



Genetic evidence for sexual selection in black ratsnakes, *Elaphe obsoleta*

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We investigated benefits of polyandry and phenotypic correlates of male mating success in black ratsnakes. We used 10 microsatellite loci to identify the sires of 375 hatchlings from 34 clutches with known mothers. Multiple paternity occurred in 88% of clutches, despite the fact that ratsnakes mate when dispersed. The incidence of multiple paternity did not vary with female size or with clutch size. Hatching success tended to be higher in multiply sired clutches, but offspring from these clutches were not longer or heavier. We identified 34 males as the sires of 144 hatchlings. Most instances where sires could not be identified resulted from exclusion of all 426 potential sires that we screened. Unidentified sires were probably males that hibernated outside our study area and thus were not included in the intensive sampling at hibernacula in the study area. Among males that sired offspring, body size but not tail length contributed to success, consistent with male-biased sexual size dimorphism and male–male combat shown by this species. Large males were more successful primarily by siring more offspring per clutch rather than by siring offspring in more clutches. This may be a result of greater success in sperm competition. Neither body condition nor genetic similarity to the female was related to male mating success. Our results, in conjunction with published data on movement patterns, suggest that snakes of both genders can benefit from actively seeking multiple matings.

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In many animals, males can increase their reproductive success by mating with multiple females (polygyny). This leads to intrasexual competition and reproductive skew, where phenotypically superior males are more successful. Females, on the other hand, should not benefit from multiple mating because copulations are costly and the ejaculate of one male can normally fertilize all of the female's ova (Orsetti & Rutowski 2003). Contrary to expectation, however, multiple mating by females (polyandry) is common in many animals (Andersson 1994). The benefits females might derive from polyandry can be divided into two broad categories: (1) material benefits,

often in the form of nuptial gifts or parental care, and (2) genetic benefits, often in the form of intrinsic quality of the sperm or of compatible sperm (Konior et al. 2001; Zeh & Zeh 2001). While there is convincing evidence that polyandry can increase fitness through material benefits, the fitness increase associated with genetic benefits remains controversial (Andersson 1994; Fedorka & Mousseau 2002; Kamimura 2003). Females could derive intrinsic genetic benefits from polyandry by mating selectively with high-quality males (Møller 1994), by mating with multiple males and using sperm selectively (Olsson et al. 1996; Birkhead & Pizzari 2002), or by mating with multiple males and letting sperm competition determine which sperm is best (Madsen et al. 1992). Also, multiple mating by females could ensure against sperm limitation, male infertility, or male incompatibility (Gromko et al. 1984; Osikowski & Rafinski 2001). Although underrepresented in studies of vertebrate mating systems (Shine & Bonnet 2000), snakes offer advantages as a model for understanding polyandry. Because male snakes do not provide parental care or nuptial gifts, females do not obtain material benefits from multiple

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mating. Therefore, if female snakes mate multiply, they should derive genetic benefits by mating with, or by biasing reproduction towards, superior or compatible males. Our general goal in this study was to investigate the fitness consequences of polyandry and the phenotypic correlates of male reproductive success in black ratsnakes. We estimated male reproductive success and polyandry using DNA-based parentage analysis, an approach that has been rarely used with wild populations of snakes.

In our study population, black ratsnakes mate for several weeks in June, a month after dispersing from communal hibernacula (Blouin-Demers & Weatherhead 2002). When the snakes are dispersed, their densities are low: the average male home range (25 ha) contains 3.0 mature females, and the average female home range (17 ha) contains 2.5 mature males (Blouin-Demers & Weatherhead 2002). Low densities appear to shape the nature of mating competition in snakes. In some species, mating occurs in aggregations in which males compete by scramble competition (e.g. *Thamnophis sirtalis*, Gregory & Stewart 1975; *Nerodia sipedon*, Weatherhead et al. 1995, 2002). In ratsnakes, however, females are only courted by one male at a time (Blouin-Demers & Weatherhead 2002). When males encounter each other during the mating season they compete through ritualized physical combat (Rigley 1971; Gillingham 1980), with the victor presumably realizing a reproductive advantage. European adders, *Vipera berus*, also engage in combat, and success in combat increases male mating success (Madsen et al. 1993). Snake species that engage in male combat also tend to show male-biased sexual size dimorphism (Shine 1978), as has been found in black ratsnakes (Blouin-Demers et al. 2002).

Multiple paternity within litters has been documented in both garter snakes (Gibson & Falls 1975; Schwartz et al. 1989) and watersnakes (Barry et al. 1992; Prosser et al. 2002). Because these snakes mate in aggregations, multiple mating, and thus multiple paternity, might simply be a function of the spatial distribution of males and females. However, Madsen et al. (1992) found that multiple mating was common in European adders, a species that mates while dispersed. The low density of ratsnakes during the mating season might limit opportunities for multiple mating by females, so multiple paternity could be low. In ratsnakes, however, reproductive females are more active than nonreproductive females during the mating season, which could indicate that they are actively seeking multiple mates (Blouin-Demers & Weatherhead 2002). Active effort to obtain multiple matings would predict a high incidence of multiple paternity and would also strongly suggest that females obtain substantial benefit from multiple mating.

