

**MULTIPLE MATING AND FEMALE CHOICE IN RICHARDSON'S GROUND
SQUIRRELS (*UROCITELLUS RICHARDSONII*)**

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DEDICATION

To my mom for putting up with scarce phone calls and to my dad for fixing up the best
little car so I could come to Alberta.

And to the ground squirrels for being so interesting.

ABSTRACT

I studied the mating behaviour of Richardson's ground squirrels (*Urocitellus richardsonii*) in 2007 and 2008, near Picture Butte, AB, Canada with the goals of evaluating multiple paternities within litters, sperm competition, and female mate choice. Eleven microsatellites and use of behavioural data to eliminate males who could not be putative sires based on their spatial and temporal remoteness from estrous females enabled exclusive assignment of paternity to 82.8% of 598 offspring with known maternity. The frequency of multiple paternities (26.4%, n=87 litters) in my population was lower than that reported for Richardson's ground squirrels in a Manitoba population (80.0%, n=15 litters). Sperm competition was absent except in litters weaned by those females that mated again within 5 minutes of the first copulation, in which case sperm displacement was evident. Female choice was observed through avoidance of copulation, termination of copulation, and hide-then-run behaviour.

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LIST OF ABBREVIATIONS

- DMSO: Dimethyl sulfoxide
- dNTP: Deoxyribonucleotide triphosphate
- EDTA: Ethylenediaminetetraacetic acid, disodium salt dihydrate
- H-W: Hardy-Weinberg Equilibrium
- H_E: Expected heterozygosity
- H_O: Observed heterozygosity
- LOD: Log of the likelihood
- MST: Mountain Standard Time
- OSR: Operational sex ratio
- P. Id.: Probability of identity
- P. Excl.: Probability of exclusion
- PCR: Polymerase chain reaction
- PIC: Polymorphic information content
- PTT: 2-tailed paired t-test
- r: Coefficient of relatedness
- SDS: Sodium dodecyl sulphate
- SEM: Standard error of mean
- T_a: Annealing temperature
- T_d: Denaturing temperature
- TE: Tris-EDTA buffer
- WSR: Wilcoxon signed rank

Chapter I

INTRODUCTION TO POLYANDRY IN GROUND SQUIRRELS (TRIBE: MARMOTINI)

Mating systems can be classified as monogamous, polygynous, polyandrous, polygynandrous, or promiscuous depending on whether males or females mate with a single mate or multiple mates. Polygynous mating systems, where males mate with multiple females, occur in over 90% of mammalian species (Trivers 1972, Kleiman 1977, Clutton-Brock 1989). Polygyny can be further divided into “male-defense polygyny” and “non-defense polygyny” (Emlen and Oring 1977) in which males either defend territories that contain resources (including females) or they compete for status within a dominance hierarchy, thereby gaining differential access to females (Dobson 1984, Davis and Murie 1985).

Research has focused on male mating systems because mating with multiple females enhances a male’s breeding success and lifetime reproductive success (Clutton-Brock 1989). In contrast, because a female possesses a set number of eggs and a small amount of ejaculate should be sufficient to fertilize them, it is less clear why mating with multiple males (polyandry) is beneficial to females (Trivers 1972). None-the-less, females have been described to mate with multiple males in at least 133 species of mammals (Wolff and Macdonald 2004).

Numerous reviews are available on the costs and benefits of mating with multiple males (see Halliday and Arnold 1987, Hunter et al. 1993, Keller and Reeve 1995, Yasui 1997, Wolff and Macdonald 2004). Costs of polyandry include: loss of time with respect

to feeding and nest/burrow maintenance; metabolic costs devoted to courtship; disease or parasite transmission; increased susceptibility to predation; and negative effects of seminal fluid products transferred by males during copulation such as beta-endorphins that suppress sexual receptivity in rats (Forsberg et al. 1990).

The benefits of polyandry can be divided into two categories: direct benefits and indirect or genetic benefits. Direct benefits tend to be environmental whereas genetic benefits refer to the heritable characteristics of mating success. Direct benefits include: reduced infanticide by increased male tolerance of offspring; reduced disruption in the population caused by reduced male-male competition; gain of paternal care; protection from conspecifics and predators at the time of courtship and copulation; material benefits such as access to food resources; reduced sexual harassment; stimulation of reproduction; assurance of fertilization to offset short-term sperm depletion in males and male sterility; and increase in litter size. Indirect genetic benefits include: good genes in offspring due to genetic diversity, sperm competition and probability of mating with higher-quality males; reduced chance of genetic incompatibility; increased offspring survival; and female choice of paternity. One would expect polyandry to occur when the benefits of mating with multiple males outweigh the costs.

The direct benefits of polyandry can be determined by observations and simple calculations; however, genetic benefits require molecular analysis and remain controversial (Jennions and Petrie 2000). Jennions and Petrie (2000) suggest that females are more likely to mate with multiple males for genetic benefits than for non-genetic benefits implying that non-genetic benefits always have the possibility of an underlying genetic benefit.

The good genes hypothesis states that if a male possesses better genes than another, he will pass those genes on to his offspring who will in turn have a better overall fitness than offspring sired by a lower-quality male (Howard et al. 1994). Good genes can be represented by a male's heterozygosity given a known set of polymorphic microsatellite loci (Cohas et al. 2006). Heterozygosity based on microsatellite loci is thought to represent fitness in a natural population given 2 different hypotheses (Hansson and Westerberg 2002): the local effect and the general effect hypothesis. The local effect hypothesis applies to recently bottlenecked populations and the general effect hypothesis applies during partial inbreeding; however, both contrast heterozygosity to linked recessive deleterious alleles in homozygotes (Zouros 1993). For example, female alpine marmots (*Marmota marmota*), a socially monogamous species, mate with males other than their pair-mates and these extra-pair males are most often more heterozygous than the dominant pair-mate (Cohas et al. 2006). Another way to examine good genes is via sperm competition. If 2 males mate with the same female during the same reproductive period, the sperm of the genetically superior male will be more successful at fertilizing the female's ova than the sperm of the less superior male (Parker 1970). The genetically superior sperm may be physiologically superior as well and get to the ova first because they are faster swimmers, they may get through the zona pellucida faster because of a faster acrosome reaction, or kamikaze sperm from one male may kill the sperm of a competing male (Poiani 2006). Sperm competition is widespread in polyandrous mammals despite early scepticism that sperm competition would be unlikely in mammals because sperm only survive a short time after ejaculation and, except in bats, sperm are

not stored for subsequent fertilizations (Parker 1970, Ginsberg and Huck 1989, Crighton et al. 1994).

In mammals in general, females are predicted to be the choosier sex because they contribute larger, more energetically costly gametes than males and they incur the costs of gestation and lactation (Parker et al. 1972, Trivers 1972). Males on the other hand provide relatively cheap gametes and often provide little or no parental care (Trivers 1972). Females can have an influence on which males sire their offspring by exercising choice either pre-copulation, during copulation, or post-copulation, either before fertilization or after fertilization (Birkhead and Moller 1993). Pre-copulatory female choice refers to events leading up to copulation only and post-copulatory female choice refers to processes from the start of copulation, including during copulation, and following copulation (Eberhard 2000). Pre-copulatory female choice is the more commonly examined process and includes active choice, in which the female encounters several prospective mates and she chooses only some of them to mate with (Gibson and Langen 1996). Forms of post-copulatory female choice reviewed by Eberhard (2000) include removal of copulatory plugs and termination of copulation before sperm transfer. Copulatory plug removal is seen in female fox squirrels (*Sciurus niger*) and eastern gray squirrels (*S. carolinensis*; Koprowski 1992), both medium-sized tree squirrels found in eastern and central United States (Koprowski 1994a, b). Termination of copulation before sperm transfer is observed in grey mouse lemurs (*Microcebus murinus*), small nocturnal arboreal primates found in the forests of western Madagascar (Radespiel and Zimmermann 2003). Cryptic female choice in the form of post-copulatory sperm choice, in which the female distinguishes between and chooses the sperm of a specific male

within her reproductive tract in order to fertilize her ova (Kempnaers et al. 2000), is difficult to determine and will not be further discussed.

Multiple mating by females is not uncommon in North American ground-dwelling squirrels (Family: Sciuridae, Tribe: Marmotini, previously Genus: *Spermophilus*; Helgen et al. 2009). Polyandry has been documented, on the basis of paternity analyses, in the following species: *Ictidomys tridecemlineatus*, *Otospermophilus beecheyi*, *Urocitellus beldingi*, *U. brunneus*, *U. columbianus*, *U. parryii*, and *U. richardsonii* (Hanken and Sherman 1981, Foltz and Schwagmeyer 1989, Sherman 1989, Boellstorff et al. 1994, Murie 1995, Lacey et al. 1997, Hare et al. 2004). Multiple mating by females can be common and occur 100% of the time as in *U. columbianus* (Murie 1995) and *O. beecheyi* (Boellstorff et al. 1994) or lacking altogether as in *Spermophilus citellus* (Millesi et al. 1998). In all of the species with multiple mating, polyandry sometimes results in shared paternity of litters (Hare et al. 2004), from as low as 8% of litters in *U. parryii* to as high as 89% of litters in *O. beecheyi* (Boellstorff et al. 1994, Lacey et al. 1997, Hare et al. 2004).

Sperm competition, either in the form of sperm precedence (first-male advantage) or sperm displacement (last-male advantage), is known for 6 of the 7 species of ground squirrels with known multiple paternities (Hanken and Sherman 1981, Foltz and Schwagmeyer 1989, Sherman 1989, Boellstorff et al. 1994, Murie 1995, Lacey et al. 1997). There is a strong tendency for the first male to sire a higher proportion of offspring than subsequent mates within the same estrous period (sperm precedence). First males can sire as many as 92.4 % of offspring within litters of *U. parryii* (Lacey et al. 1997) or as few as 60.0% of offspring in *U. beldingi* (Sherman 1989). In *U. brunneus*

however, there is a tendency for the last and longest mate-guarding male to sire a higher proportion of the offspring than previous mates within the same estrous period (sperm displacement; Sherman 1989).

Sperm competition in the form of sperm displacement is positively correlated with mate guarding (Sherman 1989) implying that a male's share of paternity will be reduced if he does not prevent the female from mating with additional males. In *U. brunneus*, 26.9% of females were guarded by multiple males. These females were sequentially guarded by heavier males for a longer period of time with each male and therefore the last and longest mate guarding male tended to sire the highest percentage of offspring. An exception to this mate guarding-sperm displacement trend is *U. parryii*, in which only the first male to mate with a female mate guarded her and therefore sperm precedence was prominent (Lacey et al. 1997). This suggests that mate guarding may not be related to sperm displacement but to male mating success instead.

U. richardsonii have been observed to mate with multiple males (Michener and McLean 1996), and multiple paternities within litters have been confirmed by molecular analysis (van Staaden et al. 1994, Hare et al. 2004); however, patterns of sperm competition have not yet been established. Richardson's ground squirrels are medium-sized, semi-fossorial, obligate hibernators (Michener 1998). They prove to be an ideal study species for behavioural studies because they are diurnally active and they inhabit short- and mixed-grass prairie, which makes them readily visible. Individuals are relatively easy to trap and Richardson's ground squirrels are so abundant in the prairies that they are commonly perceived as agricultural pests (Bell and Piper 1915). Their short gestation period (23 days) provides a relatively short wait in order to sample litters for

parentage studies in captivity, but in nature litters do not emerge above-ground until juveniles are 29-30 days old (Michener and Koepl 1985). The non-ideal problems that arise when studying the mating behaviour of this species is that individuals usually mate underground near sunset when light conditions are poor. Although Columbian ground squirrels also typically mate underground, they usually mate in the morning when they are readily visible. However, they have small litters (2-4 pups; Murie and Dobson 1987) compared to Richardson's ground squirrels (6-8 pups; Risch et al. 2007).

In southern Alberta, female Richardson's ground squirrels emerge from hibernation in early March, by which time the majority of males have been above-ground for 2-3 weeks (Michener 1998). Although the mating period lasts 2-4 weeks, individual females have only a 2- to 3-hour period in the late afternoon in which they are receptive to copulation (Michener and McLean 1996). In spite of this time limit, about 50% of females mate with 2-3 males (Michener and McLean 1996, Hare et al. 2004).

Although the sex ratio at birth and weaning is 1:1 for Richardson's ground squirrels, the sex ratio among adults (≥ 1 year-old) is significantly biased to females, usually with more than 3 times as many females as males (Michener 1989). This female-biased sex ratio makes polygyny a statistical necessity rather than a male mating strategy (Michener 1983a).

Richardson's ground squirrels have been described to have both male-defense polygyny and non-defense polygyny with some individual males switching from one strategy to the other within the same mating period (Davis and Murie 1985). Male-defense polygyny appears to be the more common form of mating system present in Richardson's ground squirrels and its presence is negatively correlated with the daily

operational sex ratio (OSR), which is the number of reproductively available females with respect to the number of reproductively available males (Emlen and Oring 1977, Davis and Murie 1985). When exhibiting male-defense polygyny, male Richardson's ground squirrels increase their effort to acquire mates and increase their territoriality (male-male conflict; Davis and Murie 1985, Michener and McLean 1996). When males are displaying non-defense polygyny, they tend to mate with 1 female and move on to the next (Davis and Murie 1985).

Darwin proposed that sexual selection consists of male-male competition (intra-sexual competition) and female choice (inter-sexual competition) (as reviewed in Trivers 1972). A loss in weight, extensive wounding as the result of male-male fights, and high mortality have been documented for adult male Richardson's ground squirrels during the mating season (Michener 1983, Michener 1998). Weight loss, aggression, and death are indicators that males compete for access to females during the mating period and that intra-sexual selection is common. Female choice is suspected to occur in Richardson's ground squirrels based on the observations that some estrous females use a hide-then-run behaviour tactic in which females sneak away from local males and sometimes actively seek out unfamiliar males away from their usual home range (Michener and McLean 1996).

Female Richardson's ground squirrels give birth to an average of 7 pups in a single litter a year (Michener 1989) and pups increase in mass ten-fold during the 29-30 days that they are dependent on mother's milk (Michener 1989). Meanwhile, males provide no paternal care. Therefore, females would benefit from choosing their mates and/or the order with which they mate with each male in order to bias the paternity of

their litter towards the more preferred male.

In the first part of my study (Chapter II), I examine 11 microsatellites and their usefulness in assigning paternity to offspring at a strict confidence interval. The second part of my study (Chapter III) reports the frequency of multiple mating over 2 mating seasons (2007 and 2008) in a free-living population of Richardson's ground squirrels in southern Alberta and the frequency with which multiple mating results in multiply sired litters. I also address the occurrence of female choice and sperm competition with respect to the daily operational sex ratio and mate guarding in Chapter III.

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Chapter II

ASSIGNMENT OF PARENTAGE FROM MICROSATELLITE LOCI AND BEHAVIOUR IN RICHARDSON'S GROUND SQUIRRELS (*UROCITELLUS RICHARDSONII*)

Abstract

The proportion of juvenile Richardson's ground squirrels (*Urocitellus richardsonii*) assigned paternity by Hare et al. (2004) using 6 polymorphic microsatellite loci was 38% (n=85). I used 11 microsatellites originally developed for Columbian ground squirrels (*U. columbianus*) and Idaho ground squirrels (*U. brunneus*), in conjunction with the elimination of candidate sires based on their temporal and physical remoteness from estrous females, to improve assignment of paternities in Richardson's ground squirrels. Allelic diversity across loci averaged 4.5 ± 0.3 alleles per locus (range 2-7 alleles). The average expected heterozygosity was 0.5742 and the mean PIC value was 0.5309. The probability of 2 individual Richardson's ground squirrels having the same genetic identity was 3.95×10^{-7} . Behavioural observations permitted assignment of maternity to 576 juveniles in 92 litters; these assignments were confirmed from microsatellite data. Juveniles in 4 additional litters, comprised of 2 pairs of inter-mixed litters, were unambiguously assigned to their dam using microsatellite information. Given that maternity was known, the probability of excluding a male Richardson's ground squirrel as a sire across all loci was 0.9892. Of 598 juvenile Richardson's ground squirrels from 96 litters with known mothers, 46.7% were assigned paternity to one of 36 potential sires by exclusion. After observational data were used to eliminate males deemed unavailable to mate with a female on her day of estrus, 82.8% of offspring could

be assigned to one specific male. The likelihood method at the 95% confidence interval in conjunction with elimination of impossible sires due to temporal remoteness permitted paternal assignment to 97.6% of juvenile Richardson's ground squirrels. By assigning paternity at such a high rate I determined that 23 of 96 litters were multiply sired.

