Male Reproductive Success and Multiple Paternity in Wild, Low-Density Populations of the Adder (Vipera berus)

SYLVAIN URSENBACHER, CECILE ERNY, AND LUCA FUMAGALLI

From the Laboratoire de Biologie de la Conservation, Département d’Ecologie et Evolution, Biophore, Université de Lausanne, Switzerland (Ursenbacher, Erny, and Fumagalli); and the School of Biological Sciences, University of Wales—Bangor, Bangor LL57 2UW, UK (Ursenbacher).

Address correspondence to S. Ursenbacher, Department of Environmental Sciences, Section of Conservation Biology, University of Basel, St Johanns-Vorstadt 10, CH-4056 Basel, Switzerland, or e-mail: s.ursenbacher@unibas.ch.

We studied for the first time the occurrence of multiple paternity, male reproductive success, and neonate survival in wild, low-density adder (Vipera berus) populations using 13 microsatellite loci. Paternity was assigned for 15 clutches, collected during 3 years. Our data demonstrated that multiple paternity can occur at a high level (69%) in natural populations of V. berus, even if the density of adults is low. The high proportion of multiple sired clutches was comparable to the proportion observed in captive populations. Male reproductive success significantly increased with body length, and only the largest males successfully sired entire clutches. Finally, no relationship was detected between the number of fathers per clutch and neonate survival. These results suggest that multiple matings could be beneficial in populations with high level of inbreeding or low male fecundity.

Key words: Male reproductive success, multiple paternity, reptile, Vipera berus

Multiple paternity has been documented in numerous invertebrate and vertebrate taxa (Birkhead and Møller 1998). This occurs even though a single copulation is usually sufficient to fertilize an entire clutch (Orsetti and Rutowski 2003; but see Madsen et al. 1992). In the absence of direct benefits for females, such as paternal care, indirect genetic advantages might outweigh the costs associated with polyandry (e.g., Simmons 2005). It has been hypothesized that the genetic benefits of polyandry in such species is the acquisition of more good genes as well as an increase in genetic diversity within clutches (for a review, see Yasui 1998). For males, the benefit of multiple copulations with different females is generally an increase in fecundity, especially in species without postcopulatory investments (Garner et al. 2002). However, the search for females is time and energy consuming, and population density could have an impact on mating opportunities. Consequently, low-density populations show sometimes reduced levels of multiple paternity (Garner et al. 2002; but see also Gromko and Gerhart 1984; Ochando et al. 1996). In addition, male reproductive success is often related to different morphological characters, such as body size as observed in mammals (e.g., Le Boeuf and Reiter 1988) or bird species (e.g., Bryant 1988; Grant PR and Grant BR 2000).

Multiple paternity studies and male reproductive success are numerous for birds and mammals (see Reynolds 1996; Birkhead and Møller 1998), whereas snakes have been less studied (Höggren and Tegelström 1995; Garner et al. 2002; Prosser et al. 2002).

The adder (Vipera berus) is a viviparous snake widely distributed throughout northern Eurasia. The female reproductive cycle is bi- or triennial, therefore the operational sex ratio is biased toward males (Madsen and Shine 1993). Males actively court females 3–4 weeks per year. During this limited period, vigorous combats for access to receptive females occur and are generally won by the largest males (Madsen 1988; Madsen and Shine 1992; Luiselli 1993b). Short-term postcopulatory mate guarding is common (Andrén 1986); however, multiple matings (with the same or different male) do occur (Andrén and Nilson 1987; Madsen et al. 1992; Luiselli 1993a), mainly due to sneaky males (males which copulate with females while the other are fighting). Although the presence of a “copulatory plug” preventing further mating has been suggested by Nilson and Andrén (1982), multiple paternity has been observed in captivity (Stille et al. 1986; Höggren and Tegelström 1995, 2002). Based on molecular techniques, Höggren and Tegelström (2002) have shown that the first male to mate with a female sired the highest number of her offspring. Contrary to several other snake species, long-term sperm storage in adders is unlikely (Luiselli 1993a; Höggren and Tegelström 1996). Moreover, newborn viability in wild
significant genetic differentiation was detected (migration was observed between 1997 and 2004 and are considered to be isolated from each other because no coming). These 2 populations (separated by about 1.5 km) (Populations CH1 and CH2 in Ursenbacher et al., forthcoming) have only been documented in captive, high-density conditions. In natural, low-density populations, we rather expected a limited level of multiple paternity due to a presumed difficulty of finding mates. In addition, we investigated whether the number of fathers per clutch impacted neonate survival rate.