Here we determine the incidence of multiple paternity in black ratsnakes and compare our results with those from northern watersnakes, the only other snake species for which exact measures of multiple paternity have been obtained (Prosser et al. 2002). In addition, we assess two potential benefits of polyandry. First, if polyandry promotes sperm competition, which in turn favours high-quality or compatible sperm, then multiply sired clutches should have fewer fertilized but nonviable eggs (Madsen et al. 1992). Second, if sperm quality affects offspring

quality, then hatchlings from multiply sired clutches should be phenotypically superior (e.g. longer, heavier).

High-quality males could realize higher reproductive success just through sperm competition, as argued above. In addition, however, superior males may also be more successful because they mate more. Based on the ecological and behavioural patterns associated with mating in ratsnakes, we predicted that male mating success should increase with body size and condition (body mass corrected for size). This could arise because larger males or males in better condition are more successful in combat or in mate searching and thus gain greater access to females (Madsen et al. 1993), or because females preferentially mate with such males, or both. Preference for larger males could arise if size is associated with quality. Larger males have survived longer on average than smaller males (Blouin-Demers et al. 2002), and viability should be a particularly valuable quality in long-lived species. Male body size could also affect mating patterns if males display mate choice. Males are rarely selective about their mates, but if pursuing a given mating opportunity decreases the probability of mating in the future (e.g. if sperm is costly to produce or if mating requires a substantial time investment), then males should be selective. Larger female snakes often are more fecund and have also survived longer, both of which could make them more attractive to males. If larger males are competitively superior, they should preferentially mate with larger females. Male preference for larger females has been shown in garter snakes (Shine et al. 2001, 2003; LeMaster & Mason 2002). Therefore, we predicted that there should be positive assortative mating by body size.

In most snake species, males and females differ in tail length relative to body size (King 1989). King (1989) proposed two hypotheses to explain tail dimorphism. First, males may require longer tails because their hemipenes (copulatory organs) are housed in the tail. Second, in species that mate in aggregations, males compete by 'tail wrestling' to gain access to the female's cloaca, so longer tails in males could be a product of sexual selection. Shine et al. (1999) found support for both hypotheses in garter snakes (males with longer hemipenes had longer tails and higher mating success). Combat appears to preclude tail wrestling as a form of male competition in ratsnakes because only the victor copulates with the female. Thus, in addition to documenting the extent of sexual dimorphism in tail length in ratsnakes, we test the prediction that male reproductive success is unrelated to tail length.

A final factor that we consider that could affect mate choice by both male and female ratsnakes is genetic similarity. Snakes may not mate with closely related individuals to avoid the cost of inbreeding, or sperm from unrelated males may have an advantage in sperm competition (Olsson et al. 1996). In either case, we predicted that the genetic similarity of mates should be lower than expected by chance.

Meeting our general goal of understanding the factors affecting male and female mating success in black ratsnakes allowed us to address a secondary objective. In our study population, adult ratsnakes are highly faithful to

their hibernacula (Prior et al. 2001), a characteristic that could lead to genetic differentiation between hibernacula populations. Blouin-Demers & Weatherhead (2002) studied the ecological factors affecting gene flow between hibernacula populations and concluded that the movement patterns of adults allowed ample opportunity for mating between members of different hibernacula. However, genetic data are necessary to determine the extent to which this potential is realized. We use parentage data to quantify the extent of extrahibernacula mating and thus the potential importance of extrahibernacula population mating to gene flow. We predicted that mating between members of different local hibernacula should be common, explaining in part why such populations are not genetically distinct (Lougheed et al. 1999).

METHODS

We conducted this study on black ratsnakes from 1996 to 2001 at the Queen's University Biological Station, approximately 40 km north of Kingston (Ontario, Canada). Adult and juvenile black ratsnakes of both sexes were captured during spring emergence from communal hibernacula (April–May; Blouin-Demers et al. 2000a) or opportunistically during the active season (June–September; Prior et al. 2001).

Location of Nests and Incubation of Eggs

We located black ratsnake nests by radiotracking gravid females until they oviposited (Blouin-Demers & Weatherhead 2002). Radiotransmitters (SI-2T, Holohil Systems, Carp, Ontario) were surgically implanted in the body cavity of each female using sterile techniques and under isoflurane anaesthesia (Blouin-Demers et al. 2000b). Over the 6 years of the study, we followed 61 females for periods ranging from 3 weeks to 3 years. Twenty-six of the 61 radiotracked females were gravid at least once (four females were gravid twice). Females with transmitters led us to 12 communal nests and six nests used by individual females. At communal nests, we regularly encountered gravid females without transmitters.

