



Sexual selection favours large body size in males of a tropical snake (*Stegonotus cucullatus*, Colubridae)

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Information on the phenotypic correlates of male reproductive success can provide important insights into the operation of sexual selection, and the nature of evolutionary forces on phenotypic traits such as male body size. We combined results from a long-term mark–recapture field study with genetic analyses for identifying paternity of offspring to quantify male reproductive success in a colubrid snake species from tropical Australia. Because previous work has shown that male slatey-grey snakes, *Stegonotus cucullatus*, attain larger body sizes than do conspecific females, we predicted that larger males would have higher reproductive success. Our paternity assignments of 219 offspring (24 clutches) supported this prediction: larger males fathered more offspring, not because they obtained more matings, but because they fathered a higher proportion of offspring within the clutches to which they did contribute. Multiple paternity was common (mean of 2.3 fathers per clutch, range 1–5). Our results demonstrate the utility of molecular approaches to clarify mating systems in field populations of snakes, and suggest that the evolution of extreme male-biased sexual size dimorphism in this species is attributable to enhanced reproductive success afforded by larger body size in males.

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Variation among individuals in reproductive success is a fundamental prerequisite for selection and adaptation. Causal effects of phenotypic variation on individual reproductive success thus provide a critical basis for evolutionary interpretations of inter-specific and intraspecific diversity in phenotypic traits. The central importance of individual reproductive success for selection and the immense diversity of factors that influence the relationship between phenotypic traits and reproductive success have spawned a major research field in the analysis of mating systems (e.g. Carson 2003; Peterson & Husak 2006; Ramm et al. 2008).

Sexual selection theory predicts strong selection for traits that increase reproductive success (in both sexes). In males, mate acquisition is often the most severe limitation on reproductive success and, consequently, male strategies to obtain matings with more than one female are widespread (e.g. Jehl & Murray 1986; Birkhead & Møller 1998; Say et al. 2003; Uller & Olsson 2008). In reptiles, male reproductive success is mostly driven by the interaction between (1) rates of copulation, (2) cryptic female choice and (3) sperm competition, which could occur via sperm storage

(e.g. with selection on longevity of spermatozoa: Uller & Olsson 2008). In addition, the pressures for mate acquisition have resulted in intense male–male competition for females and, in many species, female-defence or resource-defence polygyny (e.g. Stamps 1977; Martins 1994; Shine 2003).

Because a male's body size may often influence its ability to defeat rival males and/or to obtain copulations with females, male-biased sexual size dimorphism might be expected to evolve in taxa where male mating success is strongly enhanced by larger body size (e.g. in lizards and snakes: Wikelski & Romero 2003; Fearn et al. 2005). The mechanism linking body size to male reproductive success may be straightforward: for example, larger males are presumably stronger, so are more likely to win combat bouts with smaller males, and may be better able to obtain matings via coercion of females or via female choice (Shine et al. 2005). In addition, larger males may have access to a larger subset of the reproductive females within the population, and consequently can select females likely to produce more (or more viable) progeny (e.g. females that are larger or are in better physical condition, and thus produce larger litters: e.g. Shine et al. 2001).

In addition to such advantages, larger males may also have an enhanced reproductive success via sperm competition, by possessing larger ejaculates. Data to test this idea are lacking for many groups of animals, including snakes. Nevertheless, the high incidence of multiple paternity within clutches of many reptile species,

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coupled with the prevalence of multiple mating in female reptiles (reviewed in Uller & Olsson 2008), suggests that strong selection to maximize male reproductive success within multiply sired clutches should influence traits that enhance success in sperm competition.

Despite increasing scientific interest in snake ecology and behaviour (Shine & Bonnet 2000), most studies on this lineage are based on species from cold climates, where thermoregulatory needs render snakes more readily observable. Therefore, our knowledge of mating systems in snakes is skewed and woefully incomplete compared to other taxa, such as birds (see Griffith et al. 2002). For example, there has probably been more detailed mating-system analysis for a single population of garter snakes, *Thamnophis sirtalis parietalis*, at the extreme northern limit of their range in Canada (e.g. Mason 1993; Shine et al. 2000a,b,c,d,e) than for all other snakes combined. Other intensively studied snake species include European adders, *Vipera berus*, and grass snakes, *Natrix natrix*, in Sweden (Madsen & Shine 1993, 1994; Madsen et al. 1992, 1993), northern water snakes, *Nerodia sipedon*, in Canada (Weatherhead et al. 1995) and prairie rattlesnakes, *Crotalus viridis*, in the northern U.S.A. (Duvall & Schuett 1997).

