

## Multiple paternity in wild populations of the garter snake, *Thamnophis sirtalis*

James M. Schwartz<sup>1,\*</sup>, Gary F. McCracken<sup>2</sup>, and Gordon M. Burghardt<sup>2,3</sup>

<sup>1</sup> Life Science Program (Ethology), University of Tennessee, Knoxville, TN 37996, USA

<sup>2</sup> Department of Zoology, University of Tennessee, Knoxville, TN 37996, USA

<sup>3</sup> Department of Psychology, University of Tennessee, Knoxville, TN 37996, USA

Received July 15, 1988 / Accepted June 6, 1989

**Summary.** Many adaptations have arisen in response to sperm competition. In garter snakes, males produce copulatory plugs that are thought to prevent such competition. We tested this assumption using paternity exclusion techniques with electrophoretic data from 32 litters of snakes collected from two populations. Multiple paternity was confirmed in 50% of the litters examined (13/22 Michigan litters; 3/10 Wisconsin litters), and we estimate that as many as 72% of the litters may have been multiply sired. The presence of multiple paternity was also confirmed using the technique of genotypic regression. Mean relatedness in litters with evidence of multiple paternity was  $0.390 \pm 0.053$ ; in litters lacking such evidence mean relatedness was  $0.498 \pm 0.022$ . Thus our data reveal a high incidence of multiple paternity in these snakes and suggest that their adaptations to prevent sperm competition are surprisingly ineffective.

### Introduction

Except in species where females are completely monogamous, the reproductive success of males often is tied to the competitive abilities of their sperm (Parker 1984). As a result, sperm competition can dramatically alter the action of sexual selection and, in turn, the reproductive behavior, physiology, and morphology of males (Parker 1970; Parker and Smith 1975). Such selection has led to adaptations that either increase sperm competitive abilities or that prevent sperm competition. Adapta-

tions to increase sperm competitive abilities include sperm removal (Waage 1984), displacement (Lefevre and Jonsson 1962), and morphological and physiological changes in sperm (Sivinski 1984). Those which serve to limit or prevent multiple mating and the consequent competition with another's sperm include mating plugs (Parker 1970; Devine 1984), anti-aphrodisiac pheromones (Ross and Crews 1977), prolonged copulation (Parker 1970), and mate guarding (Parker 1970).

Although sperm storage and multiple matings are common in reptiles, direct evidence of sperm competition has not been shown in natural populations of any reptilian species (Devine 1984). Yet many reptiles are known to have adaptations for preventing sperm competition that presumably arose from such competition. The eastern garter snake *Thamnophis sirtalis* is perhaps the best studied example in the reptiles. In these snakes, a copulatory plug is formed within 20 min of intromission (Blanchard and Blanchard 1942) and lasts from 4 days to 2 weeks depending on temperature (Devine 1975, 1984). In well-studied populations in Manitoba, garter snakes mate on emergence from communal hibernacula, and females move to summer feeding grounds within a few days (Gregory 1974). Thus the plug, deposited by a successful suitor, is in place while the female is in the vicinity of the den where matings occur. It has been suggested that the plug functions as a physical block against additional matings (Devine 1975, 1977; Ross and Crews 1977, 1978; Halpert et al. 1982). In addition, the plug contains an anti-aphrodisiac pheromone that further inhibits the reproductive activity of other males so that recently mated females are avoided (Ross and Crews 1977, 1978; Devine 1984).

Several studies suggest that copulatory plugs usually are effective in preventing females from re-

*Present address:* Hawaiian Evolutionary Biology Program, Pacific Biomedical Research Center, University of Hawaii, Honolulu, HI 96822, USA

*Offprint requests to:* J.M. Schwartz (at his present address)

mating (Parker 1970; Martin and Shepherd 1976; Ross and Crews 1977; Devine 1984; but see Parker 1984). In snakes, it is thought that plugs should be particularly effective because the hemipenes lack solid structure and cannot be used to dislodge the mass (Devine 1984). Therefore, formation of the copulatory plug, its associated anti-aphrodisiac pheromones, and the rapid movement of females away from the mating grounds provide especially strong evidence for single paternity in *T. sirtalis*. This evidence evidently has served as the basis for assuming single paternity in this species, and many authors state that female garter snakes mate only once (Gregory 1974; Halliday 1980; Garstka et al. 1982; Halpert et al. 1982; Garstka and Crews 1985; Garstka et al. 1985; Joy and Crews 1985; Whittier et al. 1985; Halliday and Arnold 1987). In addition, single paternity has been assumed in studies on the heritability of numerous behavioral and physiological features in *T. sirtalis* (Garland 1988), and in closely related *T. elegans* (Arnold 1981a, b, 1988; Ayres and Arnold 1983), and *T. radix* (Arnold and Bennett 1984).

