Mating behaviour and the effects of parasites on reproductive success in male Columbian ground squirrels (*Spermophilus columbianus*)

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Thesis presented 3rd of June 2009 in presence of

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IMPRIMATUR POUR LA THESE

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La Faculté des sciences de l'Université de Neuchâtel,
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General Abstract

Multiple mating is widespread among animals and leads to sexual competition among males and females for access to the other sex. Sexual selection arises in response to either female choice (intersexual selection), in which females choose males based upon e.g. elaborate ornamentation, male behaviours or parasite infestation, or male-male competition (intrasexual selection), in which males compete for territories, access to females, or areas on mating grounds where displays take place. Females can either choose with which male to mate with (pre-copulatory choice) or use post-copulatory mechanisms such as cryptic female choice. On the other hand, males compete for access to females (pre-copulatory) or via sperm competition (post-copulatory). In the latter case, mating order (or the timing of ejaculate release) has been shown to play often an important role in determining a males' reproductive success.

I examined the influence of mating order and male mating behaviour using Columbian ground squirrels (*Spermophilus columbianus*) as study species. The data were collected in the Sheep River Provincial Park, Alberta, Canada including field seasons from 2005 to 2008. Field work provided trapping and observational data of free-living ground squirrels. The observational data were combined with paternity analyses to assess detailed information concerning male reproductive success.

In chapter (1) I tested whether mating order affects mating success in males and whether order effects are influenced by the number of mating partners a female had (i.e. due to increased sperm competition). In chapter (2) I examined the mechanisms involved in shaping mating order effects e.g. the duration of copulations, the occurrence of mate guarding, the age of the individuals and whether mating associations were age-assortative or not. Finally in chapter (3) I evaluated whether experimental parasite removal influenced mating success in Columbian ground squirrels (*Spermophilus columbianus*).

I found (1) that the majority of all litters were multiply sired, while singly sired litters did occur as well and were mainly produced by the first mating partner. The first position within a mating sequence was the most successful position in terms of reproductive success. Nevertheless, subsequent males up to the fifth position did fertilise offspring. The first male advantage diminished with increasing number of male mating partners, indicating that sperm

competition plays an important role. And a male's position in the mating sequences was not consistent within and across seasons.

(2) The time a male spent with a female in a burrow (where mating take place) and mate guarding durations were positively correlated. Both durations positively correlated with male reproductive success, but only for the first and the second male to mate. Mate guarding by the first male significantly reduced, but not excluded, the number of additional males a female mated with. The time interval from the start of the female's mating sequence appeared a good predictor of copulation duration, mate guarding duration, mating order and the likely period of female receptiveness, and therefore a good predictor of overall male siring success. Finally, male investment in reproductive behaviours increased with male and female age, but was also increased when partners mated age-assortatively.

(3) Contrary to our expectations, our findings showed that the parasite removal treatment did not significantly affect male reproductive behaviour (mating frequency, mating order, copulation and mate guarding duration), and hence did not change male reproductive success. Furthermore, parasite-free males did not gain more body mass (both within the season and post-hibernation) than control animals.

Taken together, the results support the idea that male-male competition and/or mate choice are of primary importance for shaping reproductive strategies in Columbian ground squirrels. The role of parasites for variation in male reproductive success, however, remains elusive.

General Introduction

Mating systems influence reproductive characteristics across a wide range of taxa (Birkhead and Møller, 1998; Krebs and Davies, 1996; Wolff and Sherman, 2007). Animals exhibit a diverse array of mating systems, from socially monogamous pairs, to highly polygynous harems, to polygynandrous systems in which multiple matings occur for both sexes (Birkhead and Møller, 1998; Clutton-Brock, 1989; Kleiman, 1977; Thornhill and Alcock, 1983). To explain the evolution of such a diversity of mating systems, Emlen and Oring (1977) proposed two main factors to be important 1) competition for environmental resources resulting from the distribution and abundance of these resources, and 2) competition for mates. In short, the distribution of resources (shelter, food, territory) is critical for the allocation and competition for females which in turn affects the behaviour of males (Clutton-Brock, 2007; Clutton-Brock, 2009; West-Eberhard, 1983). Since males and females differ in the size and energetic investment of their gametes, with females producing larger and more costly eggs, females more commonly compete with each other for access to resources necessary for successful reproduction (including breeding sites, parental care, and social rank) than for access to gametes produced by the opposite sex (Andersson, 2004; Clutton-Brock, 2007; Hauber and Lacey, 2005). Because, particularly in mammals there is an asymmetry in the cost of reproduction males try to mate with many females while females may increase their fitness by choosing the best available male partner, causing sexual selection in males (Anderson et al., 2005; Bateman, 1948).

Mating with many different females is assumed to be advantageous for males since it increases the number of their offspring, and therefore their reproductive success (Bateman, 1948). For females on the other hand the advantage of multiple mating is not obvious since they have a given maximal number of offspring they can produce. Females can improve their reproductive success primarily by optimising offspring quality through mate choice (e.g. choosing “good genes”, parasite resistant genes) (Andersson, 1994; Hamilton and Zuk, 1982; Jennions and Petrie, 1997; Kokko et al., 2003; Partridge, 1980; Welch et al., 1998). Male-male competition could be a measure for females to estimate male quality (Candolin, 1999; Candolin, 2000; Howard et al., 1998; Montgomerie and Thornhill, 1989; Pizzari, 2001). One consequence of females mating with more than one male is sperm competition among males. A sexual conflict arises because males aim for quantity (fertilisations) and females for quality (father of offspring). Multiple male mating may lead to sperm competition (Parker, 1970a) may result in sperm precedence (non-random differential fertilization success: Lewis and Austad, 1994), which may be critically affected by mating order. These effects can be detected by differential male reproduction success within a litter, where not all copulations during a mating sequence fertilise eggs with equal probability (Moller and Birkhead, 1989; Parker, 1970a; Parker, 1984; Parker, 1990).

Recent use of microsatellite DNA to reveal paternity suggests that in many species, social mating systems and actual reproductive benefits can produce a complex array of strategies in both males and females to maximize the number and quality of offspring (Birkhead and Møller, 1998; Jennions and Petrie, 1997; Jennions and Petrie, 2000; Westneat and Webster, 1994; Zeh and Zeh, 2001). Pre- and post-copulatory mate choice, physiology and mating behaviour can influence reproductive success of both sexes (Arnquist and Rowe, 2005; Birkhead and Møller, 1998; Simmons, 2001; Wolff and Sherman, 2007). While copulating, males are inseminating their ejaculate in the female tract and the sperm are transported to the site of fertilisation where the gametes interact with each other. Females and males can choose, manipulate and influence fertilisation at different stages (Birkhead and Møller, 1998; Eberhard, 1996; Simmons, 2001). Males' reproductive success can depend on: the position in which they mate in a female's multiple mating sequence, the interval between copulations, whether they can delay or prevent the female from re-mating with other partners and the timing of insemination relative to when a female ovulates (Baumgardner et al., 1982; Birkhead and Møller, 1998; Dewsbury, 1982; Linn et al., 2007; Moller and Birkhead, 1989; Parker, 1970b; Parker, 1984; Parker, 1990; Simmons, 2001; Wolff and Sherman, 2007).

Parasites can significantly influence mating success. Parasites can be a proxy of health status (low transmission during the copulation) (Abel, 1996), male quality and good genes (for the next generation) (Hamilton and Zuk, 1982). Choosing healthy or parasite resistant partners would obviously provide important fitness benefits (Hamilton and Zuk, 1982). Males with low parasite load may be more successful than infested ones either because they can invest more in male-male competition or because females choose them preferentially as mates.

While a lot is known about insects, fishes and birds in terms of mating behaviour and reproductive success, we lack detailed studies concerning order effect and the impact of parasites on wild mammals for comparison. Combining behavioural observations and paternity analyses allowed me to examine mating behaviour and mating strategies in male Columbian ground squirrels (Spermophilus columbainus). I have chosen ground squirrels as study species, because, compared to many other species the genus Spermophilus offers an ideal opportunity to study patterns of mating behaviour in the wild. Many ground squirrels are polygynandrous, thus males and females mate frequently with two and more partners. The observation of the exact mating behaviour of these animals is possible, because of a short oestrous cycle and the relatively easy handling, manipulation and observation in the field. Although mating often occurs underground, there are certain criteria which allowed determining copulation events (e.g. Foltz and Schwagmeyer, 1989; Hanken and Sherman, 1981; Lacey et al., 1997). I therefore use the term "copulation" to refer to behavioural evidence that mating occurred (Lacey et al., 1997) and to the time a female and male spent together in the burrow. Even though some females and males were present during several seasons (years) and therefore copulated and weaned litters successfully repeatedly, we treated the variables (litters, copulations and mate guarding) independently and each year like an independent data set so that year is the measure of repetition.

In chapter (1), the aim was to identify whether litters were sired by single or multiple fathers and whether a male's position in the mating order was related to male reproductive success. Further I examined the patterns of male mating order and mating strategy to determine whether age and/or body weight of males affected their reproductive success.

In chapter (2) I focused on exploring the underlying mechanisms for the observed first male advantage in this species. I evaluated whether duration of copulation, mate guarding and the total interval between copulations affected male reproductive success.

In chapter (3) I removed parasites in half of reproductive, free-ranging male ground squirrels by using chemical agents. I examined whether the different treatments had an impact on body weight loss and copulation frequency which would translate into reproductive success during the mating season.

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Mating order and reproductive success in male Columbian ground squirrels (*Spermophilus columbianus*)

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Abstract

Multiple mating by females is common in many mammalian species, often resulting in mixed paternity litters. In such mating systems, mating order frequently plays an important role in determining male reproductive success. To evaluate whether mating order has an influence on male reproductive success in Columbian ground squirrels (*Spermophilus columbianus*), we studied five colonies over four breeding season in Alberta, Canada. The mating activity of oestrous females was observed and the occurrence of sperm precedence was tested using microsatellites to determine paternity in a total of 110 litters (334 offspring). Females mated with two to eight males per litter, while paternity analyses revealed that only the first five males to mate actually sired offspring. The number of offspring sired significantly decreased with a later position in the mating sequence, showing a strong first male advantage. The extent of this first male advantage diminished with an increasing number of male mating partners, indicating that sperm competition plays an important role. A male's position in the females' mating sequences was not consistent within and across seasons, suggesting that individual males did not follow distinct reproductive strategies (i.e. targeting the first mating position in a few litters vs. targeting later mating positions in multiple litters). Even though the majority of all litters were multiply sired, singly sired litters did occur and were mainly produced by the first mating partner. Males of intermediate age were more successful than young and old males, and corrected for age effects, heavier males were more likely to mate first. We conclude that males gain the largest part of their seasonal reproductive output from mating first with a female due to a first male advantage, but gain considerable additional fitness from mating with additional, already mated females.

Keywords: multiple mating, multiple paternity, first male advantage, sperm precedence, mating strategy, Columbian ground squirrel, *Spermophilus columbianus*

Introduction

Animals exhibit a diverse array of social systems, including monogamous pairs, harem and lek-systems, polyandry, and polygynandry (Krebs and Davies, 1996; Wolff and Sherman, 2007). Parentage assignment using microsatellite markers has proven invaluable in determining the mating system of different species (Burke and Bruford, 1987; Kempenaers et al., 1992; Wetton et al., 1987). Parentage analyses have shown that the interplay between the social system and the mating system may produce a complex array of evolutionary tactics for both males and females (Birkhead and Møller, 1998; Clutton-Brock, 1989; Kleiman, 1977; Thornhill and Alcock, 1983).

Mating with numerous females is likely to be advantageous for males, since male reproductive success usually closely matches the number mates (Bateman, 1948). While links between multiple mating and increased reproductive success are more subtle in females, female multiple mating has been observed in many species (Jennions and Petrie, 1997; Zeh and Zeh, 2001). Multiple mating by females selects on more complex male mating strategies, since sperm from different males compete for fertilization of the eggs (‘sperm competition’, Parker, 1970). Sperm quality and quantity, the timing of mating relative to ovulation, position in the mating sequence, or copulatory plugs may engender differential fertilization success among males (Baumgardner et al., 1982; Birkhead and Møller, 1998; Eberhard, 1996; Hartung and Dewsbury, 1978; Jennions and Petrie, 1997; Linn et al., 2007; Parker, 1970; Simmons, 2001).

Typically, male fertilization success is biased to the first or the last male to mate with the female (‘first or last male mating advantage’ Birkhead and Møller, 1998). The key question has been whether there is any first or last male advantage in a study species (Birkhead and Møller, 1992; Dziuk, 1965; Kock and Sauer, 2007; Kraaijeveld-Smit et al., 2002a; Levine, 1967; Martan and Shepherd, 1976; Oglesby et al., 1981; Parker, 1970; Pitcher et al., 2003; Simmons and Siva-Jothy, 1998) and how this might come about (e.g. due to variation in sperm investment and copulatory plugs, or due to cryptic female choice, Eberhard, 1996; Thornhill and Alcock, 1983). As a consequence, the pattern of sperm precedence (non-random differential fertilization success in perspective of sperm number: Lewis and Austad, 1994) may select for one or more male mating strategies (Oliveira et al., 2008; Waterman, 2007). Male mating strategies should incorporate both mating frequencies and timing of copulations (e.g. mating order) to maximise reproductive success (Birkhead and Møller, 1998; Lacey et al., 1997; Schwagmeyer and Foltz, 1990; Schwagmeyer and Parker, 1990; Sherman, 1989). Access of males to females might also be determined by male age and male body mass (Manno and Dobson, 2008; Oliveira et al., 2008).

Ground squirrels (*Spermophilus*) of the tribe Marmotini are highly suitable to assess how different degrees of polygynandry affect male control over reproductive success. Ground squirrels represent the mammalian clade with one of the most available information on male mating strategies (Allainé, 2000; Boellstorff et al., 1994; Foltz and Schwagmeyer, 1989; Hoogland, 1995; Lacey et al., 1997; Schwagmeyer and Parker, 1990; Sherman, 1989). These species are excellent for studies on mating behaviour because the females exhibit a very brief receptive phase of up to twelve hours in which they typically copulate with several males (e.g. Hanken and Sherman, 1981; Schwagmeyer and Parker, 1990). This short period allows the documentation of complete mating sequences for oestrous females and the identification of mating partners using certain behavioural criteria (e.g. Hanken and Sherman, 1981; Lacey et al., 1997; Murie, 1995; Schwagmeyer and Parker, 1990). We therefore use the term "copulation" to refer to behavioural evidence that mating occurred (Lacey et al., 1997) and to the time that a male and female spent together in a burrow. Complementary data on male mating behaviour and reproductive success are available for four species of ground squirrels: *S. tridecemlineatus*, thirteen-lined ground squirrel (Foltz and Schwagmeyer, 1989; Schwagmeyer and Foltz, 1990), *S. beldingi*, Belding's ground squirrel (Hanken and Sherman, 1981; Sherman, 1989), *S. parryii plesiu*s, Arctic ground squirrel (Lacey et al., 1997) and *S. brunneus*, Idaho ground squirrel (Sherman, 1989).

The present study has three aims. First, we provide data on the effect of male mating order on male reproductive success for Columbian ground squirrels. Field observations and paternity analyses allowed us to evaluate how a male's mating position in a female's mating sequence affected his reproductive success with multiply mated females.

Second, if there is strong decline in male paternity with male mating order, as found in other species, this would suggest males should try to be the first (or at least second) to mate in as many litters as possible. All else being equal, male-male competition should lead to the males distributing them evenly over the possible mating positions (the 'ideal free distribution hypothesis' Fretwell and Lucas, 1970). Alternatively, the mating order advantage might need to be traded-off against the number of females the male might gain access to. Therefore, all else being equal, males might follow distinct reproductive strategies with equal pay-off: i.e. first mating position in a few litters vs. later mating postition in many litters (the ‘alternative male strategy hypothesis’ Oliveira et al., 2008).

Third, because individual males are not equal, high quality males may mate when fertilisation probability is highest, whereas lower quality males may be forced to accept later mating positions in the mating sequence that yield a lower success (the ‘ideal despotic distribution hypothesis’ Fretwell, 1972). Male quality is expected to correlate with male age and male body mass (Adrian et al., 2008; Berteaux et al., 1999; Elgar et al., 2003; Hoogland, 1998; Schwagmeyer and Parker, 1987). Repeated measures of the mating position of individual males within and across the seasons allowed us to test whether males followed distinct reproductive strategies and assess how reproductive success per mating position contributes to the male’s overall seasonal reproductive output and whether it depends on male quality (age and body mass).

Methods

Study area

Five neighbouring colonies of Columbian ground squirrels (Spermophilus columbianus) were studied in the Sheep River Provincial Park, Alberta, Canada (110°W, 50°N, and 1500m a.s.l.). Observations of individual behaviours were recorded from April to mid July 2005-2008. Columbian ground squirrels are diurnal, colony-living rodents inhabiting subalpine and alpine meadows. Adult males emerge first from hibernation in mid-April, followed by females a few days to a week later (Murie and Harris, 1982). Females breed on average 4 days after emergence from hibernation and are in oestrus for about 5-7 hours during a single day (Murie, 1995; Murie and Harris, 1982). Twenty four days later females give birth to a litter averaging 3 (1-7) naked and blind juveniles in a specially constructed nest burrow (Murie et al., 1998). The offspring emerge above ground about 27 days postpartum (Murie and Harris, 1982).

Sampling of adults

Columbian ground squirrels were caught within the first two days of emergence from hibernation with live traps baited with peanut butter (National live traps, WI, USA; 15×15×48 cm and 13×13×40 cm). Thereafter, animals were trapped weekly and weighted with Pesola®

spring scales to the nearest 5 g. Individually numbered fingerling fish tags (National Band & Tag Company #1) were attached in both ears for permanent identification. In addition, each ground squirrel was uniquely marked with hair dye on their back (Clairol, Hydriance - black pearl No. 52) for identification from a distance. We captured unmated, pre-oestrus females daily to evaluate their reproductive status until they had mated. The degree of swelling and the openness of the vulva indicate the upcoming day of mating (Murie, 1995).

Observations of mating associations

Animals were observed from 3 m high wooden observation towers with binoculars. Columbian ground squirrels in our colonies usually mated underground. Mating activity began in the morning between 7:00 and 10:00 lasting until 14:00 to 17:00 in the afternoon. A mating sequence of an oestrous female contained all consortship partners. Although we are confident that the behavioural criteria allowed us to identify correctly when mating occurred, they did not allow us to determine precisely the number or duration of copulations, or the interval between successive copulations. In another population of *S. columbianus* aboveground copulations were often observed and lasted on average 35 min. (range 1- 90 min.; Murie, 1995), an indication that copulations can last this long. We assumed that underground copulations took place when the oestrus female and a male went down the same burrow system and remained there for at least five minutes. Oestrus was concluded when the focal female increased her feeding activity and avoided or chased potential mating partners away (Murie, 1995).