In the present study, we explored the mating system of a secretive tropical snake species by using molecular analyses based on tissue samples from offspring, their mothers and their potential fathers, to document fundamental parameters of the mating systems, such as the incidence of multiple paternity and the correlates of male reproductive success.

Slatey-grey snakes, *Stegonotus cucullatus* (Colubridae) show more extreme male-biased sexual size dimorphism than most other snake taxa (Shine 1994). Under the hypothesis that male-biased sexual size dimorphism results from a strong enhancement of male reproductive success by larger body size (e.g. Shine 1978, 1994), we predicted that larger body size would increase reproductive success for male slatey-grey snakes. Previous studies on mating systems have identified at least three (not mutually exclusive) pathways by which such a relationship might arise, and distinguishing between those pathways can clarify the nature of the selective forces working on body size in males. Specifically, if larger male body size is an adaptation to enhance success in male–male contests for mating opportunities, we would expect to find that larger males mate with more females. If larger males are more successful at sperm competition (perhaps by producing a larger ejaculate), they may sire a higher proportion of offspring in the clutches to which they contribute. Lastly, if larger body size enables a male to obtain ‘better’ mates, we may see larger males mating with females that produce larger clutches.

METHODS

Study Species and Area

The slatey-grey snake is a large (to 1.5 m snout–vent length, SVL), slender, nonvenomous snake with a wide geographical distribution in coastal and near-coastal habitats of tropical Australia and New Guinea (Cogger 2000). Slatey-grey snakes consume a range of vertebrates (fishes, frogs, reptiles, mammals), and frequently eat reptile eggs (Shine 1991). Males grow larger than females (in our study area, mean adult SVLs = 117.1 and 98.6 cm, respectively: Brown et al. 2005) and have larger home ranges (based on radiotracking: Brown et al. 2005). Males also disperse further from their natal sites than do females, based on genetic analyses (Dubey et al. 2008b).

Our field study was focused on the earthen wall of Fogg Dam (12°34'13"S, 131°17'53"E), 60 km east of the city of Darwin in the Northern Territory of Australia. Almost every night over the period 1998–2007 (total of 3099 nights), we walked or drove along the entire length of the 2.2 km dam wall, using a spotlight to locate

active snakes. The animals were captured by hand, and returned to the field laboratory for processing (measurement, individual marking by scale clipping, removal of tissue sample) before release (typically 1 or 2 days postcapture) at their original capture site. Gravid females were detectable by abdominal palpation, and maintained in captivity until they laid their eggs (median interval capture to oviposition = 7 days). They were kept individually in plastic cages (40 × 30 cm and 20 cm high) at room temperature. Cages were lined with paper and provided with a water dish and a plastic nest/refuge box. Females in the late stages of gravidity are areorectic and thus no food was provided. The eggs were incubated in the laboratory under a variety of substrate moisture regimes (to assess incubation effects on hatchling phenotypes), and hatchlings were measured, marked and tissue-sampled, as for the adult snakes. The hatchlings were then released at their mother's site of capture. The intense sampling schedule meant that most resident snakes in this population were marked animals (e.g. in 2007, 112 of 123 adult animals were already marked when collected, as were 7 of 12 juveniles).

Tissue Sampling

We collected 380 tissue samples from slatey-grey snakes from 2001 to 2007, including samples from 144 males and 17 different gravid females (in 2001–2004 and 2006–2007) which provided 24 different clutches and 219 hatchlings (some females were captured while gravid in more than 1 year; Table 1).

