Multiple Mating Results in Multiple Paternity in Richardson's Ground Squirrels, *Spermophilus richardsonii*

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Microsatellite DNA primers developed from Columbian Ground Squirrels (*Spermophilus columbianus*) were used to establish paternity in a Manitoba population of Richardson's Ground Squirrels (*Spermophilus richardsonii*). Primers resolving variation at six microsatellite loci allowed ascription of paternity to 32 of 85 offspring born among litters of 15 breeding females sampled. While the failure to unambiguously document paternity for all juveniles precludes the use of these data to address questions of sperm competition and male mating success, the results do provide direct evidence that multiple mating by female Richardson's Ground Squirrels results in multiple paternity within litters.

Key Words: Richardson's Ground Squirrel, *Spermophilus richardsonii,* mating, microsatellite DNA, multiple paternity, Manitoba.

Differences in gamete size, and hence the differential investment made in gametes by males and female ultimately define the sexes (Parker et al. 1972) and have promoted the evolution of disparate reproductive strategies. Males commonly enhance their fitness by obtaining copulations with more than one female (Bateman 1948), maximizing the propagation of like copies of their genes by siring as many offspring as possible. Conversely, the enhancement of female fitness is typically achieved through choosiness, whereby females mate only with males providing the greatest access to resources (Verner and Willson 1966; Thornhill 1976; Stanford 1995) and/or who are the most fit and contribute good genes to the female's progeny (Zahavi 1975; Weatherhead and Robertson 1979; Hamilton and Zuk 1982). At a proximate level, anisogamy also promotes sex-differential mating behaviour. The relatively few, large, nutrient-rich eggs produced by females necessitate only a single male ejaculate containing vast numbers of small, nutrient-poor spermatozoans to achieve fertilization, and thus female reproductive success may not increase through multiple mating.

Despite these fundamental predictions following from anisogamy, multiple mating by females is common in animals and can prove beneficial to females for various reasons. Obtaining multiple male mates may ensure fertilization (Hoogland 1998), enhance access to resources (Gray 1997), promote parental care on the part of males (Davies et al. 1996), confuse males as to paternity and thus reduce the probability of infanticide (Hrdy 1977), increase female fitness by ensuring genetic compatibility between the female's eggs and at least some of the sperm (Zeh and Zeh 1996) or incite sperm competition (Gomendio and Roldan 1993), whereby the fitness of the female's offspring is enhanced by virtue of the good genes contributed by the victorious spermatozoans (Evans and Magurran 2000).

Where multiple mating occurs, it is critical to understand its implications to both males and females. Advances in molecular biology have revolutionized the study of reproductive behaviour, and revealed that observations of male/female association and mating behaviour may not be representative of parentage in free-living animal populations (Quinn et al. 1987). Methods for determining parentage (see Fleischer 1996 for a review) have also become more powerful and accessible, thereby providing researchers with an opportunity to obtain comparative data which address both the proximate and ultimate questions pertaining to reproductive behaviour.

Such a comparative picture has begun to emerge for the ground-dwelling squirrels (*Spermophilus* spp.), where multiple mating commonly results in multiple paternity within litters, and the first male to mate with a given female often sires a disproportionate number of offspring within a litter. Hanken and Sherman (1981) reported that 78% of 27 Belding's Ground Squirrel (*S. beldingi*) litters for which paternity could be determined using polymorphic blood proteins were multiply sired by between two and three males. Similarly, 50% of eight Thirteen-lined Ground Squirrel (*S. tridecemlineatus*) litters were sired multiply, with the first male to mate siring 75% of offspring in those litters (Foltz and Schwagmeyer 1989). Boellstorff et al. (1994) detected multiple paternity in 88.9% of nine litters of California Ground Squirrels (*S. beecheyi*) and Murie (1995) reported multiple paternity in 15.8% of 165 Columbian Ground Squirrel (*S. columbianus*) litters. Murie (1995), however, predicted that his data likely underestimated the incidence of multiple paternity given that only five polymorphic loci were used, and only one of those had more than two alleles. Indeed, using a more comprehensive and variable series of microsatellite primers (Stevens et al. 1997), multiple paternity was detected in 64% of litters born to female Columbian Ground Squirrels that had mated multiply (Stevens, Strobeck and Murie unpublished data). Of those litters, 77% were sired by two males, 21% by three males, and 2% by four males. Further, there was a distinct first-male advantage, with 65% of the offspring sampled from among 77 litters sired by the first male to mate, and a progressively declining proportion of paternity success for subsequently mating males (Stevens, Strobeck, and Murie unpublished data).

