

GENETIC EVIDENCE FOR FIRST-MALE MATING ADVANTAGE IN THE ADDER (*VIPERA BERUS*)

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ABSTRACT: Female Adders (*Vipera berus*) will copulate multiple times in the spring, either with one or several males. If copulations take place within several days of the female oestrus period, multiple copulations with different males can result in litters with mixed paternity. Reduced sperm competition in *V. berus* has been suggested as an effect of a first-male paternity guard in the female uterus. In order to investigate whether mating order influences paternity, we performed a controlled breeding experiment where eight female *V. berus* were each allowed to copulate sequentially with three unmated males, a different male each day for three consecutive days. DNA fingerprinting of all males, females, and offspring revealed a pronounced first-male advantage in all eight litters. We propose a mechanism of sperm precedence: paternity assurance induced by first-successful insemination. Although six of the litters exhibited mixed paternity (two or three sires), proportions of offspring were skewed toward the first male. From these results we show that the response does not completely prevent sperm transfer and fertilization by rival males if subsequent matings take place at the frequency provided in our experiment.

INTRODUCTION

Studies of reproductive strategies in polygamous mating systems have focused primarily on male benefits (Smith, 1979, 1984). More recently, however, emphasis is in understanding why females copulate repeatedly with the same or with different males (Andersson, 1994; Jennions and Petrie, 2000; for a critical review of the roles traditionally attributed to the sexes see Cunningham and Birkhead, 1998). Consequently, we have much better understanding of pre- and postcopulatory mechanisms of paternity control in females (reviewed by Birkhead and Møller, 1993; Hunter et al., 1993). Possible mechanisms include sperm competition (Parker, 1970) as well as various physiological and behavioral traits for female mate selection and avoidance of sperm competition (Knowlton and Greenwell, 1984). Thus, female control may involve trade-offs for negotiating conflicts between male and female interests (for an overview see Gowaty, 1994) in accordance with the sexual “arms race game” model (Dawkins and Krebs, 1979; Parker, 1984).

Research on multiple mating in females and sperm competition (and its consequences) is skewed toward two groups: insects and birds (Smith, 1984; Birkhead and Møller, 1998). Fortunately, current advances in DNA techniques offer prerequisites for studies of reproductive strategies in animal systems previously regarded intractable (Avise, 1994, 2000). Additional empirical approaches across all animals and plants are considered crucial for determining components of mating systems, as well as for further addressing the

sexual selection theory (Birkhead and Møller, 1993, 1998; Arnold and Duvall, 1994; Burke, 1994).

In a narrow sense, sperm competition is defined as sperm from rival males competing for ova to fertilize (Parker, 1970). This is an effect of a female mating with different males, either within a season or as a result of long-term sperm storage (Smith, 1984). In a broader sense, sperm competition also may include an array of paternity assurance mechanisms induced by a previous or subsequent copulation. These mechanisms can operate both before and after sperm transfer (Birkhead, 1994). Thus, in some instances, postcopulatory male-male competition can occur prior to an intra-uterine lottery (selection), but still at the gametic level. Specific mating order among rival males has proven decisive in numerous polygynous taxa. In birds, last-male sperm precedence seems to be obligatory, but paternity is also determined by the interval between copulations. Although detailed physiological data are lacking, limited sperm storage and indirect sperm displacement has been suggested as a plausible paternity assurance mechanism in birds (reviewed by Birkhead and Møller, 1992). In contrast, a first-male sperm precedence has recently been demonstrated in a mammalian mating system (Lacey, et al., 1997).

Unlike many other vertebrates, snakes do not typically form lengthy pair-bonds (with a few possible exceptions of biparental care; Shine, 1988), and multiple mating in females can make paternity assignment impossible without resorting to molecular methods. The difficulty, however, is finding genetic markers sufficiently powerful to discriminate between a comparably large number of potential sires (Tegelström and Höggren, 1994; Weatherhead et al., 1995; Gibbs and Weatherhead, 2001). Awaiting future development of molecular tools for large-scale screening of

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identity and relatedness in natural populations, controlled breeding experiments followed by DNA-profiling may reveal selected variables of snake mating systems.

