

REPRODUCTIVE ECOLOGY OF FEMALE GOLDEN-MANTLED GROUND SQUIRRELS

By  
Caitlin Pratt Wells  
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Approved:

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Dirk H. Van Vuren, Chair

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John M. Eadie

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Andrea K. Townsend

Committee in Charge

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## ABSTRACT

Understanding the life-history decisions of female vertebrates is critical to predicting individual- and population-level processes such as individual fitness, genetic diversity, population dynamics, and the evolution of sociality. In mammals, female-biased philopatry generates the potential for cooperation and conflict among female kin who remain in their natal range, and these relationships are likely to affect life-history decisions such as when and how females reproduce. Yet despite abundant research into the role of kinship in the reproductive ecology of social mammals, we have little understanding of its role in solitary species, such as the small-bodied ground squirrels. This dissertation consists of four studies that investigate the reproductive ecology of female golden-mantled ground squirrels (*Callospermophilus lateralis*), paying particular attention to the effects of social and environmental factors on age of first reproduction, drivers of multiple paternity, and allocation of offspring sex.

For hibernating squirrels, the amount of energy stored as fat may influence several components of female fitness, including timing of first reproduction, reproductive success, and offspring sex allocation, but fat stores are difficult to quantify in living animals. In chapter one, I use a non-destructive method to characterize body composition (lean mass versus fat mass) and condition (fat stores) of adult females at multiple stages in the circannual cycle. Body mass explained a high proportion of the variation in fat mass during the emergence and pre-hibernation stages, but less during the reproduction stage. Additionally, I found a strong effect of mass even after accounting for variation among individuals and years. These results suggest that body mass is a good estimate of body condition during the periods of emergence and pre-hibernation fattening,

and therefore may be useful to predict important components of fitness such as female reproductive success and overwinter survival.

Selection for early reproduction should be strong when lifespans are short, because early-maturing individuals are more likely to successfully reproduce before they die, yet females often delay reproduction beyond what appears to be optimal. In chapter two, I use 21 years of demographic data from a free-living population of golden-mantled ground squirrels to evaluate the developmental and social factors that may constrain early reproduction in this short-lived, asocial species. I found that females who would reproduce as yearlings were weaned earlier in the summer than females who waited until age 2 to reproduce, and that early weaning was associated with higher growth rate during the natal summer. Females were more likely to reproduce as yearlings when there were more adult males present, possibly because exposure to males accelerates reproductive maturity or due to the increased likelihood of conception with more male mates. Maternal presence had no facilitating or inhibitory effect on yearling reproduction, but yearlings with a littermate sister present were only 22% as likely to reproduce as females without a sister present. Competition among siblings before or after weaning may reduce growth rates in female rodents, and if golden-mantled ground squirrel sisters remain in close proximity in their natal range, developmental delays may extend to delays in reproductive maturation.

Female mammals often mate with multiple males, resulting in multiple paternity within their litters. While rates of multiple paternity appear quite stable in some species, other species show substantial variation among populations or among years within populations. In chapter three I

use 18 years of genetic and demographic data to examine the mating system and patterns of multiple paternity in a free-living population of golden-mantled ground squirrels. The mating system was polygynandrous, but opportunity for sexual selection was lower for females than for males. Annual reproductive success of males was low for yearlings and new immigrants, and increased with breeding tenure in the population. Multiple paternity was evident in 62% of litters. I found little support for the null model of multiple paternity, but in accordance with the socioecological model of male monopolization, rates of multiple paternity decreased with female spatial clustering, unless male-male competition, as indicated by male density, was also high. From Bateman gradients, I found no fitness benefit of multiple paternity for females, and in support of the female choice model, multiple paternity decreased with maternal age and peri-oestrous mass. Together, these results suggest that variation in the rate of multiple paternity in golden-mantled ground squirrels was determined primarily by the active strategies of males and females. High rates of multiple paternity in high-density years may increase genetic diversity during population peaks, particularly in years when more yearling females reproduce.

Evolutionary theory predicts that parents should bias investment in offspring toward the sex that will yield the higher fitness returns, and one outcome may be biased offspring sex ratios. In chapter four, I use 18 years of pedigree and demographic data to investigate variation in offspring sex ratios in a free-living population of golden-mantled ground squirrels. Despite overall parity in offspring sex ratio, I found predictable changes in offspring sex ratio in response to female density at the population level: squirrels produced more daughters when female density was low, and more sons when female density was high. At the individual level, females adjusted litter sex ratios similarly, but only in response to high densities of kin, and not in response to

density of nonkin. This effect was reversed for older females ( $\geq 3$  years) in dense kin neighbourhoods, who were more likely to produce daughters, perhaps because older females had the behavioural dominance necessary to recruit daughters at high densities. By contrast, litter sex ratios did not vary with adult sex ratios, maternal condition, or food availability. These results support the local resource competition theory of offspring sex allocation for this species, and are consistent with socially-induced physiological mechanisms that bias offspring sex in ground squirrels. Knowledge of female relatedness revealed patterns of sex ratio adjustment that otherwise would have been obscured, highlighting the importance of interactions with kin in an asocial species.

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**CHAPTER 1: BODY MASS AS AN ESTIMATE OF BODY CONDITION IN A HIBERNATING  
SMALL MAMMAL, THE GOLDEN-MANTLED GROUND SQUIRREL (*CALLOSPERMOPHILUS  
LATERALIS*)**

## **1.1 Abstract**

In hibernating squirrels, the amount of energy stored as fat may influence several important demographic traits, but is difficult to quantify in living animals. Thus, several non-destructive indices of body condition are in use: simple indices that use body mass, and scaled indices that correct body mass for structural size. However, the accuracy of these indices for hibernating squirrels is poorly known. We used measurements of total body electrical conductivity (TOBEC) from adult female golden-mantled ground squirrels (*Callospermophilus lateralis*) to characterize body composition (lean mass versus fat mass) and condition (fat stores) at multiple stages in the circannual cycle. Body mass explained a high proportion of the variation (93-96%) in fat mass during the emergence and pre-hibernation stages, but less during the reproduction stage (84%). Additionally, we found a strong effect of mass even after accounting for variation among individuals and years. Contrary to expectation, correcting for structural size did not markedly improve the condition index. Our results suggest that body mass is a good estimate of body condition during the periods of emergence and pre-hibernation fattening, and therefore may be useful to predict important components of fitness such as female reproductive success and overwinter survival.

## **1.2 Introduction**

Seasonal variation in energy supply is a central problem for many mammals, who may respond to periods of environmental energy shortage by storing energy, reducing energy expenditure, or

both (Humphries et al. 2003). Hibernation, which reduces metabolic demands during winter, is one life-history adaptation to seasonal energy scarcity, and sufficient energy stores are essential to its success (Pulawa and Florant 2000).

In hibernating squirrels, the amount of energy stored as fat may influence several important demographic traits such as overwinter survival (Murie and Boag 1984; Lenihan and Van Vuren 1996), timing of reproductive maturity (Barnes 1984), male breeding effort (Delehanty and Boonstra 2011), female reproductive success (Dobson and Michener 1995; Rieger 1996), offspring sex allocation (Allaine et al. 2000), and natal dispersal (Nunes and Holekamp 1996; Neuhaus 2006). Additionally, estimating fat stores is essential for bioenergetic models of hibernation, which can be used to project distribution changes of hibernating species under changing climatic conditions (Humphries et al. 2002). However, quantifying body condition (defined here as fat stores, in grams; Kiell and Millar 1980; Dark et al. 1989) is difficult to do non-destructively. Because determining the effects of body condition on future life-history outcomes requires that the animal survive measurement, several non-destructive indices for estimating condition have been developed (Schulte-Hostedde et al. 2005; Peig and Green 2010). These include simple condition indices that use body mass, and scaled condition indices that attempt to correct body mass for structural size. Many studies use total body mass as a simple condition index, with the implicit assumption that greater mass reflects greater relative fat stores (Barnes 1984; Sauer and Slade 1987; Lenihan and Van Vuren 1996; Neuhaus 2003; Lane et al. 2011). However, larger animals may have greater mass due to larger structural size (skeleton and associated lean tissue) instead of greater fat stores (Dobson 1992). Thus, some studies use a scaled condition index based on residuals derived from a regression of body mass on structural

size, with the expectation that correcting body mass by the structural size of an individual improves the estimate of its condition (Bachman 1993; Dobson and Michener 1995; Dobson et al. 1999; Allainé et al. 2000). Positive residuals suggest the animal contains more tissue (presumably fat) than predicted for a given structural size, while negative residuals suggest the animal contains less tissue than predicted for a given structural size. For both simple and scaled condition indices, a log transformation of mass or length has been proposed to convert the power relationship of allometric scaling ( $y = \alpha x^\beta$ , where  $y$ =mass and  $x$ =length) to a linear relationship (Green 2001; Peig and Green 2009).

Scaled indices are appealing because they correct for variance in body mass that is unrelated to energy stores, but available evidence indicates that size-corrected measures do not necessarily improve estimates of body condition compared to use of body mass alone (Krebs and Singleton 1993; Green 2001; Schamber, Esler, and Flint 2009). However, most evaluations of condition indices have focused on mammals that do not store fat for hibernation or energy reserves, and the poor relationship between the scaled condition index and measured fat content may occur because residuals of these relatively lean species primarily reflect differences in protein or water content rather than fat (Schulte-Hostedde et al. 2001). Scaled indices might be more appropriate for species in which fat content is a greater proportion of body mass, such as hibernators (Schulte-Hostedde et al. 2001), but the accuracy of simple versus scaled condition indices for hibernating squirrels is poorly known.

Fat storage in hibernating squirrels follows circannual cycles of accumulation and depletion (Buck and Barnes 1999), reflecting seasonal changes in the balance between energy acquisition and

expenditure (Kenagy et al. 1989). For an index to be an appropriate estimate of body condition, it should explain a high proportion of the variation in fat storage, preferably across multiple stages of the circannual cycle. Adult females are often excluded from condition index validation because of the confounding effect of fetal lean tissue elaboration during gestation (Krebs and Singleton 1993; Schulte-Hostedde 2005), yet energetic costs associated with hibernation and reproduction deplete fat stores, and therefore affect body condition, in adult females as well as males (Kenagy 1989; Michener and Locklear 1990; Buck and Barnes 1999). Total body electrical conductivity (TOBEC), a non-destructive method to analyze the body composition of animals (Speakman et al. 2001), has been used to obtain estimates of lean and fat mass from free-living small mammals (Walsberg 1988; Koteja 1996), including ground squirrels (Nunes and Holekamp 1996; Pulawa and Florant 2000). The TOBEC method uses electrical current, which travels differentially through fat versus lean tissue, to generate measures of electrical resistance; resistance measures are then converted to fat mass using species-specific calibration equations (Bachman 1994; Koteja 1996; Walsberg 1998; Scott et al. 2001). In this paper we use TOBEC measurements from adult female golden-mantled ground squirrels (*Callospermophilus lateralis*) to evaluate fat stores during four major stages (emergence, reproduction, post-reproduction, and pre-hibernation) in their circannual cycle. Our goal is to assess the accuracy of body mass as a simple index of body condition in each stage, and determine if using a scaled index or log transformations in simple or scaled indices improves estimates of body condition.

### 1.3 Methods

We studied golden-mantled ground squirrels over 3 years (2003 - 2005) in the northern Sierra Nevada of California. These squirrels are locally abundant, medium-sized (200-300 g), and relatively well-known both ecologically and physiologically (Bartels and Thompson 1993). Our study was conducted in the Plumas National Forest near Quincy, California, at an elevation of ~2100 m. In this area, adults emerged from hibernation in May, and pups were weaned in late July; all squirrels gained weight during September before immersing for hibernation in October. Gestation in golden-mantled ground squirrels is 28 days (Cameron 1967) and weaning occurs when pups reach 30 days of age (Phillips 1981). We divided the active season into four circannual stages; emergence, 15 May - 15 June (emergence from hibernation through mating and early gestation); reproduction, 16 June - 31 July (late gestation through lactation); post-reproduction, 1 August – 31 August (after lactation but before late summer fattening becomes pronounced); and pre-hibernation, 1 September – early October (when pre-hibernation fattening occurs).

We captured adult female squirrels with Tomahawk live traps (Model 201) baited with rolled oats and black oil sunflower seeds coated with peanut butter, set in the early morning and checked at mid-morning. Our methods were conducted according to a protocol approved by the Animal Care and Use Committee of the University of California, Davis, and followed guidelines approved by the American Society of Mammalogists (Sikes et al. 2011). We attempted to capture all squirrels on a monthly basis, but due to differential trappability, not all squirrels were captured each month. We transported captured squirrels to a laboratory near Quincy, where we chemically immobilized them with an intramuscular injection of ketamine hydrochloride (100 mg/ml). We recorded body mass to the nearest 0.1 g using a portable electronic balance, and

body length (measured as tip of nose to anus) to the nearest 0.1 cm (Pulawa and Florant 2000). We used body length as a measure of structural size (Bachman 1993; Allainé et al. 2000); our measurements of body length showed good repeatability for individuals recaptured in the same stage (Pearson correlation  $r = 0.83$ ,  $n = 5$ ). We quantified body fat using an EM-SCAN SA-3000 body composition analyzer (EM-SCAN, Springfield, IL, USA). EM-SCAN readings are known to vary with animal movement during measurement, differences in gut contents, changes in ambient temperature, and changes in body temperature greater than 4°C (Walsberg 1988; Speakman 2001). To minimize variation due to movement, we placed immobilized squirrels on a plastic sample tray and lightly restrained them with rubber bands to maintain each squirrel in the same position (dorsoventrally, ventral side down, with the tail tucked under the body). To minimize variation due to gut contents, we only trapped squirrels early in the morning (as foraging began) and did not provide food or water until after TOBEC measurement. To minimize variation due ambient temperature, we performed measurements in a laboratory at a field station. Anesthesia often causes a drop in body temperature; throughout our study, however, the mean change in body temperature was -1.5°C, and no individuals lowered their body temperatures more than 4°C. Body composition was calculated as the mean of five replicate measurements; we recorded seven replicate measurements and then discarded the highest and lowest values, though variation in measurements was minimal (coefficient of variation = 0.03). We determined lean mass ( $M_L$ ) using the calibration curve for golden-mantled ground squirrels:  $M_L = 18.0 + 0.3M_B + 1.2\sqrt{L_B \times EM}$ , where  $M_B$  is body mass,  $L_B$  is body length, and  $EM$  is the EM Scan measurement ( $r^2 = 0.98$ , Pulawa and Florant 2000). We calculated fat mass by subtracting lean mass from body mass.