Despite the widespread occurrence of multiple paternity and a first-male mating advantage, deviations from that pattern are apparent among ground squirrels. Using both protein isozymes and DNA fingerprinting (see Burke 1989), Lacey et al. (1997) revealed that multiple paternity among Arctic Ground Squirrels (*S. parryii*) was relatively rare; it occurred in only 1 (9%) of 11 litters examined and the first male to mate sired the majority (90%) of the offspring in that litter. A departure from the first-male mating advantage has been detected in Idaho Ground Squirrels (*S. brunneus*), where Sherman (1989) reported a last-male mating advantage (a minimum of 66 – 100% of pups sired by the last/longest attending male) in the five of seven litters (71.4%) where multiple paternity was evident. Additional data for other *Spermophilus* species will prove useful in determining whether these species are unique in their apparent departures from the general pattern, and thus how these expressions of multiple mating and sperm precedence impact the behaviour and fitness of males and females.

Richardson's Ground Squirrels (*S. richardsonii*) are locally abundant throughout grazed areas of the Great Plains (Michener and Koeppl 1985). Females mate multiply (Michener and McLean 1996), and although this may result in multiple paternity within litters (van Staaden et al. 1994, Michener personal communication), direct evidence of multiple paternity is lacking. Such data would contribute to our understanding of reproductive behaviour, and open new avenues for research into the contributions of paternal kinship to social interactions (e.g., Holmes and Sherman 1982) and patterns of dispersion among relatives in nature. We applied primers developed by Stevens et al. (1997) to prospective parents and offspring from litters of 15 breeding females in a Manitoba population of Richardson's Ground Squirrels to test whether multiple mating results in multiple paternity within litters.

Methods

Study site, research subjects, and sample collection

Research was conducted between 8 April and 26 July 2002 on a 1.5-ha section of a larger 5-ha cattle pasture near Westbourne, Manitoba (50°10.190'N, 98°38.103'W). Richardson's Ground Squirrels on that site were live-trapped in National or Tomahawk traps baited with peanut butter and were marked for permanent identification with numbered metal ear tags (Monel #1 fish fingerling tags). Each squirrel was also given a distinctive mark on its dorsal pelage with black hair dye (Clairol Hydrience 52 Black Pearl). Trapping was conducted during the mating season (12 through 25 April 2002) to determine mating dates of females and to monitor the reproductive condition of all squirrels. After mating had ended, trapping continued to assess gestational progress in females, and a 2-mm tissue punch was taken from one pinna of each adult (10 males**,** 18 females). Samples were placed in 1.5-ml microcentrifuge tubes in 95% ethanol, and stored at 4 – 7°C prior to DNA extraction and subsequent molecular analysis. Fifteen females for which mating and parturition dates were known, and for whom ear punches had been obtained, and nest-burrow entrances located were chosen to provide litters for paternity analysis. All juveniles in those litters were trapped within three days of their first emergence from their natal burrow, marked with ear tags and dye marks, and tissue sampled as described for adults. Animal research was conducted in accordance with the guidelines for the use of animals in research set forth by the Canadian Council on Animal Care.

Molecular analysis of paternity

Individual ear punches were placed in 1.5-mL microcentrifuge tubes, centrifuged in a speed-vac for 5 min to remove the excess ethanol and digested at 55°C for 4 – 6 hrs in 70 µL of a sterile cell lysis solution (10 mM Tris-HCL, 100 mM EDTA, 2% SDS, pH 8.0) containing 3 – 4 µL Proteinase K (10 mg/mL). Following the addition of 4 µL of RNase A, the tubes were mixed by inversion and incubated at 37°C for 30 min. Proteins were precipitated by adding 23.5 µL of 7.5 M ammonium acetate to each tube of cell lysate and placing the samples on ice for 30 min. Tubes were centrifuged at 14000 rpm for 3 min and the supernatant was transferred to a new 1.5-mL tube containing 70 µL isopropanol. Samples were mixed by inversion, stored at -20°C for several hours and centrifuged at 14000 rpm for 2 min. Following the removal of the supernatant, the pelleted DNA was washed in 75 µL of ice-cold 75% ethanol and centrifuged at 14000 rpm for 1 min. The ethanol was removed with a pipette and the tubes containing the DNA were placed in speed-vac for 5 – 10 min. The dried pellets were rehydrated in 40 µL of sterile TLA buffer (10 mM Tris-HCL, 0.1 mM EDTA, pH 8.0) and stored at -20°C.