We collected clutches from natural nests used by radio-implanted females and brought them to the laboratory for artificial incubation. In addition, gravid females without transmitters that we encountered in the field were brought to the laboratory where they were housed alone and provided with a nesting box filled with a mixture of moist peat and sphagnum moss in which they laid after a few days. From 1996 to 2001, we obtained a total of 87 clutches (1155 eggs), 34 of which (438 eggs) were from 32 known mothers (females that nested in the laboratory or in nests used by a single female in the field). The mean clutch size was 13 eggs (range 7–23 eggs). Our subsequent analyses are based only on the 34 clutches from known mothers. We incubated the eggs at 30°C in two incubators (120 × 90 × 30 cm) built with fibreboard laminated with melamine. Temperature was maintained in each incubator with a 100-W light bulb activated by an electronic thermostat (CT3300, Honeywell, Morristown, New Jersey,

U.S.A.). Eggs were grouped by clutch in plastic containers (15 × 15 × 6 cm) with perforated lids and were half-buried in 3 cm of moist vermiculite (2:1 ratio by mass of water and vermiculite). The containers with the eggs and incubation medium were weighed at the onset of incubation, and to compensate for evaporation, water was added weekly to maintain total mass. Towards the end of incubation in late August, we checked eggs regularly to determine hatching success and to remove hatchlings. In total, 984 of the 1155 eggs (85.2%) hatched. Eggs that did not hatch were not consistently examined, which precluded differentiating infertile eggs from those with embryos that were not viable.

Processing of Animals

We sexed adult snakes by probing the cloaca for the presence of hemipenes with a clean cloacal probe. Adult snakes were measured for snout–vent length (SVL) to the nearest 1 cm with a flexible measuring tape run along the body (Blouin-Demers 2003). We measured tail length (TL) by laying the tail against a metal ruler taped to a table. We weighed adult snakes to the nearest 5 g with a calibrated spring scale. Adults were measured multiple times during the study and, in our analyses, we used the measurements closest to when females laid eggs and to when males sired offspring. Within 2 days posthatching, we removed hatchlings from the incubators and determined their sex by everting the hemipenes. Hatchlings were weighed to the nearest 0.1 g on an electronic scale. SVL and TL were measured to the nearest 1 mm by laying the hatchlings against a metal ruler taped to a table (Blouin-Demers 2003). We marked snakes of all ages individually by PIT-tagging and, for genetic analyses of parentage, we drew 100 µl of blood (50 µl for hatchlings) from the caudal vein with a 0.5 ml sterile insulin syringe. The blood was suspended in 800 µl (500 µl for hatchlings) of Queen's lysis buffer (Seutin et al. 1991) and refrigerated before DNA extraction. Within 24 h following processing, adults were released at their capture location and juveniles were released where the eggs were collected.

Genotyping

DNA was extracted following established protocols (Lougheed et al. 1999) from blood samples of 32 known mothers and their 375 hatchlings (34 clutches) and of 426 potential fathers. Because male size at maturity had not been inferred from genetic parentage data previously and because male ratsnakes can cover great distances, we considered all males captured anywhere in the study area as potential fathers. All individuals were genotyped at eight microsatellite loci (Eob_μ1, Eob_μ10, Eob_μ13, Eob_μ16, Eob_μ34, Eob_μ358, Eob_μ366, Eob_μ373; Blouin-Demers & Gibbs 2003). Probabilities of paternal exclusion for each locus, when the mother was known, ranged between 0.41 and 0.84, and the combined paternal exclusion probability for these eight loci was greater than 0.99. When more than one father could not be excluded, we genotyped the mother, and the multiple potential fathers of offspring at

an additional two loci (Eob μ 360, Eob μ 379; H. L. Gibbs & G. Blouin-Demers, unpublished data) in an attempt to exclude one or more of the remaining males. Blouin-Demers & Gibbs (2003) provide details of the genotyping procedure. Briefly, we amplified the microsatellite loci via PCR with the forward primer labelled with $\gamma^{33}\text{P-ATP}$ or a fluorescent dye. The radioactively labelled products were resolved on 6% polyacrylamide gels and scored via reference to a known size clone. The fluorescently labelled products were run on an ABI 310 genetic analyser with an internal size standard and analysed using Genescan software (Applied Biosystems 2000).

Parentage Assignment

We used conventional exclusion analysis to identify the fathers of offspring. Because of the relatively small percentage of hatchlings for which we could identify the father (see Results), we estimated the number of sires contributing to each clutch using the alleles of the eight loci at which all mothers and hatchlings were genotyped. To do this, we assumed that all sires were heterozygous based on the high observed mean heterozygosity of 0.788 (range 0.664–0.868) for these eight loci (Blouin-Demers & Gibbs 2003). The number of sires per clutch was inferred to be the smallest whole number greater than or equal to one-half the number of paternal genotypes in that clutch (Parker & Kornfield 1996; DeWoody et al. 1998; Kellogg et al. 1998). For each hatchling in a clutch, alleles contributed by the father were deducted by subtraction of the maternal alleles, a procedure that always yields an unambiguous allelic assignment for the father, except when a hatchling displays the same heterozygous genotype as its mother. In these rare ambiguous cases, we assigned the two alleles as one possible allelic contribution and tallied this contribution only if none of the two paternal alleles had been detected unambiguously in other hatchlings of the same clutch. This method of assignment is highly accurate when three or fewer fathers contribute to a clutch (as was always the case in this study), but increasingly underestimates the true number of fathers for clutches with larger parental numbers (Fiumera et al. 2001).

Analyses

We obtained equations relating mass to size of males and females captured between 1996 and 2001 by regressing the log of body mass on the log of snout–vent length for the first capture (to avoid pseudoreplication) of 448 males and 329 females with snout–vent lengths of at least 800 mm. The data were log transformed because growth trajectories in snakes are not linear (Blouin-Demers et al. 2002). We then obtained an index of relative body condition for all potential sires and mothers by calculating the residuals of the regression equations using SVL and mass (log transformed) measurements of sires and mothers that were closest to the mating season when they produced offspring. Because reptiles grow throughout life, there can be a four-fold mass difference between mature animals.