We characterized the body composition (lean mass versus fat mass) and condition (fat mass) of adult female ground squirrels during emergence, reproduction, post-reproduction, and pre-hibernation stages. Because female energetic needs shift throughout the active season from expenditure on reproduction to acquisition before hibernation (Kenagy et al. 1989), potentially changing the relationship between body mass and fat mass, we considered each circannual stage separately in linear regressions, and entered stage as a fixed factor in linear mixed models. We assessed fat stores of 23 adult female golden-mantled ground squirrels; seven were measured in a single circannual stage, six were measured in two circannual stages, eight were measured in three circannual stages, and two were measured in all four stages. Sample size varied by stage, and each female was included only once per stage for linear regressions. If females were measured more than once within the same stage, we randomly selected a single measurement from those taken in the same year ( $n = 5$  females), and considered measures to be independent if taken in different years ( $n = 2$  females) (Broussard et al. 2005). We also considered averaging measurements for the same female within a year, but the results were similar whether we averaged or chose measurements at random.

We used linear regression to evaluate the relationship between body mass and fat mass for each circannual stage, and also the relationship between mass-length residuals, calculated from regressing body mass on body length, and fat mass. In addition, because percent fat is sometimes used as a measure of body condition in hibernating squirrels (Barnes 1984; Nunes and Holekamp 1996; Neuhaus 2003) we performed the same regressions for percent fat as we did for fat mass.

Because our data contained substantial individual and annual variation in fat mass, which may confound relationships between condition indices and fat mass inferred through linear regression, we fitted linear mixed models with individual female identity and year as random effects, and circannual stage and condition index specified as fixed effects. We developed four models (one for each condition index) with fat mass as the response variable. Our simple condition indices were body mass (“mass”) and log-transformed body mass (“log-mass”). Our scaled condition indices were residuals from a regression of body mass on body length (“residuals”), and residuals from a regression of log-transformed body mass on log-transformed body length (“log-residuals”). We included all measurements ( $n = 61$ ) of the 23 adult females in this analysis.

We used a model comparison approach to evaluate the ability of each index to predict fat mass. We used 2<sup>nd</sup>-order Akaike information criterion corrected for small sample sizes ( $AIC_c$ ) to rank models, based on AIC differences ( $\Delta AIC_c$ ) and Akaike weights ( $w_i$ ; Anderson et al. 2001). All analyses were run in R version 3.0.2 (R Core Team 2013); we used the package *lme4* (Bates et al. 2013) to fit mixed models, *lmerTest* (Kuznetsova et al. 2013) to compute p-values, *rethinking* to compare models (McElreath 2013), and *ggplot2* (Wickham and Chang 2013) to plot results.

## 1.4 Results

Lean mass of adult female golden-mantled ground squirrels varied among circannual stages ( $F_{3,51} = 3.52$ ,  $p = 0.02$ ; Table 1), and was lowest at emergence from hibernation and highest before immergence. Fat mass also varied among circannual stages ( $F_{3,51} = 7.35$ ,  $p < 0.001$ ), and was lowest at emergence from hibernation and highest before immergence. Percent fat varied

among circannual stages ( $F_{3,51} = 5.9, p = 0.002$ ), and appeared stable throughout the first three stages before showing a sharp increase in the pre-hibernation stage.

Body mass explained a very high proportion of the variation (93-96%) in fat mass during the emergence ( $F_{1,10} = 143.4, p < 0.001$ ), post-reproduction ( $F_{1,14} = 230.6, p < 0.001$ ), and pre-hibernation stages ( $F_{1,10} = 271.8, p < 0.001$ ), but a lesser proportion (84%) during the reproduction stage ( $F_{1,13} = 67.91, p < 0.001$ ; Fig. 1). Correcting for structural size, as measured by head and body length, did not improve fit within any stage: the proportion of variation explained by mass-length residuals was less than that for the simple index based on body mass during the emergence ( $F_{1,10} = 13.51, p = 0.004$ ), reproduction ( $F_{1,13} = 30.73, p < 0.001$ ), and post-reproduction stages ( $F_{1,14} = 22.02, p < 0.001$ ), and equivalent to that explained by body mass during the pre-hibernation stage ( $F_{1,10} = 258.8, p < 0.001$ ).

Overall, a similar pattern was evident for the analysis based on percent fat. Body mass explained a moderate to high proportion of the variation in percent fat during the emergence ( $r^2 = 0.79, F_{1,10} = 37.33, p < 0.001$ ), post-reproduction ( $r^2 = 0.69, F_{1,14} = 31.32, p < 0.001$ ) and pre-hibernation stages ( $r^2 = 0.91, F_{1,10} = 106.6, p < 0.001$ ), but a lesser proportion during the reproduction stage ( $r^2 = 0.56, F_{1,13} = 16.55, p < 0.001$ ; Fig. 1). Correcting for structural size did not markedly improve fit within most stages, though mass-length residuals did explain a significant proportion of the variation in percent fat (emergence  $r^2 = 0.61, F_{1,10} = 15.8, p = 0.003$ ; post-reproduction  $r^2 = 0.46, F_{1,14} = 11.72, p = 0.004$ ; pre-hibernation  $r^2 = 0.91, F_{1,10} = 103.9, p < 0.001$ ; Fig. 1). Correcting body mass by body length improved model fit only in the reproduction stage ( $r^2 = 0.86, F_{1,13} = 24.48, p < 0.001$ ).

The linear mixed-effect model containing the simple index of body mass greatly outperformed models containing the simple index of log-transformed mass (log-mass) or scaled indices (residual or log-residual) (Table 2). The body mass model was ranked highest, and large  $\Delta AIC_c$  values for the other models suggested that they did not compare favorably; additionally, all of the Akaike weight ( $w_i$ ) was assigned to the model containing mass, indicating strong support in the data.

For the random effects in the highest ranked model (body mass), more variance in fat mass was attributable to differences among individuals (30%) than to differences among years (7%, Table 3); however, substantial variance (63%) remained unexplained. After controlling for individual and year effects, body mass still had a strong positive effect on percent fat: there was a small but precise influence of increased mass on increased fat mass, where fat mass increased 0.45 g for each additional gram of body mass ( $t = 26.07$ ,  $p < 0.001$ , Table 3). There were no significant effects of circannual stage on fat mass.

## **1.5 Discussion**

Our study faced two limitations, besides small sample sizes. First, we did not directly measure fat content of squirrels through destructive sampling and chemical extraction. Second, although the TOBEC method has been validated for golden-mantled ground squirrels (Pulawa and Florant 2000), we did not calibrate the TOBEC machine for our population of the species. TOBEC equations derived from one population have been successfully applied to new individuals in another rodent species (Dickinson et al. 2001), but the accuracy of TOBEC among populations

of ground squirrels is unknown. Consequently, our measurements of fat content are estimates only.

Adult female golden-mantled ground squirrels showed seasonal changes in body composition consistent with other hibernating squirrels, gaining both lean mass and fat mass during the active season (Kiell and Millar 1980; Rickart 1982). Ground squirrels appear to catabolize both lean and fat tissues to fuel hibernation, but restore these once the vegetative growing season begins (Jameson and Mead 1964; Kiell and Millar 1980; Pulawa and Florant 2000). A late season gain in fat mass is characteristic of hibernating species (Kunz et al. 1998; Buck and Barnes 1999; Hilderbrand 2000).

Our results suggest that body mass is a useful estimate of body condition during the critical periods of emergence from hibernation and pre-hibernation fattening, and perhaps during the post-reproductive period, supporting the use of body mass as a simple index to predict important components of fitness such as female reproductive success (Rieger 1996) and overwinter survival (Murie and Boag 1984). Body mass was less reliable during the reproductive stage, when females varied in their reproductive status. Because variation in reproductive status is associated with differences in body composition (Holekamp and Nunes 1989), combining non-reproductive, pregnant, and lactating females likely weakened the relationship between body mass and fat mass; this pattern was more pronounced when condition was defined as percent fat. In pregnant females, increased mass reflects additional lean fetal tissue instead of fat mass (Boswell et al. 1994). Reproductive females also vary in litter size (McKeever 1964), and hence fetal mass.

In general, our findings are consistent with those of previous studies: body mass explained 76% of the variation in fat mass in Belding's ground squirrels (*Urocitellus beldingi*) collected throughout the active season (Morton and Tung 1971), and 82% of the variation in percent fat in arctic ground squirrels (*U. parryii*) held in captivity (Lee et al. 2011).

Surprisingly, adjusting for structural size using body length did not improve estimates of body condition, except during the reproduction stage for estimates of percent fat. Body length and body mass were linearly related in our squirrels at emergence ( $r^2 = 0.55$ ,  $n = 12$ ,  $p < 0.01$ ), supporting the premise that greater mass in some stages was due, at least in part, to larger structural size; however, the relationship between body length and mass almost disappeared by the pre-hibernation stage ( $r^2 = 0.01$ ,  $n = 12$ ,  $p = 0.98$ ). Because the regression of body mass on length had a slope of zero in the pre-hibernation stage, and the magnitude of residuals was equal to relative body mass, the fit of mass-length residuals was identical to that of body mass in this stage.

We suggest three reasons why scaled indices may have performed poorly. First, measures of structural size may be particularly susceptible to measurement error (Yezerinac et al. 1992; Blackwell et al. 2006; Martin et al. 2013). Although we reduced this error by measuring body length on anesthetized squirrels, which are more amenable to measurement than active, unanesthetized squirrels, and this measurement displayed high repeatability within stage, some measurement error remained. Second, measures of structural size such as body length may sometimes be a poor indicator of lean mass that is not associated with energy storage; as with the relationship between body length and total mass noted above, the strength of the relationship between body length and lean mass declined over the active season (from  $r^2 = 0.64$  at emergence

to  $r^2 = 0.01$  before hibernation). Some individuals that emerged from hibernation with lower body mass than expected for their body size still had substantial fat stores; perhaps these individuals preferentially lost lean mass during hibernation (Pulawa and Florant 2000) in order to retain fat stores necessary for initiating reproduction. Finally, though body length commonly has been used in ground squirrel studies (Morton et al. 1974; Kiell and Millar 1980; Pulawa and Florant 2000), body length may simply be a poor measure of structural size. Other studies have used breadth across the zygomatic arches as a measure of structural size (Dobson et al. 1999; Viblanc et al. 2010). Zygomatic arch breadth has a significant but not especially strong linear relationship with body length for adult females of this species ( $r^2 = 0.44$ ,  $p < 0.01$ ,  $n = 18$ ; C.P. Wells, unpublished data), however, highlighting the uncertainty of using a single measure to quantify as complex a trait as structural size.

Log transformation of either mass or the scaled metrics did not improve estimates of body condition. Log transformation of mass linearizes the relationship between mass and percent fat, but did not make log-transformed mass a better predictor than mass alone. Log transformation of both mass and length standardizes measurements made on two different scales, and each unit residual from transformed measurements is standardized for individuals of different sizes; in this way, condition is not overestimated for smaller individuals and underestimated for larger individuals (Kotiaho 1999). However, log transformation did not increase the amount of variation in fat mass explained compared to untransformed measurements, which may explain why it is rarely used in studies of hibernating squirrels (but see Dobson 1992).

Body condition is an important trait in the life history of ground squirrels, but measuring condition directly requires sacrificing the animal. Our results suggest that the simple measure of

body mass is a useful indicator of body condition, especially early and late in the active season, and that scaled indices do not improve on mass estimates during most stages in the circannual cycle.

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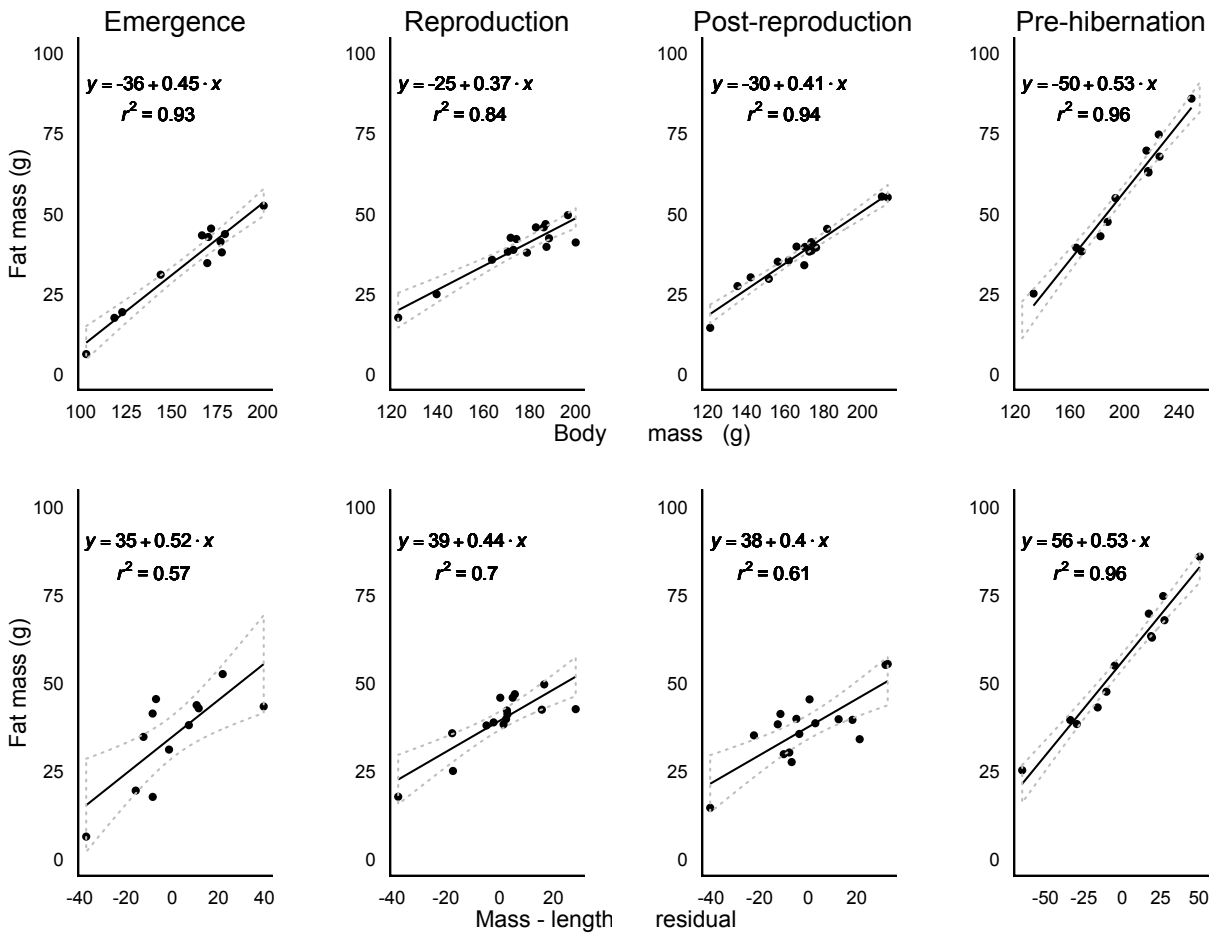
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## 1.7 Figures

**1.7.1** Linear relationships between golden-mantled ground squirrel (*Callospermophilus lateralis*) total body mass and fat mass (top row), and mass-length residuals and fat mass (bottom row), by circannual stage (columns).



Note: Dashed lines represent a 95% confidence interval (two standard errors).