Introduction

Microsatellites, short tandem repeats of nucleotide sequences (1-6 base pairs) found throughout the eukaryotic genome, are highly variable molecular markers with low mutation rates from one generation to the next (Queller et al. 1993). Because microsatellites are co-dominant markers that can differentiate between homozygotes and heterozygotes, they are extremely useful for assignment of parentage.

Paternity can be assigned using 3 different methods: exclusion, likelihood/probability, and genetic reconstruction. Exclusion takes into account the Mendelian inheritance properties of microsatellites implying that a diploid offspring must have 2 alleles at a locus, one from its mother and one from its father. Exclusion is most effective when highly variable microsatellite loci are used. Due to sampling limitations, most studies cannot sample all of the candidate parents and therefore are unable to achieve complete parentage assignments using exclusion alone (Jones and Ardren 2003). Even if all potential parents are known, exclusion has its limitations. Parentage cannot be assigned exclusively when too few microsatellites with too few alleles are used or when potential parents in the population are genetically similar due to common ancestry and recent bottlenecks. In addition, microsatellites are subject to genotyping errors, null alleles, and mutations (Webster and Reichart 2005). Genotyping errors can arise when a polymerase chain reaction (PCR) has not been fully optimized and thus scoring of alleles

due to stutter has become questionable. Null alleles occur when a mutation has occurred at the PCR priming site of an allele, such that it does not get amplified, resulting in over-representation of homozygotes (Webster and Reichart 2005). Mutations, although rare, automatically cause the real parent to be excluded erroneously; however, these are easy to detect as they mismatch at only a single locus.

Likelihood calculations relate parentage to homozygosity using the statistical assumption that homozygous individuals are more likely to have a common allele with offspring than heterozygous individuals. Likelihood is calculated by using the logarithm of the likelihood ratios (LOD score; Meagher and Thompson 1986) where the male with the greatest LOD score is the most likely (or probable) sire. Statistical confidence for this method is represented by delta (Δ), the logarithm of the ratio of the likelihood values of the most probable male with respect to any other probable males (Kalinowski et al. 2007). The higher the Δ value the greater the confidence that the most likely male is indeed the father of the offspring.

Pedigree reconstruction is useful when the genotype of one parent is known and multiple mating may have occurred (Jones and Ardren 2003). Pedigree reconstruction reveals whether 3 or more individuals must have contributed parentage to a litter and thus permits detection of multiple matings. A limitation of pedigree construction is that parentage cannot be assigned unless genotypes of all possible parents within the population are known and those genotypes are sufficiently variable among the possible parents that all other individuals in that population can be eliminated as parents.

One advantage of microsatellites over other molecular markers is that markers developed for one species can be used in closely related species (Primmer et al. 1996).

Numerous microsatellites have been developed for Sciurids (May et al. 1997, Stevens et al. 1997, Hanslik and Kruckenhauser 2000, Garner et al. 2005, Jones et al. 2005, Gondek et al. 2006) in order to perform parentage and kinship studies and to increase knowledge on population structuring. Six of the loci developed for Columbian ground squirrels (*Urocitellus columbianus*) amplify in Richardson's ground squirrels (*U. richardsonii*; Stevens et al. 1997, Hare et al. 2004).

Richardson's ground squirrels are medium-sized, semi-fossorial rodents native to the short- and mixed-grass prairies of Canada and the northern United States. They hibernate through the winter, resuming above-ground activity in southern Alberta in late February (males) to mid-March (females). The single annual mating season occurs shortly after females emerge from hibernation. Typically, each female is in behavioural estrus for 2-3 h in the afternoon of her third or fourth day after emergence (Michener and McLean 1996).

Observational data have established that some female Richardson's ground squirrels mate with 2-3 males (Michener and McLean 1996) and, based on allozyme and microsatellite data, respectively, van Staaden et al. (1994) and Hare et al. (2004) confirmed that litters of Richardson's ground squirrels can have multiple sires. The 6 microsatellites used by Hare et al. (2004) permitted assignment of paternity by exclusion to 38% of 85 juveniles in 15 litters and confirmed the occurrence of multiple paternities in Richardson's ground squirrels. Increasing the number of polymorphic microsatellite loci included in exclusion analysis should increase the proportion of juveniles assignable to a male, but 100% assignment can only be achieved if enough microsatellites with sufficient allelic variability are available to permit every possible parent to have a unique

genotype. Another approach to parental assignments is to eliminate as many candidate parents as possible through behavioural observations to leave a small pool of putative parents from which to assign paternity from genetic data.

Initial parentage studies on ground squirrels (Hanken and Sherman 1981, Foltz and Schwagmeyer 1989, Sherman 1989, Boellstorff et al. 1994, Murie 1995, Lacey et al. 1997) and prairie dogs (Genus: *Cynomys*; Foltz and Hoogland 1981) used starch gel electrophoresis and DNA fingerprinting techniques to assign potential sires to offspring. Microsatellites developed more recently have been used to examine population structuring (Roach et al. 2001, Garner et al. 2005) and parentage (Haynie et al. 2003, Hare et al. 2004). Haynie et al. (2003) used 7 microsatellites and a combination of observational data, exclusion, and likelihood analysis to unambiguously assign 46% of 261 offspring to a putative sire in Gunnison's prairie dogs (*C. gunnisoni*) and 48% of 223 offspring in Utah prairie dogs (*C. parvidens*). Although Haynie et al. (2003) tried to incorporate observational data, the assignment rates were low because either all potential sires were eliminated or multiple males remained as possible sires. Additionally, not all offspring could be assigned to their mothers. Differences due to marker choice are unlikely based on expected heterozygosities because values in the 2 studies (0.534 and 0.327-0.490, from Hare et al. 2004 and Haynie et al. 2003, respectively) were similar to that in the current study (0.574).

Maternity is easily assigned by behavioural means in Richardson's ground squirrels; however, assigning paternity by observations is less feasible. Because squirrels usually mate underground near dusk, when light conditions for observation are poor, it might not be possible to unambiguously identify potential males. Also in the event that a

female mates with several males, knowing which male sired what proportion of the female's litter remains unknown. The objective of my study is to determine whether increasing the number of microsatellites currently known to amplify in Richardson's ground squirrels is sufficient to assign paternity by exclusion and to establish the extent to which elimination of candidate sires based on their temporal remoteness to estrous females further improves assignment of paternity.

Methods

Study Area, Capture Techniques, and Tissue Sampling

The study population of Richardson's ground squirrels, located 5 km east and 1 km south of Picture Butte, Alberta, Canada, has been under investigation since 1987 (Michener 1992, 1996). The 3.4-ha Study Site contains a 1.7-ha portion, herein referred to as the Main Site, where my study was focused. The Study Site, which is bordered on 2 sides by agricultural fields and on the other 2 sides by a farmyard and a road with agricultural fields beyond, is isolated by at least 1.5 km from the next nearest suitable habitat. All adult (≥ 1 year-old) males in the population were live-trapped (Tomahawk Live Trap Co., single-door squirrel traps) on emergence from hibernation in 2007 (20 males) and 2008 (25 males) and a tissue sample obtained from 16 and 23 males, respectively. The other 6 males disappeared prior to the mating season therefore they could not have sired any offspring. An ear punch (B.Y.T. Co.) was thoroughly cleaned with isopropanol then used to remove a 2-mm diameter piece of tissue from one ear immediately after that ear had been carefully wiped with isopropanol. Each tissue sample

was stored in 20% DMSO, 0.25 M-EDTA, salt saturated solution (Seutin et al. 1991, Kilpatrick 2002) for 2 weeks at room temperature then frozen (-20°C) until analyses.

All adult male Richardson's ground squirrels were ear-tagged (National Band and Tag Co.) for permanent identification. Before release at the capture site, each male was individually dye-marked with human hair dye (Clairol Hydrience, #30 spiced bronze) for visual identification from a distance. Daily censuses were taken throughout the mating season to confirm that no additional males entered the population.

Adult female Richardson's ground squirrels resident on the Main Site were captured on emergence from hibernation in 2007 (38 females) and 2008 (74 females). All females had been ear-tagged in previous years, and all were individually identifiable from dye marks (Clairol Hydrience, #52 pearl black). Most females were captured again 3-5 days after emergence to inspect their external genitalia and to collect a vaginal lavage. Mating date of each female was determined from a combination of: observed above-ground copulation, inferred underground copulation, colour and degree of swelling of the vulva, presence of sperm in the lavage, and vaginal cytology (Michener and McLean 1996). Females were trapped again 1-2 days before and after the expected time of parturition (23 days after mating) to confirm that mating resulted in pregnancy and parturition. Rarely (6 of 108 females), pregnancy did not result from mating in the first estrus and re-mating occurred 6-8 days later. Based on the mother's known parturition date, the expected date (29-30 days postpartum) of litter emergence could be anticipated for every litter (32 in 2007 and 74 in 2008). Because adult females were censused daily and because each female Richardson's ground squirrel rears her litter in a separate underground chamber (Michener 2002), maternity of 104 of 108 litters could be reliably

assigned from the location of the litter at first emergence from the natal nest. For the remaining 4 litters, 2 mothers in adjacent burrow systems had similar-aged litters that emerged in such close temporal and spatial proximity that maternal assignment could not be made with complete confidence.

Trapping of juvenile Richardson's ground squirrels commenced as soon as a litter first appeared above-ground; 38 of 108 litters were trapped in their entirety within a day of first emergence and all were trapped within 6 days (mean=2.0±0.10 days). Of 38 litters on the Main Site in 2007, 22 litters and their dams were tissue sampled. Dams tissue sampled in 2007 included 10 females for whom mating observations were available and 12 additional females selected because they were close to focal females (n=4) or because they weaned large litters (range 6-10 offspring, mean=8.3±0.4, n=8). Litters in close proximity to focal females were chosen to assure that litters were complete and not mixed. Dams and litters tissue sampled in 2008 included 73 females that weaned a litter on the Main Site and 1 adjacent female with mating observations. A 2-mm-diameter sample of ear tissue obtained from each juvenile and each dam was preserved in the same manner as for tissue samples from males.

All procedures with animals were approved by the Animal Welfare Committee at the University of Lethbridge and were in accordance with the guidelines of the Canadian Council on Animal Care.

Behavioural Observations

I used scan and focal observations to determine potential mates for female Richardson's ground squirrels located on a 0.68-ha portion of the Main Site. This observation area had good visibility and included 24 of 38 females resident on the Main Site in 2007 and 41 of 74 females in 2008. I performed scan samples from a 2-m elevated observation booth in order to evaluate pre-estrous relationships. Focal observations of estrous females were conducted from the 2-m elevated observation booth, 2 ground-level observation sheds, and a 2-storey farm house, all located around the periphery of the Main Site and within 50 m of target animals. With 10 x 25 or 8 x 32 binoculars, individually dye-marked ground squirrels were identifiable up to 90 m away. Depending on the number of Richardson's ground squirrels in estrus on a given day, each of 2-4 observers followed 1-3 females nearest to that person's observation location.

Scan sampling of all visible female and male Richardson's ground squirrels occurred at 10-minute intervals for approximately 1 hour in the morning and 1 hour during mid-day each day during the mating period, defined as the set of consecutive days on which female Richardson's ground squirrels mated (6-14 March in 2007 and 1-15 March in 2008). On each scan I recorded every squirrel's behavioural activity and location on a map of the Study Site to determine male-female proximities. Interactions were classified as male-female, male-male, or female-female. Additional male-female interactions occurring between scans were also recorded opportunistically. As the number of emerged females increased in 2008, I dropped post-estrous females from the scan samples in order to record all pre-estrous and estrous females.

The main purpose of focal observations was to identify females likely to mate later that day and identify the sequence of these females' mates (Chapter III).

All-occurrence sampling (Altmann 1973) was used to start the focal observation by recording male-female interactions and the identity of all males within 20 m of females expected to be in estrus that day. Female Richardson's ground squirrels were expected to be in estrus if they had been out of hibernation for 2-3 days, had a swollen, pink vulva earlier in the day or the day before, and/or behaved in a shy and submissive way during scan samples.

All males that interacted with a specific female or were within 20 m of the female during scan samples prior to and during estrus were considered potential sires.

Underground consortships were inferred if a male and female spent >2 min underground and met at least 2 of the following criteria, based on above-ground copulations: male and female enter the same tunnel within 30 s of one another; mate calling by male before and/or after spending time underground; genital grooming by male or female on re-emergence above-ground; mate guarding of female by male either before or after going underground together; darkened patch of fur on the female's neck on re-emergence, presumed to be damp from the male holding the female during copulation (Michener 1998). I had partial or complete observations for 10 females in 2007 and 53 females in 2008.

Microsatellite Genotyping

Tissue samples were transported to the Museum of Vertebrate Zoology at the University of California, Berkeley, and stored at -80°C. Half of each ear punch was

digested by inverting overnight at 55°C with an extraction buffer (0.01 M Tris, 0.1 M NaCl, 0.01 M EDTA), 2% SDS, and 2 mg Proteinase K. The MacManes salt extraction protocol (MacManes 2008) was used to extract nuclear DNA from each ear punch. Salts were precipitated by centrifuging with ice cold 5M NaCl for 7 min (4°C at 13,000 rpm). The supernatant was transferred to a new tube and DNA was precipitated by adding ice cold isopropanol, cooled on ice for 10 min, then centrifuged for 10 min (4°C at 13,000 rpm). The DNA was washed twice with ice cold 80% ethanol, gently shaken, cooled on ice for 4 min, and centrifuged for 10 min (4°C at 13,000 rpm). The DNA pellet was dried using a vacuum centrifuge (SpeedVac, Savant) and rehydrated in 50 µL of TE buffer. The concentration of DNA was quantified using a spectrophotometer (Nanodrop technologies) and diluted to 10 ng/µL.

Polymerase chain reaction (PCR) conditions for the 8 *U. columbianus* and 3 *U. brunneus* loci that amplified in Richardson's ground squirrels were individually optimized (Table 2.1), with individual reactions containing a master mix [10 mM Tris-HCl pH 8.6, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.2 µM of Primer 1, 0.2 µM Primer 2], 7.5 U of Taq DNA polymerase (New England Biolabs), and 150 ng of DNA. The thermal profile consisted of 3 cycles (Table 2.1): 1) 5 min of 94-95°C denaturation; 2) 28-33 cycles for 30 sec each of 95°C denaturation, 30 sec of annealing at 51.5-68°C, and 45 sec of elongation at 72°C; and 3) 5 min of elongation at 72°C. All primers used were available at the Museum of Vertebrate Zoology, University of California at Berkeley.

Variation at individual microsatellite loci was quantified using an automated 48-capillary DNA analyzer (Applied Biosystems 3730). PCR products were compared

Table 2.1. Primer sequences and PCR conditions including denaturing temperature (T_d), annealing temperature (T_a), and number of cycles for 11 microsatellite loci used for parentage analysis in Richardson's ground squirrels. Eight microsatellite loci were developed for Columbian ground squirrels (GS; Stevens et al. 1997) and 3 for Idaho ground squirrels (IGS; May et al. 1997).

Loci	Primer sequences	T_d (°C)	T_a (°C)	# Cycles
GS 3	5'- GTT AAG TGT GTA TGA TGT GGA-3' 5'- TCA CCT AAA GAA GTG TCG TAT-3'	95	52	31
GS 0910*	5'- TTG ATA AAT GAG TGT CCT GAA-3' 5'- CCC TAG CTG TAA ATA AGT GTT-3'	94	52	33
GS 12	5'- CCA AGA GAG GCA GTC GTC CAG-3' 5'- TCA GAG CAG AGC ACT TAC AGA-3'	95	58	30
GS 14	5'- CAG GTG GGT CCA TAG TGT TAC-3' 5'- TTG TGC CTC AGC ATC TCT TTC-3'	94	51.5	33
GS 17	5'- CAA TTC GTG GTG GTT ATA TCA-3' 5'- CTG TCA ACC TAT ATG AAC ACA-3'	95	52	33
GS 2122*	5'- ATG CCC ACC GAG AAA AGA CA-3' 5'- GCC CAG CCA TCA CCC TCA CC-3'	95	62	28
GS 25	5'- CCA GCA TGG GGG AGA GAG AG-3' 5'- CTT GTC ATT TAT CCA TTC ATA G-3'	95	58	30
GS 26	5'- TCC CAG AGA ACA ACA TCA ACA G-3' 5'- AGG ACT GGG GTT GTA GGT GAG T-3'	95	68	31
IGS-1	5'- ATA ACA GCA CCC TGC TCC AC-3' 5'- AAT CCA TCC TCT ACC TGT AAT GC-3'	95	58	30
IGS-6	5'- GGG CAT TAA TTC CAG GAC TT-3' 5'- GGG CTG GAA TTA AAG GTA TCA-3'	94	57	32
IGS-1106	5'- CCA TGG AAG CAT GTC TGG TG-3' 5'- TGC TTC CTG ATT TCA AAG TTG C-3'	95	55	31

* Using the microsatellite name assigned in my study because the original is unavailable

against a sizing standard (Liz500, Applied Biosystems) and the resulting genotypes were scored using GeneMapper 4.0 (Applied Biosystems).