Therefore, being 5 g above the predicted mass should be more important for a small snake than for a large snake and we standardized our condition index by dividing it by the predicted mass (again log transformed) multiplied by 100. Our condition index thus represents a percentage above or below the predicted mass for each sex.

We calculated relative clutch masses for females by expressing the mass of the clutch (measured on an electronic scale) as a percentage of the postoviposition mass of the female. The radioimplanted females were captured within 1 week after laying to measure their postoviposition mass. We considered all eggs (completely shelled or not) laid by a female to be part of her clutch, although only four of the 34 (12%) clutches used in our analyses contained some eggs that were incompletely shelled (smaller and more yellow than normal eggs). Incompletely shelled eggs do not appear to be a consequence of laying in captivity; among all clutches we have collected, including those used here, clutches with incompletely shelled eggs were more common in the field than in the laboratory (16% versus 11%).

Relative tail length was tail length expressed as a proportion of snout–vent length. To quantify sexual dimorphism in tail length we used measurements from the first capture (to avoid pseudoreplication) of all mature individuals (greater than 800 mm SVL) between 1996 and 2001. Individuals that were clearly missing part of their tails were excluded.

We calculated coefficients of relatedness (r ; Queller & Goodnight 1989) between each mother and all potential fathers using the program Relatedness (Goodnight 2003). Our tests of the effects of genetic similarity are conservative because the standard errors associated with estimates of relatedness are generally very large given the number and variability of loci we used.

We inspected box plots and residual plots to verify the assumptions of normality and homogeneity of variance. All statistical analyses were two-tailed and were conducted using JMP version 5 (SAS Institute 2002). We considered statistical results significant at $P = 0.05$. When statistical results were marginally significant ($0.05 < P < 0.20$), we calculated standardized effect size (D) (Cohen 1977) and power to aid their interpretation. We adopted Cohen's (1977) criteria regarding standardized effect size: 0.20 was deemed small, 0.50 was deemed medium, and 0.80 was deemed large.

RESULTS

Occurrence and Consequences of Polyandry

Based on the number of paternal alleles in the 34 clutches for which we knew the maternal genotypes, four clutches (12%) were sired by a single male, 21 clutches (62%) were sired by two males, and nine clutches (26%) were sired by three males. Therefore, 88% of clutches were multiply sired. The number of sires did not increase with female size (multiple ordinal logistic regression: Wald $\chi^2_1 = 0.053$, $N = 34$, $P = 0.817$) or with clutch size (Wald $\chi^2_1 = 0.507$, $N = 34$, $P = 0.476$). Hatching success

increased with the number of sires, but not significantly (ANOVA: $R^2 = 0.141$, $F_{2,31} = 2.547$, $N = 34$, $P = 0.095$, $D = 0.387$, power = 0.471). The power of this test was low despite a large effect size, because the sample size of singly sired litters was small. Thus, the nonsignificant relationship was attributable to lower mean hatching success of singly sired litters (58%) relative to those sired by two (86%) or three males (88%; Fig. 1). This relationship did not appear to be caused by a reduced ability to detect multiple paternity in clutches with low hatching success because the number of neonates genotyped per clutch did not affect the number of sires detected (ordinal logistic regression: $R^2 < 0.001$, Wald $\chi^2_1 = 0.008$, $N = 34$, $P = 0.927$). The number of sires was not associated with the mean mass of hatchlings in a clutch (ANOVA: $R^2 = 0.021$, $F_{2,29} = 0.313$, $N = 32$, $P = 0.734$), but there was a marginal association with the mean SVL of offspring (ANOVA: $R^2 = 0.110$, $F_{2,29} = 1.792$, $N = 32$, $P = 0.185$, $D = 0.335$, power = 0.344). The marginal relationship between number of sires and mean SVL of offspring is difficult to interpret, however, because litters sired by two males had longer mean offspring SVL (280 mm) than those sired by one (274 mm) or three (272 mm) males.

Male Attributes and Paternity

After conventional paternity exclusion analysis, 221 hatchlings (58.7%) had no matching sire among the sampled males, 144 hatchlings (38.3%) had one sire, and 11 hatchlings (2.9%) had two to eight sires. The mean coefficient of relatedness within groups for each of the 11 groups of multiple sires averaged 0.37 ± 0.10 (range 0.23–0.58, the range expected for groups made up of varying numbers of full and half-siblings). We restricted our analysis of male attributes to fathers of singly sired hatchlings ($N = 39$ sires, $N = 144$ hatchlings). Thus, in the following analysis, we address variance in success between successful