Methods

Sampling

Snakes were caught by hand, measured (snout–vent length [SVL]), weighed, marked with a microchip (DataMars SA, Bedano, Switzerland) and tissue samples collected in the field (50 µl of blood) over a 3-year period (2000–2002) from 2 distinct populations in the Swiss Jura mountains (Populations CH1 and CH2 in Ursenbacher et al., forthcoming). These 2 populations (separated by about 1.5 km) are considered to be isolated from each other because no migration was observed between 1997 and 2004 and significant genetic differentiation was detected ($F_{ST} = 0.041$, $P < 0.005$). Moreover, the estimated population densities are very low (about 1 adult/ha, Ursenbacher and Monney 2003), whereas populations in the Alps usually harbor 3–11 adults/ha (see Neumeyer 1987; Luiselli 1993b). To obtain clutches, all pregnant females captured just before parturition were released at their exact capture point. Only one clutch per female was analyzed.

Microsatellite Amplification and Parentage Analyses

DNA extraction and amplification of 13 microsatellite markers have been conducted following Ursenbacher et al. (forthcoming). Parentage assignments were performed by a likelihood approach using the program CERVUS 2.0 (Marshall et al. 1998), which is particularly sensitive to the proportion of candidate adults sampled (Jones and Ardren 2003). This proportion was consequently estimated using the module CAPTURE (Otis et al. 1978) from the software MARK 3.2 (White and Burnham 1999). The proportion of candidate adults sampled was estimated for each population and each year between 1997 and 2002. The mean percentage of captured males was estimated to 82% using the model $M[t]$ in CAPTURE. Estimation of genotyping errors was performed by repeating the genotyping procedure 2–5 times for samples showing doubtful results, by counting incompatibilities between the genotypes of the mother and her offspring, and by CERVUS which calculates the ratio of the number of mismatches to the number compared, scaled by the average probability of detecting a mismatch. Because the 2 former approaches probably give an over- and underestimation, respectively, genotyping error rate of 1.6% estimated by the latter approach was used in the CERVUS simulations to assess assignment reliability. Finally, the occurrence of null (nonamplifying) alleles, which might appear in some of the 13 microsatellite markers (see Ursenbacher et al. forthcoming), was evaluated by CERVUS. Testing for the presence of null alleles is important because they can cause false exclusions in the assignments (see Jones and Ardren 2003). Although CERVUS can estimate their frequency for each locus and accommodate for their occurrence, Jones and Ardren (2003) suggest considering all homozygous genotypes as heterozygotes possessing the null allele, in order to prevent false exclusions. Paternity assignments were performed with and without this adjustment and results were compared.

Reproductive Success of Males

The relationship between SVL of males and their annual reproductive success was tested via regression. The SVL was also compared between fathers and males without assigned offspring as well as between fathers detected in monopaternal and in multiple paternity clutches (both using $t$-tests or Mann–Whitney $U$ tests depending on the normality of the data). Because some males were not captured during the year, they succeeded in mating; SVL was evaluated for all fathers a posteriori, using the following approach: The SVL was corrected by 0.01 cm per day between the mean date of the mating and the nearest capture (when the capture was done the year before, only 210 days were added due to hibernation). For juvenile males, a growth rate of 0.05 cm per day was used up to the maturity (37 cm). These growth rates were based on capture–recapture data obtained in the 2 populations (Ursenbacher S, unpublished data). We also tested whether the number of fathers for a clutch had an impact on number of offspring or neonate survival rate via linear regression with an angular transformation. In order to avoid pseudoreplication (multiple presence of a male in a test), each year was tested separately or only the first detected paternity was taken into account.