However, there is evidence that challenges the assumption of single paternity. First, field observations suggest the potential for multiple paternity in natural populations. Whittier and Crews (1986) recently observed approximately one-third of the female garter snakes in a wild population remating. Second, multiple paternity has been documented in captive populations of *T. sirtalis* and three other snake species using either genetically based morphological characters (*Agkistrodon contortrix*, Schuett and Gillingham 1986; *T. sirtalis*, Blanchard and Blanchard 1941), or electrophoretic evidence (*Lampropeltis getulus*, Zweifel and Dessauer 1983; *Vipera berus*, Still et al. 1986). Third, Gibson and Falls (1975) found evidence that up to 55% of litters were multiply sired in a natural population of melanistic *T. sirtalis*. However, the validity of this field study of paternity relies upon the questionable assumption that melanism is inherited as a single Mendelian gene (Halpert et al. 1982). Here we utilize paternity exclusion techniques to examine the frequency of multiple paternity in wild populations and demonstrate that apparent adaptations to prevent sperm competition in *T. sirtalis* are not effective in preventing multiple paternity.

## Methods

Thirty-two gravid females were collected from two populations (22 from Wayne Co., Michigan and 10 from the Wolf River drainage, Wisconsin) and held in the laboratory until they gave birth. These females gave birth to 442 young (litter sizes ranged

from 6–40). The genotypes of offspring and females were examined using standard horizontal starch-gel electrophoretic and staining techniques (Selander et al. 1971; Harris and Hopkinson 1978). Both blood and muscle tissue were collected and freshly processed for subsequent analysis. Blood was collected in heparinized capillary tubes and separated into cellular and plasma fractions by centrifugation. Muscle tissue was ground in a 1:1 weight to volume ratio of tissue to grinding buffer (Selander et al. 1971) and held for 90 min at 4° C. The homogenate was then centrifuged at 13000 rpm for 40 min at 4° C, and the resulting supernatants saved for electrophoresis. All samples were stored at –80° C and electrophoresed within 6 months of collection. A survey of 45 protein loci identified four easily scored polymorphic loci; one in blood plasma (Transferrin, Tff) and three in muscle tissue (6-phosphogluconate dehydrogenase, 6-PGD, Enzyme Commission number 1.1.1.44; Lactate dehydrogenase, LDH, E.C. 1.1.1.27; Mannose phosphate isomerase, MPI, E.C. 5.3.1.8). MPI was resolved on PGI phosphate (pH 6.7) buffer system; 6-PGD on tris citrate (pH 8.0); and Tff and LDH on Poulik discontinuous buffer system (Selander et al. 1971). Two triallelic loci, LDH and MPI, had a mean common allele frequency of 71% and 57.5%, respectively. Tff and 6-PGD had five and seven alleles, respectively, with a mean common allele frequency of 64% and 28%, respectively. Each allelomorph was scored independently by two researchers.

An examination of both the number of alleles and the number of distinct genotypes in each litter can be used to conservatively assess the incidence of multiple paternity (Akin et al. 1984; Avise and Shapiro 1986; Levine et al. 1987). When the genotype of the female parent is known, as it is in this study, the ability to detect multiple paternity greatly increases. For example, if the female is homozygous at a locus, the presence of three distinct alleles in the offspring indicates multiple paternity. It is even possible to detect the contributions of three or more males to a litter if a sufficient number of alleles (five or more) are segregating at a locus.