Amplifications were performed on a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA) in a 25-µL volume containing ~ 200 ng DNA, 0.8 mM dNTP, PCR buffer (0.1 M Tris-HCl pH 8.3, 0.5 M KCl, 15 mM MgCl, 0.005 g gelatin/10 mL buffer), 0.5 units of Taq polymerase, and 0.2 µM concentrations of each of seven primers (GS3, GS12, GS14, GS20, GS22, GS25, GS26) described by Stevens et al. (1997). The cycling conditions were 94°C for 2 min followed by two cycles of 94°C for 30 s, 58°C for 20 s, 72°C for 5 s, and 33 cycles of 94°C for 15 s, 54°C for

20 s and 72°C for 5 s followed by a final extension at 72°C for 30 s. PCR products were diluted (1:1) with sterile water and 1 µL of each diluted product was resolved by polyacrylamide gel electrophoresis on an ABI 373 Automated Sequencer (Applied Biosystems). Microsatellites were visualized and sized using GeneScan® 3.1 and Genotyper® 2.1 software (Applied Biosystems).

Results

Of the seven primers employed, one (GS22) resulted in multiple banding patterns and was excluded from subsequent analysis. The remaining six primers resolved meaningful variation among individuals and were used in establishing familial relationships among the individuals sampled. Based upon the observed frequencies of the three to six alleles evident at those six loci (Table 1), the probability that two randomly drawn unrelated individuals will be identical at all loci is 1 in 9577 (from equation in Patekau and Strobeck 1994). Further, the probability that an unrelated male will be excluded as sire given knowledge of the mother's genotype is 0.91 (from equation in Chakravarti and Li 1983). All calculations assume that the loci examined are not linked, and provide highly conservative estimates of exclusion given that they are based on pooled data from dams, sires, and their offspring.

Paternity was definitively ascribed to at least one juvenile within litters of all 15 dams included in the study. In total, however, unique identification of the male sire was achieved for only 32 of the 85 juveniles that emerged in those litters. Based upon those identifications, multiple sires were detected in 12 of the 15 litters examined (80%), with a minimum of two sires in 11 and a minimum of three sires in one of those 12 litters (2.1 ± 0.1 sires/litter; mean ± SE).

Discussion

The microsatellite primers developed by Stevens et al. (1997) proved effective in resolving multiple paternity within litters of Richardson's Ground Squirrels. Indeed, with multiple sires in 80% of the 15 litters examined, multiple paternity is at least as common in Richardson's Ground Squirrels as it is in other *Spermophilus* species with the exception of California Ground Squirrels.

Definitive genetic evidence of multiple paternity in the present study suggests that the use of the primers described by Stevens et al. (1997) in future studies of the reproductive and social behaviour of Richardson's Ground Squirrels would prove quite fruitful. In concert with data on mating behaviour of the sort presented in Michener and McLean (1996), such studies could address the outcome of sperm competition and its relation to mating order (Sherman 1989), the role of aggression and male territoriality in affecting male mating success (Schulte-Hostedde and Millar 2002), and the factors underlying mate choice such as the effects of symmetry (Penton-Voak et al. 2001), body size (Gwynne 1981), paternal effort (Huber et al. 2002), and genetic relatedness (Bateson 1983; Chapman and Crespi 1998). The use of these microsatellite loci to assess relatedness among individuals (Queller et al. 1993) would also allow insight into the role paternal kinship plays in influencing social behaviour (Widdig et al. 2002). Additional markers, however, would be necessary to comprehensively document the paternity of each and every individual. Microsatellite primers that were not employed in the present study, such as GS17 and GS34 (Stevens et al. 1997) or the 13 Idaho Ground Squirrel microsatellite primers described by May et al. (1997), would likely prove useful in this regard.

The alleles identified in our Manitoba population (Table 1) differ from those described for the Richardson's Ground Squirrels examined by Stevens et al. (1997) in evaluating the efficacy of their primers on other Sciurids (see Table 2 in Stevens et al. 1997). Given that geographic variation, the primers employed here should also prove useful for studies of dispersal (Chapuisat et al. 1997), interpopulation differences related to conservation efforts (Patekau and Strobeck 1994), and perhaps even in refining the taxonomy of the ground-dwelling squirrels (Gill and Yensen 1992).

TABLE 1: Characteristics of microsatellite loci (from Stevens et al. 1997) applied in paternity analysis of Richardson's Ground Squirrels. Results are based upon allele frequencies from 10 adult male, 15 adult female, and 85 juvenile squirrels. Expected heterozygosity and probability of identity were calculated according to formulae presented in Patekau and Strobeck (1994), and probability of exclusion was calculated following the methods of Chakravarti and Li (1983).

Locus	Alleles	Expected heterozygosity	Probability of identity	Probability of exclusion
GS3	227, 229, 231, 233	0.630	0.178	0.347
GS12	147, 149, 152, 154	0.672	0.128	0.416
GS14	242, 244, 246	0.549	0.298	0.253
GS20	220, 222, 237	0.105	0.803	0.053
GS25	138, 142, 144, 146, 148, 154	0.769	0.057	0.557
GS26	109, 111, 113, 115	0.480	0.336	0.232

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