Data Analyses

Paternity was assigned by exclusion or by the Δ LOD at the 95% confidence given the offspring-mother-father trio, with certain males eliminated based on physical or temporal distance from estrous females. The Δ LOD was calculated using CERVUS 3.0 (Kalinowski et al. 2007) and the parameters given in Table 2.2. All mother-offspring relationships in 92 litters were verified and juveniles in 2 sets of mixed litters were rightfully assigned to their respective mothers. Genotypes of all juveniles were then compared with those of all males in the population. If a male was the only possible sire given genotypes of the mother and offspring, he was assigned as the father. In cases where multiple sires were possible for a given offspring, any males that were not seen within 20 m of the female prior to or during estrus, based on both scan and focal observations, were eliminated. Analysis was re-run with the remaining subset of males in order to adjust the Δ LOD. When more than one male remained with a genotype compatible with offspring in a litter, paternity was assigned using the 95% confidence Δ LOD of the offspring-mother-father trio provided by CERVUS 3.0 (Marshall et al. 1998, Kalinowski et al. 2007).

CERVUS and similar paternity software operate under the assumptions that the study populations are in Hardy-Weinberg equilibrium and that the adults in the population are unrelated (Jones and Ardren 2003, Kalinowski et al. 2007). When these assumptions are not met or when potential sires of offspring include close relatives such

Table 2.2. Parameters used for the simulation of paternity analysis in order to perform likelihood calculations using CERVUS 3.0 (Kalinowski et al. 2007). Parameters include the number of simulated offspring, proportion of fathers sampled, proportion of loci typed, proportion of loci mistyped, minimum typed loci an offspring must have in order to perform calculations, and the number of candidate Richardson’s ground squirrel fathers in 2007 (n=154 offspring) and 2008 (n=444 offspring).

	2007	2008
Offspring	100,000	100,000
Prop. ♂ sampled	0.99*	0.99*
Prop. loci typed	1.0	1.0
Prop. loci mistyped	0.0001	0.0001
Min. typed loci	7	7
Candidate fathers	16	23

* The Study Site was monitored daily from near dawn until dusk during the mating period. Any unmarked individuals were immediately trapped, ear tagged, and dye-marked, therefore I am confident that all possible males were genotyped. The proportion given is for a conservative estimate because 4 males in 2007 and 2 males in 2008 disappeared prior to the mating season before a tissue sample could be obtained.

as full or half-siblings, these close relatives are more likely to be assigned as the most probable sire than the true father (Jones and Ardren 2003). Considering the genetic data of all males on the Study Site, 19.2% of males in 2007 and 18.2% of males in 2008, were related at least at 0.25 level (e.g.: half brothers, grandfather-grandsons) with 12.5% and 7.9% of these males, respectively, related at the 0.5 level (e.g.: full brothers, father-son). Based on observed above-ground copulations and paternity assigned by exclusion, litters were sired by 1-2 males on average and rarely by 3 males; however, likelihood results suggested that some litters were sired by as many as 6 males, which seemed improbable. Many of those assigned males were not possible sires given spatial remoteness from the female, and removing such males resulted in more parsimonious results. Because exclusion alone is prone to data entry errors, genotyping errors, and null alleles, all assignments were checked against mating observations and all anomalies were assessed. Questionable alleles were re-analyzed in order to avoid errors.

Allele frequencies, expected heterozygosity (H_E), probability of exclusion based on one known parent (1-non-exclusion probability; Chakravarti and Li 1983, Tarof et al. 2005), and the polymorphic information content (PIC; a value related to the H_E , which also measures the informativeness of the loci) were calculated by CERVUS 3.0 given the adult population only (Kalinowski et al. 2007). The probability of 2 adult Richardson's ground squirrels within the population sharing the same genotype (probability of identity) was calculated from the formula given by Paetkau and Strobeck (1994) using the program GENECAAP (Wilberg and Dreher 2004). GenePop 4.0 (Raymond and Rousset 1995) was used to calculate Hardy-Weinberg equilibrium (H-W) of the adult population

using the strict Markov chain method. Values are given as p-value \pm SEM, with p-values less than 0.05 significantly deviating from H-W.

Normality was assessed using Shapiro-Wilk's W test; when the assumption could not be satisfied, non-parametric tests were used. Unless specified, two-tailed probabilities are reported and descriptive statistics are expressed as mean \pm SEM. P-values less than 0.05 indicate a significant difference in the means. Data were analyzed using JMP-IN statistical software (version 6.0 for Mac, SAS Institute).

Results

Allelic Diversity

For the 11 microsatellite loci used to assign parentage in Richardson's ground squirrels, allelic diversity ranged from 2 to 7 alleles per locus in a given year (mean=4.5 \pm 0.3; Table 2.3). The average expected heterozygosity over loci for the adult population was 0.5742 and the mean PIC value was 0.5309 (Table 2.3). Combined probability of 2 adult individuals sharing the same genotype (probability of identity) was 3.95×10^{-7} . Paternity exclusion probability given one known parent for all loci combined was 0.9892; however, even with high exclusion probabilities, multiple males may be genetically compatible with offspring (Marshall et al. 1998). Although 11 microsatellites were used, 3 loci had only 2-3 alleles in a given year (Table 2.3), of which 2 were common and 1 occurred less frequently, with PIC values of \leq 0.21 (Table 2.4). Therefore, these alleles were not very useful in assigning parentage because potential sires could only be excluded if they exclusively shared this third rare allele with their offspring. An additional locus with 4 alleles also had PIC \leq 0.21. Although GS 14, with only 2 alleles and PIC of 0.116, was not very useful for paternity exclusion in my population of

Table 2.3. Characteristics of 11 microsatellite loci applied to resolve paternity in a population of Richardson’s ground squirrels near Picture Butte, AB, in 2007 and 2008. The population includes an individual male from Gladmar, SK, that was released into the population in 2007. The number of alleles, expected heterozygosity (H_E , CERVUS 3.0), observed heterozygosity (H_O , CERVUS 3.0), polymorphic information content (PIC, CERVUS 3.0), probability of identity (P. Id.; GENECAAP), and probability of exclusion (P. Excl.=1-non-exclusion probability; CERVUS 3.0) are averaged over 2 years. The p-values for Hardy-Weinberg equilibrium (H-W, Genepop 4.0) are shown for both years. All characteristics are given for the adult population only.

Loci	# Alleles	H_E	H_O	PIC	P. Id.	P. Excl.	H-W	
							2007	2008
GS 3	3	0.170	0.172	0.180	0.700	0.090	0.100±0.00	1.000±0.00
GS 0910	6	0.753	0.698	0.688	0.097	0.501	0.802±0.01	0.659±0.01
GS 12	4	0.228	0.212	0.216	0.624	0.112	0.566±0.01	0.043±0.00*
GS 14	2	0.135	0.178	0.148	0.778	0.074	1.000±0.00	1.000±0.00
GS 17	6	0.723	0.661	0.628	0.113	0.451	0.585±0.02	0.078±0.01
GS 2122	7	0.751	0.724	0.709	0.110	0.520	0.023±0.00*	0.740±0.01
GS 25	7	0.620	0.536	0.582	0.228	0.403	0.016±0.00*	0.393±0.02
GS 26	6	0.709	0.749	0.677	0.152	0.480	0.000±0.00*	0.255±0.01
IGS-1	5	0.578	0.607	0.540	0.221	0.340	0.024±0.00*	0.084±0.01
IGS-6	4	0.671	0.753	0.594	0.192	0.372	0.650±0.00	0.400±0.01
IGS-1106	4	0.216	0.298	0.258	0.614	0.143	0.818±0.01	0.582±0.01

* Significant deviation from Hardy-Weinberg equilibrium.

Table 2.4. Variation in alleles between 4 populations of Richardson’s ground squirrels. The Picture Butte population represents the present study. The Gladmar population is represented by a single atypical male, with white fur and black eyes, released into the Picture Butte population before the mating season began in 2007; he did not produce any offspring and did not reappear in 2008. The Westbourne population is described in Hare et al. (2004) and the Edmonton population from Stevens et al. (1997) consisted of 3 specimens that were collected near Edmonton (pers. comm.) and used to assess the cross species amplification of specific loci. Sample sizes include adults and juveniles.

Loci	Alleles			
	Picture Butte, AB (n=733)	Gladmar, SK (n=1)	Westbourne, MB (n=110)	Near Edmonton, AB (n=3)
GS 3	228, 232, 234	232	227, 229, 231, 233	221, 223, 243
GS 0910*	203, 205, 207, 211, 213, 215	203		
GS 12	144, 146, 154, 156	144, 154	147, 149, 152, 154	157, 159, 161, 167
GS 14	240, 244	240	242, 244, 246	236, 248, 252, 254, 286
GS 17	162, 166, 168, 170, 172, 174	166, 170		155, 159, 161, 165
GS 20			220, 222, 237	227, 231, 233, 241
GS 2122*	183, 184, 195, 199, 201, 213, 215	183, 195		
GS 25	139, 143, 148, 149, 150, 152	145	138, 142, 144, 146, 148, 154	124, 144, 152
GS 26	107, 109, 115, 117	111, 113	109, 111, 113, 115	117
IGS-1	91, 93, 97, 99, 101	97, 99		
IGS-6	128, 136, 138, 140	128, 136		
IGS-1106	130, 142, 146	130, 140		

* Using the microsatellite name convention given by the present study because the original is unavailable

Richardson's ground squirrels, 5 alleles were detected in just 3 individuals near Edmonton, AB (Table 2.4).

Paternity Analyses

When all male Richardson's ground squirrels resident on the Study Site during the mating season were included in analysis, exclusive paternity could be assigned to 85 of 154 (55.2%) offspring in 2007 and 194 of 444 (43.7%) offspring in 2008. After removal of males that could not feasibly have mated with a given female, an average of 6 (range 2-16) males remained eligible as potential mates per female (n=96) and assignment of exclusive paternity increased to 82.5% and 82.9% of juveniles in 2007 and 2008, respectively. Assigning paternity by the likelihood method, using Δ LOD at the 95% confidence interval, in conjunction with elimination of improbable males, further increased paternal assignment to 98.7% and 96.4 % of juveniles in 2007 and 2008, respectively. The results of the Δ LOD prior to eliminating males were dismissed because of the abundance of results that contradicted observations.

Exclusion based on all males or the reduced number of males based on scan and focal observations identified 23 litters as multiply sired, 64 litters as singly sired, and 9 litters remained ambiguous. Of the multiply sired litters, paternity of all juveniles in 10 litters was assigned by exclusion, and another 12 litters had paternity assigned by exclusion to enough juveniles to be unambiguously multiply sired. One other litter was assigned as multiply sired because the female was observed to mate with 2 males and the second male was the more probable sire for 1 of the offspring given the Δ LOD score. Of the 64 singly sired litters, 36 litters were assigned a single male as the father by exclusion

and an additional 19 litters were classed as singly sired because paternity of at least half the offspring was attributable to a particular male and remaining offspring were assigned that same male by Δ LOD. Two more litters were assigned single paternity even though only 1 of 6 and 7 offspring respectively were assigned parentage by exclusion because all remaining offspring were assigned to the same male by Δ LOD and mating observations supported the assignment. Seven females were observed to mate with only 1 male, and although the observed male was not the most likely sire for the entire litter given the Δ LOD, he was a possible genetic match for the entire litter and therefore the remaining 7 litters were also assigned as singly sired. Three litters in 2007 and 6 litters in 2008 did not have enough paternity assigned by exclusion to determine if they were singly or multiply sired and observations did not exclude enough males to assign paternity unambiguously.

Multiple paternity was detected in 6 of 19 litters in 2007 and 17 of 68 litters in 2008. Litter size, which varied from 2 to 10 offspring, did not influence the likelihood of detecting multiple paternity (Logistic regression, $X^2=0.04$, $R^2=0.0004$, $p=0.84$, $n=87$). Inclusion of 8 large litters (6-10 offspring) in 2007, which might have introduced a bias towards multiple paternities, did not affect the number of multiply sired litters with respect to 2008, when all litters on the Main Site were sampled (Fisher's exact test, $p=0.57$). The proportion of offspring sired by each male in 22 multiple sired litters did not follow a binomial distribution (Shapiro-Wilk W Test, $W=0.86$, $p=0.0001$); the 1 female with multiply sired litters in both years was randomly excluded from one of the years. From the known sequence of mates for 9 multiply mated female Richardson's ground squirrels in 2008, lack of a mating-order effect was confirmed; the frequency with

which the male that sired the greater number of offspring was a female's first or last mate did not differ significantly (0.58 ± 0.12 and 0.39 ± 0.12 , respectively; Paired t-test, $t=0.86$, $p=0.41$, $n=9$).

The 13 males that resided on the Main Site in 2008 for over 50% of the mating period sired all 401 offspring in 68 resident litters for which paternity could be assigned. Each male, on average, sired at least one offspring in 6.6 ± 0.7 litters (range 4-12) and fathered 31.3 ± 3.6 offspring (range 15-59). Six of those males resided exclusively on the Main Site, so I suspect they did not sire offspring outside the Main Site. These males sired at least one offspring in 7.0 ± 1.1 litters (range 4-11) and fathered 31.8 ± 5.3 offspring (range 15-51) on average in 2008.

Discussion

By using 6 microsatellites and litters of known maternity, Hare et al. (2004) were able to assign paternity by exclusion to 37.6% of 85 juvenile Richardson's ground squirrels in 15 litters. I used 11 microsatellites, including 5 used by Hare et al. (2004), and my additional 6 primers had an average of 5.0 alleles per locus (range 3-6 alleles). None-the-less, exclusive assignment of paternity in litters of known maternity increased to only 46.7% of 598 juveniles in 96 litters of Richardson's ground squirrels, a modest increase relative to Hare et al.'s study. Despite having 4 moderately polymorphic loci with 6 alleles and almost double the number of microsatellites as Hare et al. (2004), my paternity assignments increased by only 10% compared with Hare et al. (2004) because of genetic similarity among spatially proximal males; 18.7% of male Richardson's ground squirrels were related to each other at least at a coefficient of relatedness of 0.25.

Hare et al. (2004) had no behavioural basis on which to exclude male Richardson's ground squirrels as possible sires in their population, whereas I assessed behavioural and physical proximity during the mating period to identify males that had no opportunity to mate with a given female. After I eliminated such males as putative sires, exclusive paternal assignment increased from 46.7% to 82.2% of 598 juveniles. Thus behavioural observations can provide a powerful method to improve assignment of paternity. In contrast, Haynie et al. (2003) could only assign 46% of Gunnison's prairie dog offspring and 45-53% of Utah prairie dog offspring to their respective parents after eliminating males that were not potential parents based on observational data. Paternity of litters could not be assigned either due to ambiguity of maternity or the inadvertent exclusion of all potential sires. Shurtliff et al. (2005) used more relaxed data than Haynie et al. (2003) and the present study, using spatial relationship data along with exclusion and likelihood to assign paternity in Canyon mice (*Peromyscus crinitus*). They could not use mating observations because Canyon mice are small, nocturnal rodents that live in patchy habitats and thus are impossible to observe in nature. They used linear distances between males and pregnant females to eliminate males deemed unlikely to have mated with a female resident in another rocky outcrop. Ten pregnant females with 38 embryos were sacrificed to unambiguously assign maternity and 44 males were sampled as potential sires. Of 38 offspring, 74% were assigned paternity.

One advantage my study had compared with the studies of Haynie et al. (2003) and Shurtliff et al. (2005) was the ability to assign maternity accurately and non-invasively. However information on maternity is not always necessary for successful parentage assignments. In a study on gilthead seabream (*Sparus aurata L.*), an important

species for aquaculture in the Mediterranean, all 996 larvae with 62 potential mothers and 86 potential fathers were unambiguously assigned parentage (Castro et al. 2007). However, breeding fish were held in 8 tanks containing 13-38 fish each with sex ratios (females/males) ranging from 0.2-3.3. Because Castro et al. (2007) were trying to evaluate the set of loci for accurate parentage analysis, they also included the genotypes of 55 wild individuals as potential sires. Using all adult fish as potential sires they could assign 93.3 % of offspring to their parents, but evaluating parentage on a tank by tank basis they were able to assign parentage to all offspring. This shows that the most efficient way to assign parentage, in addition to having highly polymorphic loci, is to decrease the pool of candidate parents.