DNA Extraction and Microsatellite Analysis

Total cellular DNA was isolated from scales. Tissue of each individual was placed in 200 µl of 5% Chelex containing 0.2 mg/ml of proteinase K, incubated overnight at 56 °C, and boiled at 100 °C. Nine microsatellite loci isolated and characterized from *S. cucullatus* (Dubey et al. 2008a; Steg_A4, Steg_A5, Steg_A105, Steg_B104, Steg_B105, Steg_C109, Steg_D1, Steg_D2, Steg_D114) were amplified and scored. Amplified products were genotyped with an ABI Prism 377 DNA Sequencer using Genescan Analysis 2.1 software (Applied Biosystems Inc., Foster City, CA, U.S.A.). PCR amplifications were performed in a 9800 Fast thermal cycler (Applied Biosystems) as 5 µl reactions containing 0.075 U Taq Ti DNA polymerase (Biotech, West Perth, Australia), 0.1 mM dNTPs, 0.4 mM of each primer, 20 mM Tris-HCl, pH 8.5, 50 mM KCl, 1.25 MgCl₂ (2.0 mM for Steg_D2) and 15 ng of DNA. Cycling conditions included a hot start denaturation of 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 57 °C (58.5 °C for Steg_A5) annealing temperature for 30 s, 72 °C for 30 s, and a final extension of 72 °C for 30 min. Amplified products were genotyped with a 3130 xl Genetic Analyzer (Applied Biosystems) using Genemapper software V3.7 (Applied Biosystems).

Table 1

Sample sizes of free-ranging slatey-grey snakes for which genetic data were gathered

Year	Clutch	Juvenile	Potential father sampled
2001	2	21	47
2002	6	60	67
2003	7	71	84
2004	4	24	106
2006	4	34	116
2007	1	9	110
Total	24	219	144

The number of clutches, juveniles and potential fathers (number of adult males tested) sampled for each year are shown. The total number of potential fathers is the number of individual males, as some were sampled in more than 1 year.

Statistical Analyses

Gene diversities comprising observed (H_O) and expected heterozygosities (H_E) were estimated following the methods of Nei & Chesser (1983). Genotypic disequilibrium between loci in each sample was tested based on 10 000 randomizations to check for potentially linked loci (e.g. situated on the same chromosome). Deviations from Hardy–Weinberg equilibrium within samples were tested based on 10 000 randomizations, to check for the presence of allelic dropouts, founder effects, substructure within populations (Wahlund effect), inbreeding or null alleles. Wright's fixation indices for within-population deviation from random mating (F_{IS}) were estimated following Weir & Cockerham (1984). Deviations from random mating within populations (F_{IS}) per locus and sample were computed with a bootstrap procedure (10 000 randomizations). All summary statistics and tests mentioned above were computed using FSTAT version 2.9.3.2 (Goudet 1995). Significance values were corrected for multiple tests using the sequential Bonferroni correction (Rice 1989). Detection of null alleles was tested according to Chakraborty & Jin (1992).

Paternity Analyses

We determined paternity on the basis of a maximum likelihood method via the computer program CERVUS 3.0 (Marshall et al. 1998; Kalinowski et al. 2007). It conducts a comparison between an infant–mother pair and all the potential fathers in the data set. A LOD score (i.e. the logarithm of the likelihood ratio) is calculated for every potential father. The difference between the LOD scores of the male with the highest value and the male with second highest value is the Δ -criterion (Δ LOD; Marshall et al. 1998). Δ LOD is compared with the critical Δ values calculated after a simulation and provided with a statistical confidence level. The simulation was based on allele frequency data from the adult samples, the juvenile sampling being skewed (219 juveniles for 24 clutches). Parameters used for the simulation differed from year to year and we based our choices on the following criteria: (1) total number of candidate males = 47–110; (2) mean proportion of candidate males sampled = 0.80; (3) mean proportion of data typed = 0.95 (i.e. 5% of data missing); and (4) rate of typing errors = 0.00. We chose the confidence level proposed by Marshall et al. (1998) (strict confidence $\geq 95\%$). The candidate males were determined using two criteria: (1) their body size in the relevant year (adult size, >80 cm) and (2) their presence in the population in the year that those progeny were produced, or the year before. These strict criteria were chosen to minimize incorrect assignments, which could otherwise lead to erroneous interpretations of the mating system of the studied species.

Ethical Note

To mark adult snakes permanently, we excised the lateral edge of up to four ventral scales from the posterior body using a sterile scalpel blade. Other methods of permanently marking snakes (i.e. coelomic implantation of PIT tags) are more invasive and prone to failure. The scales excised from each individual were retained to provide a tissue sample. Juvenile snakes were permanently marked in a similar manner, but rather than excising the edge of a scale, we ablated it using a fine-tipped cautery device. The terminal scale of the tail was excised from juvenile snakes with a sterile scalpel blade to provide a tissue sample. The study was approved by the University of Sydney Animal Care and Ethics Committee and the Northern Territory Parks and Wildlife Service.