## 1.8 Tables

**1.8.1** Mean body composition ( $\pm 1$  S.E.) of adult female golden-mantled ground squirrels (*Callospermophilus lateralis*) near Quincy, California, from 2003-2005, by circannual stage.

|                           | <b>Emergence</b>    | <b>Reproduction</b>  | <b>Post-reproduction</b> | <b>Pre-hibernation</b>     |
|---------------------------|---------------------|----------------------|--------------------------|----------------------------|
|                           | May 15 –<br>June 15 | June 16 –<br>July 31 | August 1 –<br>August 31  | September 1 –<br>October 1 |
| <i>n</i>                  | 12                  | 15                   | 16                       | 12                         |
| <b>Mean lean mass (g)</b> | 124 $\pm$ 4.8       | 135 $\pm$ 3.4        | 130 $\pm$ 3.6            | 142 $\pm$ 4.5              |
| <b>Mean fat mass (g)</b>  | 35 $\pm$ 3.9        | 39 $\pm$ 2.1         | 38 $\pm$ 2.5             | 56 $\pm$ 5.1               |
| <b>Mean percent fat</b>   | 21 $\pm$ 1.7        | 22 $\pm$ 0.8         | 22 $\pm$ 0.8             | 28 $\pm$ 1.4               |

**1.8.2** Comparison of linear mixed models predicting fat mass of adult female golden-mantled ground squirrels, with two simple condition indices (mass, log-mass) and two scaled condition indices (residuals, log-residuals) used as predictor variables.

| Predictor    | k | AIC <sub>c</sub> | $\Delta\text{AIC}_c^a$ | $w_i^b$ |
|--------------|---|------------------|------------------------|---------|
| Mass         | 8 | 349.25           | 0.00                   | 1.00    |
| Log-mass     | 8 | 379.08           | 29.83                  | 0.00    |
| Residual     | 8 | 419.31           | 70.07                  | 0.00    |
| Log-residual | 8 | 432.29           | 83.05                  | 0.00    |

<sup>a</sup> Difference from best model in set. Models with  $\Delta\text{AIC}_c < 2$  are considered with the best model, and models with  $\Delta\text{AIC}_c > 10$  are not supported.

<sup>b</sup> Akaike weight.

### 1.8.3 Parameter estimates from the best-fit model<sup>a</sup> in Table 1.7.2

| Fixed Effects     | Coef $\pm$ 1 S.E  | t-value             |
|-------------------|-------------------|---------------------|
| Intercept         | -36.21 $\pm$ 2.93 | 12.32*              |
| Reproduction      | -1.88 $\pm$ 1.10  | -1.71               |
| Post-reproduction | -1.29 $\pm$ 1.21  | -1.06               |
| Pre-hibernation   | 2.50 $\pm$ 1.58   | 1.58                |
| Body Mass         | 0.45 $\pm$ 0.02   | 26.07*              |
| Random Effects    | Variance          | Variance Proportion |
| Female (n=23)     | 3.52              | 0.30                |
| Year (n=3)        | 0.80              | 0.07                |
| Residual          | 7.54              | 0.63                |
| Total             | 11.84             |                     |

<sup>a</sup> Analyzed as linear mixed model in the form Fat mass =  $\alpha + \beta$ Body Mass +  $\beta$ Stage + Female + Year, where Body mass was fitted as a fixed effect, Stage (circannual stage) was fitted as a fixed effect, and Female and Year were fitted as random effects.

Note: \*,  $P < 0.001$

## CHAPTER 2: DEVELOPMENTAL AND SOCIAL CONSTRAINTS ON EARLY REPRODUCTION IN AN ASOCIAL MAMMAL

### 2.1 Abstract

For short-lived species, selection for early reproduction should be strong, yet females often delay reproduction beyond what appears to be optimal. Delay in age of first reproduction due to developmental constraints, such as food availability, or social constraints, such as the inhibitory presence of breeding adults, has been documented for social ground-dwelling squirrels, but their effects on the reproductive maturation of asocial ground squirrels are unknown. We used 21 years of demographic data from a free-living population of golden-mantled ground squirrels to evaluate the developmental and social factors that may constrain early reproduction in this short-lived, asocial species. We found that females who would reproduce as yearlings were weaned earlier in the summer than females who waited until age 2 to reproduce, and that early weaning was associated with higher growth rate during the natal summer. Females were more likely to reproduce as yearlings when there were more adult males present, possibly because exposure to males accelerates reproductive maturity or due to the increased likelihood of conception with more male mates. Maternal presence had no facilitating or inhibitory effect on yearling reproduction, but yearlings with a littermate sister present were only 22% as likely to reproduce as females without a sister present. Competition among siblings before or after weaning may reduce growth rates in female rodents, and if golden-mantled ground squirrel sisters remain in close proximity in their natal range, developmental delays may extend to delays in reproductive maturation.

## 2.2 Introduction

The age at which females first reproduce is an important component of vertebrate life-histories, with effects on individual fitness (Lewontin 1965, Oli et al. 2002), population dynamics (Cole 1954, Tkadlek and Zejda 1995, Dobson and Oli 2001), and social organization (Armitage 1981, Bekoff et al. 1981). Selection for early reproduction should be strong when lifespans are short (Stearns 1992), because early-maturing individuals are more likely to successfully reproduce before they die, and this expectation is well supported among mammals (Promislow and Harvey 1990, Fisher et al. 2001). However, considerable variation exists in timing of first reproduction even within short-lived species (Bronson and Rissman 1986), and individual females may delay reproduction beyond what appears to be optimal (*sensu* Stearns 1992). Despite substantial theoretical work in this field, our understanding of the proximate factors that may constrain optimal early reproduction is incomplete.

Optimal early reproduction may be constrained by environmental or social conditions that delay reproductive maturity. In small mammals, the timing of reproductive maturity is controlled by the hypothalamic-pituitary-gonadal (HPG) axis, which is sensitive to energetic and social factors (Adkins-Regan 2005): limited energy availability may preclude ovulation (Bronson 2000), and the presence of conspecific adults can inhibit (Whitten 1959, Drickamer 1977) or facilitate (Vandenbergh 1967) female maturation. In addition, characteristics of the natal litter can slow the rate of juvenile development: individuals from larger litters tend to exhibit lower mass at weaning (Phillips 1981), which may delay maturation, and females from male-biased litters, exposed to fetal androgens produced by their male siblings, tend to delay reproduction (Ryan and Vandenbergh 2002). Among ground squirrels, the Armitage (1981) model of sociality emphasizes the limited energy available in highly seasonal environments, which can delay

reproductive maturity, and this link is supported by several studies of food availability and maturation in ground squirrels (Dobson and Kjelgard 1985, Ritchie and Belovsky 1990, Neuhaus et al. 2004). Among long-lived, social ground-dwelling squirrels, male-bias in the natal litter has been implicated in delayed reproduction (e.g. in yellow bellied marmots, *Marmota flaviventris*, Monclus et al. 2012, Monclus et al. 2014), and so has the presence of related females and adult males in the social environment (in black-tailed prairie dogs, *Cynomys ludovicianus*, Shier 2006; Cape ground squirrel, *Xerus inaurus*, Pettitt and Waterman 2011). However, the effects of natal sex ratios and features of the social environment on the reproductive maturation of short-lived, asocial ground squirrels are unknown.

We used 21 years of demographic data from a free-living population of golden-mantled ground squirrels to evaluate the developmental and social factors that may constrain early reproduction in a short-lived, asocial ground squirrel. The golden-mantled ground squirrel is a hibernating species that usually reaches reproductive maturity at age 1 year, but is less likely to do so in high-elevation environments with a short active growing season (Bronson 1979). If exposure to fetal androgens in utero or neonatal competition for maternal resources affected female development, we expected that females that were born into male-biased litters, or showed a low mass at weaning, would be less likely to breed as yearlings. Golden-mantled ground squirrels are primarily herbivorous, and availability of green vegetation for food begins at weaning and ends at the first killing frost during late summer (Van Vuren and Armitage 1991); further, summer rainfall may reduce aboveground activity, hence foraging opportunities (Kneip et al. 2011). Consequently, we expected that females that were weaned later than other females, experienced extensive summer rainfall, or encountered an early killing frost, would be less likely to breed at age 1 year. Finally, because growth of vegetation the following spring is initiated by

snow melt, delayed snow melt when females emerge from hibernation as yearlings could reduce the likelihood of reproduction.

Female golden-mantled ground squirrels tend to live solitarily, but can be in close proximity to both unrelated and related females (Wells 2016). In rodents, the presence of adult females can delay reproductive maturation in young females (Drickamer 2007). However, it is unclear whether it is related females, particularly the mother, or unrelated females that suppress reproduction: in some species the presence of female kin inhibits reproduction of young females (Clark and Galef 2001, Pettitt and Waterman 2011), and in other cases it does not (Wolff et al. 2001, Schradin and Pillay 2014). We expected that either the presence of unrelated females, or the presence of related females (including mother and sisters), would reduce the likelihood of golden-mantled ground squirrels reproducing at age 1. Additionally, due to substantial variation in annual population density (Kneip et al. 2011), female golden-mantled ground squirrels may be exposed to many or few potential male mates. The presence of adult males has been shown to accelerate reproductive maturity in female mice (Drickamer and Mikesic 1990) and voles (Batzli et al. 1977, Carter et al. 1980). If a scarcity of males can delay female reproduction, either by delaying reproductive maturity or by decreasing the likelihood of conception, we expected that in years of low density of adult males, or a low ratio of adult males to females, yearling females would be less likely to reproduce.

## **2.3 Methods**

### *2.3.1. Study area and species*

We studied golden-mantled ground squirrels at the Rocky Mountain Biological Laboratory (2900m), in the East River Valley, Gunnison County, Colorado (38°58'N, 106°59'W), from

1995-2015. A detailed description of the study site can be found in McEachern et al. (2011). We live-trapped squirrels using Tomahawk traps baited with black-oil sunflower seeds and peanut butter, and marked individuals with numbered eartags for permanent identification and a unique pattern of black Nyanzol dye on the dorsal pelage for visual identification. At capture, we recorded mass (to the nearest g), location, and reproductive status (positive for females with swollen nipples and for males with scrotal testes). We censused the population each year beginning in late May, after squirrels emerged from hibernation, and continued until all individuals were identified and marked. Females give birth to a single litter per year, and nurse pups underground for approximately 30 days (Phillips 1981). We trapped litters as they emerged aboveground at weaning during June and July, and recorded emergence date, weight, and sex of each pup. We attempted to trap and weigh all squirrels each month until squirrels immersed for hibernation during August and September.

### *2.3.2 Statistical analysis*

We defined “early reproduction” in this study as positive reproductive status at one year of age, regardless of whether the female successfully weaned a litter. We excluded females for which reproductive status as a yearling was uncertain (i.e. yearling females who died or dispersed before reproductive status could be assessed). We included immigrant females who settled as juveniles at the end of their natal summer and could be confidently aged by body weight. Our final dataset included 93 yearling females, observed in 1995-2015; no juvenile females survived their first hibernation in 1999 and 2001, so those years are not represented in the dataset. We used the yearly census data to determine number of adult males, breeding females, and operational sex ratio (number of breeding females per adult male). We reasoned that if exposure to the sight or smell of males affected reproductive maturity, then a higher number of adult males

would predict early reproduction, whereas if additional mating opportunities increased likelihood of conception, a lower ratio of females to males (i.e. an increase in the relative number of males) would predict early reproduction. We used litter data to determine litter sex ratios, weight at weaning, and female kin relationships (mother-daughter, littermate sisters) of yearling females, and matriline pedigrees constructed from litter data to identify all female kin for each yearling female (Wells 2016).

To explore the effect of rainfall during the natal summer on individual females, we obtained rainfall records for Gothic, CO (weather station GTH161, <http://www.epa.gov/castnet>). We used total amount of rain (mm) that fell between emergence of each female from the natal burrow and the date of the first hard freeze, defined as the first date on which temperatures fell below -2°C on two consecutive days.

To model likelihood of early reproduction (0 or 1), we fit Bayesian generalized linear mixed effects models (bglmer, package bmle, Dorie 2015), with a binomial error structure and logit link. We did not have complete data for all females, so for each group of hypotheses (developmental constraints, social environment), we constructed an *a priori* model of factors affecting yearling reproduction. For developmental constraints, we modeled likelihood of yearling reproduction as a function of date of emergence from natal burrow, weight at emergence from natal burrow (g), natal litter sex ratio (ratio of male/female pups), natal summer rainfall (mm), and onset of spring the next year (defined as date of bare ground in the yearling spring). For our social environment analysis, we used population level factors for male presence, because males are not territorial and roam throughout the entire study site (C. P. Wells, unpublished data), and local level factors for female presence, because females are thought to be territorial (Michener 1983) and at our study site occur in one of six localities containing persistent burrow

systems. Localities contained both related and unrelated females, with 1-6 breeding females per locality (Wells 2016). We modeled likelihood of yearling reproduction as a function number of adult males (2 years and older; yearling males rarely breed, Wells 2016), operational sex ratio, maternal presence, littermate sister presence, number of related breeding females, and number of unrelated breeding females. We specified “year” as a random effect with Wishart covariance prior in both models, to account for cohort effects and unspecified interannual variation in environmental conditions.

We lacked data on late-summer weight for sufficient females to be able to include pre-hibernation weight as a fixed effect in our developmental model. However, to determine if females weaned late could compensate with higher growth rates, we calculated growth rate beginning at weaning for a subset of juvenile females for which we had weight measurements for late August or early September. We calculated the average growth rate for these females (g/day), and fit a linear model to determine how rate of weight gain varied with date of natal emergence.

## **2.4 Results**

Overall, 44% of 93 yearling female golden-mantled ground squirrels became reproductive. Emergence date had a significantly negative effect on the likelihood of early reproduction ( $\beta = -0.17 \pm 0.06$ ,  $z = -2.67$ ,  $p = 0.008$ ; Figure 1): the date of weaning for females who would reproduce as yearlings was 5 July ( $\pm 10.8$  days), compared to 12 July ( $\pm 10.9$  days) for females who would not reproduce. There was no significant effect of emergence weight ( $\beta = 0.06 \pm 0.04$ ,  $z = 1.64$ ,  $p = 0.102$ ) or natal sex ratio ( $\beta = -0.90 \pm 0.64$ ,  $z = -1.42$ ,  $p = 0.157$ ). The amount of rainfall during the natal summer ( $\beta = -0.02 \pm 0.01$ ,  $z = -1.59$ ,  $p = 0.113$ ) did not significantly affect likelihood of early reproduction, and neither did the date of the first hard freeze ( $\beta = 0.00 \pm$

0.04,  $z = 0.11$ ,  $p = 0.915$ ). However, the effect of the date of bare ground in the yearling spring was negative and approached significance ( $\beta = -0.08 \pm 0.04$ ,  $z = -1.89$ ,  $p = 0.059$ ). Rate of weight gain during the natal summer declined significantly with later emergence from the natal burrow ( $\beta = -0.04 \pm 0.01$ ,  $F_{1,27} = 16.01$ ,  $p < 0.001$ ,  $R^2 = 0.35$ ; Figure 2).