Although the probability of detecting multiple paternity within a litter should increase with litter size, I found that for Richardson's ground squirrels not only did evidence of multiple siring not depend on litter size, but there was no between-year difference in frequency of multiple paternity even though half the litters in 2007 were specifically tissue sampled due to their large size (6-10 pups per litter). I cannot relate whether litter size is related to paternal assignment rates in other species of ground squirrels because microsatellites have not been used to assess parentage in species other than Richardson's ground squirrels.

Because observations are not always feasible for a given species and because some loci are more polymorphic in one geographic region than another, testing additional, more-polymorphic loci for Richardson's ground squirrels may increase the ability to assign parentage without observations and opens up new possibilities that require genetic information such as studies of dispersal, inbreeding and

between-population differences. In addition to the 11 microsatellites I used for Richardson's ground squirrels, 14 other loci have been developed for black-tailed prairie dogs (*Cynomys ludovicianus*; Jones et al. 2005), 6 for European ground squirrels (*Spermophilus citellus*; Hanslik and Kruckenhauser 2000), 9 for spotted susliks (*S. suslicus*; Gondek et al. 2006), and 3 for southern Idaho ground squirrels (*Uroditellus brunneus endemicus*; Garner et al. 2005) providing a large number of prospective loci.

For organisms with limited numbers of microsatellite loci currently available, combining mating observations with paternity analysis and retaining the minimum number of potential parents may be a better alternative than developing new microsatellites.

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Chapter III

MULTIPLE PATERNITY AND FEMALE CHOICE IN RICHARDSON'S GROUND SQUIRRELS (*UROCITELLUS RICHARDSONII*)

Abstract

Female Richardson's ground squirrels (*Urocitellus richardsonii*) located in southern Alberta were observed during the mating period in 2007 (n=10) and 2008 (n=47) to determine the frequency of multiple mating and the frequency with which multiple mating resulted in multiple paternities within litters. Female choice of mates, although not as evident and frequent as expected, was assessed based on a displacement index that quantified a male's ability to maintain proximity to estrous females by displacing neighbouring males, male-female familiarity, male heterozygosity, genetic relatedness, and male weight. Eleven microsatellites were used to evaluate paternity within 96 litters. Paternity was discernable in 87 litters of which 23 showed evidence of multiple paternities. Thirteen of 43 dams with known mating sequences mated with 2-3 males, and 7 produced litters with multiple sires. Neither the first nor last male to mate with the female gained a paternity advantage, but mate guarding increased a male's chances of siring offspring. Mate guarding occurred in 24 of 35 consortships involving 22 of 28 females. Of these mate-guarded females, 18 were guarded by 1 male and had singly sired litters, 2 were guarded by 2 males and had correspondingly mixed litters, and 2 were guarded by 1 male but had multiply sired litters. The 32 females for which genetic and observational data were available were no more likely to mate with some males than others, based on their familiarity, heterozygosity, genetic relatedness, or

weight. Of 21 females in 2008 with observational data available, 9 mated only with the male that had the greatest displacement index among the males in her vicinity (displacing male), 5 mated with the displacing male first then with other males, and 1 female did not mate with the displacing male. Multiple paternities within litters were less frequent than previously reported for Richardson's ground squirrels, and female choice based on familiarity, heterozygosity, relatedness, and male weight was not evident.

Introduction

The mating systems of 10 species of ground squirrels (Family: Sciuridae, Tribe: Marmotini; previously Genus: *Spermophilus*; Helgen et al. 2009) have all been described as polygynous, with males mating with multiple females in the annual mating period (see Schwagmeyer 1990 for summary, Millesi et al. 1998, Titov 2004, Titov et al. 2007). Because populations of most species of ground squirrels have female-biased sex ratios among adults and all females are inseminated, polygyny is a statistical necessity, not necessarily a male mating strategy (Michener 1983). Furthermore, describing the mating system exclusively from male behaviour provides virtually no information on female mating behaviour. Seven species of ground squirrels described as polygynous are also polyandrous (i.e.: females mate with multiple males; Hanken and Sherman 1981, Foltz and Schwagmeyer 1989, Sherman 1989, Boellstorff et al. 1994, Murie 1995, Lacey et al. 1997, Hare et al. 2004). Polyandry in the remaining species of ground squirrels has not yet been studied. The main objectives of my study are to examine a species of ground squirrel (Genus: *Urocitellus*) for which polyandry has been established both by observation (Michener and McLean 1996) and multiple

paternity of litters (van Staaden et al. 1994, Hare et al. 2004) in order to establish benefits of polyandry, sperm competition patterns, and female choice.

Because the costs of mating for female Richardson's ground squirrels (*Urocitellus richardsonii*) can involve loss of time spent feeding and maintaining burrows, metabolic costs associated with reduced time feeding and increased activity (Michener 1984b, Michener and McLean 1996, Michener 1998), predation (Michener 2001), and possibility of parasite transmission (Yensen et al. 1996), why would a female risk mating with multiple mates when a single ejaculate should be enough to fertilize her eggs? Polyandry is predicted to occur when the benefits of mating with multiple mates outweigh the costs. Possible benefits of polyandry for females include direct benefits such as reduced disruption in the population caused by male-male competition, gain of paternal care, protection from conspecifics and predators, material benefits such as access to food resources, reduced sexual harassment, stimulation of reproduction, assurance of fertilization due to short-term sperm depletion in males and male sterility, and increase in litter size (Halliday and Arnold 1987, Hunter et al. 1993, Keller and Reeve 1995). Possible indirect or genetic benefits include good genes in offspring due to genetic diversity, sperm competition, probability of mating with genetically higher quality males, reduced chance of genetic incompatibility, and female choice of paternity.

Among ground-dwelling sciurids, Richardson's ground squirrels are classified with a low social-system ranking of 2 on a 5-point scale (Armitage 1981, Michener 1983). Richardson's ground squirrels live in single-family female kin clusters with no males permanently associated with the cluster (Michener 1983). Females live in

proximity to close female kin, but they do not form a defined social group comparable to the coterie that typify black-tailed prairie dogs (*Cynomys ludovicianus*; Michener 1983). Male Richardson's ground squirrels usually disperse as juveniles, they tend to move around during the mating season, they rarely settle near the females with whom they have mated, and they usually survive for only 1 or 2 mating seasons (Michener 1983). Therefore, submitting to a male in order to reduce disruption within the social group caused by male-male competition and to increase male tolerance of offspring (Halliday and Arnold 1987) is unlikely. Additionally, males do not defend females from predators or conspecifics, nor do they provide resources for females or paternal care (Michener 1983). Non-aggressive interactions between male and female Richardson's ground squirrels are limited to when a female is in pre-estrus or estrus, with females rarely incurring physical injuries (Michener 1983). Because about 50% of females in a given mating period have been observed to mate with multiple males (Michener and McLean 1996) yet almost no females incur physical injuries, it is unlikely that females mate with multiple males in order to reduce sexual harassment and/or probability of injury.

Stimulation of reproduction in induced ovulators (Hunter et al. 1993) and assurance of fertilization due to short-term depletion of sperm (Hunter et al. 1993) or sterility (Keller and Reeve 1995) may also account for multiple mating, but no information is currently available for Richardson's ground squirrels. The reproductive physiology of males and females was not examined in the present study.

In mammals, increasing litter size at birth by mating with multiple males is unlikely because a female cannot produce more offspring than the number of ova she ovulates (Trivers 1972), but polyandry might increase assurance of fertilization without

increasing litter size (Hoogland 1998) or it might increase litter size at weaning by reducing pre-weaning infant mortality via genetically more viable sperm (Fisher et al. 2006). In Gunnison's prairie dogs (*Cynomys gunnisoni*), not only did copulation with more than 2 males guarantee a female's pregnancy and parturition, it also directly increased litter size at weaning (Hoogland 1998). In *Antechinus stuartii* the difference in litter size at weaning between singly and multiply sired litters was due to a difference in pre-weaning mortality, rather than litter size at birth (Fisher et al. 2006). Michener (1989a) showed that litter size at birth in captivity and at weaning in the field are not statistically different in Richardson's ground squirrels; however, she did not have information on number of mates. Multiple mating does not increase litter size at weaning in thirteen-lined ground squirrels (Schwagmeyer 1986) or Columbian ground squirrels (Murie 1995) and I do not expect multiple mating to affect litter size in Richardson's ground squirrels. However, Michener (1980a) showed that female Richardson's ground squirrels increase their reproductive success, measured as the number of female descendants present in the population for 3 consecutive years, by producing female-biased litters rather than by producing large litters. Therefore I investigated whether multiple mating affects litter size in Richardson's ground squirrels and I examined the possibility of sex biases between singly and multiply sired litters by comparing the female sex ratio of litters.

Direct benefits of polyandry, with the exception of the type of ovulation, can be easily observed in the field; however, genetic benefits have only recently become amenable to study with the advent of molecular markers such as microsatellites (Queller et al. 1993). Although I address direct benefits such as assurance of fertilization,

increased litter size, and female sex bias in multiply sired litters, I am especially interested in indirect genetic benefits. Do female Richardson's ground squirrels that mate with only a single male, mate with the highest quality male available, and do females that mate with multiple males choose a higher-quality male for subsequent matings in order to obtain good genes for their offspring? Do females facilitate sperm competition by mating with multiple males? Do females increase genetic diversity within their litters, measured as standard heterozygosity (Cohas et al. 2006), by mating multiply and are subsequent mates genetically less similar to the female than the first mate in order to produce more genetically diverse offspring? These concerns lead me to my next question: are female Richardson's ground squirrels choosy about mates?

Females make greater parental investment in offspring, including size of gametes and amount of parental care, than males for the majority of species (Trivers 1972). The cost for females to produce eggs is much greater than the cost for males to produce sperm (Parker et al. 1972) and males of most mammals provide little or no parental care (Trivers 1972). Female Richardson's ground squirrels for example are the sole provider of nourishment and protection for their litters for 52 days, from conception until litters emerge above-ground (Michener and Locklear 1990). Even if the parental roles are reversed, as in the purple sandpiper (*Calidris maritima*) where females leave the nest at hatching and males provide all the parental care until hatchlings fledge 24-34 days later (Pierce 1997), it is to a female's advantage to be choosy. If females invest greater parental care than males, females ought to choose males with ideal genes. If females invest less parental care than males, females ought to choose males that provide quality paternal care (Trivers 1972). Female Richardson's ground squirrels reportedly exhibit

pre-copulatory female choice by a hide-then-run behaviour tactic in which females sneak away from resident males and sometimes seek unfamiliar males away from their usual home range (Michener and McLean 1996). Even so, the criteria female Richardson's ground squirrels use in order to choose a mate has yet to be established. What constitutes a superior male?

In many species with sexual selection, extreme secondary sexual characteristics, such as the extravagant caudal plumage of peacocks, provide a means for females to choose a high-quality male (Andersson and Simmons 2006). Richardson's ground squirrels are sexually dimorphic, with males slightly larger and heavier than females (Michener 1984b). Besides size, perhaps age (e.g.: Fricke and Maklakov 2007), dominance rank (e.g.: Waterman 1998), genetic compatibility (e.g.: Penn and Potts 1999), familiarity (e.g.: Randall et al. 2002), or genetic relatedness (e.g.: Cohan et al. 2006) is used by females to choose a superior male.

In addition to female choice, I also examine whether males can influence their share of paternity within a litter. Male Richardson's ground squirrels sometimes mate guard a female pre-copulation and/or post-copulation, preventing her from mating with another male (pers. obs.). Additionally, a male might increase his chances of siring offspring if he mates with the same female multiple times (Hunter et al. 1993) or prolongs his copulations (Murie 1995). By preventing the female from mating with another male, he is decreasing the chance of competition from sperm of a rival male. The timing of matings with respect to ovulation, the number of ejaculations by each male, and the delay between copulations can all affect sperm competition (Foltz and Schwagmeyer 1989). Sperm competition is known to occur in 6 species of ground squirrels with either

the first or last male to mate more likely to sire a higher percentage of the offspring than the following or previous male (Hanken and Sherman 1981, Foltz and Schwagmeyer 1989, Sherman 1989, Boellstorff et al. 1994, Murie 1995, Lacey et al. 1997). Sperm precedence (first-male advantage) occurs in *Ictidomys tridecemlineatus* , *Otospermophilus beecheyi*, *Urocitellus beldingi*, *U. columbianus*, and *U. parryii* (Hanken and Sherman 1981, Foltz and Schwagmeyer 1989, Boellstorff et al. 1994, Murie 1995, Lacey et al. 1997) whereas sperm displacement (last-male advantage) occurs in *U. brunneus* (Sherman 1989).

Another way a male may try to influence his chances of siring offspring is by producing a copulatory plug. Copulatory plugs are found in approximately 37% of female Richardson's ground squirrels the morning after copulation; for the remaining females, plugs either do not form or they only stay in place for a short period such that they are gone by the time females are trapped 15-18 hours post-copulation (Michener 1984a). Copulatory plugs, however, do not always prevent further copulations (Ginsberg and Huck 1989). Female tree squirrels (*Sciurus niger* and *S. carolinensis*), for example, often remove plugs within 30 seconds after mating (Koprowski 1992); copulatory plug removal has not been observed in Richardson's ground squirrels. Although my study was not designed to trap females after each copulation to check for plugs, the vaginas of females were inspected the day after mating. Unfortunately it is not possible to assign copulatory plugs to males based on microsatellite analysis because squirrel plugs, which are made up layers of semen and vaginal epithelia around a core of coagulated fluid (Michener 1984a), do not contain enough paternal DNA for PCR amplification (Koprowski pers. comm.).

Multiple paternities within litters have been confirmed for Richardson's ground squirrels by Hare et al. (2004) and van Staaden et al. (1994), but no information is available on how paternity is shared amongst a female's mates. If sperm competition occurs (Foltz and Schwagmeyer 1989), such that either the first or last male to mate sires a disproportionate share of the litter, then the order in which a female accepts males for mating will affect each male's paternal share. Assessment of paternal share requires assignment of paternity, which can be done using microsatellites (Hare et al. 2004; Chapter II). Because each female rears her litter in isolation (Michener 2002), offspring can be unambiguously assigned to their mothers. Once paternity is known, the relationship between mating order, sperm competition, and paternity of offspring within litters can be unravelled. In addition, the genetic relatedness between mating pairs can be determined.

The first objective of my study was to report the frequency with which multiple mating occurred in Richardson's ground squirrels and the frequency with which multiple mating resulted in multiple paternities. The second objective was to examine female choice and report any direct or indirect benefits that females gained by mating with multiple males. As my last objective, I assessed whether mate guarding increased a male's share of paternity within a litter or not and whether sperm competition occurred.

Methods

Study Area

The study population of Richardson's ground squirrels, located 5 km E and 1 km S of Picture Butte, Alberta, Canada, has been under investigation since 1987 (Michener

1992, 1996). The 3.4-ha Study Site, which is bordered on 2 sides by agricultural fields and on the other 2 sides by a farmyard and a road with agricultural fields beyond, is isolated by at least 1.5 km from the next nearest suitable habitat. In 2007 and 2008 my study focussed on 39 and 74 female ground squirrels respectively, resident within a 1.7-ha portion of the site, herein referred to as the Main Site, where a 10-m by 10-m grid of labelled cement blocks permitted locations of animals to be estimated visually to the nearest 1 m. The remaining portion of the Study Site will be referred to as the East Field.

Capture and Handling Techniques

Squirrels were live-trapped by placing unbaited traps (Tomahawk Live Trap Co., single-door squirrel traps) at the tunnel down which the target squirrel descended; nearby tunnels suspected to be part of the same burrow system were temporarily blocked with rocks. Typically every animal selected for trapping on a given day was captured, usually within 30 min of setting traps. Traps were kept under constant visual surveillance and animals were processed within 5 min of capture.

Squirrels on the Main Site were of known age because they had been trapped and ear tagged as juveniles in previous years. Squirrels in the East Field were either of known minimum age, having been tagged as adults in previous years, or were assumed to be yearlings born in the preceding year. All squirrels were permanently marked with a numbered metal ear tag (National Band and Tag Co.) in each ear, and adults were individually dye marked with hair dye (Clairol Hydrience #52 (pearl black) for females and #30 (spiced bronze) for males) for visual identification. At each capture, squirrels

were weighed to the nearest 5 g on a spring scale (Pesola) and inspected for reproductive status, and wounds.