RESULTS

Diversity of Microsatellite Markers

Within samples from the adult snakes, we did not detect any significant linkage disequilibrium or null alleles. Consequently, all loci were included in the following analyses. However, we detected a significant deviation from random mating with a slightly negative global F_{IS} of -0.05 ($P < 0.001$). For the nine microsatellite loci, there were 3–10 alleles per locus (Table 2; average = 8.00), with a total of 72 alleles across nine loci. Observed heterozygosity within the adult population (H_O) was 0.77 and expected heterozygosity (H_E) was 0.73 (Table 2).

Within the 219 juveniles, no linkage disequilibrium was detected when we analysed each clutch separately. When we combined data for all clutches into a single analysis, significant linkage disequilibrium was observed between the pairs of loci B105/B104 A105/D1, A105/A5, B104/D1, B104/D114, and A4/A5. This result clearly reflects the skewed sampling (i.e. high relatedness of juveniles within each of the 24 clutches). For the nine microsatellite loci, there were 3–10 alleles per locus (Table 2; average = 7.33), with a total of 66 alleles across nine loci. Observed heterozygosity within the juvenile population (H_O) was 0.76, compared to expected heterozygosity (H_E) values of 0.60 and 0.70 (based on calculations treating each clutch separately versus combined, respectively; Table 2). There were significant deviations from random mating ($P < 0.001$), regardless of whether we treated the data for each clutch separately (negative global $F_{IS} = -0.26$), or combined the data for all clutches (global $F_{IS} = -0.07$).

Incidence of Multiple Paternity

Of the 219 offspring with known mothers, 88 were assigned at 95% certainty or more to fathers sampled in the percentage (40.2%). The percentage of offspring to which a father was assigned at 95% or more certainly within a given clutch varied from 0% to 100%. Of the 88 offspring assigned at 95% or more certainty to fathers, 72 (32.9%) had a father for which we had data on the adult male's body size in the year of assignment.

The number of different fathers assigned with 95% or more certainty per clutch (for clutches with at least one father assigned) was 1–5, with a mean of 2.32 ($N_{clutches} = 22$; Fig. 1), and a mean of 2.53 ($N_{clutches} = 19$) when we deleted the clutches where only one juvenile had a father assigned.

Correlates of Male Reproductive Success

Overall, larger males fathered more offspring each year (male body size versus number of offspring; linear regression: $F_{1,29} = 6.90$, $r = 0.19$, $P = 0.014$; Fig. 2). In addition, in each of the five years for which we have data, the mean body size of successful males was greater than the mean body size of all adult males (criteria for adult status in males = SVL > 80 cm; Shine 1991) within the population (unpaired t test: $t_8 = 4.26$, $P = 0.003$; overall mean body size for all males = 100.3 cm, mean body size of fathers = 120.0 cm). Larger body size might increase reproductive success for male slatey-grey snakes via at least three different pathways: (1) by mating with more females, (2) by siring a higher proportion of offspring in the clutches to which they contributed, (3) or by mating with females that produced larger clutches. We can evaluate all three of these pathways.

(1) There was no significant relationship between the number of clutches sired by a father and the body size of the father (linear regression: $F_{1,29} = 1.5$, $r = 0.05$, $P = 0.23$).

(2) Larger males tended to sire a higher proportion of offspring within the clutches to which they contributed (omitting clutches

Table 2
Characteristics of microsatellite loci used for the paternity analyses

Loci	N_A Ad	N_A Ju	H_O Ad	H_O Ju	H_E Ad	H_E Ju	\hat{H}_E Ju	F_{IS} Ad	F_{IS} Ju	\hat{F}_{IS} Ju
Steg_A4	3	3	0.62	0.57	0.53	0.48	0.52	-0.17	-0.19	-0.04
Steg_A5	8	7	0.81	0.77	0.82	0.63	0.75	0.01	-0.23	-0.003
Steg_A105	8	8	0.85	0.85	0.76	0.65	0.72	-0.12	-0.31	-0.19
Steg_B104	9	6	0.79	0.76	0.75	0.60	0.72	-0.05	-0.26	-0.04
Steg_B105	9	10	0.75	0.79	0.66	0.62	0.69	-0.14	-0.28	-0.13
Steg_C109	10	8	0.75	0.71	0.75	0.55	0.68	0.00	-0.29	-0.05
Steg_D1	8	8	0.81	0.80	0.79	0.62	0.75	-0.03	-0.28	-0.05
Steg_D2	10	8	0.77	0.79	0.78	0.65	0.76	0.01	-0.23	-0.03
Steg_D114	7	8	0.80	0.79	0.75	0.62	0.72	-0.07	-0.26	-0.11
Total mean	8.00	7.33	0.77	0.76	0.73	0.60	0.70	-0.05*	-0.26*	-0.07*

Data are shown for the number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosities for the adults (Ad) and juveniles (Ju) and the wright's fixation index for within-population deviation from random mating (F_{IS}) considering each clutch separately or all clutches combined (\hat{F}_{IS}), respectively.