Number of adult males had a significantly positive effect on likelihood of early reproduction ( $\beta = 0.33 \pm 0.25$ ,  $z = 2.20$ ,  $p = 0.028$ ; Figure 3), but operational sex ratio had no significant effect ( $\beta = 0.49 \pm 0.51$ ,  $z = 0.95$ ,  $p = 0.340$ ). Maternal presence ( $\beta = 0.32 \pm 0.66$ ,  $z = 0.49$ ,  $p = 0.624$ ), number of related breeding females ( $\beta = 0.15 \pm 0.25$ ,  $z = 0.57$ ,  $p = 0.566$ ), and number of unrelated breeding females ( $\beta = -0.10 \pm 0.21$ ,  $z = -0.47$ ,  $p = 0.640$ ) had no significant effect. However, the local presence of a littermate sister had a strong negative effect on the likelihood of early reproduction ( $\beta = -1.52 \pm 0.70$ ,  $z = -2.15$ ,  $p = 0.031$ ).

## 2.5 Discussion

In our population of golden-mantled ground squirrels, less than half of females first reproduce as yearlings, and this reproductive timing appears to be influenced both by developmental and social constraints. Characteristics of the natal litter might affect early reproduction, but neither of the characteristics we tested – weight at weaning or litter sex ratio – had a significant effect. Growth rates among pups born at the same time are variable (C. P. Wells, unpublished data), hence perhaps the weight differential at weaning was inconsequential by the time of entry into hibernation. Evidence from laboratory rodents shows that natal sex ratio affects development by influencing the local uterine environment; females can become “masculinized” via exposure to the androgens produced by adjacent male fetuses (Ryan and Vandenberg 2002). The lack of an effect in golden mantled ground squirrels may be due to variation in intrauterine position: a

female fetus developing between two brothers will experience a more androgen-rich local environment, with greater “masculinizing” consequences, than will a female with one or no adjacent brothers (Ryan and Vandenberg 2002).

In free-living rodents, female reproductive maturity depends at least in part on food availability: for example, supplemental feeding accelerates reproductive maturity in female tropical mice (*Peromyscus mexicanus*, 219 days to 109 days, Duquette and Millar 1995) and Columbian ground squirrels (*Urocitellus columbianus*, from age 2 to age 1, Dobson and Kjelgaard 1985). Food availability may be limited for golden-mantled ground squirrels living at high elevations, where the vegetative growing season is short and green forbs (which make up much of their diet) begin to senesce by late summer. Hence, the number of days available for foraging before winter may determine the amount of food available for developing juveniles in their natal summer (Armitage 1981, Ritchie and Belovsky 1990). Accordingly, female Columbian ground squirrels exposed to longer periods of plant growth were more likely to reproduce as yearlings (Ritchie and Belovsky 1990). Consistent with this expectation, we found that early emergence from the natal burrow had a strong positive effect on the likelihood of early reproduction in golden-mantled ground squirrels. Further, late-emerging females were unable to compensate with higher growth rates, and in fact showed lower growth rates than early-emerging females, perhaps because of lower quality of forage late in the season. Hence, delayed emergence from the natal burrow results in both less time to forage and reduced forage quality, thereby delaying development and reproductive maturity until after the yearling spring.

Contrary to our expectation, amount of rainfall during the period of independent foraging did not reduce the likelihood of yearling reproduction. This result appears inconsistent with that of Kneip et al. (2011), who found a negative effect of summer rainfall on reproduction the next year

for yearling females in the same population. However, the differing outcomes likely result from the different time periods examined by each study; Kneip et al. (2011) considered only early-season rainfall, during June and July. Perhaps early-season rainfall affects reproduction of young females indirectly, by affecting their mothers before pups are weaned. Lactation in ground squirrels is fueled by daily food intake (Kenagy 1989), and heavy rains that limit foraging during lactation may reduce the amount of energy available to nursing pups. Reductions in milk quality or amount may slow pup development, with delays that carry over into the period of post-weaning growth and subsequent timing of reproductive maturity (Lindstrom 1999).

Longer growing seasons have been associated with increased body mass and population growth of yellow-bellied marmots (Ozgul et al. 2010), and earlier age of reproduction in golden-mantled ground squirrels (Bronson 1979). We tested two factors that influence growing season length, and hence duration of food availability: date of the first hard freeze in the natal summer, and date of the first bare ground in the yearling spring. Interestingly, only the latter was associated with early reproduction. While a later hard freeze in the natal summer should increase the number of days that vegetation is available to growing juveniles, a decline in vegetation quality late in the season may render that benefit inconsequential. By contrast, earlier onset of vegetation growth in the yearling spring, when vegetation quality is high, may allow young females to gain additional weight immediately after hibernation and reach reproductive maturity with enough time left in the season to reproduce.

High density of breeding males could contribute to the early reproduction of female golden-mantled ground squirrels in at least two ways, by advancing reproductive maturity or by increasing the likelihood of conception. The presence of breeding males has been shown to accelerate reproductive maturity in female rodents (Batzli et al. 1977, Carter et al. 1980,

Drickamer and Mikesic 1990), through exposure to the sight or scent of males (Drickamer 2007). Specifically, at least in the prairie vole (*Microtus ochrogaster*), female inhalation of chemosignals in male urine triggers an increase in leutenizing hormone, bringing females into estrus (Carter et al. 1980). In golden-mantled ground squirrels, we found that the number of adult males had a positive effect reproduction by yearling females, although the ratio of males to females did not. During the breeding season, adult males routinely leave scent marks on rocks, stumps, and other often-used perches within female home ranges (Wells, pers. obs.); chemosignals from these marks or from urine may function to accelerate reproductive maturity in females. Alternatively, yearling females may be reproductively mature, but not have as many opportunities for mating when males are scarce. In Columbian ground squirrels, males spend less time copulating with yearling females than older females, and seek them out less frequently, suggesting that they are less preferred as mates (Murie 1995). Similarly, yearling golden-mantled ground squirrels mate with dominant males less often than do older females (Wells 2016), and combined with potentially lower fertility (Wells 2016), may be at a disadvantage in conceiving a litter when males are limited.

Social inhibition of reproduction is widespread among group-living species, where dominant females reduce competition by suppressing the reproduction of subordinate females (Stockley and Bro-Jorgenson 2011), including often their own daughters (Wasser and Barash 1983, Shier 2006, Pettit and Waterman 2011). Conversely, maternal facilitation of reproduction may occur in other species, where adult females can advantage their daughters by protection or cooperation. In Columbian ground squirrels, for example, maternal presence was associated with higher reproductive output of the mother's early-maturing daughters (Neuhaus et al. 2004). Female golden-mantled ground squirrels tend to be philopatric, and hence are often found in close

proximity to their mother. However, maternal presence did not affect the likelihood of early reproduction in golden-mantled ground squirrels, neither delaying nor advancing the timing of daughter reproduction, and neither did the density of related or unrelated females. Instead, it was the local presence of a littermate sister that inhibited early reproduction: yearling females with a sister present were only 22% as likely to reproduce compared to yearling females without a sister present. Competition among neonate siblings for limited maternal resources is assumed to be universal in polytocous species (Drummond 2006), and may persist even after weaning: young female California (*Microtus californicus*) and prairie voles housed with littermate sisters, or simply exposed to their scent, had lower growth rate than females housed with nonsibling males (Batzli et al. 1977). If sisters remain in close proximity in their natal range, developmental delays may extend to delays in reproductive maturation.

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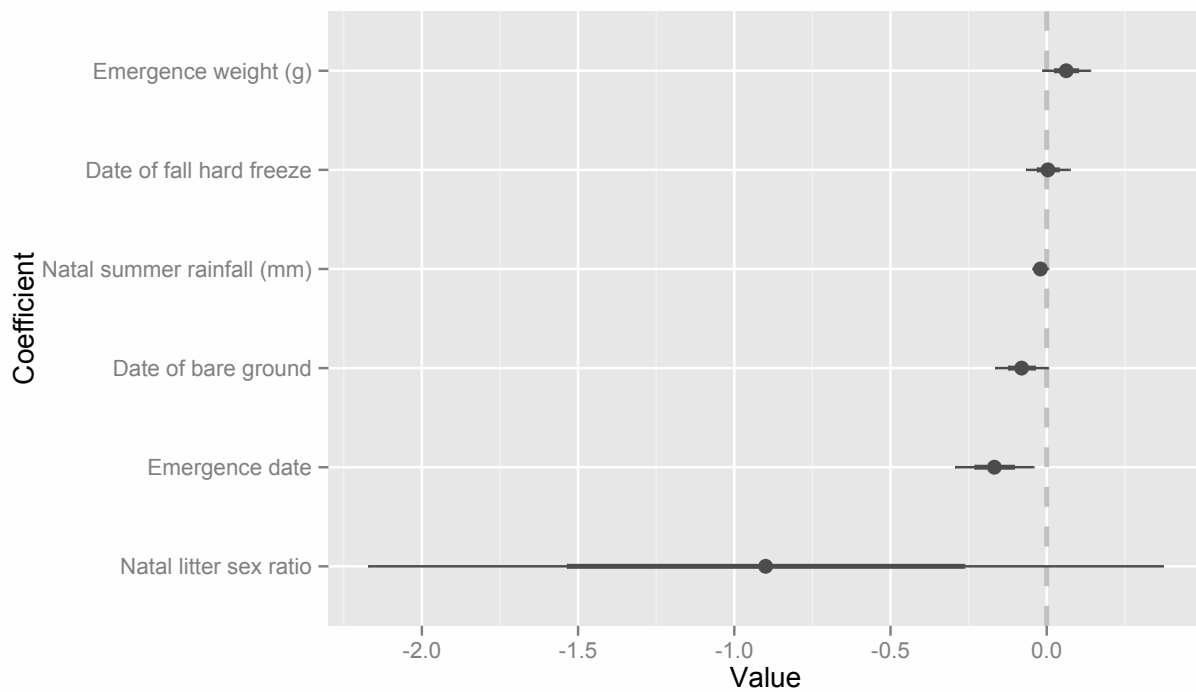
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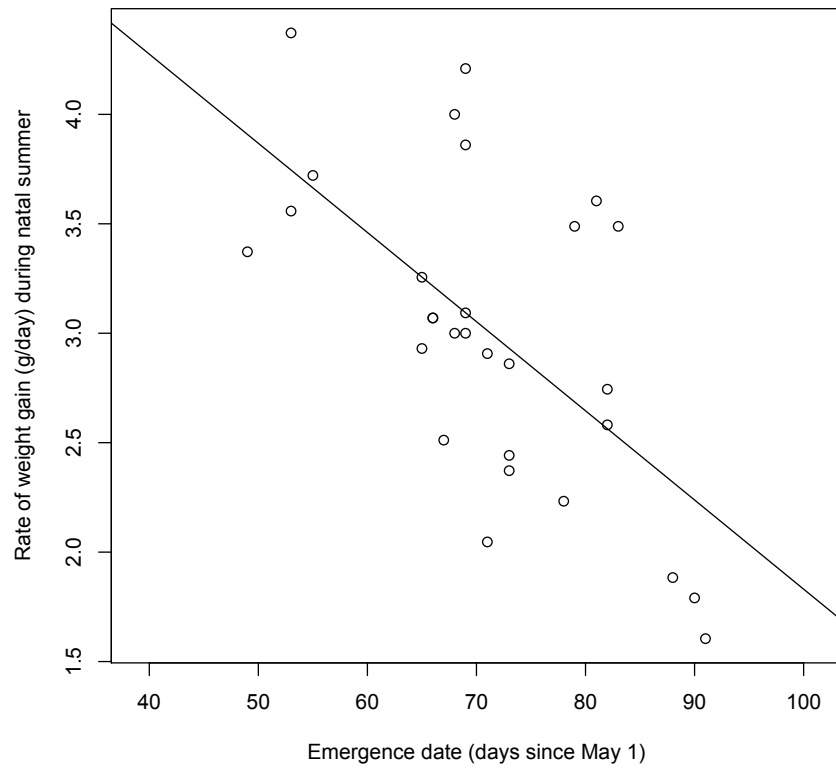
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## 2.7 Figures

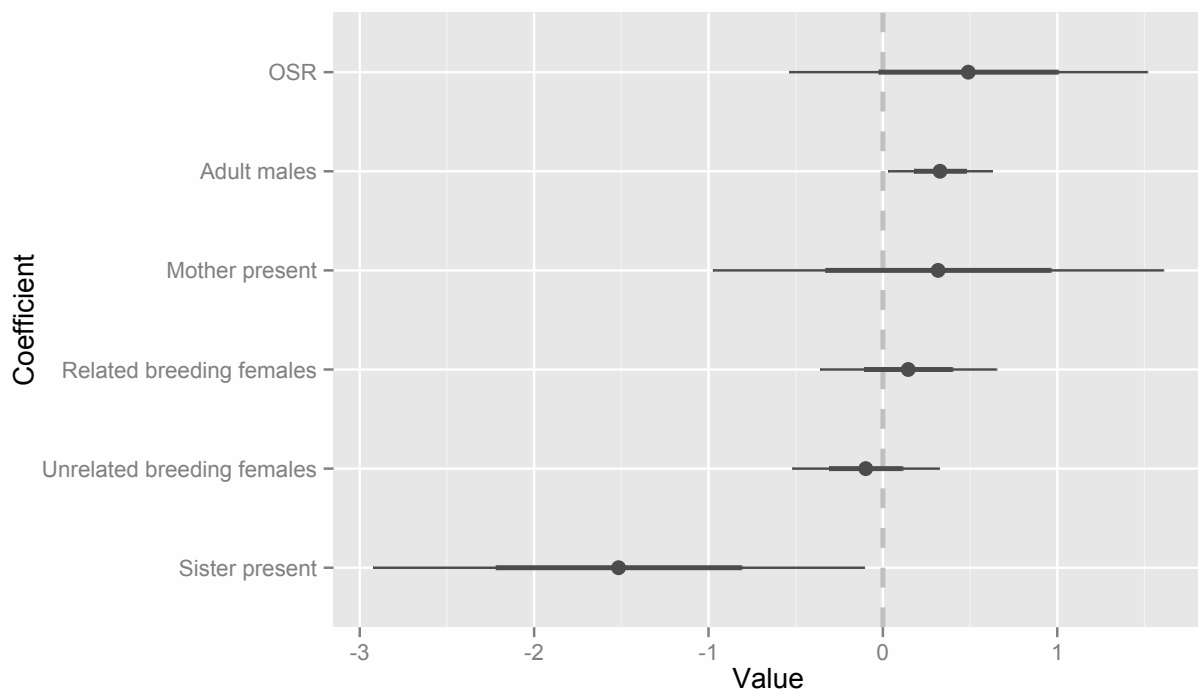
**2.7.1** Coefficient estimates for fixed effects in the developmental constraints model. Effects are ordered by magnitude of their effect on likelihood of a female reproducing (or not) as a yearling. Dots represent point estimates, thick lines represent inner confidence interval (one standard error), and thin lines represent outer confidence interval (two standard errors). Positive values indicate an increase in log-odds of early reproduction, negative values indicate a decrease in log-odds of early reproduction.