Sampling of offspring

Nest burrows were identified through observations (for details see Murie et al., 1998) and marked with coloured flags. Females from three colonies were brought to the lab where they were kept in polycarbonate cages (48 x 27 x 20 cm) for two days prior to parturition (for more details see Murie et al., 1998). Within 12h of parturition, neonates were weighed, sexed and marked individually by removing a small amount of skin tissue from an outer hind toe or the tail. Tissue samples were later used to determine paternity. Females and their litters were released back into the colony the following day (for details see Murie et al., 1998). In the fourth and fifth colony, tissue for paternity analysis was collected from the ear at juvenile emergence (age 27 days). Only offspring that successfully emerged from their nest burrows during weaning were included in analyses to standardize among the five colonies. Hence, presence at weaning was the decisive factor characterizing reproductive success for males and females. Offspring were caught within the first 2 days after emergence above ground for the first time, with either unbaited 13x13x40 cm National live-traps or with multi-capture traps (Murie, 1995). Juveniles were marked and weighed, and their sex was determined or confirmed if born in captivity.

Only mothers > 2 were included in the analysis because yearlings seldom reproduce (Dobson and Murie, 1987; Festa-Bianchet and King, 1984; Murie and Harris, 1982). Multiple copulations between the same male and female were rarely observed and were excluded in the used data-set. A yearling female raised two juveniles after mating with only one male and was also excluded from further analyses. Only females with known mating sequences were included in the mating order analyses, whereas all litters were tested for multiple paternities. Only complete mating sequences were used to calculate the average of male mating partners per female, however all observed copulations (complete and incomplete mating sequences) as well as the paternity information were included to obtain the average value for the different female partners a male had.

Paternity analyses

DNA was extracted from preserved tissue using DNeasy Tissue extraction kits (Qiagen, Venlo, The Netherlands) and 13 microsatellite loci were amplified using polymerase chain reaction (PCR). Primer pairs already developed for S. columbianus (GS12, GS14, GS17, GS20, GS22, GS25 and GS26; Stevens et al., 1997), Marmota marmota (BIBL18; Goossens et al., 1998; MS41 and MS53; (Hanslik and Kruckenhauser, 2000) and M. caligata (2g4, 2h6; Kyle et al., 2004 and 2h4 f-TGTAGGTGTTGATGTTGAATGA r-TCCCTGCCACAAGAAATA will be submitted to Genbank) amplified polymorphic microsatellite loci. We tested for deviations from Hardy–Weinberg equilibrium (HWE) at each locus within cohorts, and for linkage disequilibrium between pairs of loci within cohorts using exact tests.

We used CERVUS 3.0 (Kalinowski et al., 2007) for the parentage assignment. Maternity was determined by behavioural observation at the nest while paternity was assigned with 95% to 99% trio-confidence (assumed mother-father-offspring relationship). Note that maternity was certain for all the offspring born in captivity, as females were held in separate cages. Analyses were conducted for each year and colony separately. The input parameters for the simulation step of CERVUS were: 10000 cycles, 70 candidate fathers, 90% of the population sampled and 1% genotyping error. Consorting, mating males were listed as potential sires when mating behaviour was recorded, otherwise we included all males older than 1 year. Parental assignments were accepted when the offspring had no more than two mismatches with both parents.

Data analyses and statistics

For the statistical analyses we used SPSS Version 17. The following independent variables were used throughout: year (fixed categorical effect), colony (fixed categorical effect), mating partners (two to eight, fixed categorical effect) and mating position (one to eight, fixed continuous effect). The mating order effect was analysed as the number of offspring sired per male (poisson distribution with a log-link) using Generalised Estimating Equations (GEE) with individual male identifier as subjects (to account for repeated measures per male), mating partners, mating position nested within mating partners, year and colony as independent variables (the scaling parameter was estimated using the deviance method). Consistency in individual male mating position was analysed using Spearman rank correlations, both for within-season consistency (mating position litter_i vs mating position litter_{i+1}) and between-season consistency (average mating position year_t vs average mating position year_{t+1}). Effects of male age, male age squared and residual body mass (from the age-body mass relationship, see Results) on reproduction were analysed using GEE with individual male as subjects, corrected for differences due to year and colony effects and using various link functions depending on the type of response variable. We used a non-parametric Mann Whitney U-test to analyse differences in number of copulations or number of offspring between singly and multiply sired litters.

Results

Paternity assignment

In total, 330 adult males and females, and 432 offspring were successfully genotyped. Our genotyping success rate was 97.5%, with 77.0% of the ground squirrels genotyped at all 13 loci (*n* = 764). We retained all 13 loci in our analyses as there was no significant deviation from HWE or linkage disequilibrium, tested per colony. All 432 offspring were successfully assigned to both parents: 97% of the offspring had 99% trio-confidence, while the remaining 3% had 95% trio-confidence, suggesting that our sampling of adults was complete and un-sampled males were unlikely to be the true fathers. Note that 411 offspring (95.2%) had zero mismatches with both assigned parents, 20 offspring (4.6%) had one mismatch with an assigned parent, and only one offspring (0.2%) had two mismatches with an assigned parent (*n* = 432).

Mating order

Out of 145 litters (from 96 individual females), we observed the complete mating sequence, that is, the mating position and identities of all consorting males, for 110 litters (from 76 individual females, Table 1). The following data were excluded from analysis: 19 litters (53 offspring, from 19 individual females) where the mating sequence was not obtained and for 16 litters (43 offspring, from 16 individual females) where the assigned sire was not observed to mate with the female, furthermore the yearling which mated only once and had two offspring. Overall, 464 copulations were observed, with two to eight mating partners per litter (mean ± SE: 4.22 ± 0.11, Figure 1a). The first five males actually sired offspring, and this distribution was strongly skewed towards early mating males (Table 1, Figure 1b). Since we did not monitor all copulations a male had (complete and incomplete mating sequences were included), the minimum estimate of average (± SE) is 4.82 ± 0.3 females per season (range: 1-16, n = 71 individual males; n = 540 observed copulations or paternity within the litter), however this average seems to be underestimated.

Singly sired versus multiply sired litters

Overall, 85 of 126 litters (67.5%) were sired by more than one male. Multiply sired litters were sired on average by 2.37 ± 0.06 (mean ± SE) different fathers. Of the 110 litters with known mating sequences, 62 litters (56.4%) were multiple sired (Table 1), with the first and second male siring the majority of offspring (Table 1). Singly sired litters (*n* = 48) were sired by males from the mating positions one (*n* = 41), two (*n* = 4) and three (*n* = 3); or when excluding the 13 litters with only one offspring: one (*n* = 32), two (*n* = 0) and three (*n* = 3).

First male advantage

On average, the mean number of offspring sired by a male strongly declined with his mating position (Figure 2), with the first males taking the largest share (Table 2: significant effect of mating order) and the sharing more evenly distributed when more males were involved (Table 2: effect of mating partners). Therefore, the first male advantage significantly declined when more male partners were involved, since each successive male, up to five males, was likely to gain some reproduction (GEE on mating position 1 males only, corrected for year and colony effects; effect of mating partners fitted as a covariate: $\chi 2 = 12.8$, df = 1, $n = 110$ of 41 males, $p < 0.001$, coefficient ± SE: -0.23 ± 0.06).

First males had offspring in 93 of 110 litters (84.5%), second males were successful in 51 of 110 litters (46.4%), third males in 35 of 103 litters (34.0%), fourth males in 11 of 82 litters (13.4%) and fifth males in 4 of 41 (9.8%) litters (Table 1). In 17 litters the first males did not sire offspring. These 17 cases involved 13 different males, and all of these 13 males appeared fertile since all sired young in other mating events with other females in the same year.

Male reproductive strategies

We found no evidence that males followed distinct reproductive strategies. There was no correlation between the position in the mating order obtained in a litter and the position in the mating order obtained in the subsequent litter for individual males within a season (Figure 3a). Similarly, there was no correlation between the average mating position obtained in a season and the average mating position obtained in the subsequent season (Figure 3b). Of the 32 individual males mating at least five times, the observed mating positions did not differ from those expected from the overall probabilities (32 males x 8 mating positions cross-tabulation: Likelihood ratio $\chi 2 = 182.1$, df = 186, $p = 0.57$). We evaluated that on average each male can expect 0.85 copulations with a female in mating position one and two each (per season), 0.80 copulations in mating position three and a rapid significant decline thereafter

(Figure 4a, Friedman Test: $\chi2 = 161.7$, df = 7, $n = 71$ males, $p < 0.001$). Therefore, the male's seasonal reproductive output strongly depends on what he obtains from the first mating position (Figure 4b, GEE $n = 307$ litters mated by 71 males; effects of mating order: $\chi2 = 190.5$, df = 1, $p < 0.001$; year: $\chi2 = 3.4$, df = 3, $p = 0.34$; colony: $\chi2 = 7.2$, df = 4, $p = 0.13$).

Overall a male's reproductive success correlated positively with the achieved number of matings (Spearman rank correlation, $r_s = 0.70$, $p < 0.001$, $n = 71$). Male variation in seasonal reproductive success was tightly correlated to the number of litters he consorted in the first mating position (Spearman rank correlation, $r_s = 0.77$, $p < 0.001$, $n = 71$ males, data averaged per individual male before analyses). However, there was no indication that males needed to trade-off their number of matings in the first mating position with the total number of matings achieved. On the contrary: there was a significant positive correlation between the numbers in mating position one vs. the total number of matings (Spearman rank correlation, $r_s = 0.56$, $p < 0.001$, $n = 71$ males). Again, there was no evidence for male alternative reproductive strategies (GLM, logit, $n = 71$ males; effects of number of matings: $\chi2 = 0.15$, df = 1, $p = 0.698$). For instance, males mating one to ten times achieved to be 41 times (24%) on mating position one, whereas males mating 12 to 42 times achieved to be 52 times (22%) on mating position one.

Male age, residual body mass and reproductive strategies

In total, 44 males were both of known age and their body mass was determined in one to three seasons, given a total sample sizes of 71 cases. Males significantly increased in body mass in a linear fashion when growing older (Regression of body mass on age $n = 71$, effect of age: $F = 7.6$, df = 1, $p = 0.007$), so we entered the residual of male body mass vs. male age relationship in subsequent analyses. Male age was the most important and significant determinant of male seasonal reproductive output (Figure 5). Males of intermediate age mated most often (Figure 5a), sired the largest number of offspring per litter (Figure 5b) and therefore acquired the largest seasonal reproductive output (Figure 5c), compared to younger and older males (Table 3). The effects seemed to be augmented by intermediate males mating more often in the first and second mating position, but the overall effect of male age on mating order was not significant ($\chi^2 = 38.8$, df = 42, $p = 0.61$).

Male residual body mass significantly affected the probability of acquiring the first mating position (Figure 6, probit GEE on the number mated in first mating position / total matings, corrected for year and colony effects; effect of residual body mass: $\chi^2 = 6.1$, df = 1, $p = 0.013$; age and age squared were non-significant and removed from the model, $p = 0.69$ and 0.85 respectively). However, male residual body mass did not affect seasonal reproductive success (Table 3).

Discussion

Five main results emerged from our mating observations combined with paternity analyses of free-ranging Columbian ground squirrels: (1) there was no one-to-one matching between the number of males a female mated with and the paternity distribution between the males; which was due to (2) a strong first male reproductive advantage (first male precedence) with the sixth to the eight mating males siring no offspring at all. (3) However, the first male precedence effect declined with an increasing number of mating partners. (4) Males did not follow alternative mating strategies, where e.g. the mating frequency has to be traded-off with the position in the mating order (absence of mating position consistency within and between the seasons). (5) Males of intermediate age classes were the most successful in terms of seasonal copulation frequency and reproductive success. These males were acquiring relatively more often the first mating position, which in itself was affected by relative male body mass (body mass corrected for age effects).

Multiple paternity litters

Multiple mating in Columbian ground squirrels resulted mainly in multiply sired litters. Overall, many studies of paternity analyses in polygynandrous mammal species have shown the existence of multiply sired litters (e.g. Berteaux et al., 1999; Boellstorff et al., 1994; Dugdale et al., 2007; Foltz and Schwagmeyer, 1989; Hanken and Sherman, 1981; Hare, 2004; Hare et al., 2004; Haynie et al., 2003; Holleley et al., 2006; Hoogland, 1995; Kraaijeveld-Smit et al., 2002b; Lacey et al., 1997; Lane et al., 2008; Ratkiewicz and Borkowska, 2000; Say et al., 1999; Schenk and Kovacs, 1995; Sherman, 1989; Solomon et al., 2004; Stockley et al., 1993). Similar findings were found in related species with a polygynandrous mating system (Boellstorff et al., 1994; Foltz and Schwagmeyer, 1989; Hanken and Sherman, 1981; Sherman, 1989), a lower incidence of multiple paternity have been reported in black-tailed prairie dogs (*Cynomys ludovicianus*), Gunnison's prairie dogs (*Cynomys gunnisoni*) and arctic ground squirrels (Hoogland and Foltz, 1982; Lacey, 1991; Lacey et al., 1997; Travis et al., 1996). While a proportion of the litters in our study (about 33%) were singly sired, we also found the other extreme: two litters containing four offspring that were sired by four different males. (Murie, 1995) found a much lower proportion of multiply sired litters in the same species (16%), however, he speculated that the true value may have been underestimated due to the limited resolving power of allozyme variation (protein electrophoresis) and the low number of protein types used in his study. In our study, we found multiple paternity occurring in 67% of litters, which is a high value, for a species with relatively small litters (mean litter size is three: Dobson and Murie, 1987).

Mating order effect and comparison with other related species

Insemination by several males implies that sperm competition (Parker, 1970) may play an important role in male Columbian ground squirrels reproductive success. Our results showed a strong first male mating advantage, with relative reproductive success declining exponentially with later mating positions. Nevertheless, the relative success of the first males declined significantly with an increasing number of mating partners, since up to five males were likely to sire at least some offspring. The unsuccessful first mating males (n = 17) might be due to males transferring sperm before the female was actually receptive and their sperm might have lost their viability when she finally was receptive. Whether first male precedence is affected by the male, the female, or both, remains to be tested in the future.

Our results compare well to other species of Marmotine rodents. In three related species first male reproductive bias has been confirmed: Belding's ground squirrel *S. beldingi* (Hanken and Sherman, 1981; Sherman, 1989), thirteen-lined ground squirrel *S. tridecemlineatus* (Schwagmeyer and Foltz, 1990) and Arctic ground squirrel *S. parryii plesius* (Lacey et al., 1997). Compared to other ground squirrel species having a first male bias, female Columbian ground squirrels mated on average with a large number of different males per litter produced (see Lacey et al., 1997) for the data on four species: range 1 to 5 males). Conversely, males mating in the second and later mating positions had a relatively large likelihood of siring offspring in our study species, compared to the other species (see Lacey et al., 1997): first male advantage range 60 to 93%, compared to 57.5% in our study species). For example, males mating in fourth and later positions have virtually no reproductive success in three ground squirrel species, despite larger litter sizes (Foltz and Schwagmeyer, 1989;

Hanken and Sherman, 1981; Lacey et al., 1997). However, without an experimental approach we are unable to determine whether an increased number of mating partners lead to a lower proportion of first male advantage or an increased chance of siring offspring.

Male age and reproductive success

Columbian ground squirrels are relatively long-lived, with males reaching 9 years and females up to 12 years, compared to other rodents of similar body mass (Neuhaus and Pelletier, 2001; Wolff and Sherman, 2007). Males of intermediate age had the highest reproductive success in our study. This was not due to these males mating more often in first position (which was independent of age, but depended on body mass), but due to these males mating more often all together, which also correlate with the likelihood of mating at least once in the first position. Nine-year-old males did copulate, but never sired any offspring. One potential explanation for this result might be senescence. Spermatogenesis is known to decrease with age in several mammalian species (male mutation load, see Ellegren, 2007), and this might explain some of the unsuccessfulness of sperm from old males to acquire fertilizations. Alternatively, since female dispersal is limited (Dobson, 1982; Murie and Michener, 1984; Neuhaus, 2006), older males might progressively encounter more and more daughters from previous years as potential mating partners, and due to inbreeding avoidance or depression, progressively experience lower reproductive success.

In our study the likelihood of acquiring a certain mating position was randomly distributed within and between seasons, which meets the criteria of an ideal free distribution. At the same time, mating with more females correlated with the likelihood of male mating at least once in first position. Suggesting that mating in respect to mating position is achieved randomly (and more mating will consequently increase the likelihood of mating more often in the first mating position). Alternative reproductive tactics of males have been found in several rodents (Boellstorff et al., 1994; Koprowski, 1993; Lacey and Wieczorek, 2001; Manno and Dobson, 2008; Schwagmeyer, 1985; Schwagmeyer and Woontner, 1985; Travis et al., 1996). Male Columbian ground squirrels often exhibit post-copulatory mate guarding which may increase chances of siring success (Manno et al., 2007). This indicates that sperm precedence in *S. columbianus* may underlie behavioural mechanisms causing this effect, which will be examined in more detail.

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Table 1. Number of different sires per litter for the various numbers of mating partners and the percentage of offspring sired per mating position. Note that the minimum number of mating partners was two, the maximum number of sires was four and males in the sixth to eight mating position did not sire any offspring. Only litters with known mating positions were included.

Number of mating partners	Number of males siring offspring					Percentage offspring sired per mating position					
	n litters	One	Two	Three	Four	*n* offspring	First	Second	Third	Fourth	Fifth
Two	7	5	2	-	-	18	88.9	11.1	-	-	-
Three	21	11	9	1	-	63	66.7	22.2	11.1	-	-
Four	41	19	16	6	0	119	52.1	27.7	14.3	5.9	-
Five	28	9	9	10	0	87	50.6	23.0	17.2	4.6	4.6
Six	9	4	3	0	2	30	66.7	10.0	16.7	6.7	0.0
Seven	3	0	2	1	0	13	38.5	30.8	23.1	0.0	7.7
Eight	1	0	1	0	0	4	75.0	25.0	0.0	0.0	0.0
Total	110	48	42	18	2	334	57.5	23.1	14.1	3.9	1.5

Table 2. Effects of the male mating position and the number of mating partners on the male's reproductive success per litter (*n* = 464 events of 71 males, 1 to 42 events per male). Given are results from a GEE with male identifier as subject to account for repeated measures per male (*n* = 71 males) and fixed effects of mating position (covariate, 1 to 8, nested within mating partners), mating partners (2 to 8), year (2005 to 2008) and colony (A to E).