Intra-specific differences in snake mating strategies may be due to the effects of geographic and ecological variation on thermal and nutritional requirements (Fitch, 1985; Seigel and Ford, 1987; Duvall et al., 1992, 1993; Olsson and Madsen, 1998; Schuett et al., this volume). Differing population structures, such as effective population sizes, operational sex ratios (OSR), and genetic composition have also been suggested to cause locally observed reproductive adaptations (see Loman et al., 1988). Hence, conflicting data on a wide-ranging species may reflect spatial variability in reproductive patterns. Moreover, temporal variation between seasons in the operational sex ratio has been demonstrated to considerably affect the mating system of an isolated *V. berus* population in southern Sweden (Madsen and Shine, 1993).

Typically, wild female *Vipera berus* mate either only once per season, or repeatedly but with the same male (Nilson and Andrén, 1982; Luiselli, 1993), or several times per season and with different males (Madsen et al., 1992). In *V. berus*, the mating season begins about one month following emergence from hibernation in spring, and copulation seems necessary for inducing ovulation (Volsøe, 1944; Luiselli, 1993). Females ovulate ca. one month after mating (Nilson, 1981), and duration of pregnancy is about three months. The litter size of *V. berus* usually ranges from five to 15. Also, because the female reproductive cycle is normally biennial, the operational sex ratio (OSR) is male-biased. Males fight vigorously for the opportunity to mate, and larger males generally win (Andrén and Nilson, 1987; Madsen and Shine, 1993). Short-term postcopulatory mate-guarding is common (Andrén, 1986); however, multiple copulations with different males can result in mixed paternity, at least if occurring within a short interval of estrus (Höggren and Tegelström, 1995). Long-term sperm storage between seasons, as reported in several other temperate snake species (Saint Girons, 1975; Gist and Jones, 1987; Schuett, 1992), is unlikely in *V. berus* (Höggren and Tegelström, 1996).

Male renal sex-segment secretions of the anterior kidney have been reported to induce a sperm-inhibiting response in female *V. berus*, caused by a contraction of the posterior oviduct (Nilson and Andrén, 1982). The principal function of such a mechanism, namely trapping or ejecting sperm, however, has been

subject to conflicting opinions (Stille et al., 1986, 1987; Andrén and Nilson, 1987). Physiologically-controlled paternity guards related functionally to the adaptation described by Nilson and Andrén (1982) have been documented in several squamate taxa, although the underlying mechanisms for reducing female attractivity and/or receptivity may vary between species investigated (see reviews by Devine, 1984; Mason, 1992; Whittier and Tokarz, 1992; Olsson and Madsen, 1998).

Using DNA fingerprinting, our previous breeding experiments with subsequent paternity assignment did not allow us to unambiguously reject the hypothesis of a paternity assurance mechanism in *V. berus* (Höggren and Tegelström, 1995), but they did verify sperm competition. In the absence of behavioral data (e.g. mating order and mating frequency), sexual efforts and observed fecundity cannot be correlated. In order to investigate possible effects of mating order, we conducted a controlled experiment, followed by paternity analysis, where female *V. berus* were allowed to copulate with a different male each day for three consecutive days.

MATERIALS AND METHODS

Adult *V. berus* were collected in April at different localities in the vicinity of Uppsala ($59^{\circ}50' N$) prior to the breeding season. Throughout the experiment, snakes were kept in outdoor enclosures. All snakes were individually marked through combinations of scale-clips in the first ten ventrals. Husbandry procedures, induction of breeding, maintenance of pregnant females, blood sampling (for DNA extraction), laboratory protocols for multi-locus DNA fingerprinting, and paternity analysis follow procedures referred to or described in Tegelström and Höggren (1994) and Höggren and Tegelström (1995). Thus, methods used at the different experimental stages are only briefly outlined here.