**2.7.2** Linear regression of emergence date on rate of weight gain (fall weight-emergence weight/days between measurements).



**2.7.3** Coefficient estimates for fixed effects in the social environment model. Effects are ordered by magnitude of their effect on likelihood of a female reproducing (or not) as a yearling. Dots represent point estimates, thick lines represent inner confidence interval (one standard error), and thin lines represent outer confidence interval (two standard errors). Positive values indicate an increase in log-odds of early reproduction, negative values indicate a decrease in log-odds of early reproduction.



## CHAPTER 3: DETERMINANTS OF MULTIPLE PATERNITY IN A FLUCTUATING POPULATION OF GROUND SQUIRRELS

### 3.1 Abstract

Multiple paternity is common in vertebrates that produce several offspring in the same reproductive bout, but the rate often varies among and within populations. Three primary explanations for this variation have been advanced: null models based on encounter rate of mates, socioecological models dependent on the ability of males to monopolize females, and age- or condition-dependent models of female choice. We used 18 years of genetic and demographic data to examine the mating system and patterns of multiple paternity in a free-living population of golden-mantled ground squirrels (*Callospermophilus lateralis*). The mating system was polygynandrous, but opportunity for sexual selection was lower for females than for males. Annual reproductive success of males was low for yearlings and new immigrants, and increased with breeding tenure in the population. Multiple paternity was evident in 62% of litters. We found little support for the null model of multiple paternity, but in accordance with the socioecological model of male monopolization, rates of multiple paternity decreased with female spatial clustering, unless male-male competition, as indicated by male density, was also high. From Bateman gradients, we found no fitness benefit of multiple paternity for females, and in support of the female choice model, multiple paternity decreased with maternal age and peri-oestrous mass. Together, our results suggest that variation in the rate of multiple paternity in golden-mantled ground squirrels was determined primarily by the active strategies of males and females. High rates of multiple paternity in high-density years may increase genetic diversity during population peaks, particularly in years when more yearling females reproduce.

### 3.2 Introduction

Female vertebrates often mate with multiple males, resulting in multiple paternity within their clutch or litter (Jennions and Petrie 2000; Slatyer et al. 2012). While rates of multiple paternity appear quite stable in some species (Murie 1995; Naim et al. 2011; Thonhauser 2013; Sommaro et al. 2015), other species show substantial variation among populations (Dean et al. 2006) or among years within populations (Schwagmeyer and Brown 1983; Jones et al. 2012). Several explanations have been proposed for within-population variation in multiple paternity, both environmental and social, but the causes remain poorly understood (Solomon and Keane 2007).

Multiple mating by females may be simply a matter of opportunity, varying with the rate at which females encounter potential mates. Both increases in male density and increases in male-bias in the operational sex ratio (number of adult males per adult female, Emlen and Oring 1977) increase the number of males that females might encounter (Kokko and Rankin 2006), and barriers to male movement during the breeding season, such as spring snowfall, may decrease that number (Michener and McLean 1996; Bergeron et al. 2011; Martin et al. 2014). This scenario, in which the rate of multiple mating scales with encounter rate, has been considered the null model of multiple mating (Kokko and Rankin 2006); we will refer to it as the “encounter rate” model. Alternatively, multiple paternity may result from the active mating strategies of males or females. Males may attempt to maximize paternity by monopolizing receptive females; if so, multiple mating by females is expected to be most common when they are least monopolizable, such as when females become spatially dispersed (Emlen and Oring 1977), their receptivity becomes temporally clustered (Isvaran and Clutton-Brock 2007), or both (Emlen and Oring 1977, Shuster and Wade 2003). We will refer to this scenario as the “male monopolization” model. On the other hand, females may seek multiple paternity according to

the costs and benefits of multiple mating. Females may choose to mate multiply to gain direct (Wolff and MacDonald 2004; Hoogland 2013) or indirect benefits (Eberhard 1996; Zeh and Zeh 1997; Jennions and Petrie 2000) that enhance reproductive success. One of the proposed direct benefits of multiple mating, enhanced fertility, should be particularly important for species in which the costs of reproductive failure are high, such as those who face a short breeding season, produce only one litter, or have low survival probability. Additionally, the costs and benefits of mate choice, including multiple mating, likely vary according to a female's age, physical condition, or genetic diversity (Cotton et al. 2006). Young females with higher associated reproductive value may be more selective than older females (Kodric-Brown and Nicoletto 2001, Atwell and Wagner 2014), and hence reduce multiple mating. Females in poor physical condition may reduce multiple mating in order to minimize the costs associated with mating, such as disease transmission or enhanced vulnerability to predation (Daly 1978; Magnhagen 1991). Females with low genetic diversity may require multiple sires to produce genetically diverse offspring and avoid genetic incompatibilities that impair fitness (Jennions and Petrie 2000; Burdfield-Steel et al. 2015). This scenario, in which multiple paternity varies with maternal characteristics, we call the “female choice” model.

Multiple paternity has the potential to shape the strength of sexual selection. The opportunity for sexual selection is determined by intrasexual variation in the number of offspring produced; the strength of sexual selection is often defined as the slope of the linear relationship between offspring produced and number of mates obtained, known as Bateman's gradient (Bateman 1948, Jones et al. 2002). In vertebrates, it is assumed that sexual selection acts more strongly on males (Andersson and Iwasa 1996). However, this may not be the case in polygynandrous populations if variation in reproductive success is similar between the sexes. Specifically, if females gain

direct benefits from multiple mating, in the form of higher reproductive success (Jennions and Petrie 2000, Munroe and Koprowski 2011, Hoogland 2013), then high rates of multiple paternity can produce strong and potentially divergent selection on mating tactics of males and females (Munroe and Koprowski 2011).

Female vertebrates appear to mate multiply under many systems of social organization. Social monogamy is typical in avian species, yet female birds frequently produce multiply-sired clutches (Arct et al. 2015), and female mammals of socially monogamous species mate with multiple males as often as those of socially polygynous species (Solomon and Keane 2007).

Among mammals, multiple paternity is common in ground-dwelling squirrels, and small-bodied ground squirrels in particular exhibit polygynandrous mating systems in which males mate with multiple females and females mate with multiple males (Munroe and Koprowski 2011). We used data from a long-term study of golden-mantled ground squirrels (*Callospermophilus lateralis*) to investigate intrapopulation variation in rate of multiple paternity. The mating system of golden-mantled ground squirrels is unknown, but limited spatial overlap among home ranges of adult females (Jesmer et al. 2011) suggests that potential for polygyny is low (Emlen and Oring 1977). However, spatial clustering of females appears to increase with population density (Wells 2016), potentially generating interannual variation in mating patterns with changes in density.

We characterized the mating system of golden-mantled ground squirrels in order to estimate the opportunity for sexual selection and Bateman gradient for each sex, quantify the rate of multiple paternity, and assess factors influencing the frequency of multiple paternity. We tested two predictions based on encounter rate, that multiple paternity would increase when male density or OSR was high, and decrease when weather conditions at mating were unfavorable. We tested two predictions about the effect of male monopolization, that multiple paternity would decrease

when female breeding synchrony was high, and when female density, and presumably spatial overlap of females, was high. We tested three predictions based on female choice, that multiple paternity would be higher for mothers at the age of first reproduction (1 year) than for older mothers, higher for mothers in better condition, and higher for mothers who were less genetically diverse.

### **3.3 Methods**

#### *3.3.1 Study site and species*

We studied golden-mantled ground squirrels in the East River Valley of Gunnison County, Colorado (38°58'N, 106°59'W), at the Rocky Mountain Biological Laboratory (2900 m), from 1996 – 2015. Golden-mantled ground squirrels are a small-sized (150-300 g) diurnal species, and are considered asocial (Michener 1983; Ferron 1985; Bartels and Thompson 1993). At our study site, adult ( $\geq 1$  year old) squirrels were active above ground between late April and early September. The breeding season began shortly after females emerged from hibernation in April or May and encountered scrotal males; breeding ended usually by late May. Females gave birth to a single litter per year, after approximately 28 days gestation (McKeever 1964). Litters emerged from their natal burrows at weaning (approximately 30 days after birth, Phillips 1981), in June or July. Juvenile dispersal, which was male-biased, began in August; adult immigrants of both sexes appeared in all months of the active season. Adults began to immerge for hibernation in August, juveniles in September, and all squirrels were below ground by October. Female activity was generally concentrated on rocky slopes and in dry meadows, in one of six localities containing persistent burrow systems (Wells 2016). Density of adults at our 13-ha site ranged from 1-3 squirrels/ha among years (Kneip et al. 2011), with 1-6 breeding females per locality (Wells 2016).

We captured squirrels using Tomahawk live traps (Model 201) set in areas of squirrel activity and baited with peanut butter and sunflower seeds; our trapping protocol was approved by Animal Care and Use Committees of the University of California, Davis, and the Rocky Mountain Biological Laboratory, and adhered to guidelines approved by the American Society of Mammalogists (Sikes and Gannon 2011). We attempted to capture adults monthly. Pups were captured at weaning as they emerged from their natal burrows from mid June to mid July, and again before immergence in September; mothers typically were trapped with their pups as the litter emerged. Immigrants were captured as they appeared in the population, and again before immergence in September. At capture, we recorded mass (to the nearest 1g), sex (anogenital distance), and reproductive status. Age was known for individuals who were born into the population, or who immigrated into the population as juveniles. We marked each squirrel with numbered metal eartags for permanent identification, and with black fur dye for identification during behavioral observations. We plucked hairs from the rump of each animal, and stored the hairs in a dry envelope at room temperature until the end of the field season, when they were transferred to a chest freezer (0°C) until genetic analysis.

### *3.3.2 DNA extraction and genotyping*

Squirrels captured 1996-2005 were genotyped as described in McEachern et al. (2011). Usable DNA could not be obtained from hair samples collected in 2006 and 2007. Squirrels captured 2008-2015 were genotyped according to the following protocol. Hairs were individually inspected for the presence of a tissue bulb, and DNA was extracted from 10 hairs per individual using the DNA IQ system with Hair and Tissue kit (Promega Corp., Madison, WI, USA), following manufacturer instructions. Hair samples were stored up to 6 years before extraction. Six microsatellite loci, previously verified to amplify in this species (McEachern et al. 2011),

were amplified for 445 individuals. Forward primers were fluorescently labeled with 6-FAM (ST7, Bib36), NED (Bib18, Sgs17), or VIC (Bib4, Sgs14), and reverse primers were unlabeled (all primers Applied Biosystems, Foster City, CA, USA).

We amplified markers by polymerase chain reaction (PCR), conducted in 10ul reactions, using 1ul template DNA (approx. 2ng/ul), 0.15-0.4ul forward and reverse primers, 1ul BSA (100ng/ul), 0.15ul FastStart Taq DNA polymerase, 1ul FastStart buffer, 0.8ul dNTP (all Sigma-Aldrich Co., St.Louis, MO, USA), and 5.6-5.9ul H<sub>2</sub>O. We used a touch-down thermal profile for PCR, consisting of one denaturation cycle at 94°C for 5 minutes, 15 cycles at 94°C for 30 sec, 65°C (stepped down by 1°C each cycle) for 90 sec, and 72°C for 60 sec; 15 cycles at 94°C for 30 sec, 55°C for 90 sec, and 72°C for 45 sec; ending with 10 minutes at 72°C for final extension. We used a nested PCR protocol to increase amplification for five of the six loci (all but Sgs14): conditions for the nested PCR were the same as above, substituting 1ul PCR product for 1ul starting template DNA. Amplification was sufficient for locus SGS14 without the nested protocol, using 2ul template DNA, 0.5ul each forward and reverse primer, and 4.05ul H<sub>2</sub>O (other reagents stayed the same). We loaded 1ul PCR product with 8.7ul formamide and 0.3ul 600LIZ internal size standard, to visualize PCR fragments on an ABI 3730 DNA Analyzer (Applied Biosystems Inc., Foster City, CA, USA). Alleles were scored using GeneMapper software (v4.0, Applied Biosystems, Inc.), and confirmed by visual inspection of electropherograms.

### *3.3.3 Paternity analysis*

We used Cervus 3.0 (Kalinowski et al. 2007) to assign paternity to offspring with known mothers. We simulated the likelihood of paternity assignment separately for each year to

determine critical delta values. Simulations used allele frequencies from genotypes of all squirrels in the population for that year; parameters were set to simulate 100,000 offspring from all known mothers and 75% of potential fathers ( $n=4-20$ , depending on the year), with 90% of loci typed (minimum observed value) and 2% genotyping error (averaged across all loci from a minimum of 40 replications for each locus). We used mother-father-offspring trios that were assigned at the 80% confidence level or higher. For some litters, particularly those born 1996-2005, not all offspring in a litter could be definitively assigned to specific males; however, if one pup in a litter was assigned to a male at 80% or higher confidence, and that male was not among the top two candidate fathers for an unassigned sibling, we considered that litter was multiply sired. Litters for which no fathers could be confidently assigned were excluded from analysis. For 2002-2005, limitations of gel electrophoresis made scoring unreliable for some loci, resulting in poor paternity assignment. For each of those years, we used genotype matching of known maternal-offspring pairs in Cervus to determine error rates, and excluded the locus with the highest error rate. For 2002 this was locus ST7 (4 mismatches out of 8 parent-offspring pairs, 55.8% error rate), and locus Sgs14 in 2003 (5 mismatches/31 pairs, 13.8%), 2004 (12 mismatches/67 pairs, 16.3%) and 2005 (18 mismatches/72 pairs, 22%). For 2005, locus Sgs17 was found not to be in Hardy-Weinberg equilibrium, and was also excluded. After these loci were excluded, observed error rates dropped to 7% or lower for those years, and paternity assignment was possible for some offspring. We excluded any assignments that were based on fewer than 3 loci.

#### *3.3.4 Mating and opportunity for sexual selection*

For a subset of years ( $n=30$  litters, 168 pups from 2008-2015), we were able to assign paternity for nearly all pups. For each of those years, we tallied the number of pups sired by each male,

and the number of pups born to each female. We used these values for all reproductive adults (males that sired at least one pup, and females that produced at least one pup) to calculate the opportunity for sexual selection for each sex ( $I$ ) according to the equation:  $I = V_o / O^2$ , where  $V_o$  is the variance in offspring numbers and  $O^2$  is the squared average in offspring numbers (Shuster and Wade 2003, Munroe and Koprowski 2011). We also calculated the difference in opportunity for sexual selection between the sexes ( $\Delta I = I_{\text{male}} - I_{\text{female}}$ ), to describe the difference in strength of selection between the sexes (Shuster and Wade 2003). To determine Bateman's gradient ( $\beta_{\text{ss}}$ ) for each sex, we tallied the number of mates for each male and each female, and combined these data with the above offspring values for all reproductive adults (Andersson and Iwasa 1996). We fit a linear model for each sex, with total offspring produced per individual modeled as a function of number of mates.