	Number of offspring sired		
Parameter	df	Wald $\chi 2$	*p*
Constant	1	89.0	< 0.001
Mating order within mating partners	7	150.8	<0.001
Mating partners	6	24.1	0.001
Year	3	32.8	<0.001
Colony	4	12.9	0.012

Number of offspring sired per litter fitted as a poisson distribution, with litter size as the offset, the scaling parameter was adjusted using the deviance method.

Table 3. Effects of male age on male reproductive success per season: the frequency of mating, the average number of offspring sired per litter and the total reproductive success (n = 71 cases of 44 males, 1 to 3 events per male). Given are results from three separate GEEs with male identifier as subject to account for repeated measures per male and the independent variables male age (2 to 9) and male age squared (covariates) corrected for year (2005 to 2008) and colony effects (A to E).

		Frequency of mating		Sired offspring per litter		Total sired offspring	
Parameter	df	Wald $\chi2$	p	Wald $\chi2$	p	Wald $\chi2$	p
Constant	1	3.4	0.07	11.7	0.001	14.7	<0.001
Male age	1	61.9	<0.001[a]	10.4	0.001[c]	31.2	<0.001[e]
Male age^2	1	65.2	<0.001[b]	11.5	0.001[d]	31.6	<0.001[f]
Year	3	10.1	0.018	1.7	0.63	4.5	0.22
Colony	4	215.9	<0.001	7.4	0.12	22.3	<0.001

Frequency of mating and total sired offspring fitted as a poisson distribution with a log-link, average sired offspring per litter fitted as a normal distribution (Kolmogorov-Smirnov Test, p = 0.29) with a log-link, the scaling parameter was adjusted using the deviance method in each model. Residual male body mass was non-significant in each of the three models and removed (p = 0.71, 0.83 and 0.91, respectively).

Coefficients ± SE of the male age effects: [a]0.744 ± 0.095, [b]-0.069 ± 0.009, [c]1.107 ± 0.343, [d]-0.117 ± 0.035, [e]1.585 ± 0.284, [f]-0.154 ± 0.028.

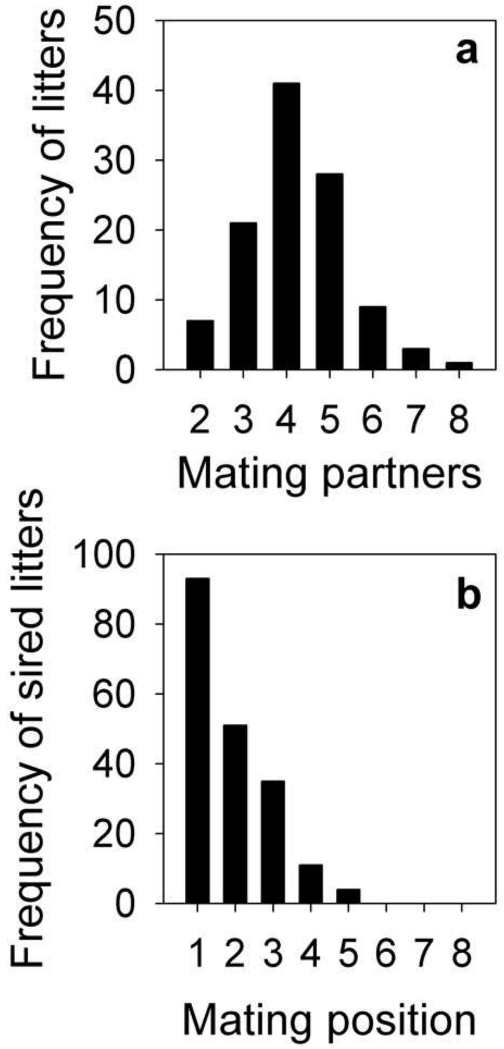

Fig. 1. (a) The number of mating partners per litter (*n* = 110) and (b) the frequency of sired litters (at least one offspring sired) per male mating position (1 = first to mate, 8 = last to mate, *n* = 194).

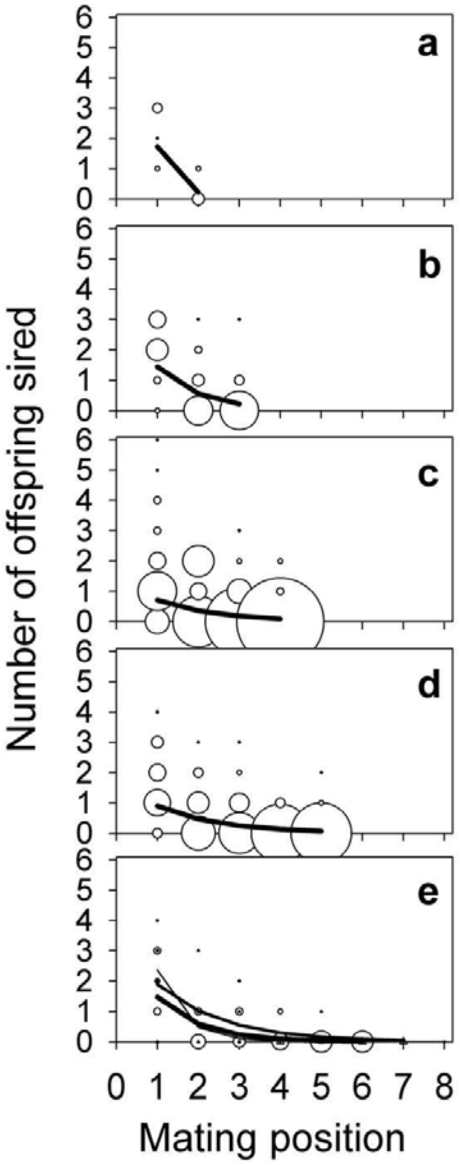

Fig. 2. The number of offspring sired per litter for each mating position for varying numbers of mating partners (number of litters sampled in brackets): (a) two (n = 7); (b) three (n = 21); (c) four (n = 41); (d) five (n = 28); (e) six (circles, n = 9), seven (triangles up, n = 3) and eight (triangles down, n = 1). Overlapping samples are indicated with different symbol sizes (1 to 36 overlapping data points). Also depicted are the fitted values from the GEE analysis given in Table 2 (corrected for a weighted year and a weighted colony effects).

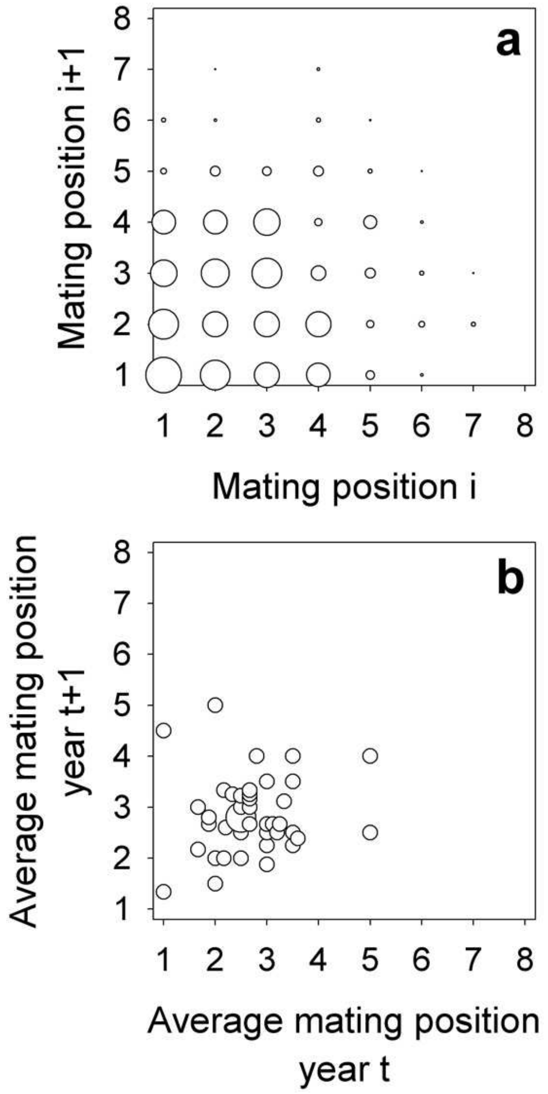

Fig. 3. The mating position of individual males was not repeatable. (a) Within-season correlation of male mating position (litter$_i$ vs next litter$_{i+1}$, Spearman rank correlation $r_s = 0.048$, $p = 0.37$, $n = 352$). Sample sizes are indicated with different symbol sizes (1 to 24 overlapping data points). (b) Between-season correlation of male mating position (average of year$_t$ vs average of next year$_{t+1}$, $r_s = 0.074$, $p = 0.64$, $n = 41$ males). Sample sizes are indicated with different symbol sizes (1 or 2 overlapping data points).

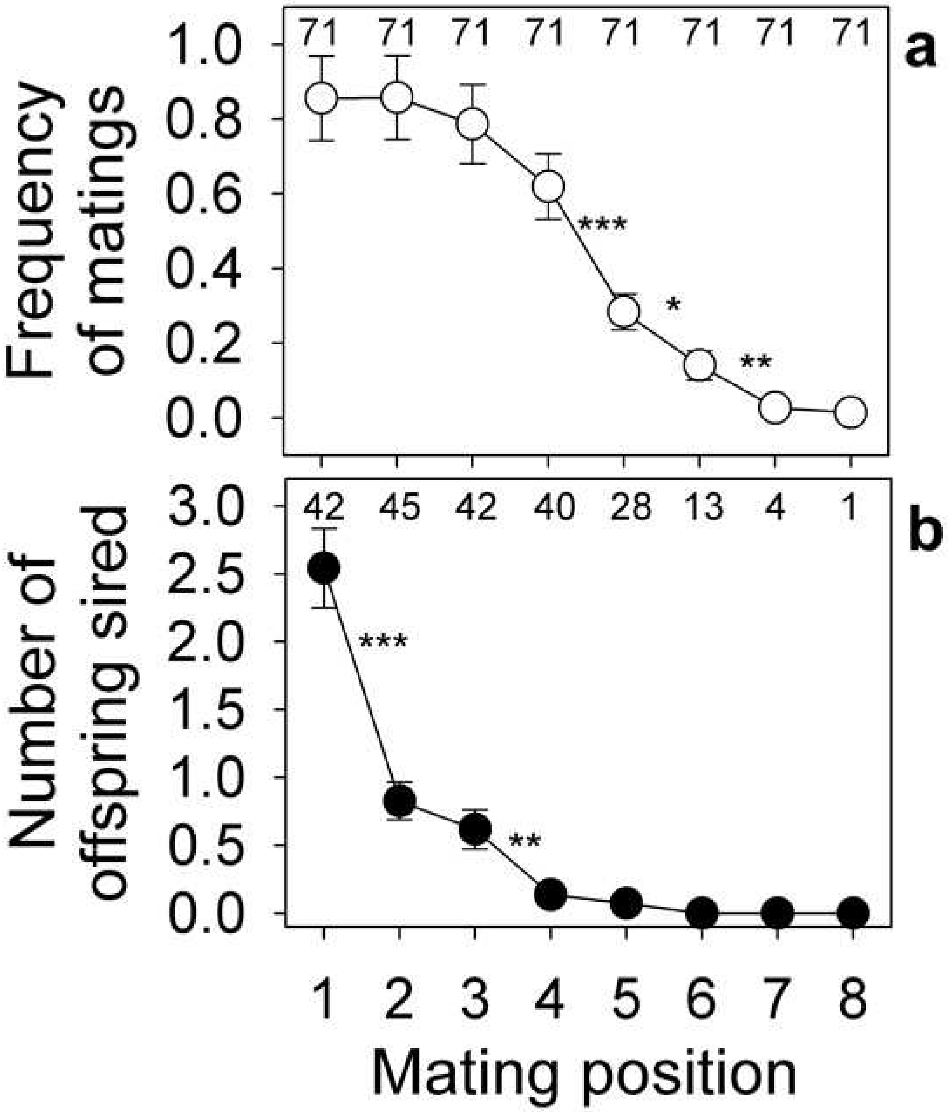

Fig. 4. Average seasonal reproductive behaviour of individual males (means ± s.e.m.). (a) Average number of litters males were in mating position one to eight (*n* = 71 individuals). (b) Average total number of offspring sired per mating position (*n* = 1 to 45 individuals per observed mating position, summed over the season). Wilcoxon's signed ranks tests: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, in (b) only for individual males observed in both mating position$_i$ and mating position$_{i+1}$ (*n* = 31, 29, 29, 24, 5, 0 and 0, respectively).

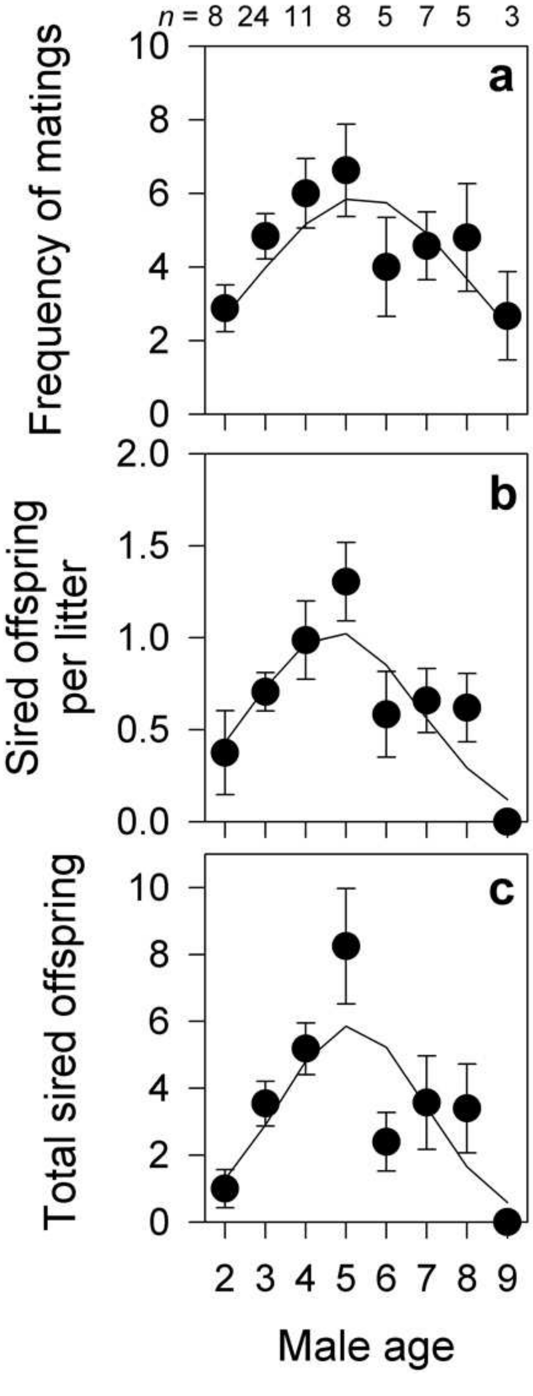

Fig. 5. Male age affected seasonal reproductive success (*n* = 71 cases of 44 males, sample sizes indicated on top of the graph): (a) the frequency of matings, (b) the average number of offspring sired per litter, (c) the total number of offspring sired. Quadratic curve fits are from the models depicted in Table 3.

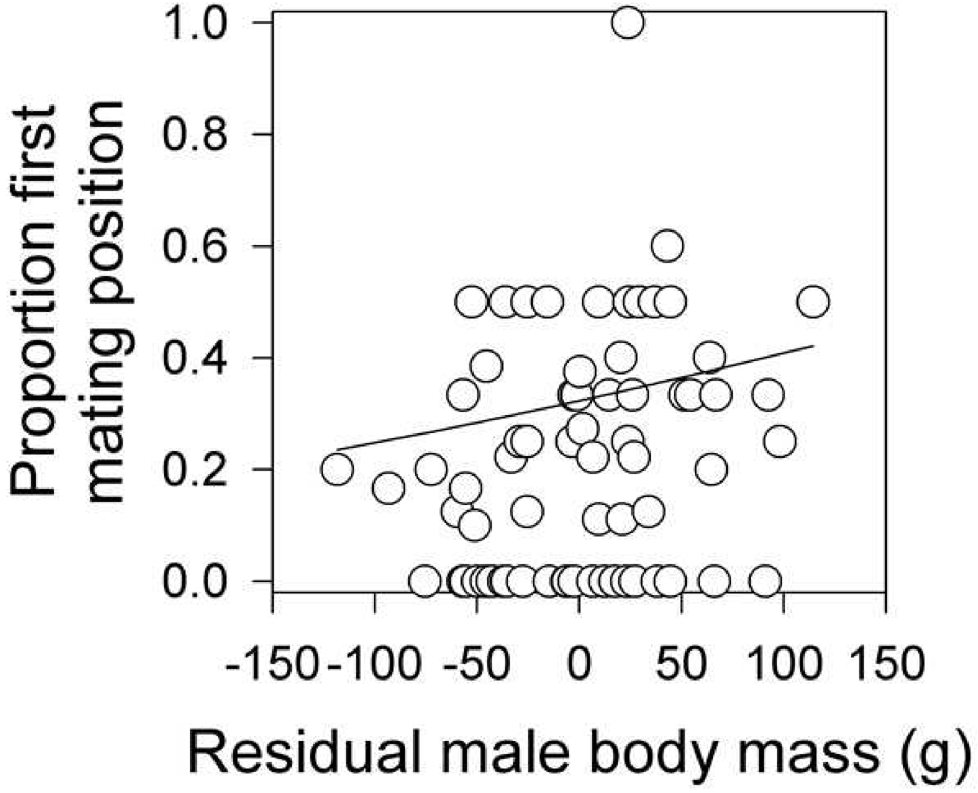

Fig. 6. The proportion of litters individual males acquired the first mating position depending on male residual body mass (mass corrected for age effect, *n* = 71 of 44 males). Also depicted is the GEE regression line described in the text (coefficient ± SE: 0.0037 ± 0.0015, *p* = 0.013).