Males and females were kept isolated until all males had undergone ecdysis. In male *V. berus* ecdysis is synchronized to occur within several days immediately preceding the beginning of the mating season. Subsequently, each of the 12 reproductive females was exposed to a different set of three males, that is, a different male on each of three consecutive days. By using each male only once, we avoided the confounding factor of sperm depletion that can occur in offering females previously mated males. Throughout the experimental period, all pairs were watched for sexual activities, and each female was allowed to copulate

Table 1. Number of offspring sired by first, second, and third males, respectively, in the eight litters of *Vipera berus*. Numbers in parentheses indicate total lengths (cm) of males and females.

Mate order	Females and litter size							
	1 (70)	2 (69)	3 (63)	4 (68)	5 (66)	6 (58)	7 (66)	8 (56)
Male 1	6 (50)	6 (56)	8 (60)	5 (48)	7 (52)	7 (58)	4 (59)	3 (59)
Male 2	1 (50)	3 (51)	0 (55)	4 (48)	0 (66)	0 (56)	1 (51)	2 (58)
Male 3	3 (51)	0 (58)	1 (48)	0 (62)	0 (50)	0 (59)	0 (58)	0 (51)
Σ offspring	10	9	9	9	7	7	5	5

once with each male, so that each female experienced three copulations, one with each male. Following coitus (which lasts for about 45–60 min), female cloacae were checked for semen to confirm ejaculation.

As in other ectothermic vertebrates, *V. berus* requires suitable weather conditions for mating to occur. In one of the study seasons, a cold front interrupted mating activities, forcing us to exclude two females from the analyses. Two additional females were omitted because their second or third male, respectively, did not accomplish mating for unknown reasons, despite observed courtship. Hence, altogether eight of the 12 females were observed to copulate once on each of the three planned mating days. After blood was sampled from all males, females and offspring, adults were released at their sampling localities.

DNA analyses.—Genomic DNA was isolated following Mullenbach et al. (1989) and digested with the restriction enzyme *Alu* I. DNA fragments were separated in agarose gels for 42–44 h and transferred to Biodyne Nylon membranes by Southern blotting. The synthetic (TG)_n polydinucleotide (250 ng, Pharmacia LKB Biotechnology) was ³²P-labelled according to the manufacturer's instructions. For hybridization, we followed methods described by Ellegren (1991), and autoradiographed the hybridized membranes at –70°C for 1–6 days using Kodak X-omat AR with or without intensifying screens, depending on the signal strength.

Maternal and paternal DNA fragments within the range of 3–20 kilobases were identified among the offspring. Comparison of band migration and intensities followed principles outlined in Westneat (1993). The band sharing statistic of Wetton et al. (1987) was used to calculate the proportion of shared bands between two individuals (Similarity Index).

To determine whether the proportion of offspring sired by the first, second, and third males was significantly different, we tested the observed distributions against a null hypothesis where each male was given equal probabilities to contribute to fertilization.

RESULTS

Eight litters from the eight females resulted in 61 offspring and litter size ranged from 5–10 ($\bar{x} = 7.63$). Multiple paternity was detected in six litters (Table 1). The 24 males sired on average 2.54 offspring each (including unsuccessful males), while the corresponding figure when restricted to the 15 contributing males was 4.07. The number of fathers per litter ranged between one and three ($\bar{x} = 1.88$). The total number of offspring sired by first, second, and third males were 46, 11 and 4, respectively (Fig. 1), which gives an average yield per brood of 5.75, 1.38, and 0.50 young for the same mating order. In the two litters with single paternity, first males sired all offspring, while in the remaining mixed broods each of the first males sired more than half of the offspring ($\bar{x}_{\text{offspring}} = 69\%$, range 56–89%, N = 6).

We tested the observed proportions of offspring sired by the first, second, and third males, respectively, against the null hypothesis that the three sequential copulations in the experiment contributed equally to litter size (number of offspring); H₀ = each consecutive male fathered one-third of a female's litter. The observed proportions deviated significantly from expected values if each male contributed equally to litter size regardless of mating order ($\chi^2 = 49.81$, df = 2, P < 0.001). Thus, effect of mating order is the most parsimonious explanation for the observed contributions to litter size by three consecutive males.