### *3.3.5 Male reproductive success*

Male reproductive success, an important component of the mating system and opportunity for sexual selection, might be determined by several factors. We used the complete paternity data from 2008-2015 to evaluate whether age, breeding tenure, overall tenure, breeding mass, reproductive investment, or genetic diversity influenced male reproductive success. Age was known for almost half of adult males (47% of 71 males). Breeding tenure was the number of years the male was present during the breeding season. Overall tenure was the number of years the male was present in the population at any time of the active season; for example, a yearling male who immigrated into the population at the end of his natal summer would be assigned an overall tenure of 2 years (year of immigration + first breeding season) but a breeding tenure of 1 year (since he was not present during the breeding season of his natal summer). We reasoned that if knowing the locations of reproductive females before hibernation increased the likelihood

of effective mating, then overall tenure would improve male reproductive success. We used size of scrotal testes, measured as anogenital distance, to estimate reproductive investment (Schulte-Hostedde and Millar 2004). Genetic diversity was defined as internal relatedness (IR, Amos et al. 2001), calculated using individual multilocus genotypes and the Rhh package in R (Alho et al. 2010).

We fit a series of generalized linear mixed-effect models (GLMMs) with male characteristics (age, genetic diversity, breeding tenure, overall tenure, breeding mass, and reproductive investment) as fixed effects, to predict the following outcomes for each breeding season: likelihood of reproduction (yes/no, modeled with binomial error structure and logit link), number of mates (Poisson error structure), and number of offspring produced (Poisson error structure). We included male identity as a random effect in all models, to control for multiple records from males who were present in the breeding season of more than one year. We used AIC to compare models predicting the same outcome variable but with different fixed effects (Burnham and Anderson 2002).

### *3.3.6 Multiple paternity*

Unless otherwise stated, we modeled multiple paternity as a binary outcome variable (0/1) in generalized linear mixed-effects models (GLMMs), with binomial error structure and a logit link. We fitted maternal ID as a random effect, to control for multiple litters produced by individual females in different years. We also included year as a random effect, to control for unspecified annual variation in environmental conditions.

### *Encounter rate*

The number of potential male mates may influence the frequency with which females mate multiply (Kokko and Rankin 2006, and citations therein). Increases in population density increase the absolute number of potential mates, while increased male-bias in adult sex ratios increases the relative number of potential mates females are likely encounter (Kokko and Rankin 2006). Hence, we modeled multiple paternity as a function of male density and operational sex ratio. Male density was defined as the total number of adult ( $\geq 1$  year old) males resident in the population, and operational sex ratio (OSR) was defined as number of adult males per adult female. Because participation of 1 year old males in breeding was unknown but potentially limited, we also tested for an effect of the density of males  $\geq 2$  years old on multiple paternity.

Deep snow and spring storms can reduce mating activity in ground squirrels (Morton and Sherman 1978, Michener and McLean 1996), which may result in lower rates of multiple paternity (Bergeron et al. 2011, Martin et al. 2014). Golden-mantled ground squirrels often mate before snowmelt, in some years when snow is quite deep (Wells 2016). Hence, we tested the prediction that deep snow or heavy precipitation reduce male mobility, impacting the likelihood that females will encounter multiple males during their estrous period, and therefore reducing the incidence of multiple paternity of their litters. We modeled multiple paternity of each litter as a function of snowpack and precipitation on date of estrous. Snowpack (depth of snow in cm) and precipitation (in cm) were measured daily, and date of estrous was estimated by subtracting 58 days (28 days for gestation, 30 days for lactation) from the observed date of litter emergence.

### *Male monopolization*

We modeled multiple paternity as a function of breeding asynchrony and local density of breeding females. Breeding asynchrony, which we used as an estimate of temporal clustering of

receptive females, was defined as the number of days between the emergence of the focal litter and the next litter closest in time (before or after): assuming a relatively fixed period of gestation+lactation, we reasoned that the number of days between the emergence of two litters should approximate the number of days between the conception of those two litters, and hence the temporal separation between mating of estrous females. However, because we excluded females who mated but did not conceive, or who conceived but lost their litter, our measure of breeding asynchrony may be an underestimate of temporal clustering. Local density of breeding females was defined as the number of reproductive females who produced a litter in the same one of the six localities as the focal female. This local density was further partitioned into local density of related females versus local density of unrelated females; there is some evidence that female kin tend to live in closer proximity (Wells 2016), thereby enhancing spatial clustering. Female relatedness was calculated by using pedigree data to construct matriline (Wells 2016); females were considered kin if estimated relatedness ( $r$ ) was  $\geq 0.125$ , corresponding to first cousins or closer. Males should be attracted to clusters of females, and because increases in male-male competition may affect the ability of males to monopolize clustered females (Emlen and Oring 1977; Shuster and Wade 2003), we included male density in the model to test for an interaction between breeding female density (spatial clustering) and male density. Clustered females should be economically defensible by a single male only when competition from other males is low (Emlen and Oring 1977): hence, we predicted that rates of multiple paternity in female clusters should be low when male density is low, and high when male density is high.

### *Female Choice*

We modeled multiple paternity as a function of maternal age, maternal condition, and genetic diversity. Maternal age was defined as a factor, where 0 represented yearling females,

and 1 represented females 2 years and older. We used body mass as an estimate of maternal condition (Wells 2016), which was measured for a subset of females and was defined in two ways: peri-oestrous condition (mass as measured within one week before or after estimated date of estrous, n=17), and post-parturition condition (mass as measured within one week after estimated date of parturition, n=35). As for adult males, genetic diversity was defined as internal relatedness (IR).

Data were analysed using R version 3.0.2 (R Core Team 2013). We used the packages lme4 (Bates et al. 2014) and rethinking (McElreath 2014) to fit mixed-effect models, and bbmle (Bolker et al. 2014) to compute AIC differences between models. Parameter estimates  $\beta$  ( $\pm$  SE) are reported in the logit form for binomial models.

### **3.4 Results**

We genotyped a total of 476 offspring from 108 litters, and we were able to assign paternity with 80% or higher confidence to 270 (56.7%) mother-father-offspring trios. Our final dataset included 244 trios (of  $\geq 80\%$  confidence) from 66 litters. Mean polymorphic information content (PIC) was 0.72 (range 0.63-0.75), and combined non-exclusion probability for a second parent was 0.011 (range 0.003-0.039; Table 1).

#### *3.4.1 Mating patterns*

In every year of the study, at least one male mated with multiple females or at least one female mated with multiple males; hence, this population meets the definition of polygamy (McEachern et al. 2009) or polygynandry (Munroe and Koprowski 2011). However, not all females mated multiply, and both monogamous and polygamous patterns were observed (Figure 1). Across all years, rates of monogamy and polygamy were 37% and 63%, respectively, for females,

compared to 49% and 51% for males. One case of polygyny extended across years, in which the same male mated with both an adult female and her adult daughter in 2009, and again in 2010. We detected one case of close incest out of approximately 100 matings, where a yearling female mated with her father.

Maximum annual reproductive success was higher for males than for females: litter size for females did not exceed seven pups, whereas a single male sired 20 pups (with five females) in one year. Opportunity for sexual selection was substantially lower for females ( $I_s = 0.06$ ) than for males ( $I_s = 0.60$ ), with a difference in relative fitness ( $\Delta I$ ) of 0.54. Bateman's gradient was strongly positive for males ( $y = 3.88x - 1.41$ ,  $r^2 = 0.78$ ,  $p < 0.001$ ), but not different from zero for females ( $y = 0.46x + 4.45$ ,  $r^2 = 0.05$ ,  $p = 0.252$ ; Figure 2).

#### *3.4.2 Male reproductive success*

Yearling males rarely bred successfully (only 2 out of 29 known-aged yearling males), and age had a significantly positive effect on likelihood of reproduction ( $\beta = 1.22 \pm 0.58$ ,  $z = 2.09$ ,  $p = 0.037$ ). Immigrant males rarely bred in their first year of residence in the population: only 3 of 28 (11%) successfully reproduced in their first season, compared to 8 of 12 (67%) in their second season, and 3 of 3 (100%) in their third season. Average male breeding tenure was 1.36 seasons ( $n=152$  males, range=1-5 seasons). Male breeding tenure strongly affected reproductive success: for each additional breeding season present, males were more likely to reproduce that season ( $\beta = 1.96 \pm 0.51$ ,  $z = 3.83$ ,  $p < 0.001$ ), gained a higher number of mates ( $\beta = 0.91 \pm 0.20$ ,  $z = 4.56$ ,  $p < 0.001$ ), and produced more offspring ( $\beta = 0.82 \pm 0.14$ ,  $z = 5.74$ ,  $p < 0.001$ ). Overall tenure positively influenced likelihood of reproduction ( $\beta = 1.13 \pm 0.38$ ,  $z = 2.95$ ,  $p = 0.003$ ), but to a lesser extent than did breeding tenure ( $\Delta AIC = 10.2$ ,  $df = 3$ ). After controlling for breeding

tenure, neither male mass ( $\beta = -0.02 \pm 0.10$ ,  $z = -0.23$ ,  $p = 0.821$ ) nor ano-genital distance ( $\beta = 0.07 \pm 0.52$ ,  $z = 0.13$ ,  $p = 0.895$ ) had an effect on likelihood of reproduction. Similarly, male genetic diversity did not significantly improve reproductive success ( $\beta = 2.33 \pm 1.80$ ,  $z = 1.30$ ,  $p = 0.193$ ).

### *3.4.3 Multiple paternity*

Over the course of this study, 62% of litters were multiply sired, but the proportion varied from year to year: multiple paternity was lowest in 2009 (25% of litters), and highest in 1998, 2004, 2005, and 2014 (100% of litters). Multiple paternity did not affect litter size ( $\beta = 0.07 \pm 0.35$ ,  $t = 0.19$ ,  $p=0.851$ ).

#### *Encounter rate*

Male density did not significantly affect the likelihood of multiple paternity ( $\beta = 0.08 \pm 0.08$ ,  $z = 0.93$ ,  $p = 0.350$ ), and neither did density of males 2 years and older ( $\beta = 0.23 \pm 0.15$ ,  $z = 1.62$ ,  $p = 0.106$ ). Operational sex ratio also did not affect multiple paternity ( $\beta = -0.08 \pm 0.66$ ,  $z = 0.13$ ,  $p = 0.895$ ). Amount of precipitation on the estimated date of conception had a negative effect on likelihood of multiple paternity that approached statistical significance ( $\beta = -0.13 \pm 0.07$ ,  $z = -1.75$ ,  $p = 0.081$ ; Figure 3). Snow depth on the estimated date of conception had no effect on multiple paternity ( $\beta = 0.00 \pm 0.00$ ,  $z = 0.33$ ,  $p = 0.740$ ).

#### *Male monopolization*

Average breeding asynchrony (an estimate of temporal clustering) was 3.41 days (range=0-29); breeding asynchrony had no effect on multiple paternity ( $\beta = -0.04 \pm 0.05$ ,  $z = -0.76$ ,  $p = 0.450$ ). Local density of breeding females did not affect the likelihood of multiple

paternity ( $\beta = 0.03 \pm 0.19$ ,  $z = 0.18$ ,  $p = 0.859$ ). However, when related versus unrelated females were distinguished, local density of related breeding females had a strong negative effect on multiple paternity ( $\beta = -5.00 \pm 2.47$ ,  $z = -2.03$ ,  $p = 0.043$ ), while local density of unrelated breeding females had no effect on multiple paternity ( $\beta = 0.24 \pm 1.24$ ,  $z = 0.20$ ,  $p = 0.846$ ). Further, there was a significant interaction between the local density of related breeding females and total male density ( $\beta = 0.42 \pm 0.20$ ,  $z = 2.06$ ,  $p = 0.039$ ; Figure 4): high female density decreased the likelihood of multiple paternity when males were relatively scarce (5 total males), but increased the likelihood of multiple paternity when males were abundant (15 total males).

#### *Female choice*

Maternal age had a negative effect on multiple paternity, with females 2 years or older being less likely than yearling females to produce multiply-sired litters ( $\beta = -1.67 \pm 0.87$ ,  $z = -1.91$ ,  $p = 0.056$ ). Though sample size was small ( $n=17$ ), the effect of maternal peri-oestrous mass was slightly but reliably negative, and approached statistical significance ( $\beta = -0.05 \pm 0.03$ ,  $z = -1.75$ ,  $p = 0.080$ ; Figure 5). Maternal post-parturition mass did not affect likelihood of multiple paternity ( $\beta = -0.01 \pm 0.02$ ,  $z = -0.30$ ,  $p = 0.761$ ), and neither did maternal genetic diversity (internal relatedness, IR) ( $\beta = -2.34 \pm 1.90$ ,  $z = -1.23$ ,  $p = 0.218$ ).

### **3.5 Discussion**

The mating system of golden-mantled ground squirrels is consistent with that of other small-bodied ground squirrels, in which mating patterns are typically polygynandrous (Raveh et al. 2010). Further, the species exhibits high rates of multiple paternity, with nearly two-thirds of litters multiply sired, which is within the range reported for other squirrels with a low level of

sociality (e.g. 54% for 13-lined ground squirrels, *Ictidomys tridecemlineatus*, Schwagmeyer and Foltz 1990; 55% for round-tailed ground squirrels, *Xerospermophilus tereticaudus*, Munroe and Koprowski 2011; 78% for Belding's ground squirrels, *Urocitellus beldingi*, Hanken and Sherman 1981; 80% for Richardson's ground squirrels, *Urocitellus richardsonii*, Hare et al. 2004). Despite multiple mating by both sexes, the opportunity for sexual selection was lower in females than in males, and the difference in opportunity for sexual selection between males and females was lower than for other ground squirrels (Munroe and Koprowski 2011); this low difference in opportunity for sexual selection may contribute to the low sexual dimorphism observed in this species (Mateju and Kratochvil 2013). Additionally, female variance in reproductive success was low, and did not depend on number of mates: this finding is in contrast to other small-bodied squirrels, where female reproductive success did increase with number of mates (yellow-pine chipmunks, *Tamias amoenus*, Schulte-Hostedde et al. 2004; round-tailed ground squirrels, Munroe and Koprowski 2011; eastern chipmunks, *Tamias striatus*, Bergeron et al. 2012). In a highly-seasonal environment, such as that inhabited by our high-elevation population of golden-mantled ground squirrels, litter sizes may be constrained by resource availability, not by sperm-limitation. Regardless, though this does not rule out indirect (genetic) benefits of multiple paternity, it appears that female golden mantled ground squirrels do not realize a direct fertility benefit from additional mates.

