Frequency of multiple paternity in the grass snake (*Natrix natrix*)

Barbara Meister*, Sylvain Ursenbacher, Bruno Baur

**Abstract.** Males can enhance their reproductive success through mating with multiple females. For females, however, one mating is usually sufficient to inseminate all of their ova. Females may benefit from multiple mating by producing genetically more diverse offspring, and by having the opportunity to choose sperm of the genetically most compatible male. We used five microsatellite loci to investigate the occurrence and frequency of multiple paternity in 11 clutches of the grass snake (*Natrix natrix*) in Switzerland. Using a very conservative estimate (program GERUD), two or more fathers were found in 27% of the clutches. However, based on the maximum likelihood estimate (program COLONY), multiple paternity occurred in 91% of the clutches with 2-5 contributing males per female. This is the first investigation demonstrating multiple paternity in a European natricine, with a frequency similar to those found in new world natricines.

**Keywords:** COLONY, GERUD, microsatellite DNA, Natricinae, paternity analysis.

In mating systems without paternal care, males can enhance their reproductive success through mating with multiple females. In these species, mate acquisition is the limiting factor for male reproduction and strategies to overcome this limitation are numerous (Shine, 2003). For females, however, one copulation might be sufficient to inseminate all ova. Nevertheless, multiple mating by females has been reported in a variety of reptile species, indicating direct and indirect benefits for the females (Zeh and Zeh, 2001). Direct benefits arising from paternal contributions to egg production or parental care are unlikely to play an important role in most reptile species (Uller and Olsson, 2008). However, indirect benefits may arise to multiply mated females from increased genetic quality, higher complementarity, and/or enhanced genetic variation (bet hedging) of their offspring (Madsen et al., 2005; Uller and Olsson, 2008).

In squamates, multiple paternity is common, and occurs at high levels in natural populations (Uller and Olsson, 2008). Oviducal sperm storage has been reported in females for numerous reptilian taxa with varying durations (Birkhead and Møller, 1993; Sever and Hamlett, 2002).

To our knowledge, the occurrence and extent of multiple paternity have not yet been examined in the grass snake (*Natrix natrix*). In this study, we used five microsatellite loci to investigate the occurrence and frequency of multiple paternity in three natural populations of *N. natrix* in Switzerland.

The grass snake has a wide distribution (northern Africa and Eurasia; Kabisch, 1999). Despite its wide potential distribution in Switzerland, the area actually occupied is relatively restricted and a decline in abundance of over 30% has been recorded during the past century (Monney and Meyer, 2005). The species is therefore registered on the red list of Switzerland as “vulnerable” (Monney and Meyer, 2005). The decline is paralleled by pronounced changes in land use and a decrease in amphibian population sizes, the primary food of *N. natrix* (Reading and Davies, 1996; Gregory and Isaac, 2004). Mating occurs in spring (April-May) shortly after emergence from hibernation. Occasionally, grass snakes mate a second time in autumn (September-October; Kabisch, 1999). Many snake species that live in temperate regions and copulate in fall store sperm in tubules until ovulation in spring (Sever and Hamlett, 2002; Uller, Stuart-Fox and Olsson, 2010). Female grass snakes can store sperm up to 180 days (Birkhead and Møller, 1993). During copulation, male grass snakes release a white viscous fluid (Olsson and Madsen, 1998). One possible function of such fluids is the formation of a copulatory plug that prevents further copulations or sperm transfer (Olsson and Madsen, 1998; Uller, Stuart-Fox and Olsson, 2010). Finally, oviposition takes place between July and August in Switzerland, and progeny hatch 7-9 weeks later (Kabisch, 1999).

In this study eleven pregnant females of *N. n. helvetica* were collected in June and July in the years 2006-2008.
Table 1. Number of fathers contributing to the clutches of *Natrix natrix* collected in three natural populations and the number of offspring sired by different fathers (indicated by different letters). The same letter in different mother-offspring arrays does not refer to the same father.

<table>
<thead>
<tr>
<th>Mother</th>
<th>Number of offspring analysed</th>
<th>Minimum number of fathers*</th>
<th>Total number of fathers‡</th>
<th>Total number of offspring sired by different fathers‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM 51</td>
<td>8</td>
<td>1</td>
<td>2</td>
<td>6a, 2b</td>
</tr>
<tr>
<td>GM 126</td>
<td>35</td>
<td>3</td>
<td>5</td>
<td>14a, 11b, 5c, 3d, 2e†</td>
</tr>
<tr>
<td>GM 240</td>
<td>12</td>
<td>1</td>
<td>1</td>
<td>12a</td>
</tr>
<tr>
<td>GM 244</td>
<td>13</td>
<td>2</td>
<td>2</td>
<td>11a, 2b†</td>
</tr>
<tr>
<td>A 117</td>
<td>16</td>
<td>1</td>
<td>3</td>
<td>8a, 6b, 2c</td>
</tr>
<tr>
<td>A 247</td>
<td>11</td>
<td>1</td>
<td>3</td>
<td>7a, 2b, 2c</td>
</tr>
<tr>
<td>G 72</td>
<td>7</td>
<td>1</td>
<td>3</td>
<td>4a, 2b, 1c</td>
</tr>
<tr>
<td>G 74</td>
<td>8</td>
<td>2</td>
<td>4</td>
<td>3a, 2b, 2c, 1d</td>
</tr>
<tr>
<td>G 76</td>
<td>6</td>
<td>1</td>
<td>2</td>
<td>5a, 1b</td>
</tr>
<tr>
<td>G 140</td>
<td>8</td>
<td>1</td>
<td>3</td>
<td>5a, 2b, 1c</td>
</tr>
<tr>
<td>G 255</td>
<td>17</td>
<td>1</td>
<td>4</td>
<td>8a, 5b, 3c, 1d</td>
</tr>
<tr>
<td>Mean</td>
<td>12.8</td>
<td>1.4</td>
<td>2.9</td>
<td>–</td>
</tr>
</tbody>
</table>