We found no correlation between body size of the first, second and third males, respectively, and the number of offspring fathered, as determined from visual inspection of the data set (Table 1). Nor was there any obvious relationship between male size and proportion of the litter sired. When males were divided into two size classes, regardless of mating order, the small-size class (length ≤ 55 cm, N = 12) sired 31 young (51%), while the large-size class (length ≥ 56 cm, N = 12) fathered 30 young (49%). The lack of a relationship between male size and reproductive suc-

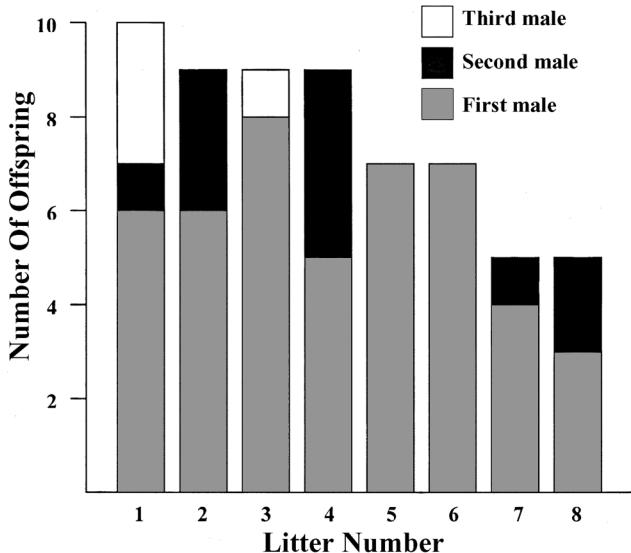


Fig. 1. Within litter proportions of offspring sired by the first, second, and third males when female *Vipera berus* were allowed to copulate with three different males each.

cess allows us to reject any size-dependent effects interfering with the observed outcome of mating order. This result is consistent with a study of sexual selection in the Sand Lizard (*Lacerta agilis*), where no male-size advantage was found at the gametic level (Olsson et al., 1996), and no allometric relationship between testis volume and body size was confirmed in males collected during the breeding season.

DISCUSSION

Our results demonstrate that, under the present experimental conditions, a first-male advantage occurs in the Adder (*Vipera berus*). Since sperm were confirmed in the cloacae of females, we propose that a sperm-inhibiting response in the female, likely by the uterus, resulted in the observed sperm precedence. We cannot, however, confirm unambiguously that sperm transfer from subsequent matings actually was reduced or omitted by sphincter muscle contractions in the female uterus, that are hypothesized to be induced by renal secretions in seminal fluids of the first male (Nilsson and Andrén, 1982). Nonetheless, our data do not contradict such a mechanism, nor do preliminary results by Stille et al. (1987) who used allozyme markers to analyse paternity in five different litters of females that in 4 d intervals mated with two males. In all litters, the first male sired all or a majority of the progeny. In Stille et al. (1987), in the five litters combined, the number of offspring sired was skewed toward the first male by a proportion of 23:14 ($\bar{x}_{\text{litter}} =$

7.40, $N_{\text{offspring}} = 37$). Despite a longer mating interval, a first-male advantage was less pronounced in the study by Stille et al., (1987) than in ours. Further investigation is needed to determine whether the difference is due to temporal variability in timing of copulations, population differences in response to mate-order, or stochastic effects.

In a previous study (Höggren and Tegelström, 1995), analysis of the progeny of a female *V. berus* that was collected shortly after she had been mated in the wild suggested a possible correlation between mating order and female receptivity in *V. berus*. Only two days after capture, she was exposed to enclosure males that exhibited intense sexual behavior, yet none of the captive males succeeded in fertilizing the female. Further, in a separate study of possible long-term sperm storage in *V. berus*, we noted a tendency toward skewed distribution of offspring between males that sired mixed broods (Höggren and Tegelström, 1996). Since mating order and mating frequency were not controlled, we made no attempts to quantify those observations, but instead proceeded with the present study.