In contrast to females, males were able to substantially increase their annual reproductive success through additional mates. In polygynandrous ground squirrels, scramble competition for fertile females is thought to involve several components: overt male-male conflict, sperm competition, and skill in locating mates (Schwagmeyer and Wootner 1986; Raveh et al. 2010). Male mass, which may confer an advantage in overt conflict, did not affect reproductive success. Neither did

reproductive investment (testes size) or genetic diversity, which may confer advantages in sperm competition. However, longer male tenure, which may influence conflict ability and skill in locating mates, had a strong positive effect on reproductive success. Specifically, both young and new immigrant males appeared to be at a reproductive disadvantage in our population, rarely acquiring mates and siring offspring. However, it is important to note that we did not directly observe copulations, and hence are reporting number of effective matings (i.e. those resulting in paternity) instead of the number of matings: females who produced singly-sired litters may have actually mated with multiple males, including yearlings and immigrant males, but only accepted sperm from older or longer-resident males. Therefore, low reproductive success of yearling males may result from low rates of reproductive maturity for males at this elevation after their first hibernation (Bronson 1979; Wells, unpublished data), female preference for older males, or the greater competitive ability of older males. Low reproductive success of immigrant males could have resulted from their difficulty in locating females in a new area, female preference for familiar males, or the greater competitive ability of resident males. Male reproductive success was enhanced more by breeding tenure than overall tenure, indicating that knowledge of female locations the previous fall was not as useful as experience during the previous breeding season. Taken together, our results suggest that male experience (perhaps associated with dominance) is important for access to female mates and siring of more offspring.

We expected that precipitation would affect multiple paternity by reducing male mobility. We found a negative effect of precipitation on multiple paternity that approached statistical significance, perhaps because wet conditions reduce aboveground activity in this species (Kneip et al. 2011). We did not find an effect of snow depth, in contrast to a study of yellow-bellied marmots (*Marmota flaviventris*) conducted in the same area, which found that snow depth during

the breeding season had larger effects on limiting multiple paternity than did social or individual variables (Martin et al. 2014). However, yellow-bellied marmots are polygynous, and females often live in discrete habitat patches defended by a single territorial male (Armitage 2014); hence, single paternity is much more common than multiple paternity (82% vs. 18% of litters, Martin et al. 2014), and deep snow may impede the movements between distant patches that are required for a female to encounter multiple males. Because male golden-mantled ground squirrels are not territorial (C.P. Wells, unpublished), females may have ready access to multiple males regardless of snow depth.

An increase of multiple paternity with increasing density has been documented in several rodent species (Dean et al. 2006; Bryja et al. 2008; Streatfield et al. 2011; but see Sommaro et al. 2015), including squirrels: a scarcity of reproductive males was thought to underlie a seasonal decline in multiple paternity in eastern chipmunks (Bergeron et al. 2011), Columbian ground squirrels exhibited higher rates of multiple paternity in years with more breeding males (Jones et al. 2012), and the number of potential male mates predicted a higher rate of multiple male mating in red squirrels (*Tamiasciurus hudsonicus*, McFarlane et al. 2011). Yet male density did not increase the rate of multiple paternity of golden-mantled ground squirrels.

Male-bias in operational sex ratio is also expected to increase multiple paternity by increasing the rate at which females encounter male mates (Kokko et al. 2006), and in socially-monogamous vertebrates, increases in male-biased sex ratios are associated with increases in extra-pair paternity (Gowaty and Bridges 1991, Kokko et al. 2006). However, the relationship between OSR and multiple paternity in species with polygynandrous mating systems is less clear. Male-biased OSR was associated with increased multiple mating by female 13-lined ground squirrels (Schwagmeyer and Brown 1983), but somewhat reduced multiple mating by

female Columbian ground squirrels (*Urocitellus columbianus*, Murie 1995). In both Richardson's ground squirrels (*Urocitellus richardsonii*, Michener and McLean 1996) and Cape ground squirrels (*Xerus inaurus*, Waterman 1998), female rate of multiple mating was independent of changes in OSR: females mated with multiple males when the OSR was strongly male-biased and when it was less so. OSR did not affect rates of multiple paternity in golden-mantled ground squirrels, though through post-copulatory female choice (Eberhard 1996), it is possible for OSR to have increased the rate of multiple mating without affecting the rate of multiple paternity (e.g. Murie 1995). Taken together, these findings suggest that, with the exception of a possible effect of precipitation on male availability, the encounter rate model is not sufficient to explain variation in the rate of multiple paternity in our system: instead of being a passive response to mate availability, variation in multiple paternity likely results from the active strategies of males or females.

The ability of males to monopolize paternity is thought to vary with the spatial and temporal availability of fertile females (Emlen & Oring 1977; Shuster and Wade 2003). Spatial clustering of females did reduce the rate of multiple paternity in golden-mantled ground squirrels, but the effect was limited to female kin, perhaps because female kin are more clustered than nonkin (Wells 2016). Competitive males, which maximize their reproductive success by maximizing their number of mates, should be attracted to clusters of females and motivated to defend them. However, the effect of female clustering was reversed at high male density, presumably when male-male competition is higher: male-male competition increases the chance of mating interference (Kokko and Rankin 2006), including disrupted copulations (Klemme et al. 2007), leading to higher rates of multiple paternity. Apparently, individual males were able to monopolize females only when competition from other males was low. Unlike spatial clustering,

temporal clustering of breeding females did not affect multiple paternity. In theory, a single dominant male should be able to take advantage of asynchronously fertile females by sequentially monopolizing each one (Emlen and Oring 1977), but in golden-mantled ground squirrels this advantage is either not present (perhaps because of intense male-male competition) or may be outweighed by female choice.

Contrary to expectations, we found that young females and those that were lighter in weight, hence presumably in poorer condition, were more likely to produce multiply sired litters, not less likely. Much of the work on age-specific changes in mating preferences has used invertebrate models, where reproductive value is highest for young females and declines with age (Gray 1999, Wilgers and Hebets 2012, Atwell and Wagner 2014), but maximal reproductive value might occur at a somewhat older age in some ground squirrels (Dobson et al. 1999). Further, similar results for both age and condition suggest the possibility of a common explanation. We suggest that females that are young or in poor condition have lower fertility than other females, and that, as in Gunnison's prairie dogs (*Cynomys gunnisoni*, Hoogland 1998), multiple mating may increase the likelihood of conception. For yearling females, there is some evidence that number of mates is positively associated with litter size (Wells 2016). Alternatively, multiple mating may be a way for yearling females to avoid inbreeding with their father (Stockley et al. 1993, Schradin et al. 2010); given the high turnover of adult males in this population, and nearly complete dispersal of male offspring, older females are unlikely to encounter male relatives as potential mates.

Age or condition of the female may have additional effects on mating, since more socially or physically dominant females may be able to choose their mate or mates. In grey mouse lemurs (*Microcebus murinus*, Huchard et al. 2012), yellow-bellied marmots (Martin et al. 2014), and

Gunnison's prairie dogs (Hoogland 1998), heavier females were more able than their lighter-weight counterparts to evade social mates and engage in additional copulations. By contrast, heavier golden-mantled ground squirrel females were less likely to produce multiply sired litters. This difference likely reflects species-specific differences in female mating preferences, shaped by the fitness benefit of multiple paternity ("Bateman gradient"): female Gunnison's prairie dogs gain fitness with additional mates (Hoogland 1998), as may female grey mouse lemurs and yellow-bellied marmots, but female golden-mantled ground squirrels do not. In free-living populations, female and male preferences for mating may come into conflict. Differences in the slope of male and female Bateman gradients in our study suggest a conflict over multiple mating (Arnquist and Rowe 2005): while females do not benefit from more than one mate, males do. Yet, multiple paternity was observed in the majority of litters produced in our population. Though the effect of female peri-oestrous mass did not quite reach significance, it is revealing that no female over 200g produced a multiply sired litter; females this size are larger than the average male during the breeding season, and hence may have been able to evade mating attempts from non-preferred males. If lighter-weight females are unable to resist mating attempts, or unwilling to expend the energy required to evade males, multiple paternity in their litters of lighter-weight females may be characterized as "convenience polyandry" (Lane et al. 2008). In support for the female choice model, older and heavier females were apparently able to exert their preference for single sires.

Female genetic diversity did not appear to affect mating strategies: relatively inbred female golden-mantled ground squirrels did not accept more sires for their offspring than did relatively outbred females. Despite case studies from monogamous species supporting extra-pair mating for genetic benefits, meta-analyses show the pattern is weak at best (Akçay and Roughgarden

2007; Slatyer et al. 2012; Arct et al. 2015); females of polygynandrous species also may not choose mates according to their own or partner's genetic diversity (Thonhauser et al. 2014), and genetic diversity of offspring may not be a goal of multiple mating.

The rate of multiple paternity has consequences for the demography and evolution of populations. By increasing the genetic diversity of offspring (e.g. Cohas et al. 2007, Thonhauser et al. 2013) and the effective population size (Sugg and Chesser 1994, but see Karl 2008), higher rates of multiple paternity may promote population persistence. Our population of golden-mantled ground squirrels has experienced at least one genetic bottleneck during a period of low density, but recovered within a few generations (McEachern et al. 2011). It is possible that high rates of multiple paternity in high-density years, coupled with an influx of allelic diversity from immigrants (McEachern et al. 2011), may have restored genetic diversity. Immigration is generally credited with preserving the high genetic variability observed in rodents with strong population cycles (Erich and Jorde 2005; McEachern et al. 2011), but immigrants must successfully mate to contribute to gene flow; multiple paternity may complement immigration as an important mechanism underlying the genetic resilience of fluctuating rodent populations.

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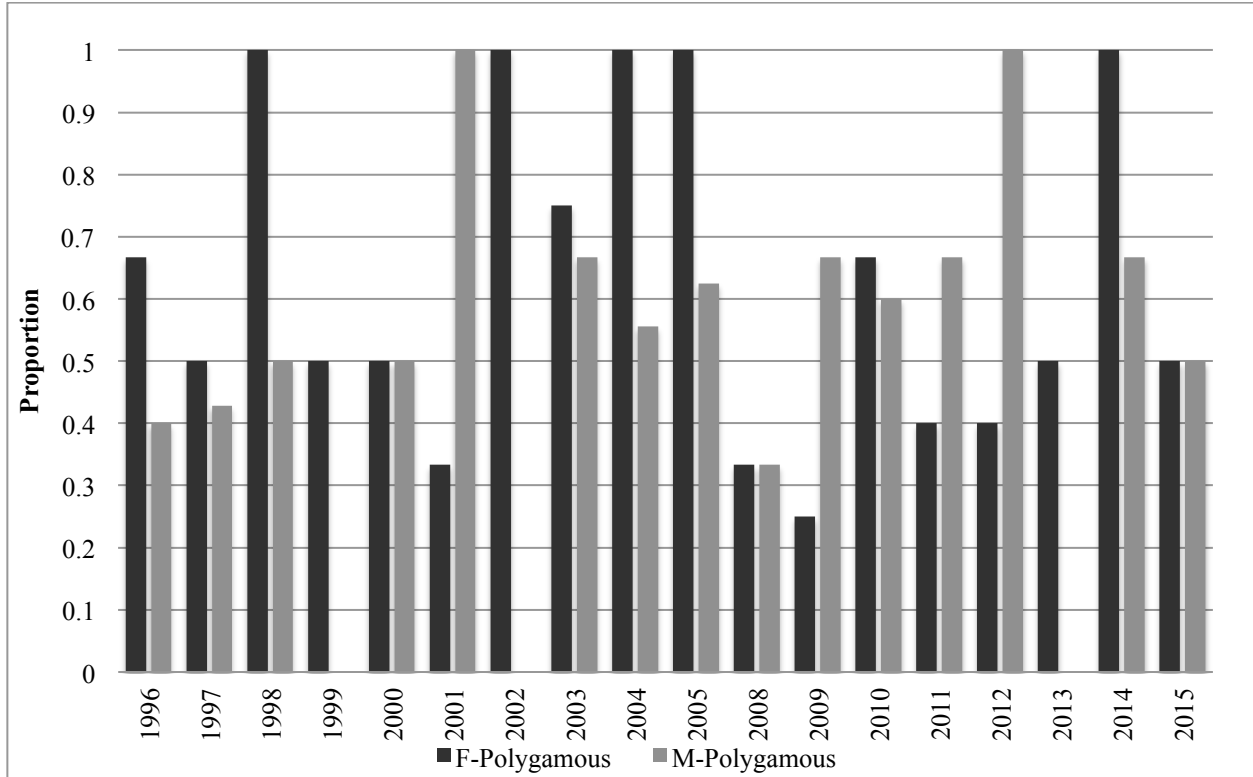
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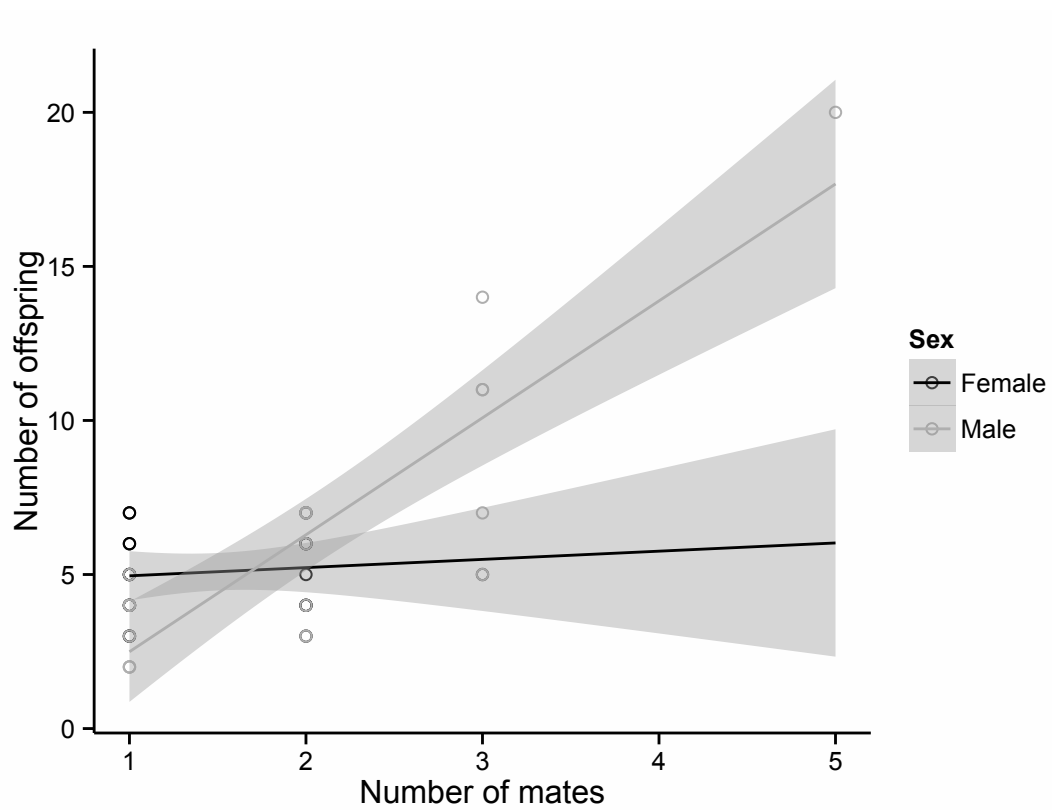
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### 3.7 Figures