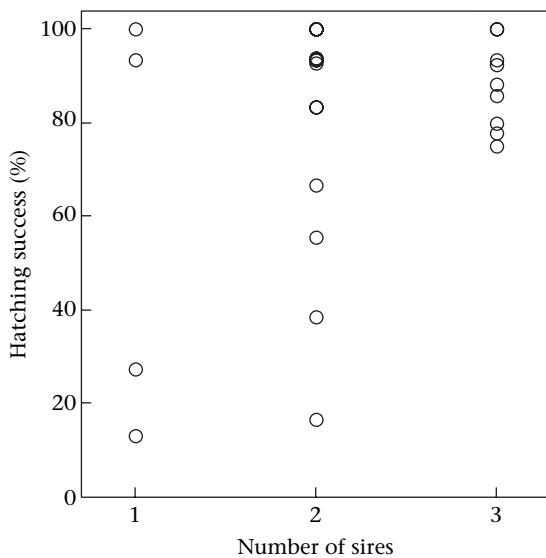


Figure 1. Hatching success of black ratsnake clutches as a function of the number of sires ($N = 34$ egg clutches).

males because we could not identify with certainty which males were unsuccessful. Among the 39 males identified as having sired offspring, the smallest sire was 813 mm SVL, and condition varied from -3.4% to 4.5% . We determined sexual dimorphism in tail length (TL) from 227 females ($\bar{X} \pm SE$ TL = $17.5 \pm 0.01\%$ of SVL, range 15.0–20.5%) and 247 males ($19.6 \pm 0.01\%$ of SVL, range 16.7–22.7%). The difference was highly significant (t test: $t_{472} = 19.29$, $P < 0.001$). Consistent with our predictions, larger males sired more offspring than smaller males (multiple ordinal logistic regression: Wald $\chi^2_1 = 3.878$, $N = 39$, $P = 0.049$; Fig. 2), but number of offspring sired by males with longer and shorter tails did not differ (Wald $\chi^2_1 = 0.353$, $N = 39$, $P = 0.552$). Inconsistent with our prediction, however, males in better condition did not sire more offspring than males in poorer condition (Wald $\chi^2_1 = 1.036$, $N = 39$, $P = 0.309$). Similarly, larger males sired a larger proportion (log transformed) of offspring per clutch (multiple linear regression: $R^2 = 0.120$, $F_{1,42} = 6.299$, $N = 46$, $P = 0.016$; Fig. 2), whereas males with longer tails ($R^2 < 0.001$, $F_{1,42} = 0.003$, $N = 46$, $P = 0.956$) or males in better condition ($R^2 = 0.004$, $F_{1,42} = 0.216$, $N = 46$, $P = 0.644$) did not.

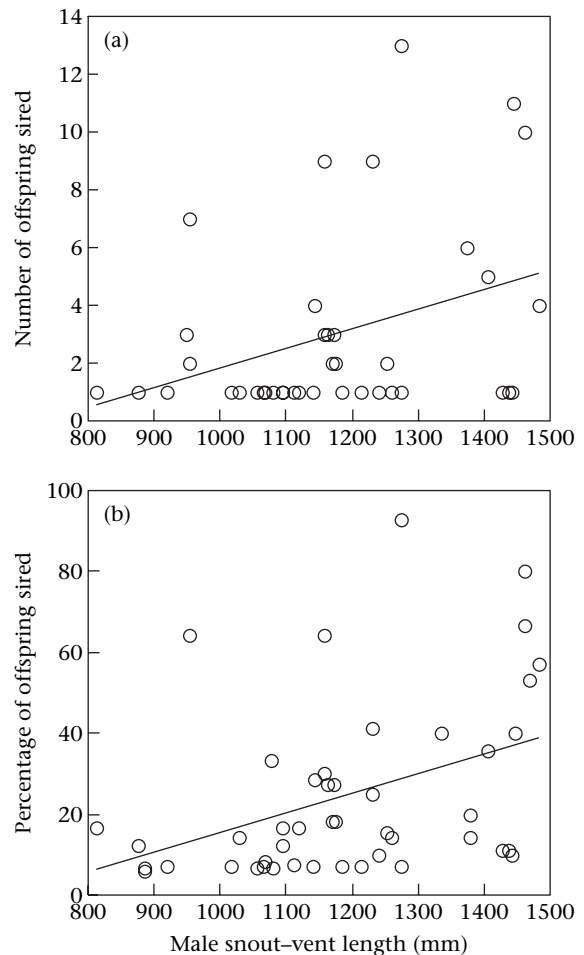


Figure 2. Number of offspring (a) and percentage of clutch (b) sired by individual black ratsnakes ($N = 39$) as a function of snout-vent length of the sire.

In total, 34 males (87%) sired offspring with a single female, four males (10%) sired offspring with two females, and only one male (3%) sired offspring with three females. Consistent with our prediction, males with longer tails did not mate with more females than did males with shorter tails (multiple ordinal logistic regression: Wald $\chi^2_1 = 0.059$, $N = 39$, $P = 0.809$). Inconsistent with our predictions, however, larger males and males in better condition did not mate with more females than did smaller males (Wald $\chi^2_1 = 0.693$, $N = 39$, $P = 0.405$) or males in poorer condition (Wald $\chi^2_1 = 0.229$, $N = 39$, $P = 0.632$). The total number of offspring sired by a male increased with number of mates (Pearson moment correlation: $r_{37} = 0.428$, $P = 0.007$).