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Multiple mating and male reproductive tactics to increase paternity in the Columbian ground squirrel (*Spermophilus columbianus*)

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Abstract

In species were females are likely to mate with multiple males for each litter, males are expected to show reproductive behaviours to maximise their paternity. Columbian ground squirrels (*Spermophilus columbianus*) show a first male paternity advantage ('sperm precedence'), but there is no specific position in which individual males mate with receptive females. This suggests males might need to strategically alter their investment in pre- and post-copulatory behaviour, depending on their position in the mating order, to maximise their fitness. We address the underlying mechanisms of sperm precedence mediated by male behaviour in this species. We found that the durations of copulation and mate guarding declined with increasing mating position. Number of male partners declined with an increase in mate guarding behaviour by the first male and the operational sex ratio (oestrus females/males). Mate guarding by the first male significantly reduced, but not excluded, the number of additional males the female accepted. Instead, both copulation and mate guarding durations appeared to shift the later mating males away from the optimal fertilisation window, inducing males mating later to reduce their copulation durations. Copulation and mate guarding durations were positively correlated. Both durations positively correlated with male reproductive success, but only for the first and the second male to mate. This suggests first male sperm precedence is both achieved through longer mating and a devaluation of the effect of copulation duration on siring success for later males. Finally, male investment in reproductive behaviours increased with male and female age, but was also increased when partners mated age-assortatively. Key word: *Spermophilus columbianus*, mating order, mate guarding, copulation duration, sperm precedence

Introduction

Optimising individual fitness has driven mating systems and mating strategies to evolve in regards of the cost and benefits suffered or acquired by each of the sexes (Jennions and Petrie, 2000; Shuster and Wade, 2003; Wedell et al., 2006; Zeh and Zeh, 1996). In polyandrous and polygynandrous systems, where females mate multiply during a single period of sexual receptivity, sperm competition and cryptic female mate choice may influence a male's reproductive success, leading to differential fertilization within a litter and ultimately variation in male reproductive success (Birkhead and Pizzari, 2002; Kraaijeveld-Smit et al., 2002; Moller and Birkhead, 1989; Parker, 1982; Parker, 1984). Sperm competition is the competition for fertilisation success amongst sperm delivered by two or more males within the female reproductive organs. The outcome of this competition may be affected by the female preferring sperm from certain males for fertilization (cryptic female choice, e.g. for genetically compatible male Eberhard, 1996). One adaptation to sperm competition is selection on males for increased sperm numbers (Parker, 1982) and sperm quality (e.g. sperm viability and swimming ability). When sperm of many males are in the female tract there will be a selective advantage to the male, which provides the higher number of sperm, because he will gain proportionally more fertilization. Consequently, elevated levels of sperm competition in mammals result in greater sperm counts per ejaculate (Gomendio et al., 1998). Assuming that sperm transfer rate is constrained (i.e. has a maximum achievable level), males need to copulate longer with the female to deliver more sperm in insects (Arnqvist and Nilsson, 2000; Bukowski and Christenson, 1997; Simmons, 2001; Snow and Andrade, 2004), and this needs to be traded-off with other investments, e.g. territory defence, searching for additional unmated females, and mate guarding of already mated females (Parker, 1974).

Sperm competition often results in certain males achieving differential success in siring offspring. That is, all else being equal, sperm from certain males are more likely to achieve fertilization compared to the sperm from other males ('sperm value', the so called 'loaded raffle': Parker, 1990). If sperm from different males are stored or processed by the females sequentially, the first sperm to come in may be the first to reach the eggs, leading to sperm value decreasing with the mating order ('first male sperm precedence' Birkhead and Møller, 1998). Alternatively, if sperm is stored in dead-end spermatheca, the last sperm to come in, may be the first to be released by the female, leading to sperm value increasing with the mating order (‘last male sperm precedence’ Birkhead and Møller, 1998; Simmons, 2001). Finally, if sperm is continuously processed, sperm delivered closest to the optimal fertilisation time point may have the highest sperm value, which may lead to any pattern of sperm precedence with the mating order.

Facing sperm competition, males are expected to develop mating and post-copulatory strategies in order to ensure their paternity and maximise their fitness. Males may compete for the best mating position, and be able to gauge their position and adapt the ejaculate size to the intensity of sperm competition (Birkhead and Møller, 1998) in insects (Simmons and Siva-Jothy, 1998) or in fish: (Petersen and Warner, 1998). Males may try to swamp or remove rival sperm; or in the extreme block rival sperm by copulatory plugs (Birkhead and Møller, 1998; Murie and McLean, 1980). Males may increase their investment in copulation (both frequency and duration) and mate guarding to decrease the likelihood of females mating with rival males (Birkhead and Møller, 1998; Linn et al., 2007; Parker, 1970; Pizzari and Snook, 2003; Simmons, 2001).

The genus Spermophilus has been well studied concerning first male sperm precedence effects and multiple paternity. These species are excellent for studies on mating behaviour because the females exhibit a very brief receptive phase of up to twelve hours in which they typically copulate with several males (Hanken and Sherman, 1981; Lacey et al., 1997; Murie, 1995; Schwagmeyer and Parker, 1987). Although mating often occurs in underground burrows, copulations are readily detected using established behavioural criteria (e.g. Hanken and Sherman, 1981; Lacey et al., 1997; Murie, 1995; Schwagmeyer and Parker, 1990). We therefore use the term “copulation” to refer to behavioural evidence that mating occurred (Lacey et al., 1997) and to the time that a male and female spent together in a burrow. Several authors have reported a male sperm precedence effect in this genus: thirteen-lined ground squirrels, *S. tridecemlineatus*;(Foltz and Schwagmeyer, 1989; Schwagmeyer and Foltz, 1990), Belding’s ground squirrels, *S. beldingi*; (Hanken and Sherman, 1981; Sherman, 1989), Arctic ground squirrels, *S. parryii plesiu*s; (Lacey et al., 1997), Idaho ground squirrels, *S. brunneus*; (Sherman, 1989) and Columbian ground squirrels (*S. columbianus*; Raveh et al., in chapter 1).

A previous study in Columbian ground squirrels revealed that first males were more likely to elicit mating calls after copulation, and this behaviour is often part of mate guarding behaviour (Manno et al., 2007). Female Columbian ground squirrels which reproduced for the first time are less successful of gestating or weaning offspring than are older, more experienced females, because of their lower body mass and/or a lack of experience (Broussard et al., 2008). Additionally, experienced females had significantly higher reproductive investment and reproductive success (number of offspring surviving to yearling age) compared to inexperienced females (Broussard et al., 2008). Body condition has proven successful at predicting reproductive success in Columbian ground squirrels (Dobson et al., 1999; Risch et al., 1995). Better body condition of these females was associated with increased reproductive success. Thus one can expect males to invest more into copulations with older, heavier and more experienced females. Moreover, older males are likely to be dominant over younger males (Manno and Dobson, 2008), so one can expect older males to invest more into copulations and mate guarding than younger males.

In this study, we investigated copulatory and post-copulatory behaviours in free-living male Columbian ground squirrels (*Spermophilus columbianus*) to identify the underlying mechanisms explaining first male sperm precedence and variation in male reproductive success. First, we tested whether first male sperm precedence correlated with copulation and mate guarding duration decreasing with the mating order, and/or whether this might be linked to copulation duration having a positive, but declining, effect on male siring success through the female's mating sequence. Second, we analysed whether first male sperm precedence might be due to first males copulating, on average, closer to the presumed female's fertile period.

Accordingly, if first male sperm precedence is due solely to differences in copulation durations amongst males in *S. columbianus*, we expected that (i) copulation duration should decrease through the mating order and copulation duration should explain the siring success irrespective of the mating order ('sperm numbers hypothesis'). If first male sperm precedence is due to males guarding their females, which might shift subsequent males away from the optimal fertilisation window or increase the likelihood of their sperm reaching the ova first (i.e. sperm stored in layers or directly swimming towards the ova), (ii) mate guarding duration should have an effect on siring success independent from the copulation duration. Mate guarding should also reduce the number of male mating partners, particularly if the operational sex ratio (oestrus females / males) is low. Using similar reasoning, if first male sperm is more valuable due to combined timing and precedence effects, (iii) the effect of copulation duration on siring success should decline through the mating order ('sperm precedence hypothesis'). In a previous study, we detected 17 litters (13 males), where the first male did not sire offspring, despite these males sired offspring in other litters, showing they were fertile. To test whether this might have been due to first males sometimes mistiming their copulations in respect to the female's ovulations, (iv) we compared the timing effect on siring success of the different males in litters where first males sired either few or many offspring. If timing in general plays a role, the relationship between time in the mating sequence and male siring success should show a single optimum (which coincides with the female's fertile period), and this optimum should shift from the right to the left on this time axis going from litters not sired by the first male to litters sired almost exclusively by the first male. Multiple optima would clearly refute the timing hypothesis. (v) Finally, we address whether male and/or female quality (age and body mass) determines the male's investment in copulation and mate guarding.

Methods

Study populations

Five neighbouring colonies (meadows A, B, C, D, E) of Columbian ground squirrels (Spermophilus columbianus) were studied in Sheep River Provincial Park, Alberta, Canada (110°W, 50°N, and 1500m a.s.l.) from 2005 to 2008. Columbian ground squirrels are colonial, hibernating rodents that inhabit subalpine and alpine meadows of the northern regions of the Rocky Mountains. The active season is short and adult males are typically the first to emerge from hibernation around mid-April, followed by females a few days to a week later (Murie and Harris, 1982). Females have their oestrous approximately 4 days after emergence. Gestation lasts for 24 days when females give birth to a litter averaging 3 (1-7) naked and blind juveniles in a specially constructed nest burrow (Dobson and Murie, 1987; Murie, 1995). The weaned offspring emerge above ground about 27 days later (Murie and Harris, 1982).

Capture of individuals

Columbian ground squirrels were caught within the first two days of emergence from hibernation with live traps baited with peanut butter (National live traps, WI, USA; 15×15×48 cm3 and 13×13×40 cm3). Animals were individually marked using numbered fingerling fish tags (National Band & Tag Co. Monel#1) attached to both ears and an ear clipping was collected for the paternity analyses. In addition, each ground squirrel was given a unique dorsal mark using black hair dye (Clairol®, Hydriance -black pearl No. 52) for identification from a distance during field observations. Thereafter, animals were trapped weekly and weighed with Pesola® spring scales to the nearest 5 g. Unmated pre-oestrus females were captured daily in order to evaluate their reproductive status, until they had mated, the degree of swelling and the openness of the vulva being indicators of the upcoming mating day (Murie, 1995).

Nest burrows were identified through observations (for details see Murie et al., 1998) and marked with coloured flags. Females from three colonies were caught two days before expected parturition and kept indoors in polycarbonate cages (48 x 27 x 20 cm) enclosed in black covers (for details see Murie et al., 1998). Animals were fed twice daily with fresh lettuce and apple, while water and grains (a molasses-enriched colt feed) were given ad libitum. Within 12 hours after birth, neonates were weighed, sexed and marked individually by removing a small amount of skin tissue from an outer hind toe or the tail. Tissue samples were stored in 99% EtOH and later used to determine paternity. Females and their litters were returned to the colony the next day (for details see Murie et al., 1998). In the fourth and fifth colony, tissue for paternity analysis was collected from the ear at juvenile emergence (age 27 days). Only offspring that successfully emerged from their nest burrows during weaning were included in analyses to standardize among the five colonies. Hence, presence at weaning was the decisive factor characterizing reproductive success. Offspring were caught within the first 2 days after emergence, with either unbaited 13x13x40 cm3 National live-traps or with multi-capture traps (Murie et al., 1998). Juveniles were marked and weighed, and their sex was determined or confirmed in the field if born in captivity. Only females > 2 were included in the analysis as yearlings seldom reproduce (Dobson and Murie, 1987; Festa-Bianchet and King, 1984; Murie, 1995). A yearling female raised two juveniles after mating with only one male and was also excluded from further analyses. Only females with known mating sequences were included in the mating order analyses.

Behavioural observations

Focal oestrus female mating observations were conducted from April to mid July 2005, 2006, 2007 and 2008. Mating squirrels were observed from a distance with binoculars, from the top of 2-3 m high wooden stands dispersed throughout the meadows. In the studied colonies, Columbian ground squirrels usually mated underground. Mating activity began in the morning between 7:00 and 10:00h lasting until 14:00 to 17:00h in the afternoon. A mating sequence of an oestrous female (focal female) contains all copulation partners, additionally the duration of copulation as well as mate guarding and duration of the post-copulation interval (all in minutes) were recorded for each male. Although we are confident that the behavioural criteria allowed us to identify correctly when mating occurred, they did not allow us to determine precisely the number or duration of copulations, or the interval between successive copulations. In another population of *S. columbianus* aboveground copulations were often observed and lasted on average 35 min. (range 1- 90 min.; Murie, 1995), an indication that copulations can last this long. We assumed that underground copulations took place when the oestrus female and a male went down the same burrow system and remained there for at least five minutes.

We had missing values (n= 83) for the copulation duration particularly for the first male to mate with the female, e.g. if they had entered the burrow before our observations started at 7:00h, but we observed them to emerge together. Mate guarding duration was defined as the time spent by the male after copulation in chasing the female into a burrow, sitting on that burrow, and/or fighting with other males to prevent the female from mating with subsequent males (Manno et al., 2007). Note that the majority of males did not guard their females at all after mating (see Results). The post-copulating interval (abbreviated 'interval') was defined as the time interval after guarding (or copulation, if the male did not guard) to the next copulation. Note that individual males were never seen to copulate with the female more than once per litter in our data set (used for these analyses), otherwise theses litters were excluded and that the interval is by default missing for the last male to mate. Oestrus was concluded when the focal female increased her feeding activity and avoided or chased potential mating partners away (Murie, 1995).

We also recorded the daily operational sex ratio (OSR; Emlen and Oring, 1977), i.e. the number of oestrous females per sexually active male. The end of the oestrus period was defined as the time when the focal female increased her feeding activity up to 30 minutes and avoided or chased away potential additional mating partners (Murie, 1995).

Genetic analysis

Details of the molecular methods for microsatellite loci isolation and paternity assignment are provided elsewhere (Raveh et al., chapter one). Briefly, DNA was extracted from preserved tissue using DNeasy Tissue extraction kits (Qiagen, Venlo, The Netherlands), and polymerase chain reaction (PCR) amplification was performed for a panel of 13 microsatellite loci. Note that maternity was certain for all the offspring born in captivity, as females were held in separate cages. Paternity was assigned at 95% to 99% confidence using CERVUS 3.0 (Marshall et al., 1998), where paternity assignment was conducted for each colony and year (2005 - 2008) separately.

Statistics

Two different data sets were used for the analyses. The complete data set contained 110 litters from 76 different females all genotyped, but with missing values for copulation duration and the interval. From this data set we selected a data set of 46 litters from 44 females, which contained only 11 missing values for the male behaviours. These missing values were replaced with the average value for the specific mating position from the complete data set: for copulation duration $n = 5$ (2 times position two and 3 times position three), and for the interval between two matings $n = 6$ (2 times position one and 4 times position two; mate guarding had no missing values). The selected data set was used to analyse the association between sperm precedence and the underlying mechanisms in more detail.

All analyses were conducted in SPSS 17. We used Generalised Estimating Equations (GEE) in the majority of analyses, with individual male identifier as subjects (which accounts for repeated measures per male), various link-functions depending on the parameter analysed and the scaling parameter estimated using the deviance method (Norusis, 2007). The following independent variables were used throughout: year (2005 to 2008, fixed categorical effect), colony (A to E, fixed categorical effect), mating partners (two to eight, fixed categorical effect) and mating order (one to eight position, fixed continuous effect). Note that year and colony effects were highly non-significant in the majority of analyses (p between 0.5-0.99), and accordingly dropped from the final models for clarity. Male behaviour (copulation and mate guarding duration, both poisson distributed) were analysed using GEE, with a log-link. The siring success (offspring sired/litter size) was analysed using GEE with mating partners, mating order nested within mating partners as fixed effects. Effects of male and female age and residual body mass (from the body masses – ages relationship, see Results) on male behaviour were analysed using GEE with individual males and females as subjects.

Results

Paternity assignment

In total, 330 adult males and females, and 432 offspring were successfully genotyped. Our genotyping success rate was 97.5%, with 77.0% of the ground squirrels genotyped at all 13 loci (n = 764). We retained all 13 loci in our analyses, as there was no significant deviation from HWE or linkage disequilibrium, tested per colony. All 432 offspring were successfully assigned to both parents: 97% of the offspring had 99% trio-confidence, while the remaining 3% had 95% trio-confidence, suggesting that our sampling of adults was complete and un-sampled males were unlikely to be the true fathers. Note that 411 offspring (95.2%) had zero mismatches with both assigned parents, 20 offspring (4.6%) had one mismatch with an assigned parent, and only one offspring (0.2%) had two mismatches with an assigned parent (n = 432).

Reproductive behaviour

The complete data-set was used for the following analyses. On average, males copulated for 57.6 ± 32.3 minutes (± SD, range = 8-261, n = 381), guarded their females for 7.3 ± 22.2 minutes (range = 0-223, n = 464), and the subsequent interval to a female's next mating was 23.9 ± 28.9 minutes (range = 0-162, n = 298, measured from the end of copulation or guarding to the next mating). Male reproductive behaviour significantly varied both with his position in the mating sequence and the number of mating partners (Figure 1, Table 1). Copulation duration and mate guarding duration decreased significantly with male mating position and the number of mating partners (Figure 1, Table 1). The interval to the next mating increased with the mating order and decreased with the number of mating partners (Figure 1, Table 1).

Male copulating and mate guarding duration were positively correlated (Spearman rank correlation $r_s = 0.24$, $n = 381$, $p < 0.001$). No correlations existed between these two behaviours and the interval ($r_s = -0.06$, $n = 354$, $p = 0.35$ and $r_s = 0.01$, $n = 298$, $p = 0.81$, respectively).

Mate guarding, operational sex ratio and the number of mating partners

The complete data-set was used for the following analyses. The number of males a female copulated with was mainly influenced by two factors: first male mate guarding behaviour and the operational sex ratio (Figure 2, poisson GEE with individual females as subjects, $\chi^2 = 7.1$, df = 1, $p = 0.008$ and $\chi^2 = 5.1$, df = 1, $p = 0.024$, respectively). Females were less likely to mate with many males when the first male guarded her for a prolonged period (Figure 2a, coefficient ± SE: -0.0023 ± 0.0009) and when the operational sex ratio was low (Figure 2b, i.e. more males per oestrus female, coefficient ± SE: -0.41 ± 0.18). However, females always mated at least with two males, so the question arises whether the second male's guarding also affected the number of additional males the female attracted (third and later males). The effect of the second males guarding on the number of additional males was negative, but not significant (similar poisson GEE as above, $p = 0.077$, whereas the operational sex ratio remained significantly negative, $p = 0.03$).

Copulation behaviour and siring success

The minimum copulation duration that resulted in any offspring sired was 15 minutes. The selected data-set was used for the following analyses. Male copulation duration and mating order both significantly affected siring success of males when tested separately (positive and negative, respectively, two GEEs, both $p < 0.001$). The crucial test of the mechanism(s) behind first male sperm precedence, however, is whether they still affected male siring success when tested in conjunction. This appeared to be the case: siring success significantly declined with position in the mating order, and copulation duration only affected siring success positively for the first two males to mate (due to the significant interaction, Figure 3, Table 2).