From late January onwards, the Study Site was inspected daily for emergence of Richardson's ground squirrels from hibernation. Newly emerged squirrels on the Main Site were captured as soon as possible, usually on the day of emergence. Of the 24 adult males (11 in 2007 and 13 in 2008) that were resident on the Main Site for at least 50% of the mating period, 15 (6 in 2007 and 9 in 2008) were recaptured within 2.0 ± 0.4 days (range 1-6) of both the beginning and the end of the mating period in order to determine pre- and post-mating weight and assess wounds. Adult females were recaptured 3-5 days after emergence from hibernation to assess reproductive status and to confirm mating, and optimally prior to estrus to confirm that mating has not yet occurred. Females were also trapped 1-2 days before and after the expected time of parturition (23 days after mating) to confirm that mating resulted in pregnancy and parturition.

All males present on the Study Site at any time during the mating season (16 in 2007 and 23 in 2008) were tissue sampled. Selected dams and all their offspring were tissue sampled as soon as juveniles first emerged above-ground at 29-31 days old. The un-sampled males disappeared from the Study Site shortly after emergence from hibernation and before female emergence. In 2007, 32 of 38 litters and in 2008, all 73 litters weaned by the 74 females resident on the Main Site were trapped; 22 and 73 of these litters, respectively, were tissue sampled. In 2008, one female from the Main Site failed to wean a litter and one female in the East Field, for whom I had mating observations, and her litter were tissue sampled in addition to the 73 resident females. Tissue was obtained by using an ear punch (B.Y.T. Co.) to remove a 2-mm piece of ear

after both the ear and ear punch had been cleaned with isopropanol. Each tissue sample was stored in a 0.5-mL microcentrifuge tube containing DMSO-EDTA salt solution (Seutin et al. 1991, Kilpatrick 2002) for subsequent microsatellite analysis.

All procedures with animals were approved by the Animal Welfare Committee at the University of Lethbridge and were in accordance with the guidelines of the Canadian Council on Animal Care.

Assessment of Reproductive Status

Reproductive status of female Richardson's ground squirrels at each capture from emergence from hibernation until post-mating was assessed on the appearance of the vulva and the cellular contents of vaginal lavages. On emergence from hibernation, the vulva is typically closed, flat, and white, but then opens, swells, and reddens over the next 2-3 days as the female approaches estrus. If the vulva was patent, a vaginal lavage was taken as described by Michener (1980b), stained using Shorr's stain (Shorr 1940), viewed under 100x magnification, and categorized as pre-estrus, estrus, 1-day post-estrus, or anestrus from the relative proportions of nucleated epithelial cells, cornified epithelial cells, leukocytes, and sperm (Michener 1980b). A copulatory plug in the vagina, dried semen around the vulva, or sperm in the lavage indicated that the female mated within the previous 18 h (Michener 1984a).

To confirm that they were impregnated on their first estrus, female Richardson's ground squirrels were captured within 1-2 days of their anticipated date of parturition, which typically occurs 23 days post-estrus. A decline in body mass accompanied by a stretched or bloody vulva and soft, pointy, or pink-tipped nipples indicated recent

parturition and initiation of lactation. For the occasional female that was not trapped immediately post-estrus or around the time of parturition, mating was confirmed by the emergence of her litter 52 days after her presumed date of estrus (Michener 1985).

Because the location of all adult females was recorded during daily censusing and because each female rears her litter in a separate burrow (Michener 2002), litters were assigned unambiguously to their mother based on date and location of litter emergence. Of 32 and 74 litters that were trapped in 2007 and 2008, respectively, inter-mixing of litters between 2 adjacent genetically related females occurred once in each year. Microsatellite data were conclusive enough to identify the correct mother for each litter, and microsatellite data also confirmed the maternal assignments of juveniles in all other litters.

Mating Period and OSR

The mating period was defined as the set of consecutive days on which the majority (>95%) of female Richardson's ground squirrels mated. The pre- and post-mating periods included a few females (3 in 2007 and 3 in 2008) that emerged from hibernation unusually early or late with respect to the majority of females. The peak mating period was defined as the shortest set of consecutive days on which >70% of females mated and average daily operational sex ratio (OSR) was >0.5. OSR was calculated daily as the number of estrous females, as determined from reproductive status, per resident male on the Main Site.

Pre-Mating Observations

Scan sampling was used to obtain information on each female's familiarity with males over the several-day interval between emergence and mating and to assess each male's displacement index. I performed scan sampling from a 2-m elevated observation booth from which dye-marked Richardson's ground squirrels could be individually identified up to a distance of 90 m. Scans of all visible female and male Richardson's ground squirrels occurred at 10-min intervals for approximately 1 hour in the morning and 1 hour at mid-day each day of the mating period: 6-14 March in 2007 and 1-15 Mar in 2008. On each scan I recorded every squirrel's behavioural activity and location on a map of the Study Site to determine male-female proximities. Interactions were classified as male-female, male-male, or female-female. Additional male-female interactions occurring between scans were also recorded opportunistically. As the number of emerged females increased in 2008, I dropped post-estrous females from the scan samples in order to record all pre-estrous and estrous females.

I calculated familiarity with nearby males for each female Richardson's ground squirrel as the proportion of her pre-mating scan samples in which a given male was within 20 m of that female. Males within 20 m of an estrous female were assigned a displacement index calculated as the number of times they chased other males away from the female relative to the number of times they were chased by other males. I refer to the male with the greatest displacement index as the displacing male for that estrous female and the other males as the displaced males. Therefore on a given day, a male could simultaneously be the displacing male relative to 1 estrous female but the displaced male with respect to another estrous female. The displacing male was always the most familiar

male on a female's day of estrus, but not necessarily the most familiar male prior to estrus. In 2008, I had enough information based on scan samples and observed matings to determine whether a female mated with the most displacing, most familiar, or heaviest male for 21 females, and enough information based on microsatellite genetic profiles to determine whether a female mated with the most heterozygous or least related male for 32 females. Based on observed matings and genetic profiles, I determined the effects of male weight loss during the mating period and male weight loss per day during the mating period on mate choice by 11 and 15 females, respectively.

Mating Observations

The goal of mating observations was to locate focal female Richardson's ground squirrels expected to mate that day, record the presence of males within 20 m of that female, and determine the identity and sequence of males that mated with the focal female. Mating observations were done by 2-4 observers from a 2-m elevated observation booth, 2 ground-level observation sheds, and a 2-storey farm house distributed so that each observer was within 50 m of focal females. Depending on their level of experience with observing mating behaviour, an observer followed 1-3 females per day, for an average of 1.0 estrous female per observer per day in 2007 (n=10 females) and 1.5 in 2008 (n=53 females). Observers used 10 x 25 or 8 x 32 binoculars and focal-animal sampling to record the activity of estrous females.

Female Richardson's ground squirrels typically mate after 15:00 h Mountain Standard Time (MST), with most copulations occurring after 17:00 h MST (Michener 2001). For my study, mating observations started on average at 16:30 h in 2007 (n=9

days) and at 16:00 h in 2008 (n=16 days) and continued until light conditions prevented further detection of ground squirrels (~19:00 h). The first copulations on the Main Site were observed on 24 February and 26 February in 2007 and 2008, respectively (Figure 3.1).

Mating observations began with all-occurrence sampling (Altmann 1973) of male-female interactions and male-male conflict until a female was identified as showing signs of behavioural estrus, such as run-then-hide behaviour (Michener and McLean 1996), solicitation of males, frequent approaches by males attempting to sniff the female's ano-genital area, or attempted mate guarding of the female by the male (Michener 1998). Observations then switched to focal-animal sampling for 10 females in 2007 and 53 females in 2008. Of these, 3 and 9, respectively, had been captured to obtain a vaginal lavage 60 ± 26 min (range=69-104 min) and 60 ± 10 min (range=19-182 min) before observations began, and 4 and 30 females, respectively, were captured the day after mating to obtain a vaginal lavage.

Because Richardson's ground squirrels usually mate underground, the following criteria, based on observation of above-ground copulations, were used to infer underground copulations: male and female entered the same tunnel within 30 s of one another; pair remained underground >2 min; male mate called before and/or after spending time underground with the female; male and/or female genital groomed on re-emergence above-ground; male mate guarded female before and/or after going underground together; female had a darkened patch of fur on her neck, presumed to be damp from the male holding the female during copulation, on re-emergence

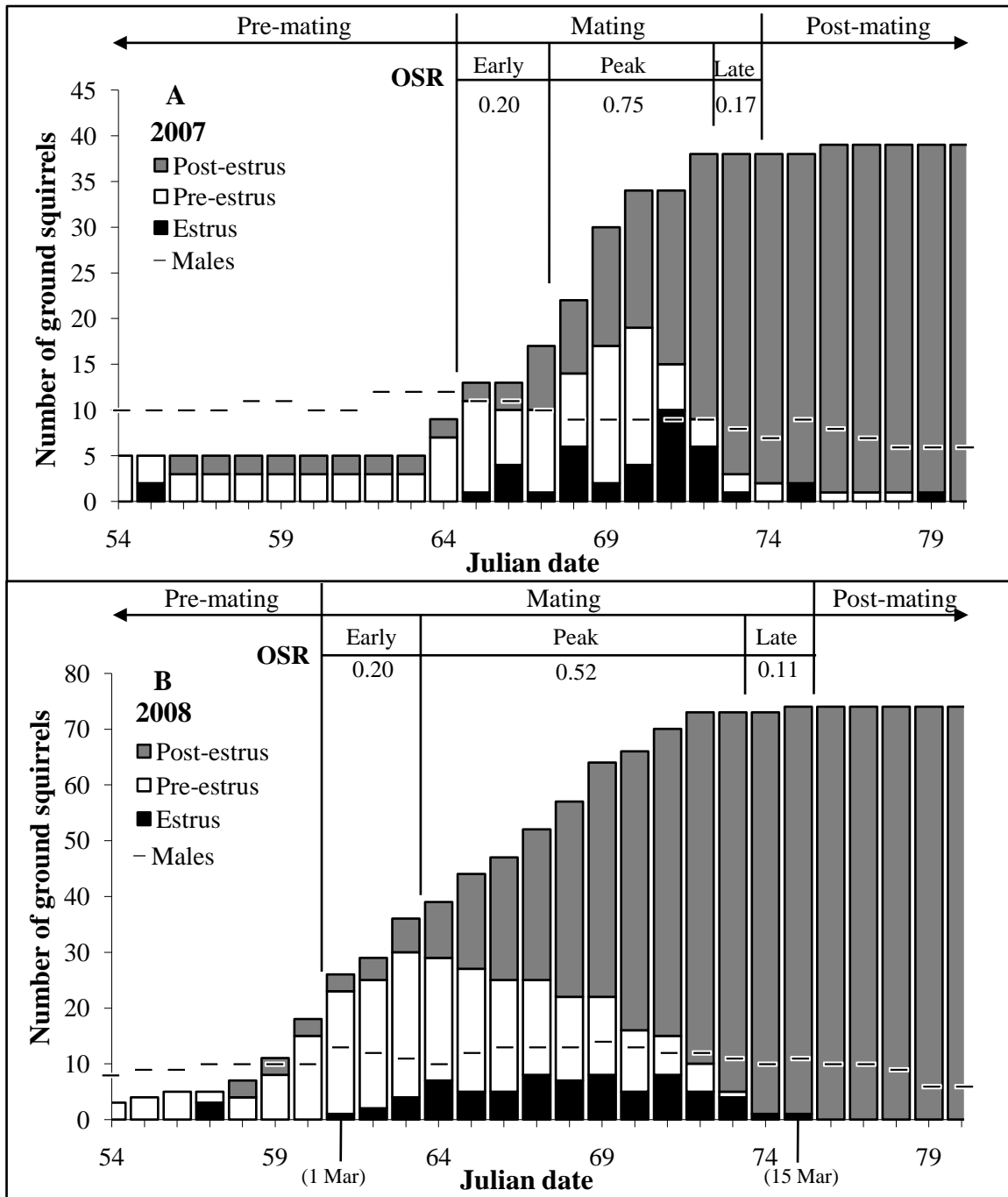


Figure 3.1. Number of adult (≥ 1 year-old) post-estrous, pre-estrous, and estrous female and male Richardson's ground squirrels resident daily on the Main Site, during pre-mating, mating, and post-mating periods in 2007 (A) and 2008 (B). Operational sex ratio (OSR), averaged across each period, is given for the early, peak, and late phases of the mating period in each year. Note that the scale on the y axis differs between years associated with greater population density in 2008 compared with 2007.

(Michener 1998). The following were also recorded: location of the tunnel the pair entered and exited to nearest 1 m; sequence in which the female and male entered and exited the underground site; mate guarding of female by male; number and identity of males within 20-m radius of the female.

I confine the term copulation to witnessed above-ground copulations and to below-ground consortships known from molecular evidence to have resulted in offspring sired by that male. I use the term consortship when a male and female spent >2 min underground and at least 2 other criteria associated with copulation were met, whether molecular evidence indicates that offspring were sired or not. Inferred copulations refer to consortships and copulations combined. Subsequent matings by a female in the same estrous period were categorized as same-male or different-male repeated matings. If a female mated with 2 different males, those were considered as 2 unique consort pairs.

Mating observations were classed as complete if the observer was confident that the focal female did not have an opportunity to mate with any other males during the observations, either because the observer had the animal in view the entire time or, if the female was out of sight, the locations of all nearby males were known. Mating observations were considered incomplete if the focal female and any of the nearby males were simultaneously out of view for >2 min, either because of visual obstruction or because the observer's priority was to watch another animal, and the female could have interacted with males during that time. When complete observations were supported by molecular data indicating that a female's litter was sired only by a male or males with whom she was known to consort or copulate, they were considered confirmed.

Observations were classified as complete for all 10 estrous females observed in 2007 and

for 47 of 53 observed in 2008. However, 14 apparently complete observations were subsequently reclassified either as definitely incomplete because the litter included a sire not seen to mate with the female (n=2 in 2007 and n=12 in 2008) or as possibly incomplete because paternity information was either not available (n=1 female that did not wean a litter) or was ambiguous (n=2). A litter's paternity was classed as ambiguous if the most likely sire for some offspring, based on Δ LOD, was seen approaching the female but never observed mating the female, meanwhile the male actually observed to mate with the female was a potential sire for the entire litter. Observations were confirmed for 8 females in 2007 and 35 females in 2008 from molecular data. Including squirrels with confirmed, complete, or incomplete data, 6 above-ground copulations and 75 underground consortships were detected for 50 focal females in 2007 and 2008 (Table 3.1). I knew the start time of 53 consortships including 42 underground copulations and the duration of 46 consortships including 37 underground copulations. The end of consortships may not have been known due to the male-female pair remaining underground until light conditions made it impossible to observe them further. Depending on the objective of calculations, I used complete, incomplete, or confirmed data. Because all males in the entire population were individually identifiable and all those resident ≥ 1 day in the mating season were tissue sampled on first appearance in the population, I am confident that the pool of putative sires was complete.

Mate Guarding

Mate guarding by male Richardson's ground squirrels was classed as absent, pre-copulation, or post-copulation. Pre-copulatory mate guarding was inferred when a

Table 3.1. Underground consortships and copulations observed in 2007 and 2008 in a population of Richardson’s ground squirrels near Picture Butte, AB. Sample sizes are given for the total number of consortships and copulations observed in a set of females. The sample sizes, means \pm SEM and ranges for duration and start time of consortships and copulations are also reported for both years. See text for definitions of consortships and copulations.

		Consortships	Copulations
2007	Number of observations	16 (n=10 ♀)	9 (n=9 ♀)
	Number of known duration	11 (n=8 ♀)	6 (n=6 ♀)
	Mean duration (min)	7.4 \pm 1.1	7.6 \pm 2.1
	Range of duration (min)	2.9-15.7	2.9-15.7
	Number with known start time	12 (n=8 ♀)	7 (n=7 ♀)
	Mean start time (MST)	18:06 h \pm 7 min	18:02 h \pm 9 min
	Range of start time (MST)	17:20-18:48 h	17:20-18:32 h
2008	Number of observations	59 (40 ♀)	49 (39 ♀)
	Number of known duration	35 (n=33 ♀)	31 (n=30 ♀)
	Mean duration (min)	11.0 \pm 1.1	10.7 \pm 1.1
	Range of duration (min)	3.4-26.2	3.4-26.2
	Number with known start time	41 (n=36 ♀)	35 (n=34 ♀)
	Mean start time (MST)	17:54 h \pm 5 min	17:54 h \pm 5 min
	Range of start time (MST)	16:27-18:47 h	16:27-18:43 h

* 2 underground consortships in 2007 and 6 in 2008 were second matings with the same male.

male was close (<10 m) to an estrous female, chased away other males that were within 20 m of the female (or an area encompassing several estrous females), or the male chased the female when she ventured outside of her usual range. Post-copulatory mate guarding was inferred by the same criteria as pre-copulatory mate guarding but also included mate calling by the male at the burrow hole where the pair consorted while the female was still in the burrow system. Occasionally the female darted from the burrow when the male stopped mate calling; when the male chased the female back to that burrow system, the behaviour was referred to as “herding” (Sherman 1989). Males were grouped according to the effort they invested in mate guarding females. If a male mate guarded a female both pre- and post-copulation, he was assigned as a high effort male. If the male only mate guarded the female either pre- or post- copulation, he was classed as medium effort. Males who did not mate guard females were classed as no effort.