Although larger females had lower relative clutch masses than smaller females (linear regression: $R^2 = 0.122$, $F_{1,32} = 4.464$, $N = 34$, $P = 0.043$), larger females tended to produce larger ($N = 35$, $R^2 = 0.089$, $F_{1,33} = 3.237$, $P = 0.081$, $D = 0.304$, power = 0.416) and heavier clutches ($N = 35$, $R^2 = 0.065$, $F_{1,33} = 2.308$, $P = 0.138$, $D = 0.257$, power = 0.314; Fig. 3). Despite medium effect sizes, power was low because of small sample size. Therefore, we believe that these results support our assumption that larger females should be more fecund, which could make them more attractive to males. The sizes of the mothers and the male(s) that sired their offspring (each pair represented only once) were positively correlated, but this relationship was not significant (Pearson moment correlation: $r_{43} = 0.205$, $P = 0.177$, $D = 0.204$, power = 0.268; Fig. 4). Again, despite a medium effect size, the power for this test was low because of small sample size.

We found little evidence that offspring phenotypes varied with those of either parent. Offspring SVL was unaffected by paternal or maternal SVL and condition (multiple linear regression: paternal SVL: $P = 0.654$; paternal condition: $P = 0.459$; maternal SVL: $P = 0.360$; maternal condition $P = 0.353$). Males in better condition

did sire significantly lighter offspring (multiple linear regression: $R^2 = 0.041$, $F_{1,113} = 5.023$, $N = 118$, $P = 0.027$). Although longer males tended to produce lighter offspring ($R^2 = 0.017$, $F_{1,33} = 2.013$, $N = 118$, $P = 0.159$, $D = 0.131$, power = 0.290), the effect size was small. Females in better condition tended to produce heavier offspring ($R^2 = 0.017$, $F_{1,33} = 2.048$, $N = 118$, $P = 0.155$, $D = 0.132$, power = 0.295), but again the effect size was small. Offspring mass was unrelated to maternal SVL ($R^2 = 0.001$, $F_{1,33} = 0.139$, $N = 118$, $P = 0.711$).

Genetic Similarity

Females tended to be slightly more related to the males that sired their offspring (mean \pm SE $r = 0.017 \pm 0.200$) than to all the other males sampled ($r = 0.001 \pm 0.059$). However, the mean coefficient of relatedness of a mother and her mate(s) and her mean coefficient of relatedness to all other sexually mature males that we sampled were not significantly different (each pair only represented once, paired t test: $t_{44} = 0.659$, $P = 0.513$). The hatching success of a clutch was not related to the mean coefficient of relatedness of the mother and her mates (linear regression: $R^2 = 0.019$, $F_{1,24} = 0.454$, $N = 26$, $P = 0.507$). The coefficient of relatedness between the male and the female did not affect the number of offspring they produced ($R^2 < 0.001$, $F_{1,44} = 0.003$, $N = 46$, $P = 0.956$), the proportion of the clutch sired by the male ($R^2 < 0.001$, $F_{1,44} = 0.008$, $N = 46$, $P = 0.928$), the mass of the offspring ($R^2 < 0.001$, $F_{1,117} = 0.018$, $N = 119$, $P = 0.895$), or the length of the offspring ($R^2 < 0.001$, $F_{1,116} = 0.062$, $N = 4118$, $P = 0.804$).

Spatial Patterns

The effect of size on mating patterns reported above could arise because home range sizes vary with the size of snakes. To assess this possibility we reanalysed the data

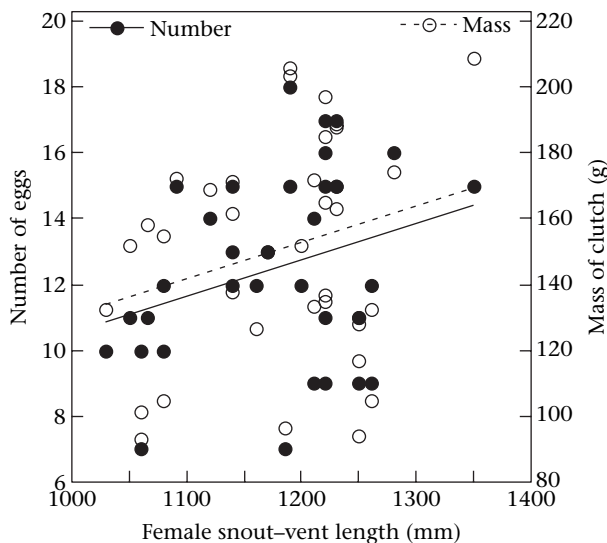


Figure 3. Size and mass of black ratsnake clutches as a function of female snout-vent length.

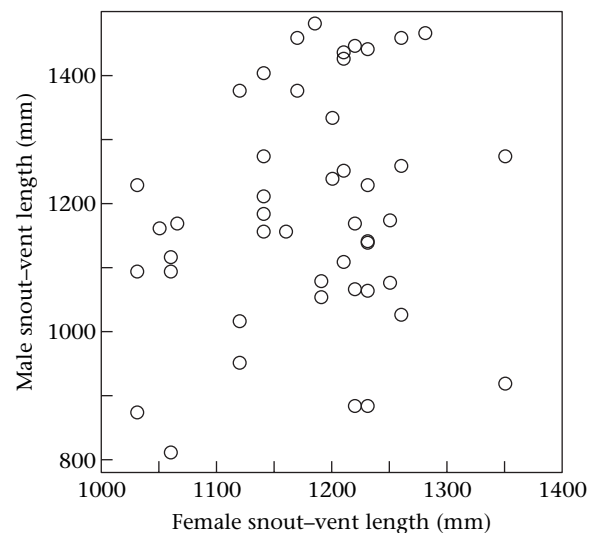


Figure 4. Correlation between the snout-vent lengths of male-female black ratsnake pairs that produced offspring.