Results

Parentage Assignments

Fifteen pregnant females (7 in the first population, 8 in the second) were captured and gave birth to 115 offspring (Table 1); 43 males (25 in the first population, 18 in the second) were considered as potential fathers. Between 2
Table 1. Reproductive females, number of offspring, estimated number of fathers within the clutch, number of offspring not assigned, and morphometric measurements of the female before laying

<table>
<thead>
<tr>
<th>Population</th>
<th>Female code</th>
<th>Number of offspring</th>
<th>Number of fathers in the clutch (P ≥ 80%)</th>
<th>Number of offspring not assigned (P &lt; 80%)</th>
<th>Female SVL (cm)</th>
<th>Female weight (g)</th>
<th>Number of dead offspring</th>
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</thead>
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<td>16</td>
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<td>3 (2)</td>
<td>0 (1)</td>
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<td>9</td>
<td>2 (2)</td>
<td>0 (0)</td>
<td>52.2</td>
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<td>48</td>
<td>10</td>
<td>5 (4)</td>
<td>5 (4)</td>
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<td>2 (2)</td>
<td>0 (0)</td>
<td>53.9</td>
<td>144</td>
<td>3</td>
</tr>
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<td>8 (5)</td>
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<td>0</td>
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<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>54.8 ± 2.83</td>
<td>127.3 ± 27.1</td>
<td></td>
</tr>
</tbody>
</table>

In brackets, the results obtained without the correction suggested by Jones and Ardren (2003). In clutches of female 48 and female 66 (italic), less than 70% of the neonates were assigned to a father, and both clutches were consequently not used in the analyses of multiple paternity and male behavior.

* Mean without females 48 and 66. Standard deviations for each means are indicated.

(D12) and 13 (D6) alleles were observed. CERVUS detected null alleles in 5 of the 13 loci (Vb-A8, Vb-B1, Vb-B18, Vb-D6, and Vb-D’13; Ursenbacher et al. forthcoming). Discrepancies in paternity assignment with and without the correction suggested by Jones and Ardren (2003) occurred for 9 neonates only. Paternity was assigned to 102 (99 without correction) of the 115 offspring sampled with 80% or 95% confidence, the majority (56%; 45% without correction) at the 95% level (Table 1). Ten (11 without the correction) of the 13 (16) unassigned offspring belonged to 2 clutches from females living at the edge of the first population. Parental assignment in these 2 clutches was low (50% and 62%, respectively), and thus, they were excluded from the behavioral analyses below.

Male Reproductive Success and Multiple Paternity

Only 4 clutches (31%) were fathered by a single male, and all belonged to the second population. For remaining clutches, 3 were fertilized by 2 males, 5 clutches by 3 males, and 1 clutch by 4 different males (Table 1). The number of fathers per clutch was not significantly different between the 2 studied populations (meanpop1 = 2.4; meanpop2 = 2.1; t-test: t11 = 0.460, P = 0.65). Twenty-one different fathers (18 without correction) were detected in the 13 clutches, 5 (5) of them in 2 different clutches and 1 (2) in 3 clutches (sometimes across different years). Fathers that sired offspring in more than one clutch were significantly larger (SVL corrected) than other fathers (meanoneclutch = 43.9; meanmore than one clutch = 49.4; U = 12, N1 = 14, N2 = 7, P = 0.006; P < 0.001 without correction). Furthermore, we found that monopaternal clutches were fathered by males larger than other fathers (data of the 3 years pooled: meanmonopaternity fathers = 51.4; meanmultipaternity fathers = 46.1; U = 67, N1 = 17, N2 = 4, P < 0.001; P < 0.001 without correction). In addition, the annual number of offspring sired by a male was correlated with paternal body length (SVL corrected; Spearman rank correlation: rS = 0.252, N = 21, P = 0.020; see Figure 1; P = 0.005 without correction). Moreover, the number of fathers of a clutch had no impact either on the number of offspring (rS = -0.174, N = 13, P = 0.549; P = 0.882 without correction) or on the neonate survival rate (rS = 0.176, N = 13, P = 0.954; P = 0.524 without correction).