Microsatellite Analyses

Eleven microsatellites with polymorphic alleles previously shown to amplify in Richardson’s ground squirrels were used to assign paternity. Methods of DNA extraction, amplification, and sequencing are described in detail in Chapter II. Briefly, half of each 2-mm tissue sample was digested overnight at 55°C with 2 mg Proteinase K, 2% SDS, and an extraction buffer (0.01 M Tris, 0.1 M NaCl, 0.01 M EDTA). DNA was extracted using the MacManes salt extraction (MacManes 2008), in which salts were precipitated with 5M NaCl and DNA was precipitated with isopropanol. Precipitated DNA was washed with 80% ethanol, dried using a vacuum centrifuge (SpeedVac,

Savant), and rehydrated in 50 μ L of TE buffer. The concentration of DNA was quantified using a spectrophotometer (Nanodrop technologies) and diluted to 10ng/ μ L.

Polymerase chain reactions were carried out in 15 μ L reactions containing 10 mM Tris-HCl pH 8.6, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.2 μ M of Primer 1, 0.2 μ M Primer 2, 7.5 U of Taq DNA polymerase (New England Biolabs), and 150 ng of DNA. The thermal profile consisted of 3 cycles: first cycle was 5 min of denaturation; second cycle consisted of 30 sec of denaturation, 30 sec of annealing, and 45 sec of elongation; and the last cycle was 5 min of final elongation.

Variation at individual microsatellite loci was quantified using an automated 48-capillary DNA analyzer (Applied Biosystems 3730). Genotypes were visualized using GeneMapper 4.0 (Applied Biosystems).

Data Analyses

Paternity was assigned using CERVUS 3.0 (Kalinowski et al. 2007) based on exclusion or a likelihood probability at the 95% confidence level using Δ LOD of the offspring-mother-father trio provided by the software. All mother-offspring relationships in 96 litters were verified, including juveniles in 2 sets of mixed litters that were rightfully assigned to their respective mothers. Genotypes of all juveniles were then compared with those of all males in the population. If a male was the only possible sire given genotypes of the father and offspring, he was assigned as the father. When more than 1 male was a potential sire based on his genotype, males that were not seen within 20 m of the female before or during estrus were excluded and paternity analysis re-run with the remaining males. If, after elimination of males too distant to have sired

offspring in a litter, more than 1 male had a genotype compatible with offspring, paternity was assigned using the 95% confidence Δ LOD of the offspring-mother-father trio provided by CERVUS 3.0 (Marshall et al. 1998, Kalinowski et al. 2007). Exclusion based on all males and on the reduced number of males enabled assignment of paternity to 279 and 495 offspring, respectively, of the 598 juveniles in 96 litters. The inclusion of results based on Δ LOD further increased assignment rates to 580 juveniles. Sixty-four litters were assigned as singly sired, 23 were assigned as multiply sired, and 9 were classed as ambiguous. Thirty-six singly sired litters were exclusively assigned to 1 male, and 19 litters had at least half the offspring sired by a single male and the remainder assigned that same male by Δ LOD. The remaining 9 litters were assigned a single male because the female's entire litter was attributable to the only male with whom she was observed consorting and other males were eliminated because they were known to be courting other females. All 23 multiply sired litters were determined so by having littermates that were sired by different males. Ten of those litters were completely assigned parentage via exclusion and the remaining 13 litters were assigned paternity using Δ LOD.

GenePop 4.0 (Raymond and Rousset 1995) was used to calculate the Hardy-Weinberg equilibrium (H-W) using a strict Markov chain. Tests of Hardy-Weinberg equilibrium were performed on the adult population only. Kinship 1.3.1 (Queller and Goodnight 1989) was used to calculate the coefficient of relatedness (r). Heterozygosity of males and offspring are represented by the standardized heterozygosity, which was calculated as the proportion of heterozygous loci per mean

observed heterozygosity of the typed loci (Cohas et al. 2006). The observed heterozygosity was calculated using CERVUS 3.0.

Statistical Analyses

Normality was assessed using Shapiro-Wilk's *W* test; when the assumption could not be satisfied, non-parametric tests were used. Student's *t*-tests assumed unequal variances. Unless specified, two-tailed probabilities are reported and descriptive statistics are expressed as mean \pm SEM. *P*-values less than 0.05 indicate a significant difference in the means. First versus last male comparisons were made for multiply mated females, whether they had 2 or 3 mates.

Data were analyzed using JMP-IN statistical software (version 6.0 for Mac, SAS Institute). A logistic regression with dichotomous criterion variables was used to assess female choice (SPSS 16.0).

Independence of data for females observed in both 2007 and 2008 varied with the question being asked, and sample sizes varied depending on completeness of information for a given individual. All times are reported as Mountain Standard Time (MST).

Results

Mating Periods and OSR

The mating period for Richardson's ground squirrels lasted 9 days (6-14 March) in 2007 and 15 days (1-15 March) in 2008 with peak mating periods of 5- and 10-days duration, respectively (Figure 3.1). Average daily OSR did not differ significantly between 2007 and 2008 for either the mating season (0.50 ± 0.14 females/male, $n=9$ days,

and 0.41 ± 0.05 females/male, $n=15$ days, respectively; Wilcoxon, $S=116$, $z=0.15$, $p=0.88$) or the peak mating period (0.75 ± 0.19 females/male, $n=5$ days and 0.52 ± 0.04 females/male, $n=10$ days, respectively; Wilcoxon, $S=49.5$, $z=1.11$, $p=0.27$). Except for 1 day in the peak mating period of 2007, daily OSR was <1.0 estrous females per male on all days in the mating seasons of both 2007 and 2008 (Figure 3.1), indicating that the number of reproductively available males typically exceeded the number of estrous females.

Average litter size at weaning was 6.9 ± 0.4 ($n=32$) in 2007 and 6.0 ± 0.2 ($n=74$) in 2008. Litter size was positively correlated between years for females that weaned litters in both years (Linear regression, $R^2=0.24$, $F=6.25$, $p=0.02$, $n=22$ females). After 1 litter was randomly omitted for each of these females, litter size was significantly larger in 2007 than in 2008 (6.9 ± 0.5 , $n=20$ and 6.0 ± 0.2 , $n=64$, respectively; Wilcoxon, $S=1047$, $Z=2.09$, $p=0.04$).

Copulations and Consortships

Above-ground copulations occurred earlier on average than underground copulations ($17:18$ h ± 10 min; range= $16:58$ - $17:49$ h, $n=6$ and $17:55$ h ± 5 min; range= $16:27$ - $18:48$ h, $n=42$, respectively; Wilcoxon, $S=57$, $Z=-2.79$, $p=0.005$). Average start times did not differ between 2007 and 2008 for either consortships (Table 3.1; Wilcoxon, $S=378$, $Z=1.13$, $p=0.26$) or underground copulations (Table 3.1; Wilcoxon, $S=165$, $Z=0.47$, $p=0.63$). The average length of above-ground copulations was 3.9 ± 1.0 min (range= 2.2 - 9.0 min, $n=6$), whereas underground consortships (10.2 ± 0.9 min; range= 2.9 - 26.2 min, $n=46$) and underground copulations (10.2 ± 1.0 min; range= 2.9 - 26.2

min, n=37) lasted significantly longer (Wilcoxon, S=53, Z=-3.02, p=0.003 and Wilcoxon, S=47, Z=-2.96, p=0.003, respectively). The average length of underground consortships and underground copulations did not differ significantly between 2007 and 2008 (Table 3.1; Wilcoxon, S=197, Z=-1.57, p=0.12 and Wilcoxon, S=85, Z=-1.17, p=0.24, respectively).

Of 6 females that copulated above-ground, 1 subsequently mated above-ground with another male, 3 subsequently mated underground with another male, and 1 mated with another male at an unknown time. An above-ground copulation that lasted 2.2 min did not result in any offspring being sired by that male; in contrast, an underground consortship that lasted 2.9 min resulted in the entire litter of 7 offspring being sired by that male.

In 2007 and 2008 combined, 35 of 63 focal females were trapped on the day following their estrus. Estrus dates were confirmed for 1 female based on the presence of a copulatory plug, 19 by the presence of sperm and 10 by the presence of leukocytes in vaginal lavages, and 5 from a combination of cell types in the lavage and backdating from the known date of parturition and/or litter emergence. The estrous dates of 8 females that were not trapped the day after estrus were also determined by backdating from the parturition date and/or litter emergence. All 43 focal females gave birth, and only the final estrus was considered for 3 females that re-mated in 2008, except in calculation of OSR.

Multiple Mating and Multiple Paternity

Three of 8 female Richardson's ground squirrels with confirmed observations in 2007 and 7 of 35 females in 2008 were observed to mate with 2 males, and 1 female in 2007 and 2 females in 2008 were observed to mate with 3 males. The frequency of multiple mating did not differ significantly between years (Figure 3.2; Fisher's exact test, $p=0.22$, $n=43$). Complete observations were available for both years for only 3 females, so the tendency to mate with single or multiple males in different years could not be tested. None of the 4 litters weaned by females that mated with multiple males in 2007 were multiply sired, whereas 7 of 9 litters weaned by multiply-mated females in 2008 had multiple sires (Figure 3.2; Fisher's exact test, $p=0.02$). Although none of the litters of focal females in 2007 were multiply sired, the litters weaned by 6 of 11 non-focal females did have multiply sired litters as did those weaned by 10 of 33 non-focal females in 2008.

Based on inferred underground copulations, length of first matings for multiply-mated females did not differ significantly from length of last matings (6.0 ± 1.2 min, $n=5$ and 9.2 ± 1.6 min, $n=6$, respectively; t -test, $t=1.61$, $p=0.14$), with random exclusion of 2008 data for a female with eligible information for both years. Seven females that mated at least twice with the same male also showed no difference between the lengths of first and last inferred copulations (4.0 ± 0.8 min and 9.6 ± 2.9 min, respectively; Wilcoxon signed rank, $t=10.0$, $p=0.11$).

For 73 litters on the Main Site and 1 litter adjacent in 2008, the proportion of matings each day that resulted in multiple paternities was not correlated with daily OSR (Linear regression, $R^2=0.13$, $F=1.98$, $p=0.18$, $n=15$ days).

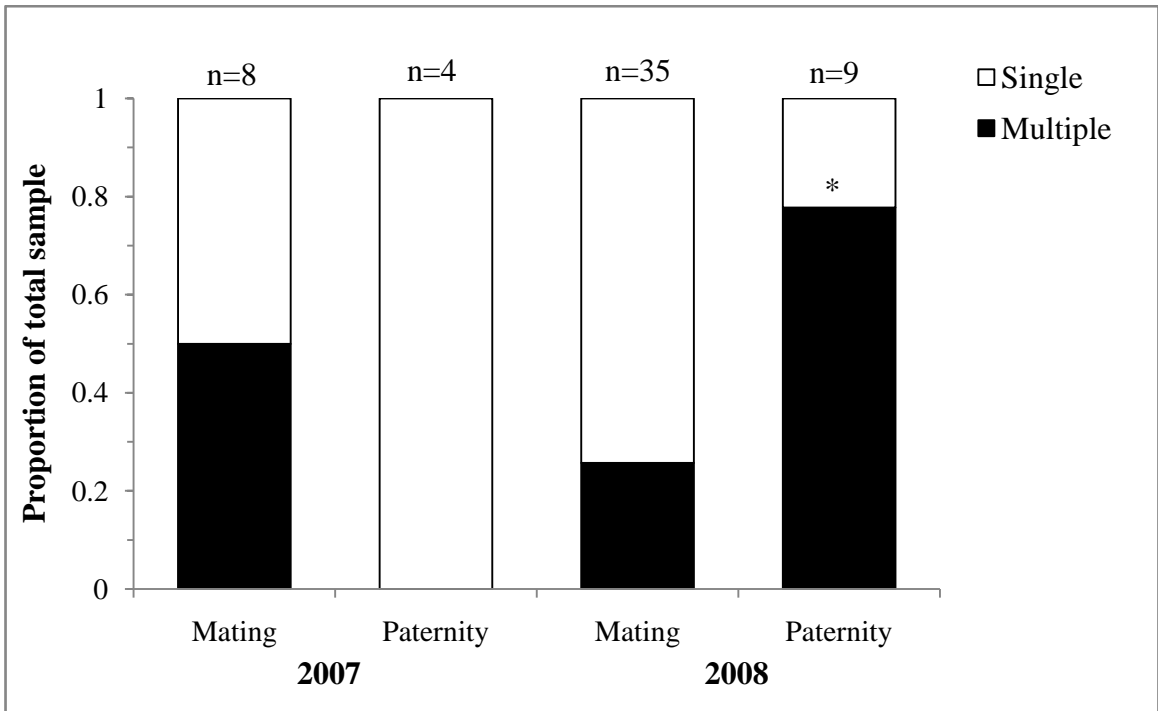


Figure 3.2. The proportion of female Richardson's ground squirrels that mated singly versus multiply (Mating) and the proportion of litters of multiply mated females that were singly or multiply sired (Paternity) in 2007 and 2008. The asterisk (*) indicates a significant difference between years in the proportion of multiply sired litters (Fisher's exact test, $p=0.02$).

Direct Benefits of Multiple Mating

Whether they mated singly or multiply, all 63 females with partial or complete observations located on the Main Site got pregnant indicating that assurance of fertilization is not a concern for Richardson's ground squirrels. Neither litter size at weaning (Figure 3.3A: 6.29 ± 0.41 , $n=24$ and 6.13 ± 0.23 , $n=64$, respectively) nor proportion of daughters in litters (Figure 3.3B: 0.58 ± 0.05 , $n=24$ and 0.52 ± 0.03 , $n=64$, respectively) differed significantly between multiply and singly sired litters (Wilcoxon, $S=1106$, $Z=0.3569$, $p=0.72$ and t-test, $t=1.20$, $p=0.23$, $n=88$, respectively). Only 4 of 21 females that were trapped both before and after mating had wounds 1 day post-estrus, but because I could not determine who inflicted those wounds I cannot assess the risks of resisting mating.