* Parentage reconstruction using GERUD.
‡ Likelihood assessment based on genetic diversity using COLONY.
† Different fathers sired different numbers of offspring.

in three different areas in Switzerland: an intensively used agricultural area called Grosses Moos, in the Aaretal, an agricultural area with remnants of wetland along the river Aare between the cities of Thun and Bern, and in Gadmental, a valley in the Alps. The three areas are situated 30-100 km apart from each other (for a detailed description see Meister, Ursenbacher and Baur, 2012). Snakes were hand-captured by walking along edges of the habitats and by controlling coverboards laid out to attract snakes (Fitch, 1992). Pregnant females were kept at the zoo “Däihölzi” in Bern until oviposition and then released at their catching places. Clutches were incubated and neonates were kept in boxes until first ecdysis after which they were released at the catching place of their mother. DNA was obtained from cut scales and shed skins of juvenile snakes. Unhatched eggs were obtained for undeveloped embryos, which were used for DNA extraction. If no embryo could be detected, the eggs were regarded as unfertilised. Tissue of female snakes was obtained by clipping ventral scales. Scale tissue was preserved in 80% EtOH and stored at 5°C until genetic analysis.

Genomic DNA was extracted using a modified cetyltrimethyl ammonium bromide-based extraction protocol (CTAB; Doyle and Doyle, 1987) and genotyped using a set of five microsatellite loci (*Natnat05, Natnat06, Natnat09, Natnat11 and µNt8new; Meister et al., 2009*). The genetic analysis was performed as described in Meister, Ursenbacher and Baur (2012). Microsatellite characteristics were assessed using GenAIEx, version 6.1 (Peakall and Smouse, 2006). Probabilities for departures from Hardy-Weinberg equilibrium expectations, linkage disequilibrium, and occurrence of null alleles, stuttering signals or large allelic dropout were previously examined for these populations in Meister, Ursenbacher and Baur (2012). The DNA of eight juveniles in four clutches did not amplify any loci. These individuals were excluded from the analyses. We genotyped a total of 141 offspring from 11 mothers (table 1). The proportions of offspring included in the paternity analyses averaged 80.2% (range: 50.0%-100.0%) of the total number of eggs produced (including eggs that were later regarded as unfertilised). Considering exclusively fertilised eggs, the proportions of offspring included in the paternity analyses averaged 93.2% (range: 72.7%-100.0%). Sibship analysis and parentage reconstruction of the mother snakes and their progeny were performed with GERUD, version 2.0 (Jones, 2005) and COLONY, version 2.0 (Wang, 2004). GERUD uses multiple-locus data for reconstruction of the contributing paternal genotype(s) from mother-offspring arrays. The software does not distinguish between potential fathers of the same genotype. Consequently, the number of paternal genotypes estimated is equal to a minimum number of involved fathers. The maximum likelihood software COLONY was used to assess the total number of fathers contributing their gametes to a progeny array. For the paternity assignment, the genotypes of the individuals sampled in the populations were used (Grosses Moos: 91 individuals, Aaretal: 62 individuals, Gadmental: 19 individuals; Meister et al., 2010; Meister, Ursenbacher and Baur, 2012). COLONY provides the most probable configuration of paternity including assignments of every offspring to one of the estimated paternal genotypes. We calculated Spearman rank correlation to test whether the number of fathers contributing to a clutch is correlated with the number of offspring analysed. Differences in levels of paternity between populations were investigated with a one-way ANOVA. These statistical analyses were conducted using PASW®.
Table 2. Summary statistics for five microsatellite loci used in *Natrix natrix* populations.