Because males may share alleles with one another or with the female, the paternity exclusion technique is apt to underestimate the actual frequency of multiple paternity that occurs in a population. Akin et al. (1984) developed a method to estimate the real frequency of multiple paternity in cases where individuals share alleles. Their approach uses progeny sample size to calculate the probability of detection of multiple paternity for all possible combinations of parental genotypes. The detection probability for each genotype combination is weighted by its probability of occurrence using the gene frequencies of alleles in the population. Females are assumed to have mated at most twice with equal paternal probabilities (i.e., no sperm displacement). We modified this method, which assumes no knowledge of parental genotypes, to include our information on maternal genotypes in order to estimate the real frequency of multiple paternity in these litters.

This approach assumes that the loci assort independently. Tests for linkage disequilibria between all combinations of locus pairs were performed as follows. Assuming that the alleles of mothers assort independently, there should be an equal probability of finding each gametic combination in the offspring. The genotypes of offspring from mothers heterozygous at both loci were then used to calculate a G test goodness of fit statistic (Sokal and Rohlf 1981) adjusted for small sample size. Samples from both populations were combined to increase sample sizes for these tests.

As a second test for the presence of multiple paternity in litters we estimated the mean relatedness of littermates using the genotypic regression technique (Pamilo 1984). The correlation coefficients obtained in this analysis can be interpreted

**Table 1.** Litters and loci for which evidence of multiple paternity was obtained. Multiple paternity was not detected in 16 other litters examined

Litters with multiple paternity					
Population	Litter	Locus (# of alleles)			
		6-PGD(7)	Tff(5)	MPI(3)	LDH(3)
Michigan	1538	X	X	X	
	1520	X	X		X
	1502	X		X	
	1510	X	X		
	1513	X		X	
	1519	X			X
	1523			X	X
	1539	X	X		
	1501	X			
	1505	X			
	1512			X	
	1514	X			
	1537	X			
	Wisconsin	1552	X	X	
1543			X		
1558		X			
Total #	16	13	6	5	3

as the average degree of relatedness of individuals within litters (Pamilo 1984).

## Results

Direct evidence of multiple paternity was found in 16 of the 32 litters examined (Table 1). Each locus scored revealed multiple paternity in 3 or more different litters, and in 9 litters multiple paternity was detected at two or more loci. As seen in Table 1, our ability to detect multiple-sired litters increased with the number of alleles resolved at a locus. Litters from both populations were multiple-sired (13/22 from Michigan, 3/10 from Wisconsin). There was no evidence of triple paternity in any litter, though only the 6-PGD and Tff loci had a sufficient number of alleles to permit its possible detection. Only one of the 14 pairwise tests for linkage (between MPI and 6PGD;  $G=8.85$ ,  $df=3$ ,  $P<0.05$ ) reached significance. Since one test in 20 is expected to be significant by chance effects alone, there is little evidence of linkage. Applying the approach of Akin et al. (1984), and combining our results from the four independent loci (assuming a mean litter size of 14), we estimate that the real frequency of multiple paternity in the 32 litters examined was 72% (variance = 0.07).

For the genotypic regression analysis (Pamilo 1984), we separated the litters into two groups:

**Table 2.** Mean relatedness as determined by genotypic regression (Pamilo 1984) in litters with and without evidence of multiple paternity

Locus	Alleles	Litters	$\bar{r}$	$\pm SE$	
6PGD	7	16	0.528	0.061	Single paternity
Tff	5	15	0.544	0.081	
MPI	3	16	0.471	0.111	
LDH	3	16	0.450	0.141	
Mean			0.498	0.022	
6PGD	7	16	0.465	0.046	Multiple paternity
Tff	5	16	0.359	0.077	
MPI	3	16	0.482	0.085	
LDH	3	16	0.254	0.110	
Mean			0.390	0.053	

those in which multiple paternity was detected and those for which single paternity could not be excluded. Assuming the parents are unrelated, when a litter is composed of full-siblings the mean relatedness ( $\bar{r}$ ) among individuals is 0.5, when composed entirely of half-siblings (i.e., each individual has a different unrelated father)  $\bar{r}$  is 0.25. The estimated  $\bar{r}$  ( $\pm SE$ ) in those litters lacking evidence of multiple paternity is  $0.498 \pm 0.022$  (Table 2). In the multiple-sired litters, the estimated  $\bar{r}$  is intermediate between the theoretical limits ( $\bar{r}=0.390 \pm 0.053$ ).