presented by Blouin-Demers & Weatherhead (2002) on the home ranges (100% minimum convex polygon, square-root transformed) of ratsnakes in the same study population. Using data for the whole active season, reproductive status (male, reproductive female, nonreproductive female) had no effect on home range size, but larger individuals had larger home ranges (ANCOVA: status*SVL: $F_{2,47} = 0.566$, $N = 53$, $P = 0.572$; status: $R^2 = 0.022$, $F_{2,47} = 0.0672$, $P = 0.516$; SVL: $R^2 = 0.142$, $F_{1,47} = 8.736$, $P = 0.005$).

The 144 hatchlings with a single matching sire were produced by 45 different pairs (26 females and 39 males). We knew the hibernaculum membership of the two parents (both the male and the female were captured at least once at their hibernaculum) for 18 different pairs (10 females and 17 males). Five of the 18 pairs (28%) were from the same hibernaculum and 13 of the 18 pairs (72%) were from different hibernacula. For these 18 pairs, the mean distance between the hibernacula of the female and male was significantly greater (Wilcoxon matched-pairs signed-ranks test: $Z = 47.50$, $P = 0.038$) than the mean distance between nearest-neighbour hibernacula (811 m: Blouin-Demers & Weatherhead 2002). For the 13 pairs from different hibernacula, the distance between the hibernaculum of the male and female averaged 2.3 km (range 0.5–4.2 km). For four of the 10 females of known hibernaculum membership, more than one sire with known hibernaculum membership was identified. For three females (75%), the multiple sires identified were from different hibernacula: in two instances the two sires were from two hibernacula and in one instance the three sires were from three hibernacula. For the other female, both sires were from the same hibernaculum.

DISCUSSION

We screened 426 males to try to identify the sires of 375 hatchlings from 34 clutches of black ratsnakes with known mothers. We successfully identified 39 males as the sires of 144 hatchlings. Although our overall success at identifying sires was low, this approach is clearly superior to what one can learn about the mating system of the species by observation alone. Blouin-Demers & Weatherhead (2002) radiotracked 82 black ratsnakes that they relocated 5657 times over 5 years but still only witnessed 11 matings. We first consider the implications of having a large number of offspring for which we could not identify the sire. Next, we consider the implications of the results for those for which sires were identified.

The most common reason that we could not identify the sire for a given offspring was that we excluded all 426 potential sires that we screened. Thus, despite our extensive capture of black ratsnakes in the study area, we failed to capture and sample the sires for 59% of offspring. Our trapping success at hibernacula in the study area was high, but opportunistic captures of black ratsnakes during the active season were scant in comparison (Blouin-Demers et al. 2000a; Prior et al. 2001). Thus, males that hibernated outside the study area but spent at least part of the mating season within the study area had a low probability of

having been sampled. This explanation is consistent with known movement patterns of males relative to the scale of our study area (2.5×9.5 km). The mean male home range size is 25 ha (equivalent to a circle of ≈ 550 m diameter), but is as large as 93 ha (equivalent to a circle of ≈ 1100 m diameter), and the mean commuting distance from the hibernaculum to the home range for males is 400 m, but is as high as 4 km (Blouin-Demers & Weatherhead 2002). Thus, it is likely that many males present in the study area during the mating season hibernated outside the study area and that, conversely, many males that hibernated in the study area (and that we screened as potential sires), spent their mating season outside the study area. Although less vagile than males, female ratsnakes also move extensively, particularly when reproductive (Blouin-Demers & Weatherhead 2002). Thus, some females that nested in the study area could have spent the mating season outside the study area, thereby increasing the likelihood of mating with males we had not sampled.

A second reason that we could not identify sires was that for 3% of hatchlings, we failed to exclude more than one male as the potential sire. We had one hatchling for which eight males had genotypes consistent with being the sire. Given the high heterozygosity of the microsatellite loci we used, and the fact that our study population is outbred (Lougheed et al. 1999), this result was unexpected. The mean genetic similarity ($r = 0.37$) among the males that were identified as the possible sire of the same hatchlings was approximately midway between that of half- (0.25) and full siblings (0.5) and below that of fathers and sons (0.5), suggesting that these males were relatives. Having this many close relatives in our study population suggests that natal dispersal may not be extensive. Assessing this possibility will require direct data on natal dispersal, something that is currently unavailable for ratsnakes, or indeed any other snake species.