<table>
<thead>
<tr>
<th>Locus</th>
<th>N</th>
<th>N_C</th>
<th>Exclusion probability*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natnat05</td>
<td>3</td>
<td>3</td>
<td>0.33-0.33</td>
</tr>
<tr>
<td>Natnat06</td>
<td>4</td>
<td>7</td>
<td>0.33-0.54</td>
</tr>
<tr>
<td>Natnat09</td>
<td>4</td>
<td>5</td>
<td>0.33-0.68</td>
</tr>
<tr>
<td>Natnat11</td>
<td>3</td>
<td>3</td>
<td>0.22-0.42</td>
</tr>
<tr>
<td>µNt8new</td>
<td>5</td>
<td>7</td>
<td>0.36-0.42</td>
</tr>
</tbody>
</table>

N: Number of alleles per locus for mother snakes; N_C: Number of alleles in clutches.
* Range for all three populations. Combined exclusion probability for all five loci ranged 0.86-0.98.

statistics 18.0 core system (SPSS Inc., 2009). We examined whether multiple fathers sire equal numbers of eggs in a given mother using contingency tests for the clutches exhibiting multiple paternity and with more than 10 offspring analysed (we considered that the statistical power in this particular test is too low in clutches with less than 10 offspring).

The polymorphism level of the five microsatellite loci ranged from three to five alleles in the mother snakes and from three to seven alleles in their progeny (table 2). Paternity exclusion probability for each microsatellite ranged from 0.22 to 0.68 (table 2) and from 0.86 to 0.98 for all microsatellite loci combined.

Occurrences of multiple paternity were detected in three of the eleven clutches using GERUD (table 1). This conservative analysis reveals the minimum number of contributing fathers, which ranged from 2 to 3 (table 1). Using COLONY, multiple paternity was found in ten of the eleven clutches. This analysis estimates the total number of fathers, which ranged from 2 to 5 per clutches (table 1). The number of fathers per clutch was not correlated with the number of offspring analysed (Spearman rank correlation, GERUD: \( r_s = 0.38, N = 11, P = 0.25 \); COLONY: \( r_s = 0.39, N = 11, P = 0.24 \), and the three populations considered did not differ in the level of multiple paternity (GERUD: \( F_{2,8} = 1.12, P = 0.37 \); COLONY: \( F_{2,8} = 0.38, P = 0.70 \)).

Patterns of sperm utilisation were examined in the five clutches consisting of more than 10 offspring and showing multiple paternity (COLONY). In two of them, the distribution of paternity was non-random (contingency-test, in all cases, \( P < 0.05 \); table 1). In the remaining three clutches, no significant difference in the number of offspring sired by each father was found (\( P > 0.05 \)).

Multiple paternity was found in at least 27% of the grass snake clutches (using GERUD), but most probably occurred in more than 90% of the clutches (using COLONY). Most studies on multiple paternity in natricines considered new world species: multiply sired litters have been reported in *Thamnophis sirtalis*, with 37.5%-75.0% of the litters exhibiting multiple paternity (Schwartz, McCracken and Burghardt, 1989; McCracken, Burghardt and Houts, 1999; Garner et al., 2002), and in *Nerodia sipedon*, in which 58.0%-85.7% of the litters were sired by more than one father (Barry, Weatherhead and Philipp, 1992; Prosser et al., 2002; Kissner, Weatherhead and Gibbs, 2005). In most natricine species investigated so far multiple paternity has been documented: *T. elegans*, *T. butleri*, *T. radix*, *T. sauritus*, *N. rhombifer*, *Regina septemvittata*, *Storeria occipitomaculata*, *S. dekayi* (Wusterbarth et al., 2010). However, this study is the first to demonstrate the occurrence and level of multiple paternity in a European natricine.

Interestingly, Madsen and Shine (1993) observed in a mating experiment with captive grass snakes that males abandoned the female after successful copulation and did not court a previously mated female. If a female mates only with one male per mating season (as observed by Madsen and Shine, 1993), the presence of multiply sired clutches, as found in this study, could be explained by mating in the previous mating season(s) or year(s), combined with long-term sperm storage. Given that grass snakes have been reported to store sperm for a maximum of six months (Rollinat, 1946; Birkehead and Møller, 1993), the presence of two sires could be explained by one autumn mating in the previous year and one spring mating in the current year, according to the obser-
vation of a single copulation per mating season (Madsen and Shine, 1993). Our study, however, demonstrated that more than two males sired eggs in the same clutch. Consequently, our findings demonstrate that males copulate with previously mated females in the wild. Otherwise the paternity by more than two males in a clutch could not be explained. Sexual unattractiveness of mated females due to a copulatory plug formed by gelatinous material has been reported in Thamnophis species (Ross and Crews, 1977; but see Shine, Olsson and Mason, 2000). In T. sirtalis, multiply mated females with two or more plugs have been found in the field (Shine, Olsson and Mason, 2000). Nevertheless, they produced multiple-sired clutches (see above). Obviously, mating plugs do not completely prevent further sperm transmission and female natrixines seem to have developed strategies to overcome forced chastity, as found in Viperidae (Ursenbacher, Erny and Fumagalli, 2009).

In the present study, different fathers sired different numbers of offspring in two out of five clutches. This could be a result of different numbers of sperm delivered by different males, the ability of the female to choose among sperm from different males (cryptic female choice), or of “topping off”, in which the female accepts a large amount of sperm from the first male, while sperm from further males are only kept in the sperm storage tubules if space allows. Once the tubules are filled, sperm from consecutive mates admix (Jones, Adams and Arnold, 2002). Our data do not allow to distinguish between these explanations.

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References


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