## Discussion

The electrophoretic data reveal a high incidence of multiple paternity in these natural populations of garter snakes despite the presence of copulatory plugs and anti-aphrodisiac pheromones. Because garter snakes store sperm, occasionally for extended periods of time (Stewart 1972), an alternative hypothesis is that the multiple paternity that we observed was due not to ineffectiveness of the copulatory plugs, but rather to an overlap of sperm in the female between subsequent mating seasons. For the following reasons, we suggest that this is not the case. First, although many females have abundant sperm in their oviduct in the spring after mating, no females have been found with live stored sperm during mid-summer, indicating that all available sperm supplies are used to fertilize the litter (Table 3; Rahn 1940; Blanchard and Blanchard 1942; Fox 1956). Several researchers have observed autumn mating in this species (Blanchard and Blanchard 1941; Blanchard 1943; Aleksiuik and Gregory 1974) and, in the fall, an increasing number of females are observed with sperm

**Table 3.** Frequency (%) of female *Thamnophis sirtalis* with spermatozoa in oviducts at various times throughout the year. An unquantified number of additional snakes were sampled in June, but none were found with spermatozoa in the oviduct (Fitch 1965). Data compiled from Fox (1956), Fitch (1965), Aleksiuik and Gregory (1974), and Halpert et al. (1982). Parturition normally occurs from late July to early August

Season	W/O Sperm	W/Sperm
Mar 1–June 6	16 (34.8)	30 (65.2)
June 7–July 30	57+ (96.6)	2 (3.4) <sup>a</sup>
July 31–Oct 8	122 (81.3)	28 (18.7)
Oct 9–Feb 29	49 (51.0)	47 (49.0)

$P < 0.001$ ,  $G$  test.

<sup>a</sup> Both females found between June and July contained dead sperm (Fitch 1965)

in their oviducts. However, there appears to be little chance that sperm from fall matings are responsible for the multiple paternity we observed. Halpert et al. (1982) have demonstrated that sperm from these autumn matings can move into specialized infundibular storage receptacles in the oviduct and remain viable throughout the winter. Yet, these sperm are quickly evacuated from the receptacles and degenerate within 6 h of a subsequent spring mating. Since ovulation and subsequent fertilization of the ova occur 6–8 weeks after mating (Gregory 1976), it is unlikely that the evacuated sperm will fertilize ova.

Our results lead us to at least two conclusions. First, the existence of adaptations to thwart multiple paternity clearly should not be accepted as proof that sperm competition does not occur. Rather these adaptations provide evidence that sperm competition is a potential problem that males must face. There should be strong selective pressure on males of most species to prevent multiple paternity if possible, but their defenses may not be absolute. In some situations, such as in garter snakes where females appear to control the number of matings, male defenses may be surprisingly ineffective.

Second, our results point to the importance of testing assumptions of paternity before calculating heritabilities. Given our results, it seems likely that the inclusion of multiple-sired litters may have led to underestimates of heritabilities in previous studies of quantitative genetics in garter snakes (e.g., Arnold 1981a, b, 1988; Ayres and Arnold 1983; Arnold and Bennett 1984; Garland 1988). The magnitude of this bias will depend upon the average relatedness of littermates. In our case, with an average relatedness of 0.39 in 72% of the litters

and 0.50 in the rest, true heritabilities would theoretically be 1.35 times larger. Empirical estimates of heritabilities of various behavioral, physiological, and morphological traits in these snakes increased by an average of 1.31 times when litters of known mixed paternity were excluded (Schwartz 1989).

**Acknowledgements.** We thank C. Sagers, M. Gustin, E. Font, and D. Layne for their assistance in the lab, and M. Kramer, S. Arnold, and D. Crews for their careful reviews. This work was supported by NSF research grants (BNS 82-17569 and BNS 87-09629) to GMB and an NIH predoctoral traineeship (HD07303) to JMS.