Indirect Benefits of Multiple Mating

Indirect benefits such as increased offspring quality gained by females from mating with multiple males were assessed. Because the proportion of offspring weaned in 2007 that recruited into the population as adults in 2008 did not differ significantly between yearling and older mothers (0.24 ± 0.05 , $n=11$, and 0.41 ± 0.10 , $n=9$, respectively; t-test, $t=1.52$, $p=0.16$), I pooled all age classes for subsequent analyses. Proportion of offspring recruited did not differ significantly between multiply and singly mated females (0.40 ± 0.12 , $n=4$ and 0.22 ± 0.11 , $n=4$, respectively; t-test, $t=1.18$, $p=0.28$) or between multiply and singly sired litters (0.22 ± 0.07 , $n=7$ and 0.37 ± 0.07 , $n=13$, respectively; t-test, $t=1.46$, $p=0.16$). Because male Richardson's ground squirrels are more likely than females to disperse from the natal population (Michener 1983), I also considered just the

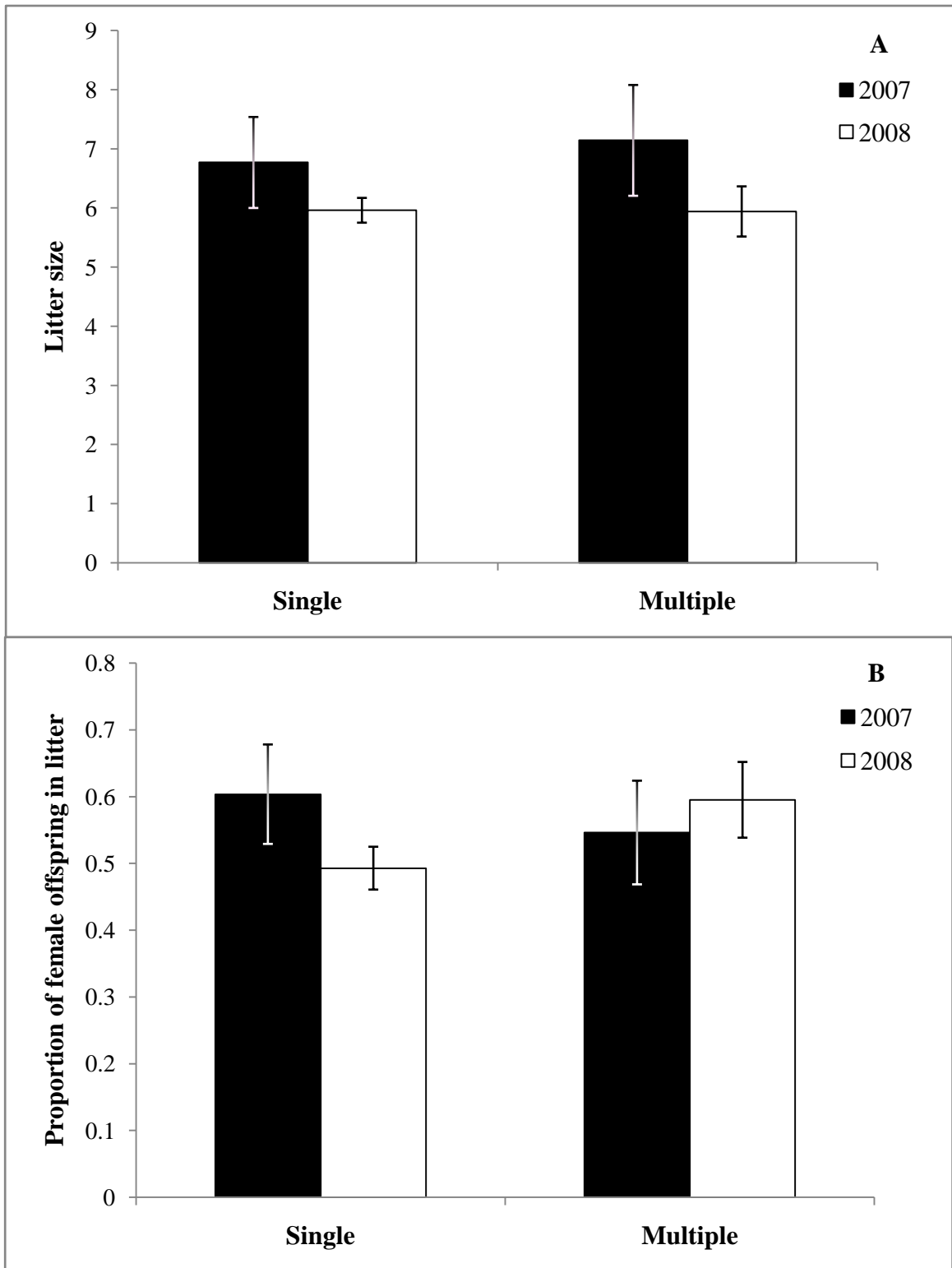


Figure 3.3. Mean \pm SEM litter size (A) and mean \pm SEM proportion of female offspring (B) in singly and multiply sired litters weaned by female Richardson's ground squirrels in 2007 (n=20 litters) and 2008 (n=68 litters).

proportion of daughters that recruited into the population for multiply and singly mated females (0.47 ± 0.15 , $n=4$ and 0.25 ± 0.10 , $n=4$, respectively) and for multiply and singly sired litters (0.23 ± 0.06 , $n=7$ and 0.41 ± 0.08 , $n=13$, respectively). Recruitment of female offspring into the population was not affected by whether offspring came from singly or multiply mated females (t-test, $t=1.18$, $p=0.29$) or singly or multiply sired litters (t-test, $t=1.74$, $p=0.10$).

Offspring quality in 2007 and 2008, as measured by standard heterozygosity of the litter, was compared for juveniles from singly and multiply sired litters. Offspring from 7 females that had a singly sired litter in 1 year but a multiply sired litter in the other year were included in both years, whereas offspring of 4 females with a singly sired litter in both years and 1 female with multiply sired litter in both years were randomly eliminated from 1 year. Standard heterozygosity did not differ between multiply or singly sired litters (1.12 ± 0.03 , $n=22$ and 1.08 ± 0.02 , $n=60$ respectively, Wilcoxon, $S=1005$, $Z=0.95$, $p=0.34$). Heterozygosity also did not differ between juveniles that did and did not survive from 2007 to 2008 (1.11 ± 0.04 , $n=38$ and 1.10 ± 0.02 , $n=100$ respectively; Wilcoxon, $S=2970$, $Z=0.24$, $p=0.8$).

Female Mate Choice

Female mate choice did not occur as often as expected. The hide-then-run behaviour tactic described by Michener and McLean (Michener and McLean 1996), where females sneak away from resident males and sometimes actively seek out unfamiliar males away from their usual home range, was observed only once. I also observed an instance in which a female escaped from the grasp of the male that was

attempting an above-ground copulation. None of the female's offspring were sired by this male indicating that females can choose to end copulation prior to ejaculation in order to prevent fertilization.

Due to the small sample of females that exhibited distinctive female mate choice, I pooled all females with sufficient observations to gain some insight into female mate choice criteria. To assess the potential indirect benefits gained by a female from being choosy in her mates, all males that were within 20 m of that female on at least 1 scan sample before and during her estrus in 2008 were evaluated for their displacement index, familiarity with the female, heterozygosity, relatedness to the female, and body mass at emergence (Figure 3.4). Too few males (3/11 in 2007 and 2/14 in 2008) were ≥ 2 years old to permit statistical analysis of the effect of a male's age on female choice.

Logistic regressions were calculated based on 2 different dependent variables: observed mates based on focal observations and genetic mates based on paternity analysis of offspring. Based on a logistic regression given dichotomous criterion variables, none of the independent variables (most familiar, familiar, most heterozygous, least genetically related, heaviest at emergence from hibernation, greatest change in weight, and greatest daily change in male weight during the mating period with respect to other males within a 20-m radius of the female) were significant in female choice, whether represented as mating with a male or conceiving offspring from that male (Table 3.2). A male's displacement index significantly influenced who females mated with, whether based on observed mates (Figure 3.4: Logistic regression, $\Delta R^2 = -0.134$, $F = 23.5$, $p = 0.000$) or genetic mates (Logistic regression, $\Delta R^2 = -0.366$, $F = 88.0$, $p = 0.000$).

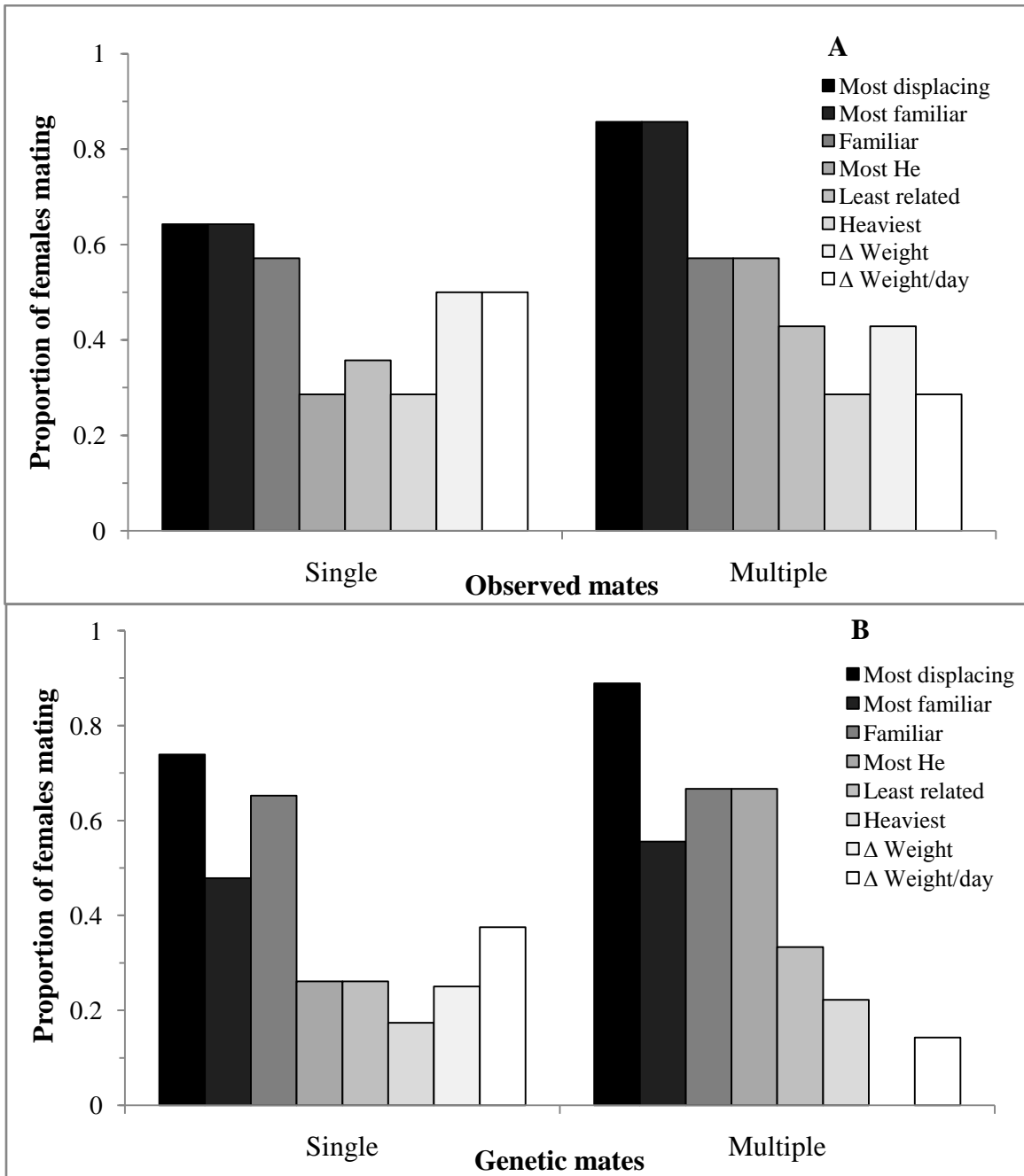


Figure 3.4. The proportion of female Richardson's ground squirrels that mated with single or multiple males in 2008 that were (A) observed to mate (n=21) with or (B) weaned offspring (n=32) from males classed as: displacing, most familiar, more familiar (familiar), most heterozygous (Most He), least related, heaviest at emergence from hibernation, losing the least weight during the mating period (Δ Weight), and losing the least weight per day during the mating period (Δ Weight/day). Criteria used to classify males are described in the Methods.

Table 3.2. Attributes of the first and last male to mate with a female Richardson's ground squirrel based on male-female familiarity, standard heterozygosity, male-female relatedness, male weight at emergence from hibernation, change in weight between the beginning and end of the mating period (Δ Weight), and daily change in weight between the beginning and end of the mating period (Δ Weight/day). Attributes are compared between first and last males by 2-tailed paired t-tests (PTT) if the data set is normal and Wilcoxon signed ranks (WSR) if normality is not met.

	First male	Last male	Statistic	n
Familiarity	0.18±0.08	0.09±0.03	WSR, t=2.00, p=0.81	7
Heterozygosity	1.41±0.04	1.24±0.09	WSR, t=11.0, p=0.18	9
Relatedness	-0.10±0.08	0.01±0.10	PTT, t=1.07, p=0.31	9
Male weight	380±14	376±15	WSR, t=2.50, p=0.80	9
Δ Weight (g)	71.4±14.1	62.1±25.8	PTT, t=0.38, p=0.72	7
Δ Weight (g/day)	4.58±0.84	4.53±1.97	PTT, t=0.02, p=0.98	7

None of familiarity, heterozygosity, relatedness, male weight at emergence, change in male weight across the mating period, or daily change in male weight affected a female's order of mates for multiply mated Richardson's ground squirrels (Table 3.2). For 9 multiply mated females for whom the displacing male was known, 6 mated with the displacing male first (Fisher's exact test, $p=0.05$), 1 female mated with the displacing male last, and 2 did not mate with the displacing male.

Mate Guarding

Male Richardson's ground squirrels could potentially ensure sole paternity of a litter by preventing a female from mating with other males by guarding her (Figure 3.5) or by occluding the vagina with a copulatory plug. I assessed the effects of mate guarding on paternity in 35 copulations by 28 females. Males that mate guarded sired a significantly higher proportion of offspring (0.88 ± 0.06 litters, $n=11$) than non-guarders (0.62 ± 0.12 litters, $n=24$; Wilcoxon, $S=128.5$, $Z=-2.90$, $p=0.004$). The greater the effort a male invested in mate guarding, the higher the proportion of offspring he sired (no effort: 0.62 ± 0.12 litters, $n=11$; medium effort: 0.80 ± 0.10 litters, $n=14$; high effort: 1.00 ± 0.00 litters, $n=10$; $X^2=11.1$, $df=2$, $p=0.004$). Only 1 of 10 females guarded by a high-effort male subsequently mated with another male (66 min later), and that male did not sire any of her litter. Two females guarded by medium-effort males re-mated with other males about 30 min later and produced litters sired by both males. The longer the interval between consecutive matings with different males, the fewer offspring were sired by the

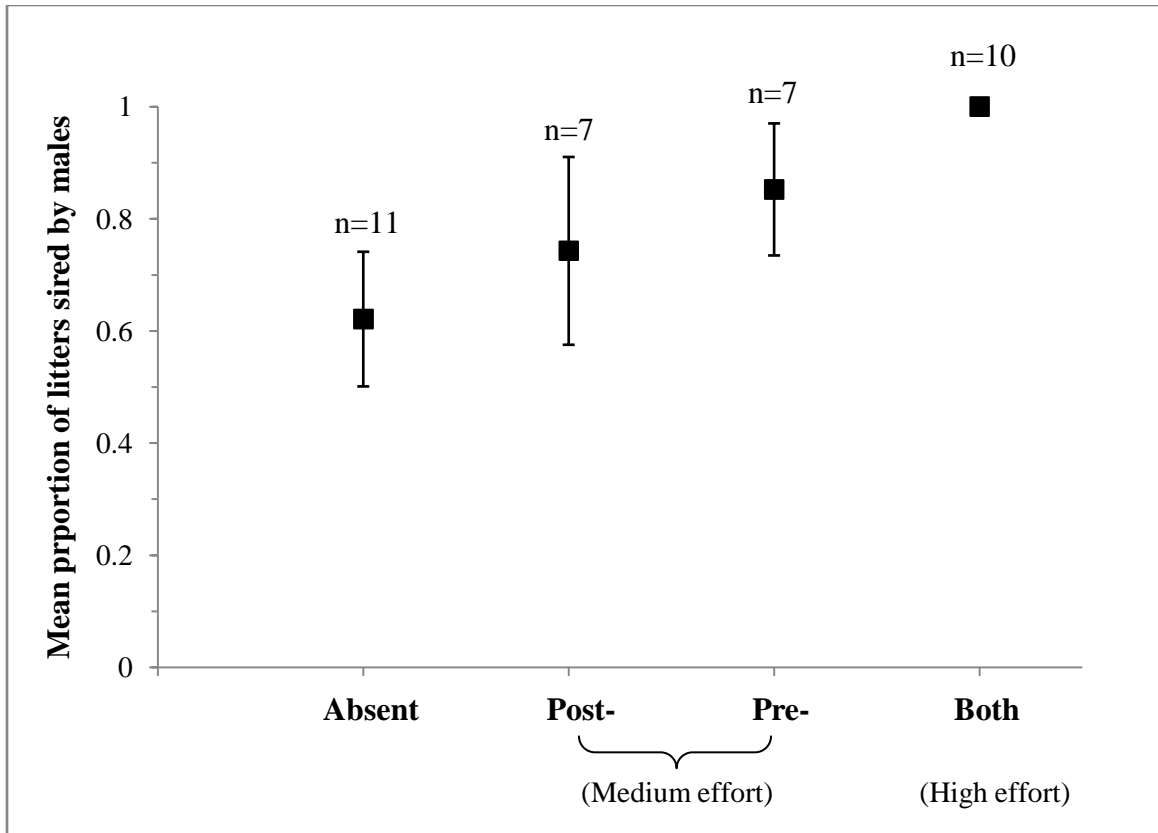


Figure 3.5. The mean proportion \pm SEM of offspring sired by male Richardson's ground squirrels in the absence of mate guarding (Absent), either with pre- or post-copulatory mate guarding (medium effort) or with both pre- and post-copulatory mate guarding (Both; high effort). Rate of siring differed significantly across all effort groups (Chi-square, $X^2=11.1$, $p=0.0004$, $n=35$).

second male (Figure 3.6; Linear regression, $R^2=0.51$, $F=6.16$, $p=0.05$, $n=8$). Displacing males (9 of 14) were no more likely to mate guard a female than displaced males (8 of 13; Fisher's exact test, $p=1.0$).