We detected multiple paternity in 88% of clutches. This is similar to what Barry et al. (1992) found, and higher than what Prosser et al. (2002) found for northern watersnakes. Unlike watersnakes, ratsnakes mate while dispersed, so the high incidence of multiple paternity is not simply a consequence of opportunity. Female ratsnakes that are reproductive increase rates of movement during the mating season, suggesting they are actively seeking mates (Blouin-Demers & Weatherhead 2002). Furthermore, the incidence of multiple paternity was unrelated to female size, although larger females had larger home ranges, suggesting that it does not arise because some females are more attractive to males: male ratsnakes appear to mate indiscriminately. Although indirect, the evidence suggests that females actively seek multiple mates. If so, they presumably pay some cost in energy and possibly risk of predation and thus should realize some benefit. In an inbred population of European adders, embryo viability was higher for females that mated with multiple mates (Madsen et al. 1992). Similarly, in an inbred population of sand lizards, *Lacerta agilis*, multiple mating appeared to enhance hatching success of eggs and subsequent offspring viability (Olsson et al. 1994). In both cases, multiple mating may increase a female's chance of obtaining sperm from a distantly related male, thereby

avoiding deleterious effects of inbreeding. Although our results were inconclusive, we found that the hatching success of black ratsnake clutches tended to increase with the number of sires. Because our study population is outbred (e.g. mean coefficient of relatedness between males and females = 0.0009 ± 0.0011), it seems unlikely that multiple mating functions to reduce costs of inbreeding. Multiple mating by female black ratsnakes may serve as insurance against male infertility or incompatibility (for reasons other than inbreeding). Of the 99 clutches that we collected, 12% contained at least one nonviable egg, and some clutches contained up to eight (62%) such eggs.

Another putative benefit of polyandry is sperm competition leading to enhanced offspring quality. Thus, it is also possible that multiple mating by female ratsnakes produced more viable offspring, a hypothesis that can only be tested by determining the fate of offspring after hatching. Our results, however, indicated that offspring from multiply sired clutches were not heavier, and probably not longer. In addition, we found no evidence that larger males (that appear more successful in sperm competition) produce offspring of higher phenotypic quality. In fact, although the results were inconclusive, females may pay a cost of mating with large males if large males actually sire lighter offspring. Thus, females may benefit from polyandry through enhanced hatching success when sperm competition promotes fertilization by high-quality or compatible sperm, but not when sperm competition promotes fertilization by large males.

Reproductive success of male black ratsnakes increased with body size, consistent with our predictions based on male-biased sexual size dimorphism and male combat characteristics in this species. Our prediction was based on the expectation that larger males should get access to more females, both because larger males have larger home ranges and because larger males should be more successful in combat with other males. However, the success of larger males was apparently attributable to their siring more young per litter rather than to siring young in more litters. We may have failed to detect the predicted pattern because our methods did not allow us to sample all of the reproductive females in the population. This had two consequences. First, we could not identify with certainty males that failed to mate, and second, we could not estimate the total reproductive success for those males that did mate. This considerably reduced the power of our analyses. Prosser et al. (2002) showed that reproductive success for male northern watersnakes was affected most strongly by the number of females with which they mated. We found that the number of offspring sired by male ratsnakes was positively correlated with their number of mates. This positive correlation and the fact that larger males have larger home ranges suggest that with more comprehensive sampling, we might have found that larger males were more successful both by mating with more females and by siring more young per clutch.

It is interesting none the less that larger males sired more young per clutch. Three factors potentially contributed to this pattern. First, although the results were inconclusive, larger males tended to mate with larger

females, which, in turn, tended to produce larger clutches. Second, larger males are likely to have larger testes (Shine et al. 1999) and thus produce larger ejaculates. Thus, sexual size dimorphism in ratsnakes could be favoured by enhanced success at sperm competition in addition to enhanced success at combat. Third, the sperm of larger males might be more competitive. The fact that male SVL was positively correlated with the percentage of a clutch sired and that the relationship between parental SVL and the number of offspring sired ($R^2 = 0.096$) was better explained by male SVL ($R^2 = 0.048$) than by female SVL ($R^2 = 0.030$) suggests that more abundant or more competitive sperm of large males is part of the explanation. Our data do not allow us to distinguish definitively between these alternatives.

Given the success of larger males, it seems surprising that some quite small males sired young. In fact, the smallest sire had a SVL of 813 mm, which corresponds to an age of 5.5 years according to the growth models of Blouin-Demers et al. (2002). Based on limited observations of mating behaviour, Blouin-Demers et al. (2002) estimated sexual maturity for male black ratsnakes to occur at 1050 mm SVL, or approximately 9 years old. Overall, approximately 18% of males that sired young were smaller than this previous estimate of size at maturity. Because female ratsnakes are dispersed during the mating season, small males must have a good chance of encountering and mating with females without having to fight other males for access. We have no direct data concerning how male reproductive effort varies with size, but males may mature sexually while quite young so they can mate opportunistically, and then increase reproductive effort as they get older and more competitive. The fact that home range area increases with male size is consistent with this suggestion.

Blouin-Demers & Weatherhead (2002) showed that the movement patterns of black ratsnakes allowed ample opportunity for mating between members of different hibernacula because the home ranges of males and females of different hibernacula overlapped widely during the mating season. In the absence of genetic data, however, they could not measure the extent to which this potential was realized. Here we showed that 72% of matings involved snakes from different hibernacula and that, on average, females mated with individuals from hibernacula approximately three times the mean distance from their own. Therefore, even if natal dispersal is low, as some of our results suggest, these mating patterns are sufficient to account for the lack of genetic structure among local hibernacula populations (Lougheed et al. 1999).

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