## References

- Akin E, Levene H, Levine L, Rockwell R (1984) A conservative procedure for the estimation of multiple insemination in *Drosophila*. *Am Nat* 124:723–737
- Aleksiuk M, Gregory P (1974) Regulation of seasonal mating behavior in *Thamnophis sirtalis parietalis*. *Copeia* 1974:681–689
- Arnold SJ (1981a) Behavioral variation in natural populations. I. Phenotypic, genetic and environmental correlations between chemoreceptive responses to prey in the garter snake, *Thamnophis elegans*. *Evolution* 35:489–509
- Arnold SJ (1981b) The microevolution of feeding behavior. In: Kamil AC, Sargent TD (eds) *Foraging behavior: ecological, ethological, and psychological approaches*. Garland Publ Inc, New York, pp 409–453
- Arnold SJ (1988) Quantitative genetics and selection in natural populations: Microevolution of vertebral numbers in the garter snake *Thamnophis elegans*. In: Weir BS, Eisen EJ, Goodman MM, Namkoong G (eds) *Proceedings of the second international conference on quantitative genetics*. Sinauer Assoc Inc, Sunderland, Massachusetts, pp 619–636
- Arnold SJ, Bennett AF (1984) Behavioral variation in natural populations. III. Antipredator displays in the garter snake *Thamnophis radix*. *Anim Behav* 32:1108–1118
- Avisé JC, Shapiro DY (1986) Evaluating kinship of newly settled juveniles within social groups of the coral reef fish *Anthias squamipinnis*. *Evolution* 40:1051–1059
- Ayres FA, Arnold SJ (1983) Behavioral variation in natural populations. IV. Mendelian models and heritability of a feeding response in the garter snake, *Thamnophis elegans*. *Heredity* 51:405–413
- Blanchard FC (1943) A test of fecundity of the garter snake *Thamnophis sirtalis sirtalis* (Linnaeus) in the year following the year of insemination. *Pap Mich Acad Sci Arts Lett* 28(1942):313–317
- Blanchard FN, Blanchard FC (1941) The inheritance of melanism in the garter snake *Thamnophis sirtalis sirtalis* (Linnaeus), and some evidence of effective autumn mating. *Pap Mich Acad Sci Arts Lett* 26(1940):177–193
- Blanchard FN, Blanchard FC (1942) Mating of the garter snake *Thamnophis sirtalis sirtalis* (Linnaeus). *Pap Mich Acad Sci Arts Lett* 27(1941):215–234
- Devine MC (1975) Copulatory plugs in snakes: enforced chastity. *Science* 187:844–845
- Devine MC (1977) Copulatory plugs, restricted mating opportunities and reproductive competition among male garter snakes. *Nature* 267:345–346
- Devine MC (1984) Potential for sperm competition in reptiles: behavioral and physiological consequences. In: Smith RL