In 2008, I had information on both mate guarding and time between copulations for 4 females. The time between copulations for these females ranged from 3.5-45.2 min, and none of them were mate guarded post-copulation. The variation in the time between matings is most likely due to the unavailability of nearby males as 3 of the 4 females were not approached by males until they re-mated. The fourth female was observed to consort with a male underground within 11 min and re-mated with a third male after observations stopped. No offspring were sired by the second male, but the third male, who was seen exiting the female's burrow system the next morning, sired 1 of her 5 offspring.

In addition to variation in latency between consortships, the length of inferred copulations varied from 2.9 to 26.2 min. For females that mated with multiple males, regardless of whether her litter was multiply sired or not, the proportion of offspring in a litter sired by a particular male did not depend on the length of inferred copulations (Linear regression, $R^2=0.01$, $F=0.45$, $p=0.50$, $n=52$ unique mating pairs). When a female consorted several times with the same male, combined time underground with that male did not correlate with percent of offspring sired (Linear regression, $R^2=0.01$, $F=0.54$, $p=0.47$, $n=52$).

Because female Richardson's ground squirrels mate just before they retire for the night, they cannot be inspected for copulatory plugs until after they resume above-ground activity the next morning, by which time few females have a copulatory plug in the

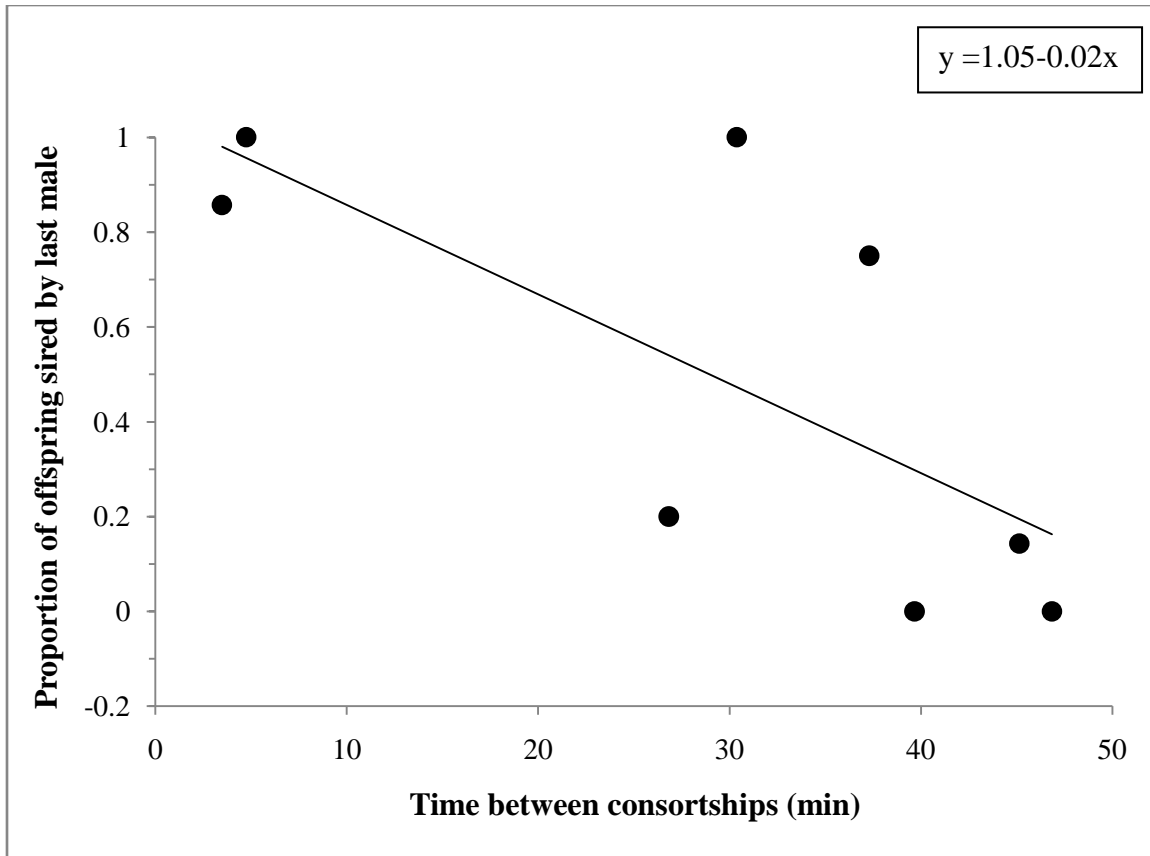


Figure 3.6. The proportion of offspring sired by the last male relative to the time between consortships of the first and the last male for litters weaned by multiply mated female Richardson's ground squirrels. Linear regression, $R^2=0.51$, $F=6.16$, $p=0.05$, $n=8$.

vagina (Michener 1984a). Indeed, only 8 of 77 females captured the morning after behavioural estrus had a copulatory plug; of those, 6 (75.0%) had singly sired litters, 1 had a multiply sired litter, and 1 litter was undetermined compared with 55 (79.7%) singly sired litters, 14 multiply sired litters, and 10 undetermined litters for 69 females without a plug the next day. There was no statistical difference in whether litters were singly or multiply sired for females with or without copulatory plugs when trapped the following morning (Fisher's exact test, $p=1.0$).

Sperm Competition

For 9 multiply mated female Richardson's ground squirrels with known mating order in 2008, the proportion of offspring sired by the first and last males did not differ significantly (0.58 ± 0.12 and 0.39 ± 0.12 , respectively; Paired t-test, $t=0.86$, $p=0.41$).

Discussion

The frequency of multiple matings I observed for Richardson's ground squirrels (30.2%, $n=43$) was less than the 53.3% ($n=15$) that was observed in another population by Michener and McLean (1996). Those frequencies of multiple mating in Richardson's ground squirrels are less than the 68.8% ($n=16$) reported for *Urocitellus parryii* (Lacey et al. 1997) and the 100% ($n=64$) reported for *U. columbianus* (Murie 1995). Some observed consortships may not have culminated in ejaculations, but because all males on the Main site were known to have sired offspring, they were all capable of producing viable sperm. In my study, none of 4 multiple matings resulted in multiple paternities in 2007, but 77.8% of 9 multiple matings resulted in multiple paternities in 2008. The frequency of multiple paternities (26.4%, $n=87$) in my study was lower than

multiple-paternity rates reported for another population of *U. richardsonii* (80.0%, n=15; Hare et al. 2004) and for *Otospermophilus beecheyi* (89.0%, n=9; Boellstorff et al. 1994), *U. beldingi* (78.0%, n=27; Hanken and Sherman 1981), *U. brunneus* (71%, n=7, Sherman 1989), and *Ictidomys tridecemlineatus* (50.0%, n=8; Foltz and Schwagmeyer 1989), but higher than *U. columbianus* (16.0%, n=168; Murie 1995) and *U. parryii* (8.0%, n=12; Lacey et al. 1997).

The potential costs of polyandry in Richardson's ground squirrels such as loss of time spent feeding and maintaining burrows, metabolic costs associated with reduced time feeding and increased activity (Michener 1984b, Michener and McLean 1996, Michener 1998), predation (Michener 2001), and possibility of parasite transmission (Yensen et al. 1996), appear to outweigh the benefits because no direct or indirect benefits were found. All females, regardless of the number of mates, were impregnated indicating that assurance of fertilization is not the reason for mating with multiple mates. All males on the Main Site sired offspring therefore avoidance of male sterility is unlikely, but I do not have direct information on short-term sperm depletion in males. In house mice, subsequent ejaculates within the same day contain reduced numbers of sperm (Huber et al. 1980). Therefore, avoiding sperm depletion in males, on a given day during the mating period, remains as a possible benefit for polyandry in Richardson's ground squirrels. To examine the reproductive physiology of females was beyond the scope of my study, and the type of ovulation present in Richardson's ground squirrels is not known. Gray et al. (1974) proposed that if an organism is an induced ovulator, longer first copulations by the female or repeated mating with the same male can be expected. Although longer or repeated copulations were not observed in Richardson's ground

squirrels, induced ovulations do not necessarily have to be induced by copulations but by male-female contact or the presence of pheromones (Bouchie et al. 2006).

Heterozygosity was not a predictor of offspring survival to yearling age. It is difficult to assess the effects of polyandry on offspring survival based on genetic differences because offspring survival strongly depends on environmental conditions and investment in growth (Tregenza et al. 2003, Fisher et al. 2006). It is also impossible to control for mating frequency and number of ejaculates in a wild population for comparison of genetic differences, but especially in a species that copulates below-ground 92.6% of the time.

Because sex ratios between multiply and singly sired litters did not differ and heterozygosity did not increase offspring quality, it appears that female Richardson's ground squirrels do not mate with multiple males in order to gain indirect benefits such as increased offspring survival or increased genetic quality of offspring. Females also did not choose higher-quality males as their second mate to sire part of their litters or to promote sperm competition. For some females that were not mate guarded by males, up to 45 min elapsed before subsequent copulations; the greater the latency between copulations, the less likely the second male sired any offspring. Although the latency between copulations was due to a lack of potential mates for 3 of 4 females, the example of a female that did not conceive from a second mate indicates that a form of post-copulatory female choice might be in place, assuming that copulation occurred while consorting. Females may choose to wait longer between copulations if the subsequent male is of lower quality than the first.

If female Richardson's ground squirrels choose mates, the characteristics that such choice was based on were not evident. Females did not preferentially mate first or last with the most familiar, least related, most heterozygous, or heaviest male. Females that mated multiply were significantly more likely to mate with the most heterozygous male than less heterozygous males indicating some importance of heterozygosity in female choice. However, for almost 50% of females, more than 1 male tied for the position of most heterozygous male; therefore it could be that this factor is statistically significant only because of the increased number of possibilities. The only characteristic that significantly characterized a female's mate was male displacement index. However, I argue that mating with displacing males does not necessarily indicate female choice; rather it indicates male mating success based on having a greater displacement index. Although the displacing male on a female's day of estrus was always the most familiar male on the same day, this male was not necessarily the most familiar male to the female prior to estrus. Michener and McLean (1996) also showed that the male Richardson's ground squirrel that spent the most time within 10 m of a female 1 day was not necessarily the same male the next day and that 19/22 (86.4%) females mated with the closest male on their day of estrus. Although the variety of male criteria I assessed did not yield insight into female mate choice, I did observe female mate choice in Richardson's ground squirrels based on termination of copulation, avoidance of copulation when nearby males were available, and hide-then-run behaviour described by Michener and McLean (1996).

The only chance a male Richardson's ground squirrel had to increase his reproductive success in addition to having a greater displacement index was to mate

guard a female in order to increase the time before the female's next mate or to prevent the female from mating with another male altogether. Males that mate guarded 1 female did not necessarily mate guard another female on the same day, whereas some males simultaneously mate guarded two adjacent estrous females. Subsequent consortships of females were longer than previous matings, but males did not increase their reproductive success by having longer consortships. In thirteen-lined ground squirrels (*I. tridecemlineatus*), the reproductive success of the first male is increased if the time between matings is increased, but decreased as the duration of the second male's copulation is increased (Schwagmeyer and Foltz 1990). Because underground consortships in my study were significantly longer than above-ground copulations, underground consortships may be another form of mate guarding. Murie (1995) noted that Columbian ground squirrels (*U. columbianus*) have unusually long copulations (35 min) compared to other ground squirrels (8-21 min; see Murie 1995 for review) yet all females manage to mate with multiple mates. He also proposed that these extended periods of mating may be an alternative form of mate guarding.

In contrast to sperm precedence in *I. tridecemlineatus*, *O. Beecheyi*, *U. beldingi*, *U. columbianus*, and *U. parryii* (Hanken and Sherman 1981, Foltz and Schwagmeyer 1989, Boellstorff et al. 1994, Murie 1995, Lacey et al. 1997) and to sperm-displacement in *U. brunneus* (Sherman 1989), Richardson's ground squirrels show no trends in sperm competition. The first or the last male to mate with the female has no distinct paternity advantage; however, the 2 females that re-mated within 5 minutes of their first copulation had almost an exclusive last male advantage (5/6 offspring and 9/9 offspring, respectively).

More male Richardson's ground squirrels showed dominance, based on male displacement index criteria, in 2008 than in 2007, and therefore defense polygyny was more evident in 2008 than in 2007; however, OSR did not differ significantly between the 2 years, whether comparing overall OSR (Davis and Murie 1985) or early, peak, and late mating periods (Michener and McLean 1996). Therefore, OSR was not indicative of male-defense polygyny as suggested by Davis and Murie (1985); they proposed that male-defense polygyny was negatively correlated with the daily OSR in Richardson's ground squirrels.

Although my study shed some light on female choice given genetic qualities of males, it would be worthwhile to examine male features such as daily body weight, fat content, body condition represented by over-all health and reproductive status (e.g.: previously unmated on a given day), and male age, although male Richardson's ground squirrels rarely live past yearling age (Michener 1989b). A long-term study gathering information on choosy females is recommended as it would be expected to yield more accurate results. A future laboratory study similar to that of Schwagmeyer and Foltz (1990) on thirteen-lined ground squirrels on sperm production and sperm depletion in males might also give insight into mating order and female choice. In addition, research on female reproductive physiology would be useful.

In summary, female Richardson's ground squirrels do not appear to gain any direct or indirect benefits by mating with multiple males, but they reportedly incur costs. Female choice was less frequent than expected, but was evident through the termination of copulation, avoidance of copulation when nearby males were available, and hide-then-run behaviour described by Michener and McLean (1996). Males did not have paternity

advantage by mating first or last with the female unless they mated within 5 minutes of the previous male, in which case sperm displacement was apparent. Lastly, males could also increase their chances of siring offspring by having a greater ability to displace males from the vicinity of estrous females or by mate guarding the female.

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CHAPTER IV

SUMMARY

I examined the usefulness of 11 microsatellite loci for assignment of paternity to juvenile Richardson's ground squirrels (*Urocitellus richardsonii*) with known maternity. Although paternity exclusion probability given one known parent for all loci combined was 0.9892, I was only able to assign paternity exclusively to 85 of 154 (55.2%) offspring in 2007 and 194 of 444 (43.7%) offspring in 2008, a slim 10% increase from assignment of paternity to 32 of 85 (37.6%) juvenile Richardson's ground squirrels by Hare et al. (2004) using 6 microsatellite loci. The utility of additional microsatellites was limited due to the genetic similarity of putative sires. Incorporation of spatial and temporal data to eliminate genetically similar males that were too remote from a specific female to have mated with her increased my paternity assignments to 82.5% and 82.9% of juveniles in 2007 and 2008, respectively.

I was able to use those high assignment rates to evaluate multiple paternities within litters and sperm competition among males. Four of 8 females in 2007 and 9 of 35 females in 2008 were observed to mate with 2-3 males. Of these multiply mated females, none had multiply sired litters in 2007 but 7 had multiply sired litters in 2008. In all tissue sampled litters combined, multiple paternities were detected in 6 of 19 litters in 2007 and 17 of 68 litters in 2008. Males did not gain a paternity advantage by mating first or last with a female unless they mated within 5 minutes of the previous male, in which case sperm displacement was apparent.

Due to evidence of female choice reported for Richardson's ground squirrels (Michener and McLean 1996), as well as personal observations of termination of copulation and avoidance of copulation while nearby males were available, I evaluated female choice of males based on displacement index, familiarity, heterozygosity, relatedness, and male weight. Females were more likely to mate with the male who displaced other males more often than he was being displaced within a 20-m radius prior to and during estrus, but the other characteristics were not relevant to female choice.

In addition to a greater displacement index, males increased their reproductive success by mate guarding a female in order to increase the time before the female's next mate or to prevent the female from mating with another male altogether. Males that mate guarded 1 female did not necessarily mate guard other females that he courted during the same day indicating that this behaviour is not specific to individuals. Because underground consortships were significantly longer than above-ground copulations, underground consortships may be another form of mate guarding.

This study showed that incorporating observational data to reduce the number of potential sires was more successful in increasing the number of paternity assignments than increasing the number of microsatellites. Although 26.4% of females had multiply sired litters and female choice was evident, female Richardson's ground squirrels did not appear to gain any direct or indirect benefits of mating with multiple males. Lastly, males increased their chances of siring offspring by having a greater ability to displace neighbouring males and/or by mate guarding the female, and patterns of sperm competition were absent unless males mated within 5 minutes of the previous male, in which case sperm displacement was apparent.

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