Timing within the mating sequence and siring success

The selected data-set was used for the following analyses. Males reacted strongly with their relative copulation duration to the timing within the mating sequence and this was independent from their actual mating position (as can be seen from the mixture of mating positions in Figure 4, see Table 3). Therefore, the cumulative effects of previous male's behaviours on a focal male's siring success can be analysed as a time-shift in the mating sequence (and thus their average mating position), which causes these focal males to copulate for a shorter time gaining less parentage. Indeed, if siring success was analysed in dependency of the relative copulation duration and the time in the mating sequence, only time was significant (GEE, $\chi^2 = 8.1$, df = 1, $p = 0.004$), whereas relative copulation duration was no longer significant ($\chi^2 = 0.5$, df = 1, $p = 0.46$).

The maximum time interval between two males successfully siring offspring was 345 minutes (10 out of 46 litters had values between 181 and 345 minutes). In litters were the first males sired the majority of offspring (60-100%), only second and third males sired any additional offspring (up to 190 min in the sequence, Figure 5a: GEE $n = 68$ of 39 males, effect of time and time squared: $\chi^2 = 19.5$ and 8.1, df = 1 and 1, $p < 0.001$ and 0.004). In litters were the first males sired medium numbers of offspring (20-60%), also later males had reasonable siring success (up to 320 min in the sequence, Figure 5b: GEE $n = 87$ of 44 males, effect of time and time squared: $\chi^2 = 5.4$ and 0.1, df = 1 and 1, $p = 0.02$ and 0.75). Finally, in the litters were the first males did not sire any offspring (although these same males sired offspring in other litters), the maximum fertilization success was achieved by both second and third males to mate, with an optimum around 200 minutes after the first male mated (up to 400 min in the sequence, Figure 5c: GEE $n = 30$ of 26 males, effect of time and time squared: $\chi^2 = 6.9$ and 5.6, df = 1 and 1, $p = 0.009$ and 0.02).

Reproductive behaviour, male age and female age

The complete data-set was used for the following analyses. Both males and females showed an exponentially declining increase in body mass with age ($R^2 = 0.11$ and 0.25, respectively), and therefore we used the residuals from these mass-age relationships in subsequent analyses and corrected for mating order effects throughout.

We related male copulation (Figure 6a) and mate guarding duration (Figure 6b) to male and female age and residual body mass (Table 4). There was a negative effect of male residual body mass on his copulation duration (but only on the verge of significance: $p = 0.051$, Table 4). Additionally, males invested significantly more in both behaviours when growing older and when mating with older females and investment also increased age-assortatively (i.e. males copulated and guarded longer when mating with females of similar age). These complicated patterns were due to the significant interaction between male and female age on both behaviours, so the main effects of male and female age (and their coefficients, see Table 4) could only be interpreted in conjunction with the interaction effects (and the coefficients of the interaction, see Figure 6).

Discussion

In chapter one we have shown that Columbian ground squirrels have a strong first male precedence in siring success, an effect that diminished with increasing number of mating partners per litter (Raveh et al. chapter 1). Additionally, males do not consistently mate in certain positions across litters within the season and between the seasons. We concluded that male alternative reproductive tactics correlating with intrinsic differences in sperm investment could not account for the sperm precedence effect (due to e.g. tactic specific differences in testis size, sperm transfer rates and sperm quality). However, we did not address whether males strategically adjusted their mating behaviour according to their mating position to achieve the highest siring success. In this paper we investigated alternative causes of first male precedence.

Male guarding behaviour, operational sex ratio and mating partners

It is in the individual male's interest to decrease the likelihood of his mating partner copulating with additional males, so his sperm will not need to compete with rival sperm for fertilizations. Copulatory plugs are found in this species (Murie and McLean, 1980), which is another male strategy to ensure paternity by assisting own sperm transfer and blocking or delaying (if the plug is lost) a successful mating by another male (Gomendio et al., 1998). However, in general these plugs appear not effective in preventing further matings in mammals (see e.g. Baumgardner et al., 1982; see e.g. Dewsbury, 1984; Gomendio et al., 1998; Koprowski, 1992; Wolff and Sherman, 2007), however might reduce the outflow of sperm. This might also explain why in our study population the majority of litters are sired multiply (Raveh et al. chapter 1). Mate guarding and the copulatory plug, especially by the first male to mate, may achieve this goal to out-compete subsequent males. In our study females mated with a minimum of two males (only one yearling female had a single mating event), thus mate guarding did not prevent second males to mate with the female squirrels.

Nevertheless, mate guarding by the first male seemed to reduce the number of additional mates the female copulated with.

Mate guarding may carry additional advantages. First, it may force additional males to mate at a suboptimal time, e.g. outside the optimal fertilization window of the female (if ovulation is non-induced). Second, if the male injects a copulatory plug, it may provide additional time for this plug to harden and effectively block the female's reproductive tract or make it less likely that the plug is lost or removed by competitors.

We found evidence that male-male competition for fertilisable females determines the number of mating partners per litter (see review Kvarnemo and Ahnesjo, 1996), but the effect was small. If the operational sex ratio was very low on a particular day (ca. 1 oestrus female per 10 males), these oestrous females mated with significantly more mating partners (on average 4.4 males). If the operational sex ratio was very high (ca. 1 oestrus female per 2 males), females mated with fewer mating partners per litter (on average 3.75 males). This suggests that males did not achieve to monopolise an oestrous female with more conspecific present due to male-male competition. Given that several females are in oestrous simultaneously, males may face a trade-off between number and duration of mating and sperm quantity and quality.

Male copulation behaviour and causes of sperm precedence

We found that (1) males decreased their copulation and mate guarding durations with the mating order, but males were more equally performing these behaviours when the female mated with more males. (2) Male siring success showed a positive relationship with relative copulation duration, but only in males mating in the first or second position. Copulation duration of the first male significantly affected his siring success, and less so for the second male, whereas copulation durations of later mating males did not affect their siring success. In a loaded raffle, one male's sperm are 'devalued' relative to his competitor, e.g. a sperm from the second male to mate counts as only half of a ticket in the fertilization lottery compared with one of the first male's sperm (Parker, 1990). Our results support the hypothesis that sperm precedence is caused mainly by sperm from the first male being more valuable than sperm from later males. In thirteen-lined ground squirrels, *S. tridecemlineatus*, a first male advantage occurs, influenced through induced ovulation (Foltz and Schwagmeyer, 1989; Foster, 1934). In this species paternity is best predicted by the delay between the males' matings, and the duration of the longest copulation achieved by the second male under lab conditions (Schwagmeyer & Foltz, 1990).

Alternatively, sperm transfer rates might correlate with the position in the mating order, and this might have caused the average pattern of sperm precedence observed. In mammals, the first portion ejaculates contains the majority of sperm (Gomendio et al., 1998) and a positive association between the ejaculate volume and the likelihood of fertilisation has been found in deer mice, golden hamsters and rats (Dewsbury, 1984; Dewsbury and Hartung, 1980; Lanier et al., 1979). However, the sperm transfer hypothesis can not explain the detailed patterns of siring success depending on mating position and relative copulation duration.

A second alternative hypothesis to explain sperm precedence in this species is that our assumption of copulating duration scaling is not valid with the "real" copulation duration. But again, to explain the patterns of siring success we detected, one would need to invoke complex relationships between copulating and actual copulation durations to explain the results. Nevertheless, calibrating actual copulation duration with the copulating duration is needed to verify this assumption (e.g. by recording what actually happens inside the burrow during the mating sequence and measure sperm transfer rates).

Male guarding behaviour and causes of sperm precedence

First, the copulation and mate guarding duration were positively correlated and mate guarding by the first male reduced the likelihood of additional males mating with the female. This result supports the hypothesis that first males guarding their females may have augmented sperm precedence. Manno et al. (2007) observed that first males in a mating order were more likely to exhibit mating calling, a behaviour that can be part of mate guarding, to prevent the oestrus females from subsequent mating. Our findings confirm that the investment in mate guarding declines through the mating sequence. This suggests that sperm from later males is progressively devalued due to mating behaviour of the previous males. Mate guarding might be a first male tactic to use less sperm, avoid competition with rival sperms and evade sperm depletion when mating later with another female. The resulting trade off between mate guarding or searching for another female may end in a war of attrition.

The mating sequence lasted up to 546 minutes (the time between the first male to start copulation, to the last male ending copulation) and the maximum duration between any two males siring offspring was 345 minutes, suggesting that female squirrels were receptive over a prolonged period of time. Nevertheless, the cumulative effects of copulation and mate guarding durations of the previous males, may have forced later males to copulate at the far end of the receptive period. This appeared to cause these males to mate for a shorter period of time, and in effect, the timing within the mating sequence was a better predictor of the copulation duration of a given male than his mating position. This again resulted in the timing within the mating sequence having a strong effect on the siring success of males, augmenting the sperm precedence patterns observed. Males invest more in the copulation and guard their success when they are in the first position, this might shift subsequent males away from the optimal period, or thicker layers of sperm may reduce the likelihood of subsequent sperm to reach the eggs.

Future studies should establish if ovulation is induced (like in *S. tridecemlineatus*), and if not, measure the receptive period of oestrus females and determine whether sperm delivered at certain time points within this period are more successful in fertilising the eggs than other sperm (i.e. based on the timings of ovulations).

Age, body mass and reproductive behaviour

We expected that male investment in copulation and mate guarding should increase with male and female age (both correlated with body mass), and with male and female residual body mass (body mass corrected for age effects). Our findings supported these predictions only for age, and the significant interaction between male and female age indicated a complicated relationship.

The longest copulations and mate guarding were found when old males were consorting old females. The shortest copulations and mate guarding were found when males were copulating females of dissimilar age. For instance, young males progressively decreased their copulation and mate guarding duration with female age, and medium aged males copulated and guarded longest when copulating medium aged females. The causes of these age-assortative patterns are unclear, but unlikely to be spurious for the copulation duration, because the interaction between male and female age was highly significant (whereas marginally significant for guarding duration). Whether these age-assortative patterns are due to the male (e.g. based on his expected siring success), due to other males (e.g. competitors interfering with copulations), or due to the female (e.g. females aborting copulations) remains to be tested in the future.

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Table 1. Male reproductive behaviour depending on his mating position and the number of mating partners, corrected for year and colony effects.

	Copulation duration (n = 381 of 69 males)			Mate guarding duration[a] (n = 381 of 67 males)			Interval (n = 298 of 61 males)		
Parameter	df	Wald χ^2	p	df	Wald χ^2	p	df	Wald χ^2	p
Intercept	1	2240.9	<0.001	1	227.8	< 0.001	1	83.3	<0.001
Mating order	7	74.511	<0.001	4	62.6	<0.001	6	26.8	<0.001
Mating partners	6	39.103	< 0.001	3	19.5	<0.001	5	21.8	0.001
Year	3	3.808	0.28	3	3.2	0.36	3	1.5	0.68
Colony	4	25.552	<0.001	4	2.2	0.69	4	10.0	<0.05

Durations (in minutes) were poisson-distributed and modeled with a log-link using three separate GEEs, correcting for random individual male effects. Mating order was nested within the number of mating partners. The scaling parameter was adjusted using the deviance method. The interval is the duration from the end of guarding (or copulation when the male did not guard) to the next mating and is by default missing for the last male to mate.

[a] Mate guarding was only performed by males in mating positions 1 to 5 and virtually absent when the female mated with more than five partners, so the analysis was conducted for two to five mating partners only.

Table 2. Male siring success depending on his relative copulation duration (duration / total copulation duration), his mating position and the interaction (*n* = 185 of 58 males).

Parameter	df	Wald χ^2	*p*	Coefficient ±SE
Intercept	1	1.1	0.29	-0.43 ± 0.41
Relative copulation duration C	1	10.0	0.002	5.35 ± 1.69
Mating order MO	1	6.4	0.011	-0.45 ± 0.18
C x MO	1	4.1	0.042	-1.94 ± 0.95

Siring success had a weighted binomial distribution and was modeled with a logit-link using GEE, correcting for random individual male effects. The scaling parameter was adjusted using the deviance method. The number of mating partners (which correlates with both relative copulation duration and mating order), year and colony effects were non-significant and removed from the model.

Table 3. Male relative copulation duration (duration / total copulation duration) depending on the time since start of the mating sequence (in minutes, 0 = first male started copulation), the number of mating partners and their interaction (*n* = 139 of 53 males).

Parameter	df	Wald χ^2	*p*	Coefficient ±SE
Intercept	1	148.5	<0.001	-0.730 ± 0.060
Time T	1	32.5	<0.001	-0.001 ± 0.0003
Mating partners MP	1	55.8	<0.001	-0.089 ± 0.012
T x MP	1	18.8	<0.001	0.0002 ± 0.00005

Relative copulation duration had a normal distribution and was modeled using GEE, correcting for random individual male effects. The scaling parameter was adjusted using the deviance method. The mating order (which correlates with the time in the sequence), year and colony effects were non-significant and removed from the model.

Table 4. Male and female age and residual body mass (g) effects on male investment in copulation and mate guarding, corrected for mating order effects (df = 1 for each effect).

	Copulation duration[a]			Mate guarding duration		
n litters	177			306		
n males, females[b]	40, 53			46, 57		
Parameter	Wald	*p*	Coeff. ± SE	Wald	*p*	Coeff. ± SE
Intercept	759.5	<0.001	4.863 ± 0.177	48.7	<0.001	4.771 ± 0.684
Mating order	53.6	<0.001	-0.172 ± 0.024	25.6	<0.001	-0.753 ± 0.149
Male age	5.1	0.025	-0.080 ± 0.036	1.3	0.25	-0.161 ± 0.140
Male residual mass	3.8	0.051	-0.001 ± 0.0007	non-significant		
Female age	4.8	0.029	-0.079 ± 0.036	7.9	0.005	-0.406 ± 0.145
Male age x female	6.9	0.009	0.020 ± 0.008	4.1	0.042	0.062 ± 0.031

Durations (in minutes) were poisson-distributed and modeled with a log-link using two separate GEEs, correcting for random individual male and random individual female effects, the scaling parameter adjusted using the deviance method. Only the final models are shown: female residual body mass was non-significant in both models.

[a] Note that copulation durations were often missing for males mating in the first position, because they were seen to leave the burrow with the female, but not seen to enter.

[b] Number of individual males and females in the analyses.

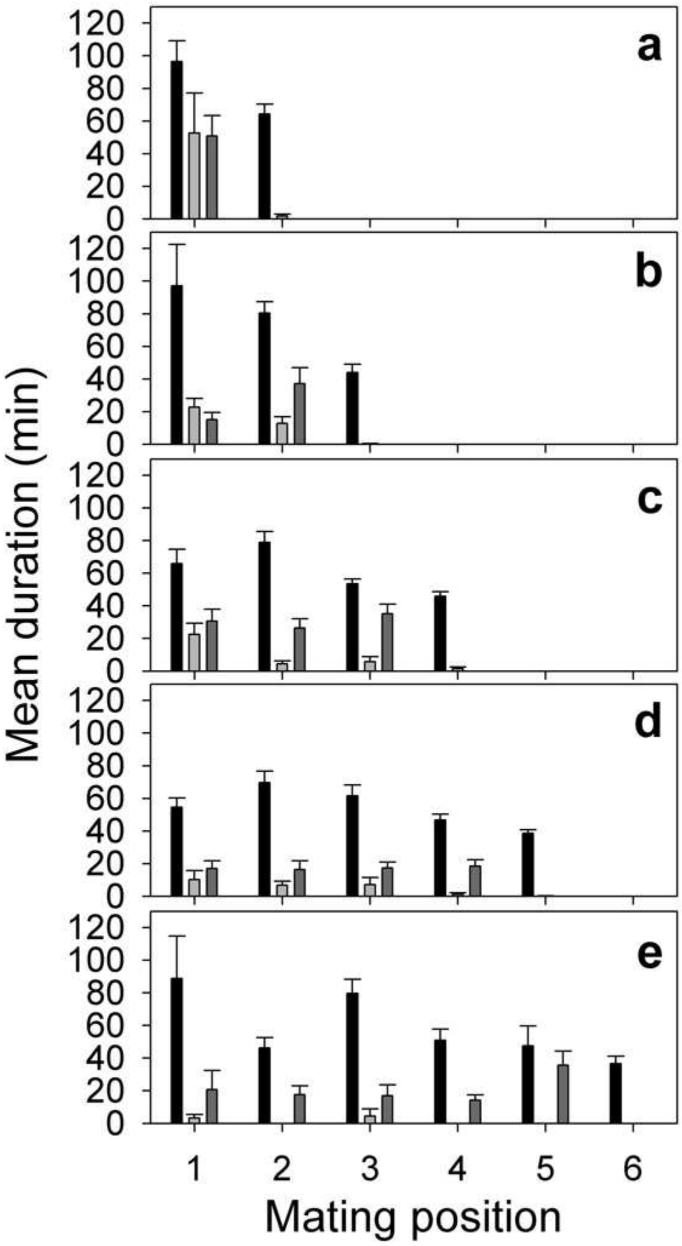

Figure 1. Male reproductive behaviour depending on his mating position and the number of males the female mated with: (a) two males (*n* = 13 litters), (b) three males (*n* = 48), (c) four males (*n* = 134), (d) five males (*n* = 116), (e) six males (*n* = 47; seven *n* = 18 and eight *n* = 5 omitted for clarity). Black bars: copulation duration, light-grey bars: mate guarding duration, dark grey bars: interval to next male. Note that sample sizes varied per bar due to missing values. See Table 1 for statistical analyses.