- (ed) Sperm competition and the evolution of animal mating systems. Academic Press Inc, Orlando, pp 509–521
- Fitch HS (1965) An ecological study of the garter snake, *Thamnophis sirtalis*. Univ Kansas Mus Nat Hist Publ 15(10):493–564
- Fox W (1956) Seminal receptacles of snakes. Anat Rec 124:519–540
- Garland T (1988) Genetic basis of activity metabolism. I. Inheritance of speed, stamina, and antipredator displays in the garter snake *Thamnophis sirtalis*. Evolution 42:335–350
- Garstka WR, Crews D (1985) Mate preference in garter snakes. Herpetologica 41:9–19
- Garstka WR, Camazine B, Crews D (1982) Interactions of behavior and physiology during the annual reproductive cycle of the red-sided garter snake (*Thamnophis sirtalis parietalis*). Herpetologica 38:104–123
- Garstka WR, Tokarz RR, Diamond M, Halpert A, Crews D (1985) Behavioral and physiological control of yolk synthesis and deposition in the female red-sided garter snake (*Thamnophis sirtalis parietalis*). Horm Behav 19:137–153
- Gibson AR, Falls JB (1975) Evidence for multiple insemination in the common garter snake *Thamnophis sirtalis*. Can J Zool 53:1362–1368
- Gregory PT (1974) Patterns of spring emergence of the red-sided garter snake (*Thamnophis sirtalis parietalis*) in the Interlake region of Manitoba. Can J Zool 52:1063–1069
- Gregory PT (1976) Life history parameters of the red-sided garter snake (*Thamnophis sirtalis parietalis*) in an extreme environment, the Interlake region of Manitoba. Natl Mus Nat Sci Ottawa Publ Zool No 13
- Halliday T (1980) Sexual strategy. Univ of Chicago Press, Chicago
- Halliday T, Arnold SJ (1987) Multiple mating by females: perspective from quantitative genetics. Anim Behav 35:939–940
- Halpert AP, Garstka WR, Crews D (1982) Sperm transport and storage and its relation to the annual sexual cycle of the female red-sided garter snake, *Thamnophis sirtalis parietalis*. J Morphol 174:149–159
- Harris H, Hopkinson DA (1978) Handbook of enzyme electrophoresis in human genetics. Elsevier, New York
- Joy JE, Crews D (1985) Social dynamics of group courtship behavior in male red-sided garter snakes (*Thamnophis sirtalis parietalis*). J Comp Psychol 99:145–149
- Lefevre G, Jonsson UB (1962) Sperm transfer, storage, displacement and utilization in *Drosophila melanogaster*. Genetics 47:1719–1736
- Levine L, Akin E, Olvera O, Gaso MI, Rockwell RF, Gonzalez F, de la Rosa ME, Guzman J (1987) Chromosomal and behavioral studies of Mexican *Drosophila*. V. Frequencies of multiple insemination in three natural populations. Am Nat 129:458–462
- Martan J, Shepherd BA (1976) The role of the copulatory plug in reproduction of the guinea pig. J Exp Zool 196:79–84
- Pamilo P (1984) Genotypic correlation and regression in social groups: multiple alleles, multiple loci and subdivided populations. Genetics 107:307–320
- Parker GA (1970) Sperm competition and its evolutionary consequences in the insects. Biol Rev 45:525–567
- Parker GA (1984) Sperm competition and the evolution of animal mating strategies. In: Smith RL (ed) Sperm competition and the evolution of animal mating systems. Academic Press Inc, Orlando, pp 1–60
- Parker GA, Smith JL (1975) Sperm competition and the evolution of the precopulatory passive phase behaviour in *Locusta migratoria migratorioides*. J Entomol (A) 49:155–171
- Rahn H (1940) Sperm viability in the uterus of the garter snake, *Thamnophis*. Copeia 1940:109–115
- Ross P, Crews D (1977) Influence of the seminal plug on mating behaviour in the garter snake. Nature 267:344–345
- Ross P, Crews D (1978) Stimuli influencing mating behavior in the garter snake, *Thamnophis radix*. Behav Ecol Sociobiol 4:133–142
- Schuett GW, Gillingham JC (1986) Sperm storage and multiple paternity in the copperhead, *Agkistrodon contortrix*. Copeia 1986:807–811
- Schwartz JM (1989) Multiple paternity and offspring variability in wild populations of the garter snake *Thamnophis sirtalis* (Colubridae). Unpublished PhD dissertation, University of Tennessee, Knoxville, Tennessee, USA
- Selander RK, Smith MH, Yang SY, Johnson WE, Gentry JB (1971) Biochemical polymorphism and systematics in the genus *Peromyscus*. I. Variation in the old-field mouse (*Peromyscus polionotus*). Studies in Genetics VI. Univ Texas Publ 7103:49–90
- Sivinski J (1984) Sperm in competition. In: Smith RL (ed) Sperm competition and the evolution of animal mating systems. Academic Press Inc, Orlando, pp 85–115
- Sokal RR, Rohlf FJ (1981) Biometry. Sec. Ed. Freeman, San Francisco
- Stewart GR (1972) An unusual record of sperm storage in a female garter snake (genus *Thamnophis*). Herpetologica 28:346–347
- Still B, Madsen T, Niklasson M (1986) Multiple paternity in the adder, *Vipera berus*. Oikos 47:173–175
- Waage JK (1984) Sperm competition and the evolution of odonate mating systems. In: Smith RL (ed) Sperm competition and the evolution of animal mating systems. Academic Press Inc, Orlando, pp 251–290
- Whittier JM, Crews D (1986) Ovarian development in red-sided garter snakes, *Thamnophis sirtalis parietalis*: relationship to mating. Gen Comp Endocrinol 61:5–12
- Whittier JM, Mason RT, Crews D (1985) Mating in the red-sided garter snake, *Thamnophis sirtalis parietalis*: differential effects on male and female sexual behavior. Behav Ecol Sociobiol 16:257–261
- Zweifel RG, Dessauer HC (1983) Multiple insemination demonstrated experimentally in the kingsnake, *Lampropeltis getulus*. Experientia 39:317–319