* F_{IS} values significantly different from zero ($P < 0.05$).

for which only a single offspring could be assigned to a known father; linear regression: $F_{1,33} = 7.51$, $r = 0.19$, $P = 0.01$, Fig. 2).

(3) There was no significant trend for larger males to contribute paternity to larger clutches (linear regression: $F_{1,37} = 0.27$, $r = 0.01$, $P = 0.60$), and no assortative mating linked to body size was evident (i.e. male body size versus female body size; linear regression: $F_{1,37} = 0.26$, $r = 0.01$, $P = 0.61$).

DISCUSSION

Although previous studies on snake mating systems have often assumed that differentials in male reproductive success are driven largely by the number of matings achieved by different males (e.g. European adders: Madsen & Shine 1994; northern water snakes: Weatherhead et al. 1995; Brown & Weatherhead 1999), our results point to a different situation. In our slatey-grey snake population, larger males had a higher overall reproductive success by achieving paternity of a higher proportion of offspring within each clutch. This pattern could be explained by a number of different pathways. The most obvious such pathway is if larger males can defeat smaller rivals in combat (as has been reported in other snake species, including adders and copperheads, *Agkistrodon contortrix*: Madsen & Shine 1992; Schuett 1997); they may thereby be able to monopolize the reproductive output of those females (e.g. through mateguarding: Luiselli 1995). However, we cannot be certain that this straightforward mechanism is responsible. For example, we might see a similar pattern if females actively choose larger males

as partners or selectively use the sperm from larger males to fertilize their eggs, as shown in sand lizards, *Lacerta agilis* (Olsson et al. 2004). Sperm competition offers another alternative explanation, whereby larger males may produce larger ejaculates (e.g. Moya-Larano & Fox 2006) that increase the proportion of the clutch fathered by the larger male (e.g. Birkhead & Møller 1993; Birkhead & Pizzari 2002).

The clear trend for larger male slatey-grey snakes to achieve higher reproductive success fits well with the prediction that we

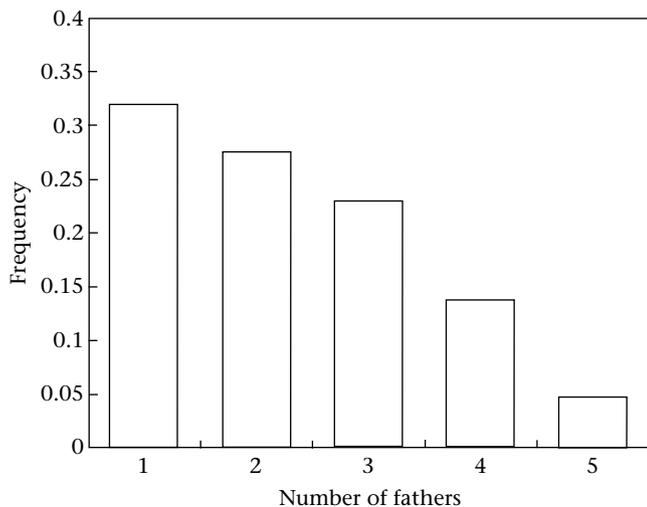


Figure 1. Frequency distribution of slatey-grey snake clutches with different numbers of fathers assigned at 95% or more certainty of paternity. All years are pooled ($N = 23$).