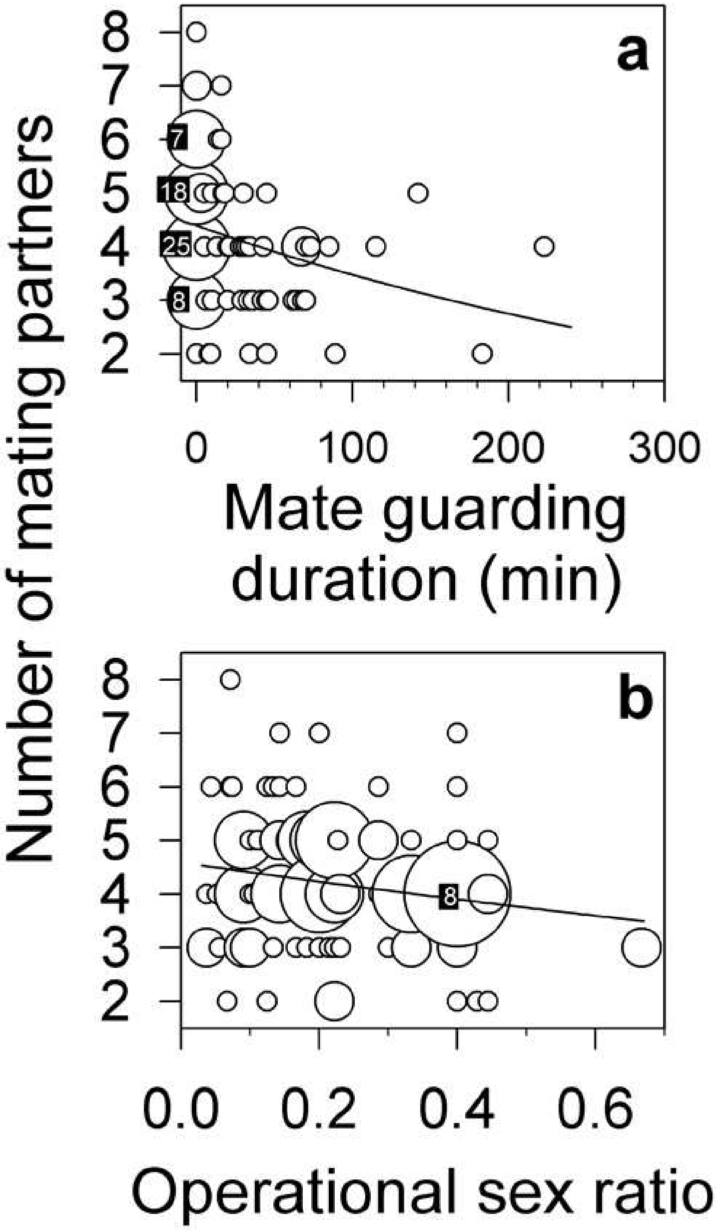

Figure 2. The number mating partners decreased with (a) the mate guarding duration of the first male to mate (*n* = 110) and (b) the operational sex ratio (oestrus females/males, *n* = 110). Overlapping values are indicated with increasing symbol sizes (exceptional large overlaps are indicated with their actual samples sizes in white).

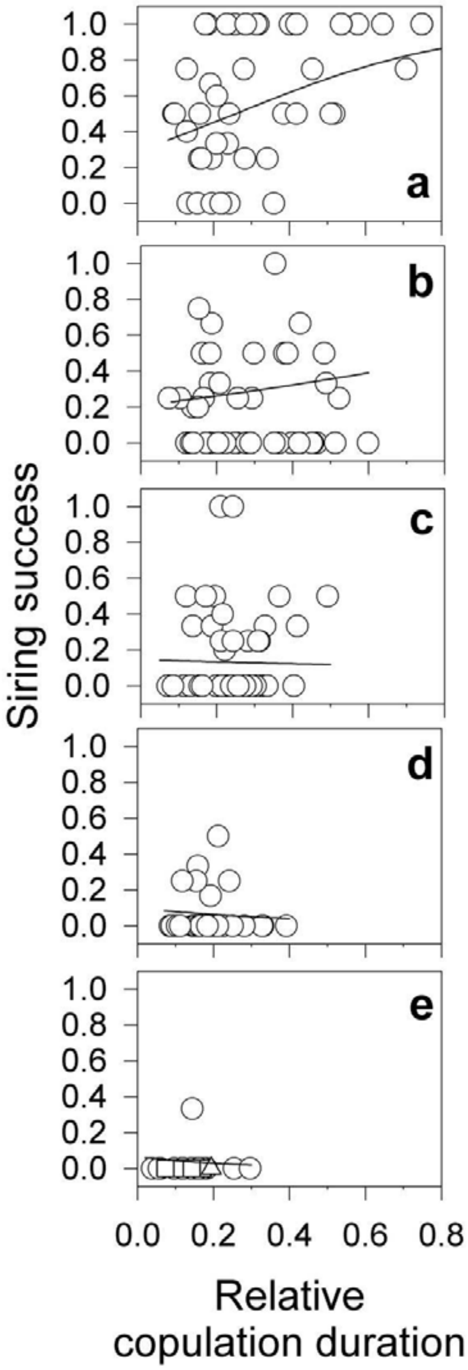

Figure 3. Male siring success (offspring sired / litter size) per mating position (*n* = 185 copulations of 58 males): (a) first; (b) second; (c) third; (d) fourth and; (e) fifth (circles), sixth (squares) and seventh (triangles) mating positions. Weighted logistic regression lines based on the model depicted in Table 2.

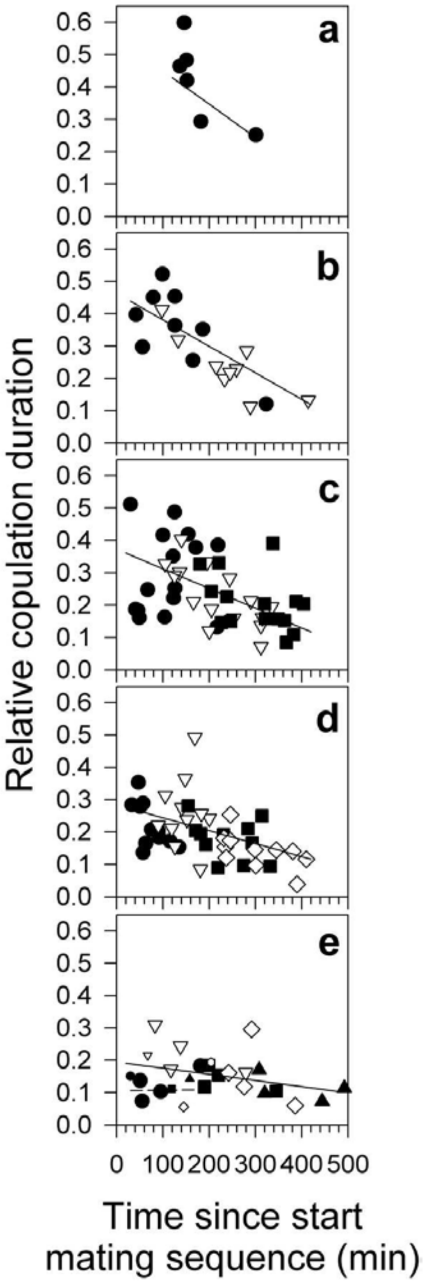

Figure 4. Male relative copulation duration (duration / total copulation duration) depending on the timing since the mating sequence started (0 = first male started copulation) and the number of mating partners (sample sizes and fitted lines from model in Table 3): (a) two, (b) three, (c) four, (d) five, (e) six (large symbols, continuous line) and seven (small symbols, dotted line) males. Note that mating order had no effect (black circles: second, triangles down: third, black squares: fourth, white diamonds: fifth, black triangles up: sixth and white circles: seventh male in the mating order).

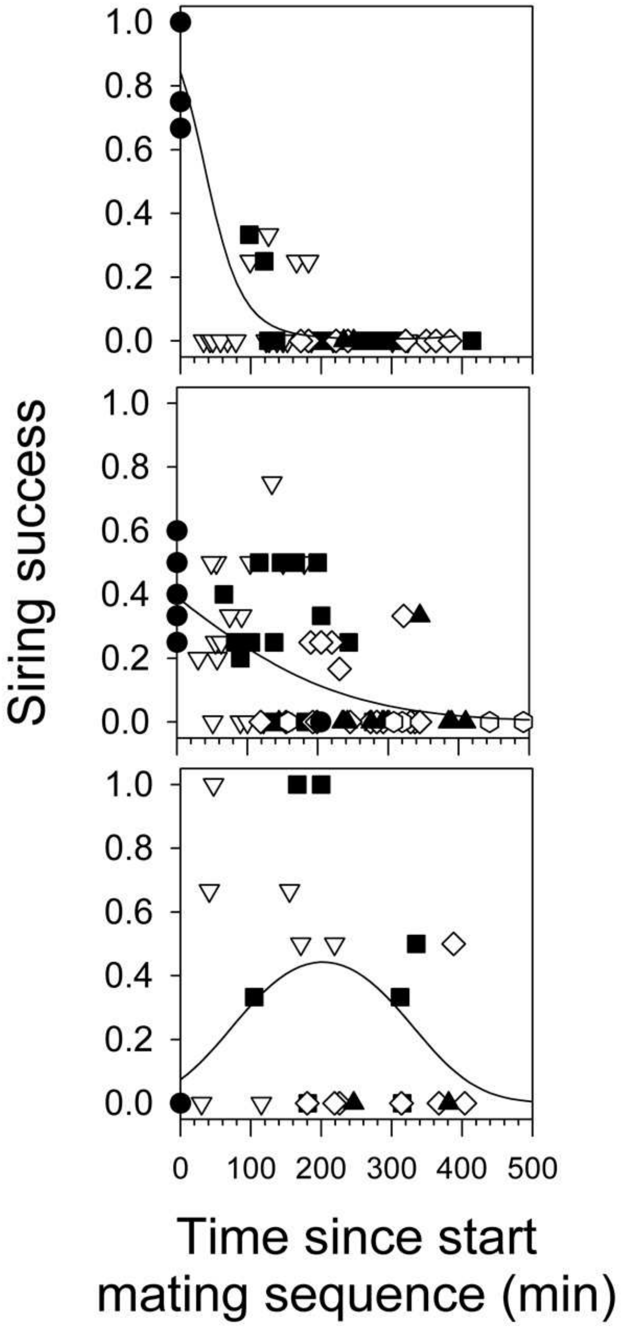

Figure 5. The timing of copulations and siring success of males of different mating positions for litters were the first males (a) sired 60-100% offspring, (b) sired 20-60% offspring, (c) sired 0-20% offspring. Note, that up to the fifth male sired any offspring. Symbols in (a-c): black circles: first; white triangles down: second; black squares: third; white rhombal: fourth; black triangles up: fifth; white diamonds: sixth to seventh mating position.

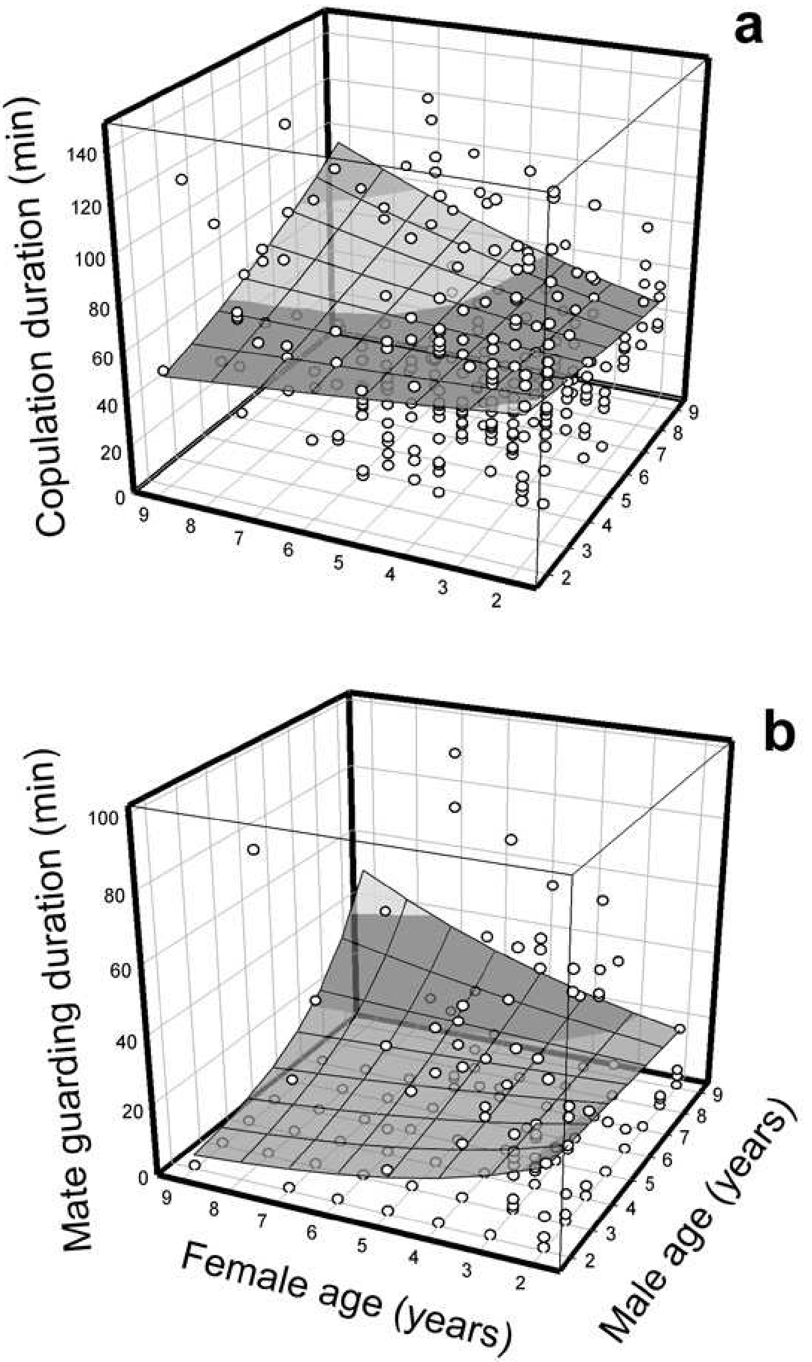

Figure 6. Male and female age had age-assortative effects on male investment in (a) copulation (*n* = 177) and (b) mate guarding (*n* = 306). The fitted planes are based on the models depicted in Table 4, corrected for the average of the other effects [coefficient x average mating position for (a) and (b) plus coefficient x average male residual body mass for (a) only].

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No experimental effects of parasite load on male mating behaviour and reproductive success in Columbian ground squirrels (*Spermophilus columbianus*)

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Abstract

Parasites can negatively affect their host's physiology and morphology in various ways and render host individuals less attractive as mating partners. The energetic requirements dealing with parasites have to be traded off against energy available for other needs such as feeding activity, territoriality, thermoregulation or reproduction. Parasites can affect mate choice and mating patterns, with females preferentially mating with parasite resistant or parasite-free mating partners. We tested experimentally whether removal of both ecto- and endo-parasites on free living, individual male Columbian ground squirrels (*Spermophilus columbianus*) led to an increase in male mating behaviour, reproductive success, seasonal weight gain and post-hibernation weight gain, compared to untreated males. We expected treated males against parasites to lose less body weight and to mate more often. In addition, we predicted experimental males to copulate early in the mating sequences of receptive females as the species exhibits a strong first male advantage. The parasite treatment significantly reduced the ecto-parasite load of males. However, contrary to our expectations, the treatment did not affect male reproductive behaviour (mating frequency, mating position, copulation and mate guarding duration), did not change male reproductive success, and finally did not result in males gaining more body mass (both within the season and post-hibernation). The potential role of parasite infestation on mating behaviour in Columbian ground squirrels will be discussed.

Key words: *Spermophilus columbianus*, parasite, manipulation, reproductive success, female mate choice

Introduction

Parasites may have detrimental effects on their hosts in various ways (Moller et al., 1999; Sheldon and Verhulst, 1996; Thompson and Kavaliers, 1994). For example, infection may lead to a reduction in host fertility (Lockhart et al., 1996), alter an animal's relative attractiveness to potential mates (Hamilton and Zuk, 1982; Moller et al., 1999; Verhulst et al., 1999) or affect whether and when to start breeding (Buchholz, 2004). Studies in various taxa have shown that parasites may impact on mate choice in both sexes (Altizer et al., 2003; Barber, 2002; Birkhead et al., 1993; Freeland, 1976; Moller et al., 1999; Moore and Wilson, 2002).

Frequent contact with conspecifics increases the likelihood of parasite transmission, thus parasites are expected to create a 'cost' of sociality (Alexander, 1974; Hoogland, 1995; Hoogland and Sherman, 1976). Many studies found that males are more parasitized than females (Zuk 1990; Poulin 1996; Schalk and Forbes 1997; Moore and Wilson 2002; Morand et al., 2004; Perez-Orella and Schulte-Hostedde, 2005; Gorrell and Schulte-Hostedde 2008), with some exceptions (Poulin 1996; Schalk & Forbes 1997; Hillegass et al. 2008). One reason for this male bias might be that males having larger home ranges and are more infested with parasites compared to females that often use smaller home ranges (Brei and Fish, 2003; Greenwood, 1980; Ims, 1987; Nunn and Dokey, 2006). Other reasons why males often are more afflicted by parasites may be due to androgenic hormones such as testosterone which suppresses the immune system (Folstad and Karter, 1992; Mougeot et al., 2006).

Parasites and resistance to parasites also plays a prominent role in sexual selection theory (Clayton, 1991; Hamilton and Zuk, 1982; Zuk, 1992; Zuk and Johnsen, 2000). Females can not increase the number of offspring by having multiple partners due to their limitation of egg production (Bateman, 1948), but they may select the best father to optimise their reproductive success (Jennions and Petrie, 1997; Zeh and Zeh, 1996). Best fathers might be males who provide compatible genes or best genes in their offspring, so these offspring can resist parasite infection or reduce the negative effects of such infections. Females which choose against the most heavily infected males avoid not only sexually or vertically transmitted parasites (Abel, 1996; Borgia and Collis, 1989; Hillgarth, 1996), but select also directly for parasite resistance genes (Hamilton and Zuk, 1982) and may receive increased paternal care (Hakkarainen et al., 1998; Milinski and Bakker, 1990). Consequently, females should prefer males who are less infested with parasites and therefore supposed to be healthier (Milinski and Bakker, 1990; Moller et al., 1999). Sexual selection for healthy partners would obviously provide choosy individuals with potentially important fitness benefits (Hamilton and Zuk, 1982). According to the theory of Hamilton & Zuk (1982) females discriminate against parasitized mates by considering costly secondary sexual traits indicative of parasite burden. This theory has been mostly tested by relating conspicuous visual or acoustic displays in male birds and fish to their parasite load or resistance (Clayton, 1991; Zuk, 1992). Hence, females can increase their fitness both directly by reducing their own risk of parasite transmission and indirectly by enhancing the parasite and/or disease resistance of their juveniles (Hamilton and Zuk, 1982). Parasite mediated sexual selection assumes that a genetic advantage is conferred by the resistant, uninfected male and that parasite resistance is heritable (Clayton, 1991).

Avoidance of infected conspecifics in experimental mate choice set-ups has been shown in animals, such as rodents, fishes and birds (Barber, 2002; Deaton, 2009; Ehman and Scott, 2002; Kavaliers et al., 2004; Kavaliers et al., 2005b; Kavaliers and Colwell, 1995; Kavaliers et al., 2003a; Kavaliers et al., 2003b; Milinski and Bakker, 1990; Penn and Potts, 1998; Zuk et al., 1998; Zuk et al., 1995). However, very few studies conducted as parasite-manipulation-experiments on free-living mammals and birds, mainly because of the difficulties in manipulation and observation in the field.