Discussion

Multiple Paternity

Our study demonstrates for the first time that multiple paternity occurs in natural populations of V. berus, even if the density of adults is low. The proportion of multiple paternity (69% of the clutches were fathered by more than one male) observed in our study is comparable to that reported by Höggren and Tegelström (2002) for captive populations. The high level of multiple paternity observed in these natural populations questions the copulatory plug hypothesis (Nilson and Andrén 1982). These authors described male renal sex-segment secretions causing a contraction of the female uterus after copulation and suggested that this phenomena could prevent fertilization by subsequent copulating males. This copulatory plug hypothesis has already been questioned by Stille et al. (1986) and Höggren and Tegelström (1995). Although male renal secretions do not seem to prevent further fertilization by other males, they could reduce the fecundity from further matings within a season as observed by Höggren and Tegelström (2002).
highlights the need for a meta-analysis in order to determine species with no parental care, such as most snakes, respectively). Therefore, the lack of a consistent pattern in studies have been published to our knowledge. For instance, species with limited interactions between sexes, only few been obtained for species that mainly live in couples. For observed discrepancy. However, these assumptions have impact on female mating strategies, which can explain the Kokko and Rankin (2006) suggested that density has an lack of correlation (e.g., Veiga and Boto 2000). In a review, Bjornstad and Lifjeld (1997), whereas others demonstrated a lack of correlation (e.g., Veiga and Boto 2000). In a review, Kokko and Rankin (2006) suggested that density has an impact on female mating strategies, which can explain the observed discrepancy. However, these assumptions have been obtained for species that mainly live in couples. For species with limited interactions between sexes, only few studies have been published to our knowledge. For instance, in the ovoviviparous fish *Heterandria formosa*, Soucy and Travis (2003) found a positive relationship between density and multiple paternity, which is probably related to a limited contact between males and females. No such correlations were observed in *Drosophila* species (Gromko and Gerhart 1984; Ochando et al. 1996). Comparing 2 populations of garter snake (*Thamnophis sirtalis*), Garner et al. (2002) suggested that the level of multiple paternity could be related to the density of males. The results stemming from our study do not confirm this hypothesis because the values observed in the adder are similar between our study (69% of multiple paternity; 100% in the first and 50% in the second population, although density is similar) and data from captive, high-density populations (84% and 75% in Höggren and Tegelström 1995 and Höggren and Tegelström 2002, respectively). Therefore, the lack of a consistent pattern in species with no parental care, such as most snakes, highlights the need for a meta-analysis in order to determine general trends concerning the relationship between density and multiple paternity levels.

### Impact of Body Size (SVL) on the Number of Offspring

Combats between males occur for access to reproductive females, and the largest males win most of the time (Andrén 1986; Madsen 1988; Luiselli 1993b) fathering more offspring (this study). Sneaky males do, however, mate and fertilize some ova. Our data show that, even though small males successfully fathered offspring, the biggest males fathered the majority of offspring or were the single fathers in a clutch. Only in rare circumstances were smaller males the dominant fathers of a clutch. This may occur when they are the first males to mate with a female, before being dislodged by bigger males during combats. Because smaller males are unlikely to father a large number of offspring and because they cannot outcompete bigger males for mating opportunities, their best option to enhance their reproductive success may be through multiple copulations.

### Neonate Survival Rate and Multiple Paternity

Madsen et al. (1992) observed a positive relationship between the number of copulations and the neonate survival rate, suggesting that sperm competition in the female’s reproductive tract occurred and that some males have a very low fecundity. We found no relationship between the number of fathers in a clutch and the survival of the neonates (confirming the observation of a lack of influence on survival rate between multiple and single mate copulations conducted by Capula and Luiselli (1994). The low neonate survival rate observed by Madsen et al. (1992) may be related to the low genetic diversity, and hence possibly low male fertility, in that particular population (Madsen et al. 1996; Madsen et al. 1999). In contrast, the Jura Mountain populations do not suffer from reduced genetic variability (Ursenbacher et al. forthcoming). Theoretically, when males have a low fertility, multiple copulations with different males have a particularly positive effect on offspring survival (see Loman et al. 1988) by avoiding deleterious effects of inbreeding and ensuring against male infertility. Consequently, multiple mating (with different males) should have a beneficial impact on the neonate survival rate particularly in populations characterized by a low genetic diversity and/or a low male fertility (see also Shine 2003; Blouin-Demers et al. 2005). Effects of multiple paternity should, however, not only be tested with respect to neonate survival but also in relation to offspring survival from birth to adult.

This study has demonstrated that in *V. berus*, the largest males father the majority of offspring. Moreover, multiple paternity appears to be common in low-density populations of this species. The influence of female sexual behavior has probably been underestimated in previous reptile studies. Further investigations focusing on female mate choice should thus be conducted to refine our knowledge of the reproductive strategies in *V. berus* and other snake species.

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**Figure 1.** Correlation between the corrected SVL of the father and its number of offspring per year (Spearman rank correlation: \( r_s = 0.252, N = 21, P = 0.02 \)).
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