In *V. berus*, female controlled sperm utilization through active selection of sperm in the reproductive tract has been suggested by Madsen's et al. (1992) demonstration of enhancement of offspring viability in females that had multiple mates (but see Capula and Luiselli, 1994). In the Sand Lizard (*Lacerta agilis*), Olsson et al. (1994a) found a positive relation between the number of males that copulated with a female and offspring fitness. Mating order, however, appears to have no effect in *L. agilis* (Olsson et al., 1994b), so their results are difficult to reconcile with mate-order effects in *V. berus*. If females control sperm, and that capability interacts with order of mating, resolving these two variables would require extensive laboratory breeding experiments. To some extent, our results provide support to the hypothesis of Madsen et al. (1992), unless each of the eight randomly selected first males in our mating series happened to be, by extreme coincidence, the most fit ones.

What are the conflicting (or mutual) benefits of multiple copulations in females and male paternity guards? From our data, we suggest that female choice in *V. berus* operates at the behavioral stages prior to insemination. As in other species of snakes, female cooperation in *V. berus* is required for successful intromission (Andrén, 1986; Gillingham, 1987). Therefore, because receptive female *V. berus* do not appear to solicit courtship or mating, successful copu-

lation is primarily a function of male persistence in courtship. Generally, a receptive female snake actively opens her vent (cloacal gaping) as a response to courtship, and cloacal gaping is not typically used for attracting passive males (for exceptions in captive animals, see Gillingham et al., 1983). Evaluating a male's persistence and capability to monopolize her may thus provide a female *V. berus* with a mechanism of estimating male qualities in the absence of investments (e.g., paternal care, territorial and nutritional resources), other than his genes (see Jennions, 1997; Yasui, 1998; Tregenza and Wedell, 2000, for discussions on mate choice and female genetic benefits).

Postcopulatory mate guarding is a significant component of reproductive behavior in male *V. berus*, and has been interpreted from the male's fitness perspective (Andrén, 1986; Madsen, 1988). Indeed, our data indicate a fairly obvious male gain in being capable of defending a recently-mated female from other males during the time sperm-inhibitor develops in her reproductive tract. But a male benefit of postcopulatory mate guarding also requires that sexual selection has favored that strategy, i.e. the net cost is lower than that of abandoned opportunities to compete for other females during the time invested in mate-guarding. Further, we suggest that postcopulatory mate-guarding in *V. berus* benefits females as well, rather than being a sexual conflict (sensu Dawkins and Krebs, 1979).

In studies of avian mating systems, theoretical benefits of mate guarding by females (i.e., the female attempts to limit a mate's chances to perform extra-pair copulations by mating repeatedly with him) have been proposed (Hunter et al., 1993; Eens et al., 1995, but see Whittingham et al., 1994). Similarly, prolonged and repeated copulations as well as mate guarding in *V. berus* may prevent a behaviorally superior male from mating additional females. Even though females do not appear to initiate or actively solicit copulations, and despite the fact that male mating behavior is more conspicuous than female mating behavior, we see no reason to reject indirect interfemale mate competition as a significant evolutionary determinant of the *V. berus* mating system, in contrast to the opinion of Madsen and Shine (1992).

Although a female's receptivity decreases after the first successful insemination, she remains attractive to males during at least the following three or four days, as we and others (e.g., Stille et al., 1986, 1987) have observed. This may reflect conflicts in male and female fitness optimization: a male can increase his

contribution of genes to future generations by a superior effort to be first to mate with a female, while the female can improve her contribution by an effective safeguarding strategy that allows potential better quality males to contribute to her fitness. The intruder's reward seemingly depends on the time elapsed since the first copulation. The present data are consistent with previous results from enclosure experiments where males competed behaviorally when snake density was high (Höggren and Tegelström, 1995). Our results indicate that subsequent copulations within a day are not pronouncedly affected by a paternity assurance mechanism. As a consequence, sneaking behavior (Madsen et al., 1993) may occasionally give subordinate (= smaller) males a small proportion in offspring.

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