We studied the relationships between parasite load, male reproductive behaviour and reproductive success on free-ranging Columbian ground squirrels (Spermophilus columbianus), where we manipulated male parasite load experimentally. Columbian ground squirrels are diurnal, allow good observations, and are tolerant of experimental manipulations in the wild (Murie et al., 1998; Neuhaus, 2000). Further, females are in oestrus for only a few hours <12 hrs, (Murie, 1995) which makes it feasible to obtain complete mating observations on focal females in oestrus. Although mating mainly occurs in underground burrows, copulations were readily detected using established behavioural criteria (e.g. Boellstorff et al., 1994; Hanken and Sherman, 1981; e.g. Hoogland and Foltz, 1982; Murie, 1995; Sherman, 1989). We therefore use the term “copulation” to refer to behavioural evidence that mating occurred (Lacey et al., 1997) and to the time that a male and female spent together in a burrow. Females mate with up to eight different males while in oestrous

(Raveh et al., chapter 1). Previous research showed that a first male siring bias occurs in this species (Raveh et al., chapter 1), indicating that male-male competition and sperm competition play a major role in generating variation in male reproductive success.

In the present study we removed ecto- and endo-parasites of half of the reproductive males on three different colonies using chemical agents (experimental males). Control males were also caught, but not treated. We compared these two groups of males to identify the impact of parasites on male mating behaviour, reproduction and body mass change. We predicted that (1) experimental males should show an increase in reproductive behaviours known to increase reproductive success such as perform a higher mating frequency, a higher likelihood of obtaining the first mating position, a higher copulation duration and a higher mate guarding duration compared to control males. We predicted that (2) experimental males should have a higher siring success and seasonal reproductive success, compared to control males. Finally, we predicted that (3) experimental males should lose less weight through the breeding season and post-hibernation, compared to the control males.

Methods

The study took place in the Sheep River Provincial Park, Alberta, Canada (110°W, 50°N, and 1500m a.s.l.). Behavioural observations were obtained from April to mid July in 2007 and 2008 on three neighbouring colonies ('meadow' A, B, C) of Columbian ground squirrels. They are diurnal, inhabiting subalpine and alpine meadows where they live in groups of a few dozen to several hundred individuals. In our study area adult males emerge first from hibernation around mid-April, followed by females emerging a few days to a week later (Murie and Harris, 1982). Females breed on average 4 days after emergence from hibernation being in oestrus on average 242 minutes during a single day (Murie, 1995). Twenty four days later females give birth to a litter averaging three (1-7) naked, blind juveniles in a specially constructed nest burrow (Murie et al., 1998). The offspring emerge above ground when they are approximately 27 days old (Murie and Harris, 1982).

Experimental procedure

Squirrels were caught within the first two days of emergence from hibernation with live traps baited with peanut butter (National® live traps, WI, USA; 15×15×48 cm and 13×13×40 cm) and weighted with Pesola® spring scales to the nearest 5 g. This first body mass measurement for each individual male and year combination was entered in the remainder of the analyses. Thereafter, animals were re-trapped weekly to obtain body weight. Individually numbered fingerling fish tags (National Band & Tag Company #1) were attached in both ears for permanent identification. In addition, each ground squirrel was uniquely marked with hair dye on its back (Clairol, Hydriance - black pearl No. 52) for identification from a distance.

On each colony all reproductive males were randomly chosen for the two treatments (experimental or control). The experimental group (E) was treated with a spot-on solution (Stronghold®) and flea powder (Zodiac®) to remove endo- and ecto-parasites (n = 31 males). The spot-on solution was applied between the shoulders on the skin, the dosage was one drop per 100 grams. Stronghold is an agent against endo- and ecto-parasites. The flea powder shaker had several holes on top, the dosage was three shakes on the back and two shakes on the belly, and then the powder was applied by rubbing it into the fur of the males. To ensure that mate choice by females was not the result of secondary treatment effects (i.e. handling or odour cues), control animals (C) were handled similarly by mocking the flea powder treatment with a massage and by applying a sham of Isopropyl-alcohol (odour carrying alcohol in the Stronghold solution; n = 31 males). The treatment with the spot-on solution and the alcohol was reapplied every 17 days, while the flea powder application respectively the massage was repeated every 6 days during the mating season. Control and experimental groups from 2007 were reversed in 2008, so that experimental males became controls and vice versa. Four males of the control and three males of the experimental group did not re-emerge in 2008, in return 14 newly males (six experimental, eight sham treatment) were present in 2008 (either from immigration from a different colony or recruitment into reproductive age). In 2007 a total of 30 males were available ($n_{\text{experimental}} = 13$; $n_{\text{control}} = 15$) and in 2008 a total number of 37 males were included into the experiment ($n_{\text{experimental}} = 18$; $n_{\text{control}} = 16$) which adds up to 40 different individual males during the two field seasons (in total 62 males treated – 22 males present in both seasons).

Parasite load

Every trapped male ground squirrel was combed to count the number of ecto-parasites (using a combination of flea comb and finger-stroking the fur). In total, four flea load categories were defined (called ‘parasite load’ throughout): (0) = no parasites detected; (1) = one to two fleas; (2) = three to five fleas or (3) = more than five fleas detected on the animal. Parasite load was determined three times for each male, time period (1): just directly after hibernation, before the treatment started; time period (2): 12 days later and; time period (3): another 12 days later. However, data for all three time periods were not available from all years and colonies. Complete and precise data collections on flea loads over all three time periods (1-3) were only available from colony A in 2008 (*n* = 13 control and 11 experimental males). Even though the spot-on solution is an effective treatment against endo- and ecto-parasite, the following analyses are considering only ecto-parasites, because we had no information about the endo-parasite load.

Observations of mating associations

Animals were observed from 2-3 m high observation towers with binoculars. Columbian ground squirrels in our colonies usually mated underground. We captured unmated, pre-oestrus females daily to evaluate their reproductive status until they had mated. The degree of swelling and the openness of the vulva indicate the upcoming day of mating (for more detail see Murie, 1995). Observations of mating behaviours were examined and recorded for each female on her annual day of oestrus.

Mating activity began in the morning between 7:00 and 10:00, and lasted until 14:00 to 17:00 in the afternoon. Although we are confident that the behavioural criteria allowed us to identify correctly when mating occurred (e.g. Boellstorff et al., 1994; Hanken and Sherman, 1981; e.g. Hoogland and Foltz, 1982; Lacey et al., 1997; Murie, 1995; Sherman, 1989), they did not allow us to determine precisely the number or duration of copulations, or the interval between successive copulations. In another population of *S. columbianus* aboveground copulations were often observed and lasted on average 35 min. (range 1- 90 min.; Murie, 1995), an indication that copulations can last this long. We assumed that underground copulations took place when the oestrus female and a male went down the same burrow system and remained there for at least five minutes. Some males exhibited mate guarding right after having copulated with an oestrous female by chasing her into a burrow, sitting on that burrow, fighting other males away and giving mate guarding calls (Manno et al., 2007). We considered that a female's oestrus had ended when she increased her feeding activity and avoided and chased away potential mating partners and other squirrels (Murie, 1995).

Sampling of litters

Females from two colonies were brought to the lab where they were kept in polycarbonate cages (48 x 27 x 20 cm) for two days prior to parturition (for more details see (Murie et al., 1998). Within 12h of parturition, neonates were weighed, sexed and marked individually by removing a small amount of skin tissue from an outer hind toe or the tail. The tissue samples were used for paternity analysis. Females and their litters were released back into the colony the following day (for more details on the methods see (Murie et al., 1998). In the third colony C, tissue for DNA paternity analysis was collected from the ear at juvenile emergence (age 27 days) rather than at birth (Murie, 1995). Only offspring that emerged from their nest burrows at weaning were included in analyses to standardize among the three colonies. Hence, reproductive success for males and females was estimated based on number of juveniles at weaning. Offspring were caught within the first 2 days after emergence, with either unbaited 13x13x40 cm National live-traps or with multi-capture traps (Murie et al., 1998). Juveniles were marked and weighed, and their sex was determined or confirmed if born in captivity. Only females older than 1 year were included in the analysis as yearlings seldom reproduce in this study area (Murie and Harris, 1982).

Paternity analyses

Details of the molecular methods for microsatellite loci isolation and paternity assignment are provided elsewhere (Raveh et al., chapter 1) Briefly, DNA was extracted from preserved tissue using DNeasy Tissue extraction kits (Qiagen, Venlo, The Netherlands), and polymerase chain reaction (PCR) amplification was performed for a panel of 13 microsatellite loci. Note that maternity was certain for all the offspring born in captivity, as females were held in separate cages and paternity was assigned at 95% to 99% confidence using CERVUS 3.0 (Marshall et al., 1998). Analyses were conducted for each colony and year (2007 and 2008) separately.

Statistical analyses

All analyses were performed in SPSS 15. The majority of analyses were conducted using generalised estimating equations (GEE), which allows for the analyses of repeated measurements of the same subjects, which in our cases were individual males (individual identifier entered as subject). Results were corrected for breeding season (‘year’, 2007 or

2008) and colony effects (meadow A, B, C) throughout. Whether the two different treatment groups (E and C) differed in their parasite loads pre-experimentally (at time period one), was tested using Kendell's τ-c. Whether the change in male parasite loads over the season (time period one, two to three) depended on the treatment was analysed using ordinal regression with treatment, time period and treatment x time period as fixed factors.

We analysed the effects on mating order, copulation duration, mate guarding duration (all three poisson distributions with a log-link) using GEE with individual male identifier as subject, including treatment, year, and colony as fixed factors; and adding mating position as a covariate for the two analyses of durations (see Raveh et al. chapter 2 for the strong effect of mating order on both durations).

The number of offspring sired per male (binomial distribution with a probit-link) and the total seasonal reproductive success (which is the total number of offspring sired, as a poisson distribution with a log-link) were analysed using GEE with individual male identifier as subjects, depending on the treatment, year, colony as fixed factors and mating order as a covariate. We added the interaction between treatment and mating order to test whether the treatment affected the relative success of the males in the different mating positions.

To evaluate whether the males did not differ in their body mass and age at the start of the experiment, experimental and control males were compared using independent t-tests. To analyse whether the treatment affected the within-breeding season weight gain and post-hibernation weight gain, independent t-tests were used. Note that experimental males and control males did not differ in their body mass and age before the treatment (mass after hibernation: mean ± SE, control males: 555.0 ± 11.06, $n = 31$; experimental males: 549.67 ± 8.49, $n = 31$, t-test $t = -.0.382$, df = 1, $p = 0.704$; age: mean ± SE, control males: 4.27 ± 0.39, $n = 22$; experimental males: 4.47 ± 0.39, $n = 21$, t-test $t = 0.364$, df = 1, $p = 0.718$).

Results

Sample sizes for paternity assignment

In total, 176 adult males and females, and 238 offspring were successfully genotyped. Our genotyping success rate was 98%, with 85% of the ground squirrels genotyped at all 13 loci ($n = 353$). We retained all 13 loci in our analyses as there was no significant deviation from HWE or linkage disequilibrium, tested per colony. All 238 offspring were successfully assigned to both parents: 98% of offspring had 99% trio-confidence, while the remaining 2% had 95% trio confidence. in 236 of 238 cases (99%) offspring had zero mismatches with both parents.

We observed the complete mating sequence for 61 of 89 litters (188 of 238 offspring). The mating order was not obtained for 7 litters (21 offspring) and in 10 cases (29 offspring) the most likely sire was not observed mating with the focal female. Furthermore, the mating sequences for an additional 11 litters were observed, however these animals did not manage to wean any young. Overall, 347 times males were observed to copulate with the females, which added up to an average of 4.25 ± 0.13 (mean ± SE) mating partners per female.

Treatment effect on parasite load

We determined the parasite load of 24 males both before and after the treatment in 2007 and 2008, load measured on an ordinal scale from 0 to 3 (see Methods). Before the experiment started (time = 1), parasite loads of males from the different treatments did not differ (Kendall's $\tau_c = -0.20$, $p = 0.29$, see Figure 1, time period 1). An ordinal regression showed a significant reduction in parasite load over time (from 1, 2 to 3) for the experimental males, but not for the control males (treatment df = 1, $p < 0.001$; time 1 df = 1, $p < 0.001$; time 2 df = 1, $p < 0.001$, treatment x time 1 df =1, $p < 0.001$; treatment x time 2 df = 1, $p < 0.001$; time 3 is the reference category, see Figure 1). The significant accompanying parallelism test showed a different reaction over time for the two treatments ($\chi^2 = 43.6$, df = 10, $p < 0.001$), supporting the result that the decrease in the experimental males was substantial and different from the change in the control males. At time period 3, when experimental males had received repeated anti-parasite treatments every week, these males were no longer infested (Figure 1: all 11 males had zero parasite loads), whereas control males were still infested (Figure 1).

Parasites and male behaviour

There were no effects of the treatment detectable on male copulation rate, male mating position, copulation duration, or mate guarding duration (Figure 2, Table 1). In contrast, copulation rates differed significantly between the colonies and male mating position differed significantly between the years (Table 1). Finally, the mating order had a significant influence on both male copulation and mate guarding duration independent from the treatments (Table 1).

Parasites and male reproduction

In total, 217 offspring were weaned during 2007 and 2008. The treatment did not affect the number of offspring sired per litter (siring success, Figure 3a, Table 2). The seasonal reproductive output did not differ between the treatments (Figure 3b, Table 2). Furthermore, the treatment did not have an influence on the siring success, when only including mating positions one to three (Table 3), which are the most promising positions to fertilise females. However, there was a trend that experimental males had a higher siring success than control animals when only including the first mating position (Table 3).

Parasites and changes in male body mass

The treatment had no influence on the within-season body mass change (Figure 3c, mass end mating season minus mass after hibernation, t-test: $t = -0.289$, $p = 0.775$).

However, the parasite treatment in the year 2007 might have affected the change in male body mass over hibernation, which would indicate a long-lasting effect of the parasite treatment on male body mass acquisition and/or loss. Nevertheless, again the treatment in 2007 did not affect the change in body mass over hibernation (Figure 3d, t-test $t = -0.352$, $p = 0.729$).

Discussion

Several studies showed that parasites have an impact on their hosts' mating behaviour and reproductive success (Deaton, 2009; Milinski and Bakker, 1990; Poulin, 1994; Rosenqvist and Johansson, 1995; Sparkes et al., 2006). We experimentally removed parasites from male Columbian ground squirrels to determine whether their mating behaviour and fitness were influenced by the treatment. Contrary to our expectations, the removal of parasites, however, did not lead to an increase in male mating behaviour, male reproductive success and body mass. This suggests that the outcome of male-male competition was not affected by our treatments, although experimental males sired 0.6 more offspring per season than control males, particularly because they tended to be more successful in siring offspring in the first mating position. Repeating our study in different populations, more years and different habitats might strengthen our conclusions. At the end of the discussion we evaluate whether female preferences independent from our treatments might have obscured our experimental results.

Males with fewer parasites are expected to be in better condition and therefore have more energy to spend in searching for females and investing into reproduction. In a study on golden hamster (*Mesocricetus auratus*) intense male copulatory activities had an immunosuppressive effect (Kress et al., 1989; Ostrowski et al., 1989). Thus, mating effort is assumed to be costly for males; e.g. infected male red flour beetles (*Tribolium castaneum*) exhibited a reduced mating vigor and consequently inseminated fewer females than did uninfected males (Pai and Yan, 2003). Conversely, our study did not find an association between copulation rate and the different treatments in males. One possible explanation for such a result might be that control males could either cope with the infestation, or the parasite load was not severe enough to be really costly for these animals. We had some evidence to suggest that parasite load carried little costs in male Columbian ground squirrels and that loads were in general too low. First, control males did not loose more weight than parasite-free animals during the mating season. However, parasite infections can cause energetic costs (Delahay et al., 1995; Scantlebury et al., 2007; Simon et al., 2005) and may decrease the motivation to feed which may lead to a reduction in physical activities (Delahay et al., 1995; Mercer et al., 2000). When emerging from hibernation only a few males were heavily infested with fleas. During the whole season we found very few fleas on adult male and female Columbian ground squirrels, only yearlings and newly emerging offspring were often heavily infested (S. Raveh, own observations).

Previous studies confirmed that some rodent females are capable to choose non parasitized males over infested males under standardized laboratory conditions (reviewed in Kavaliers et al., 2005a). Raveh et al, (chapter 1) showed that in Columbian ground squirrels mating order plays a key role in male reproductive success, insofar as first males sire substantially more offspring than subsequent partners. Thus, we expected parasite-free male ground squirrels to mate first with oestrous females, either due to these males being preferred by the females or due to these males being more successful in male-male competition. Contrary to our expectation, we found no evidence that experimental males were more successful at mating in the early (first, second or third) positions, compared to control males. Only the strong mating order effect was important and explained the variation in reproductive success while the treatment had no impact. Likewise, both durations of copulation and mate guarding were not affected by treatment, however again the males' investment in these behaviours decreased within the mating order (see Raveh et al. chapter 2). Similarly, female mate preference (choice) did not depend on male infestation rate in several other animal species (pied flycatchers: Dale et al., 1996; Drosophila sp.: Kraaijeveld et al., 1997; pipefish: Mazzi, 2004; red flour beetles: Pai and Yan, 2003).

Since Columbian ground squirrels commonly engage in sniffing and gaping behaviour before and during the mating season, it is likely that odours are important for communicating and exchanging information such as kinship or genetic compatibility for mate choice rather than only the degree of parasite infestation. Therefore, female preferences for certain mates in both the control and the experimental males might have swamped our treatment effects, rendering them non-significant. In rodents, urine and other odorous secretions, such as major histocompatibility complex (MHC), are of high importance in mate detection and selection (Brown and Eklund, 1994; Brown, 1979; Egid and Brown, 1989; Ehman and Scott, 2002; Kavaliers and Colwell, 1995; Mougeot et al., 2004 ; Penn and Potts, 1998; Penn and Potts, 1999; Potts et al., 1991). The anabolic and behavioural effects of androgens carry an energetic cost, and high levels of androgens may suppress immune function resulting in an increased susceptibility to diseases and parasites (Folstad and Karter, 1992; Grossman, 1985; Hillgarth and Wingfield, 1997; Mougeot et al., 2004 ; Zuk and McKean, 1996). Folstad & Karter (1992) postulated that these costly effects of exposure to high androgen levels would handicap the expression of androgen dependent sexual characters resulting in only high quality individuals producing these characters rendering them honest indicators of quality. Females may measure testosterone levels in urine to detect the presence of parasites in potential partners (Mougeot et al., 2004 ; Olsson et al., 2000). Willis & Poulin (2000) showed that parasitized male rats had a lower testosterone level in their blood and suggested that females used this as a cue to avoid these males to secure resistance genes for their offspring.