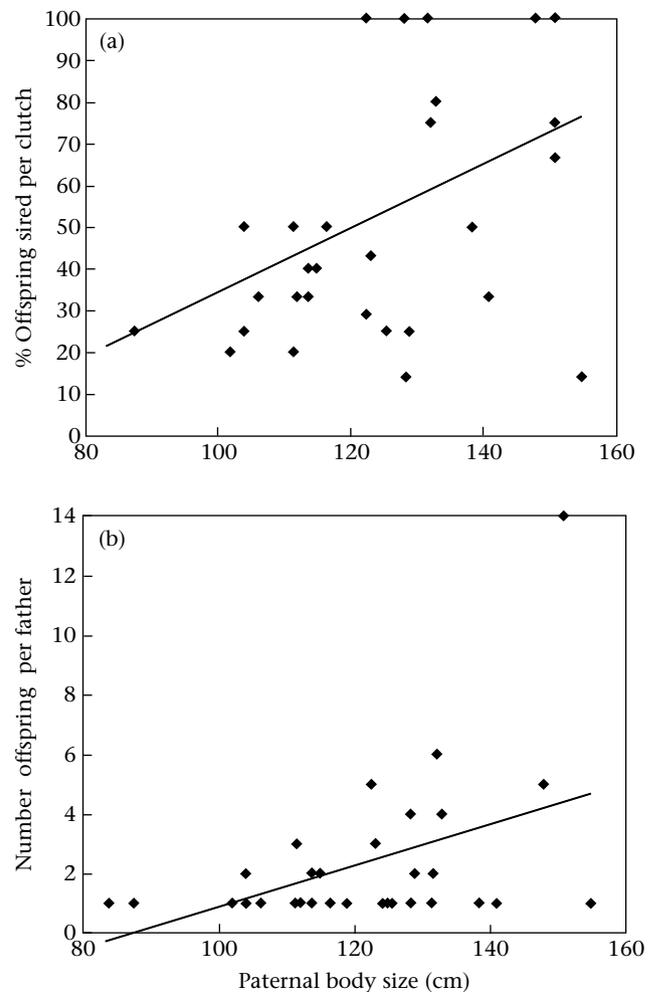


Figure 2. Relationships between paternal body size (snout-vent length) of slatey-grey snakes and (a) the percentage of offspring sired within each clutch and (b) the number of offspring per year fathered by each male.

would expect males to grow larger than females (as they do in slatey-grey snakes) only in taxa for which reproductive success increases more rapidly with body size in males than in females (Darwin 1871). Consistent with this hypothesis, snake species in which males engage in physical battles with their rivals for mating opportunities frequently have males larger than females, whereas the reverse dimorphism is widespread in snake species in which males do not engage in such battles (Shine 1978, 1994). For example, in black ratsnakes, *Elaphe obsoleta*, a species showing male-biased sexual size dimorphism and male–male combat, larger males were more successful primarily by siring more offspring per clutch rather than by siring offspring in more clutches (Blouin-Demers et al. 2005).

None the less, larger body size appears to enhance male reproductive success in most snake species, even those lacking male–male combat. For example, larger males are disproportionately successful in achieving matings in garter snakes, water snakes, grass snakes and sea kraits, *Laticauda colubrina* (Luiselli 1996; Kissner et al. 2005; Shine & Mason 2005; Shetty & Shine 2002), all species that lack male–male combat. Nevertheless, the experimental study of Kissner et al. (2005) revealed that this advantage may depend upon population parameters. Larger male water snakes were more successful only when male size varied substantially and competition among males was intense (e.g. when the operational sex ratio, was male biased), and size differences between males did not affect the proportion of offspring each male sired within multiply sired litters. Overall, the magnitude of large–male advantage is likely to be smaller in such species than in those with male–male combat (adders: Madsen et al. 1993; copperheads: Schuett 1997; black ratsnakes: Blouin-Demers et al. 2005; scrub pythons, *Morelia kinghorni*: Fearn et al. 2005). In light of this diversity, it is difficult to identify the specific pathways by which larger body size enhances reproductive success for male *S. cucullatus*.

In a recent review, Uller & Olsson (2008) highlighted the paradox that although studies on snakes and lizards provided the first empirical evidence of correlations between multiple mating and fitness in females, there have been almost no follow-up studies of such topics in reptiles. Encouragingly, some generalities are beginning to emerge; for example, multiple paternity is the norm (Stille et al. 1986; Luiselli 1995; Garner et al. 2002; Prosser et al. 2002; Blouin-Demers et al. 2005; Garner & Larsen 2005; Madsen et al. 2005). Our study reinforces this result. Although advantages of large body size appear to be widespread in male snakes, our study suggests that the pathways linking male body size to reproductive success may be more complex than are generally believed. The combination of ecological and genetic data has great potential provide a massive expansion of the phylogenetic and ecological base upon which we can draw to clarify the forces that have shaped mating systems in natural populations.

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