 2009

Revised: May 4, 2009

Accepted: May 5, 2009

Published online: June 4, 2009

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