Neuhaus (2003) showed that female Columbian ground squirrels weaned bigger litters and gained more weight during lactation when treated with flea powder if compared to untreated control females. In our study, a spot on solution was additionally used to create not only endo- but also ecto- parasite-free males, whereas in the study by (Neuhaus, 2003) only flea powder against ecto-parasites was applied. Even though this is a customary used agent for pets, we can not exclude a negative effect through light toxicity or by killing useful intestinal flora (seeVan Oers et al., 2002; for a negative effect of an ivermectin anti-endoparasite treatment on the fledging rate of oystercatchers *Haematopus ostralegus*).

For future experiments we suggests to study in more detail the role of female mating preferences in generating variation in male reproductive success. For instance, our treatment might not have affected the hormonal and odour profiles of our different males, and therefore did not alter their attractiveness to the females. Or changes might have made experimental males more attractive to some females, but less attractive to other females. Testosterone could experimentally be increased (by injection or implantation) or decreased (by blocking the receptors) in order to test for testosterone-mediated changes in health and infestation rates (Klein et al., 2002). Another interesting approach would be to apply the treatment before hibernation, since this might ensure that males are parasite-free at first emergence and test the effects on male-male competition and female preference. In this study the main focus was laid on the host’s behaviour, a next step should be to identify and determine the role of parasites themselves to learn more about their influence on their squirrel hosts. For instance, parasite loads might determine the fitness of offspring and females more than in the males, because the nest-burrows are strongly infested by ecto-parasites and heavily used by the mother and her pups.

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Table 1. Treatment effects on male reproductive behaviour. The mating order, copulation duration, mate guarding duration depending on the treatment (control or treated), corrected for year, colony and mating order effects (in the second and third analysis), using three separate GEEs with male identifier as subjects.

	Mating position (1-8) *n* = 264 of 40 males			Mate guarding (min) *n* = 264 of 40 males			Copulation duration (min) *n* = 211 of 40 males			Copulation rate *n* = 62 of 40 males		
Parameter	Wald χ^2	df	*p*	Wald χ^2	df	*p*	Wald χ2	df	*p*	Wald χ^2	df	*p*
constant	1291.886	1	<0.001	102.627	1	<0.001	2364.753	1	<0.001	658.992	1	<0.001
treatment	0.859	1	0.354	1.112	1	0.292	0.375	1	0.540	1.406	1	0.236
year	6.105	1	0.013	0.291	1	0.589	0.399	1	0.528	1.679	1	0.195
colony	1.777	2	0.411	1.153	2	0.562	4.986	2	0.083	34.039	2	<0.001
mating order				24.232	1	<0.001	20.569	1	<0.001			

Mating position, durations and copulation rate were fitted as Poisson distributions with a log-link, the scaling parameter adjusted using the deviance method. Note that the interactions between treatment x mating order were non-significant.

Table 2. Treatment effects on male reproductive output. The number of sired offspring per litter and the seasonal reproductive success depending on the treatment (control or treated), corrected for year, colony, and also mating position for sired offspring, using two separate GEEs with male identifier as subjects.

	Sired offspring / litter[1] $n = 264$ of 40 males			Total reproductive success[2] $n = 62$ of 40 males		
Parameter	Wald χ^2	df	*p*	Wald χ^2	df	*p*
constant	19.125	1	<0.001	87.943	1	<0.001
treatment	1.858	1	0.173	0.961	1	0.327
year	0.058	1	0.810	0.017	1	0.898
colony	0.065	2	0.968	8.549	2	0.014
mating order	96.650	1	<0.001			

[1] Sired offspring fitted as a weighted binomial distribution, the scaling parameter adjusted using the deviance method. Note that the interaction treatment x mating order was not significant $\chi^2 = 0.365$, df = 1, $p = 0.546$ and removed from the model.

[2] Total number of sired offspring in the season fitted as a Poisson distribution with a log-link.

Table 3. Treatment effects on male reproductive output. The number of sired offspring per litter in first to third position and first position only depending on the treatment (control or treated), corrected for year and colony effects, using two separate GEEs with male identifier as subjects.

	Sired offspring (1-3) *n* = 180 of 38 males			Sired offspring (1) *n* = 61 of 26 males		
Parameter	Wald χ^2	df	*p*	Wald χ^2	df	*p*
constant	0.003	1	0.955	56.988	1	<0.001
treatment	0.197	1	0.657	2.116	1	0.146
year	0.319	1	0.572	1.121	1	0.290
colony	4.571	2	0.102	6.167	2	0.046

Sired offspring fitted as a poisson distribution with a log link. The scaling parameter adjusted using the deviance method.

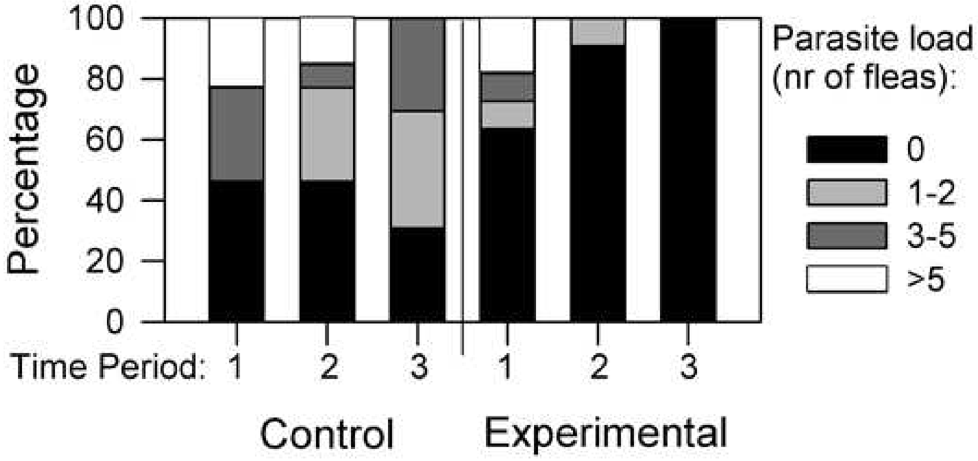

Figure 1. Percentages of the different parasite loads in control and experimental males measured during the three time periods, from emergence at hibernation (time period 1) until the end of the mating season (time period 3).

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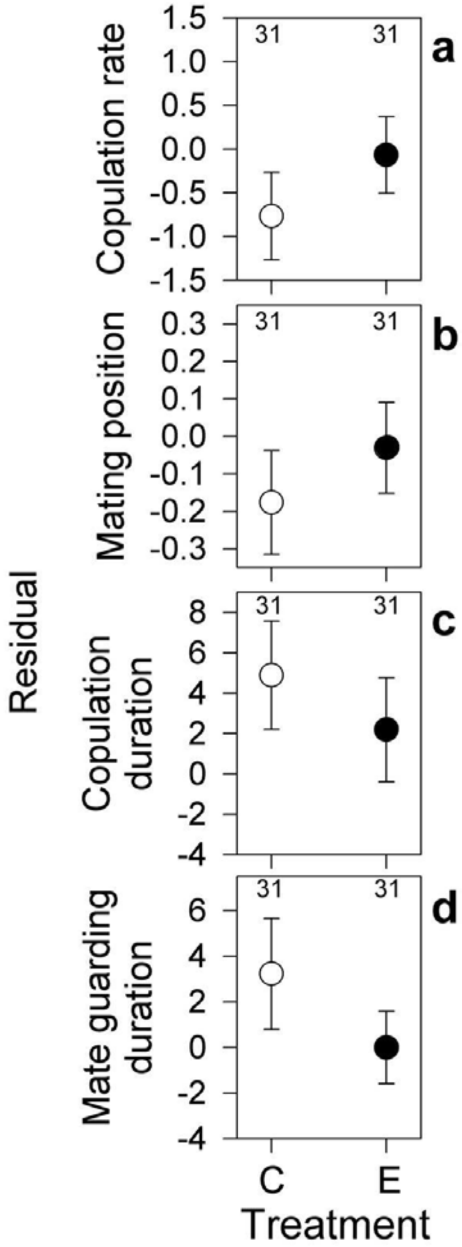

Figure 2. Male reproductive behaviour depending on the treatments (control males C: white and experimental males E: black circles): (a) copulation rate (number of females copulated per season), (b) mating order (1 to 8), (c) copulation duration (min) and (d) mate guarding duration (min). The treatment effect was non-significant in every case (see Table 1). Shown are residuals from the predicted values derived from all fixed effects in the models depicted in Table 1, without the treatment effect.

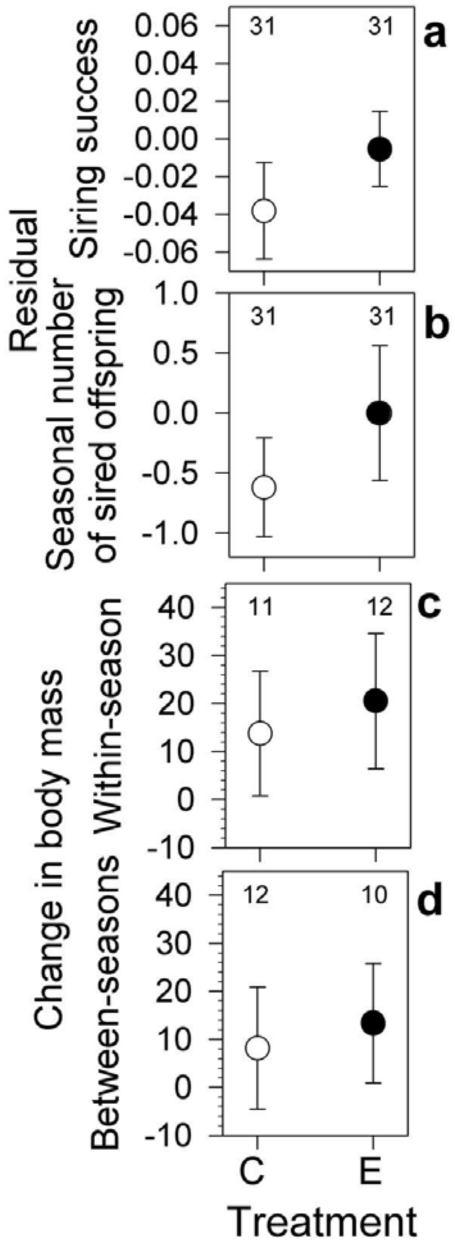

Figure 3. Male reproductive success and body mass change depending on the treatments (control males C: white and experimental males E: black circles): (a) residual siring success (offspring sired / litter size) and (b) residual seasonal number of offspring produced; and body mass change (c) within the season (end of season minus after hibernation) and (d) between-seasons (after hibernation year t+1 minus after hibernation year t, where t is the year of the treatment). The treatment effect was non-significant in every case (see Table 2). Shown are residuals from the predicted values derived from all fixed effects in the models depicted in Table 2, without the treatment effect.

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General Discussion

In the present study I investigated male mating behaviour and the impact of parasites on reproductive success in a polygynandrous system, using Columbian ground squirrels as a study system. The results of this study suggest that complex interactions between male and female strategies are involved in shaping male mating success.

Columbian ground squirrels are polygynandrous, that is males and females are mating multiply. As a direct consequence of this I found that the majority of litters were sired by multiple males. These findings are in accordance with other studies on related species with a polygynandrous mating system (Boellstorff et al., 1994; Foltz and Schwagmeyer, 1989; Hanken and Sherman, 1981; Sherman, 1989), a lower incidence of multiple paternity have been reported only in black-tailed prairie dogs (Cynomys ludovicianus), Gunnison's prairie dogs (Cynomys gunnisoni) and arctic ground squirrels (Hoogland and Foltz, 1982; Lacey, 1991; Lacey et al., 1997; Travis et al., 1996). While it seems straightforward to explain why males engage in multiple matings, this is less clear for females. The "genetic bet-hedging hypothesis" predicts that, particularly under fluctuating environments/conditions, multiple mating will be advantageous for females, because a multiple-mated female will produce a genetically more diverse litter than a single-mated female, thereby ensuring that at least some offspring within the litter will survive (Watson, 1991; Yasui, 1998). One possibility is that multiple male mating results in multiply sired litters, which in turn increases the genetic diversity of the offspring (Jennions and Petrie, 2000; Zeh and Zeh, 1996; Zeh and Zeh, 1997; Zeh and Zeh, 2001). However, many species live under fluctuating conditions, but not all engage in multiple mating. Below, I will list the results of the study and investigate male and female advantages of multiple mating that may contribute to explaining the observed mating pattern in Columbian ground squirrels.

I found (1) that the first male mating with a female generally sired the highest number of offspring, even though males could successfully fertilise offspring up to the fifth position in the mating sequence. Male mating success is largely determined by first male advantage. I suggest that males can gain the largest part of their seasonal reproductive output by being the first male to mate with a female. However, males also may gain considerable additional fitness from mating with further females that already had mated with other males. In order to choose among the females, male Columbian ground squirrels might use olfactory cues which allow them to discriminate between unmated and mated females during the mating season.

In three other related species first male reproductive bias has been confirmed: Belding's ground squirrel *S. beldingi* (Hanken and Sherman, 1981; Sherman, 1989), thirteen-lined ground squirrel *S. tridecemlineatus* (Schwagmeyer and Foltz, 1990) and Arctic ground squirrel *S. parryii plesius* (Lacey et al., 1997). Paternity in Idaho ground squirrels (*S. brunneus*) is biased in favour of a female's last mating partner and longest guarding male, that may be influenced by the number of copulations between a female and each of her mates (Sherman, 1989). Compared to other ground squirrel species, female Columbian ground squirrels mated on average with a large number of different males per litter produced. Additionally, first male advantage was lowest in this species compared to the other species, suggesting that sperm competition augments with an increasing number of mating partners. Males of intermediate age had the highest fertilisation success in our study. However, this was not because these males mated more often in first position. Instead these males mated overall more frequently, which also increased the likelihood of being in the first position of the mating order.

In a next step (2) I examined whether other possible underlying mechanisms explain the first male advantage in this species. The duration of copulations and the duration of mate guarding were positively correlated with reproductive success. Both behaviours increased the first and second males' reproductive success, while I did not find any correlation with males mating in later positions. The timing of copulation relative to the ovulation is an important factor for fertilisation success (Gomendio et al., 1998; Gomendio and Roldan, 1993). Foster (1934) showed that the thirteen-lined ground squirrel is an induced ovulator and this species shows a fist male bias which is predicted by the increased interval between the two copulations and the duration of the second mating or both. Unfortunately, there exists no further information in other species of the genus Spermophilus about whether females ovulate spontaneously or induced through multiple copulations. A next step would be to examine the timing of ovulation, since the fertile time window is crucial to understand sperm competition and the male mating behaviour to optimise their fertilisation success. Both scenarios of induced or spontaneous ovulator are likely in Columbian ground squirrel and remain to be investigated in future studies.

Male strategy can be influenced by the availability, distribution and quality of females. Male Idaho ground squirrels defend the female after copulation. This male behaviour has been explained to result from the distribution of females. As females are widely spaced, it would be too costly for males to search for another mating partner, unguarded females mate multiply (Sherman, 1989). Litters of females guarded by more than one male were multiply sired and the most successful sire was usually the last/ longest attending mating partner (Sherman, 1989). the this species the male that invests most time in mate guarding will In Belding's ground squirrels females can more easily be accessed by the males. Therefore males seek to copulate with many other females. In this species 60% of the offspring are sired by the first mating partner (Sherman, 1989). Polygynandrous red squirrels (*Tamiasciurus hudsonicus*) show no association between mating order and reproductive success (Lane & Boutin unpublished data). Additionally to male strategies, active female choice may be involved in generating the observed differences between the species. In accordance with this possibility, I observed female Columbian ground squirrels actively choosing or avoiding certain males early in the mating sequence.

Overall, it can be concluded that males in Columbian ground squirrels are mating multiply with several females to increase their reproductive success. However, it seems less clear why females mate with multiple males. The proposed hypotheses in the literature suggest that females mate multiply because they ensure fertilization as some males may be sterile (Sheldon, 1994), avoid genetic incompatibility (Zeh and Zeh, 1996), increase the genetic variability of offspring (Tooby, 1982), increase litter size (Hoogland, 1998; Pearse et al., 2002), increase offspring survival and their quality (Madsen et al., 1992; Olsson et al., 1994), avoiding inbreeding (Head et al., 2005; Hosken and Blanckenhorn, 1999; Madsen et al., 1992; Ratkiewicz and Borkowska, 2000; Tregenza and Wedell, 2002; Zeh and Zeh, 2001); genetic bet-hedging (Yasui, 1998) or need multiple copulations for stimulation of ovulation (Gomendio and Roldan, 1993; Roberts et al., 1999). Females may also mate multiply to confuse the issue of paternity in order to avoid infanticide by males (Wolff and Macdonald, 2004). Contrary to other species, Columbian ground squirrel and related species which do not recycle after loosing their offspring, infanticide is mainly committed by females and not by males (Wolff and Sherman, 2007). Our results showed that very old males sired only few or no offspring at all, fertilization assurance might explain why Columbian ground squirrel females invested into mating multiply during a single oestrous event.

(3) Copulating multiply can also be costly because it is expensive in terms of energy investment or increased danger of parasite or disease transmission, injury, or predation risk (Bateman, 1948; Daly, 1978; Magnhagen, 1991). Studies have shown that parasites limit copulation frequency and therefore reduce reproductive success (Hasu et al., 2006; Milinski and Bakker, 1990; Møller, 1990; Polak, 1996; Polak et al., 2007; Rosenqvist and Johansson, 1995; Schmid-Hempel, 1998; Simmons, 1993; Sparkes et al., 2006; Worden et al., 2000). Additionally, negative impacts on host reproductive success have been linked to parasite abundance, particularly in populations of song birds (Fitze et al., 2004; O'Brien and Dawson, 2005; Richner et al., 1993). In the present study, I did not detect any effect of parasites on any of the fitness proxies measured such as changes in weight or body condition, mating frequency, reproductive success and mating order in male Columbian ground squirrels. The interpretation of these negative results seems difficult at present. Future studies should investigate, whether interactions between males and females are indeed not affected by parasite load or whether more complex underlying interactions can explain these findings. In light of these findings, several new approaches are recommended in order to better address these questions in future studies. Although there are endless questions about these systems, here are a few questions I propose for future studies:

1) What are Columbian ground squirrels doing while underground, only copulating?
2) Is sperm quality and quantity decreasing with increased mating frequency?
3) How is ovulation triggered in *S. columbianus*?
3) How is female and male mate choice influenced?
4) Why are females mating multiply?
5) Do odour cues play a major role during the mating season (MHC)?
6) Is there a difference in age and sex distribution between infested and un-infested ground squirrels?
7) What is the exact life-cycle of fleas in the Columbian ground squirrel colonies and what are effects on host survival during hibernation?

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