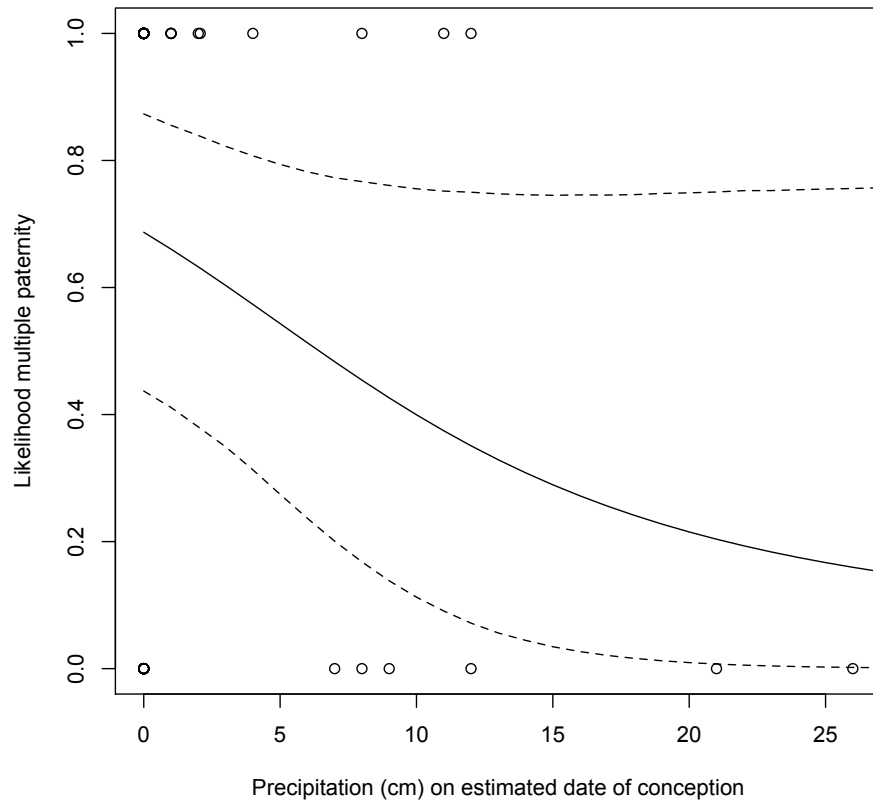
**3.7.1** Frequency of polygamous mating by year by sex: females are represented with black bars, males by grey. Individuals that did not produce at least one offspring are not included in the frequencies above. Frequency of monogamy for each sex is equal to  $1 - \text{proportion of polygamy}$ .



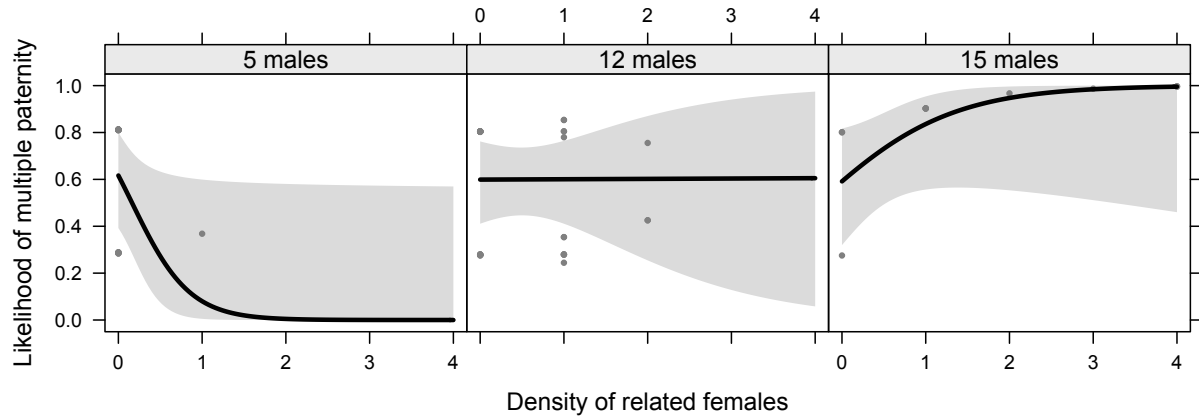
**3.7.2** Bateman's gradient for male (light grey) and female (black) golden-mantled ground squirrels in the East River Valley of Colorado (2008-2015).



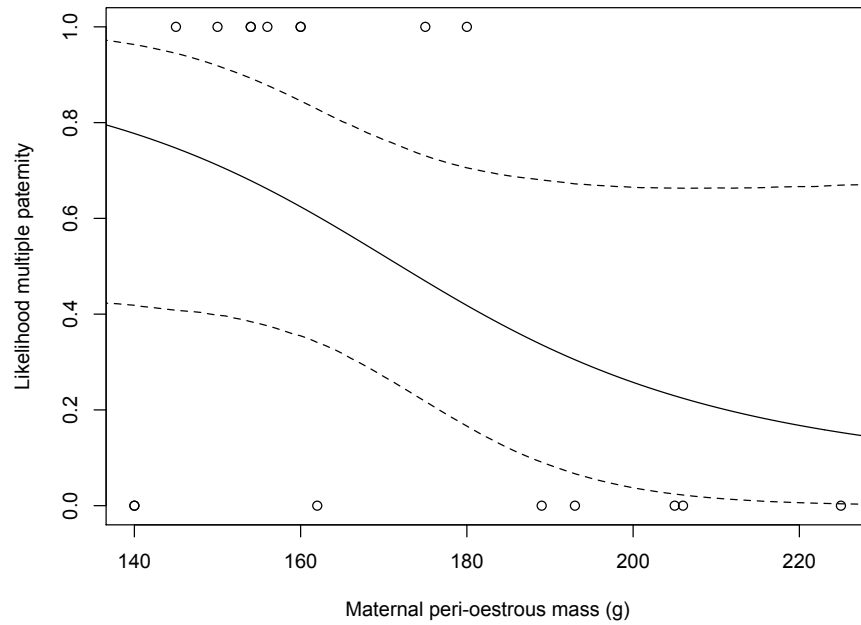
**3.7.3** The effect of precipitation on multiple paternity in golden-mantled ground squirrel litters, 1995-2014, controlling for snow depth. Open circles represent observed data. Solid line represents predictions from the model + 95% confidence interval (dashed lines).



**3.7.4** Likelihood of a litter being multiply sired, modeled as an interaction between local density of breeding female kin and total males: the left-hand panel represents the lowest male density, and the right-hand panel represents the highest male density. Lines are modeled relationship, with shaded 95% confidence intervals; dots are partial residuals from the model.



**3.7.5** Likelihood of producing a multiply-sired litter (0=no multiple paternity, 1=multiple paternity), by maternal mass near estimated date of conception. Open circles represent observed data. Solid line represents predictions from the model + 95% confidence interval (dashed lines).



### 3.8. Tables

**3.8.1** Summary statistics for population allele frequencies and paternity assignment, by year, as calculated in Cervus 3.0. <sup>a</sup>Hz=Heterozygosity, <sup>b</sup>PIC= Polymorphic Information Content.

| Year    | No. of individuals genotyped | No. of loci | Mean alleles per locus | Mean prop loci typed | Mean exp. Hz <sup>a</sup> | Mean PIC <sup>b</sup> | Combined non-exclusion probability (second parent): |
|---------|------------------------------|-------------|------------------------|----------------------|---------------------------|-----------------------|-----------------------------------------------------|
| 1996    | 56                           | 6           | 7.8                    | 0.88                 | 0.77                      | 0.73                  | 0.004                                               |
| 1997    | 65                           | 6           | 8.3                    | 0.94                 | 0.78                      | 0.75                  | 0.003                                               |
| 1998    | 27                           | 6           | 8.3                    | 0.98                 | 0.78                      | 0.74                  | 0.004                                               |
| 1999    | 24                           | 6           | 6.8                    | 0.99                 | 0.73                      | 0.68                  | 0.011                                               |
| 2000    | 22                           | 6           | 6.5                    | 0.99                 | 0.73                      | 0.67                  | 0.014                                               |
| 2001    | 29                           | 6           | 5.5                    | 0.94                 | 0.69                      | 0.63                  | 0.026                                               |
| 2002    | 33                           | 5           | 7.4                    | 0.91                 | 0.74                      | 0.70                  | 0.016                                               |
| 2003    | 62                           | 5           | 7.0                    | 0.91                 | 0.76                      | 0.72                  | 0.018                                               |
| 2004    | 123                          | 5           | 8.2                    | 0.94                 | 0.75                      | 0.71                  | 0.020                                               |
| 2005    | 132                          | 4           | 8.8                    | 0.91                 | 0.75                      | 0.72                  | 0.039                                               |
| 2008    | 53                           | 6           | 8.8                    | 0.94                 | 0.78                      | 0.75                  | 0.005                                               |
| 2009    | 65                           | 6           | 10.2                   | 0.98                 | 0.78                      | 0.75                  | 0.004                                               |
| 2010    | 73                           | 6           | 9.5                    | 0.98                 | 0.76                      | 0.73                  | 0.004                                               |
| 2011    | 53                           | 6           | 8.7                    | 0.98                 | 0.78                      | 0.74                  | 0.004                                               |
| 2012    | 72                           | 6           | 10.2                   | 0.99                 | 0.74                      | 0.71                  | 0.006                                               |
| 2013    | 49                           | 6           | 9.8                    | 1                    | 0.78                      | 0.74                  | 0.003                                               |
| 2014    | 47                           | 6           | 9.0                    | 1                    | 0.78                      | 0.74                  | 0.004                                               |
| 2015    | 33                           | 6           | 7.8                    | 0.99                 | 0.75                      | 0.71                  | 0.006                                               |
| Average |                              |             | 8.3                    | 0.96                 | 0.76                      | 0.72                  | 0.011                                               |

**CHAPTER 4: FEMALE KIN DENSITY AFFECTS OFFSPRING SEX RATIO IN AN ASOCIAL  
MAMMAL, THE GOLDEN-MANTLED GROUND SQUIRREL (*CALLOSPERMOPHILUS  
LATERALIS*)**

**4.1 Abstract**

Evolutionary theory predicts that parents should bias investment in offspring toward the sex that will yield the higher fitness returns, and one outcome may be biased offspring sex ratios. Bias in offspring sex ratios has been proposed to vary with specific social, maternal, and environmental conditions that alter the fitness returns of each sex, but empirical evidence is conflicting both within and across taxa. We used 18 years of pedigree and demographic data to investigate variation in offspring sex ratios in a population of golden-mantled ground squirrels (*Callospermophilus lateralis*). Despite overall parity in offspring sex ratio, we found predictable changes in offspring sex ratio in response to female density at the population level: squirrels produced more daughters when female density was low, and more sons when female density was high. At the individual level, females adjusted litter sex ratios similarly, but only in response to high densities of kin, and not in response to density of nonkin. This effect was reversed for older females ( $\geq 3$  years) in dense kin neighbourhoods, who were more likely to produce daughters, perhaps because older females had the behavioural dominance necessary to recruit daughters at high densities. By contrast, litter sex ratios did not vary with adult sex ratios, maternal condition, or food availability. Our results support the local resource competition theory of offspring sex allocation for this species, and are consistent with socially-induced physiological mechanisms that bias offspring sex in ground squirrels. Knowledge of female relatedness revealed patterns of sex ratio adjustment that otherwise would have been obscured, highlighting the importance of interactions with kin in an asocial species.

## 4.2 Introduction

Variation in offspring sex ratio is often observed in natural populations, but debate continues over whether this variation is adaptive (Cockburn et al. 2002; West 2009). Evolutionary theory predicts (Charnov 1982), and recent evidence supports (Thogerson 2013), fitness benefits to parents who are able to bias offspring sex ratios toward the sex with the higher reproductive value. The conditions under which parents bias offspring sex ratios are poorly known for most species, but several influential theories have been advanced. First, offspring sex ratios might vary according to social conditions. Fisher (1930) proposed that parents should adaptively vary the sex of offspring in response to local adult sex ratios as sons would gain a reproductive advantage when males are rare, and daughters when females are rare; the population result thus should be an even sex ratio maintained by frequency-dependence. Second, offspring sex ratios might vary according to maternal condition. Trivers and Willard (1973) proposed that females should vary the sex of their offspring according to their ability to physically invest in their offspring. In polygynous species, when maternal body condition positively affects offspring body condition, and when body condition influences variation in male reproductive success, females in good condition should produce sons, and females in poor condition should not.

The Fisher (1930) and Trivers and Willard (1973) theories focus on the ability of offspring to compete for mates, but offspring fitness may also be affected by competition for resources, especially among female mammals (Stockley & Bro-Jorgensen 2011). Natal philopatry in female mammals generates the potential for competition for resources between mothers and daughters, as well as other female kin (Local Resource Competition, Clark 1978). By contrast, because male mammals tend to disperse away from their natal territory (Greenwood 1980; Mabry et al. 2013), production of sons does not create the same potential competition for resources such as

food and territory. Thus, mammal mothers may gain from adjusting offspring sex in favour of daughters when local density is low and resources are abundant. At low densities, when there are available territories in which to settle, the philopatric sex should yield the highest fitness returns (Michener 1980, Armitage 1987). At high densities, when resources are scarce, females may benefit from producing more of the dispersing sex (sons) (Clark 1978).

Density alone, however, is a coarse measure of competition, because access to resources often varies with dominance and kinship (Moses & Millar 1994; Stockley & Bro-Jorgensen 2011). The reproductive consequences of competition have been well studied in group-living species, where female dominance is positively correlated with access to resources and offspring survival (Stockley & Bro-Jorgensen 2011; Clutton-Brock & Huchard 2013). By contrast, researchers are just beginning to investigate how spatial proximity might facilitate dominance and kin-directed behaviours in females of relatively asocial species, in which philopatry results in spatial overlap among females, both kin and nonkin (McEachern et al. 2007; Hatchwell 2010; Innes et al. 2012; Jones et al. 2013). Further, competition may differ among female kin as opposed to nonkin, and it is not yet clear which is more intense (Clutton-Brock & Huchard 2013). On the one hand, competition with nonkin may be more intense because interactions are not complicated by trade-offs between direct and inclusive fitness (Hamilton 1964, but see West et al. 2002). On the other hand, competition with kin may be more intense because members of a matriline may be competing for resources (e.g. burrows) within their shared natal range (Queller 1994). Despite evidence that kin presence can play an important role in sex ratio adjustment (West et al. 2005), there have been few mammal studies of offspring sex ratios that explicitly incorporate the effects of kin (Armitage 1987; Lambin 1994; Aars et al. 1995; Nunn & Pereira 2000).

In contrast to the Fisher, Trivers-Willard, or resource competition theories of adaptive adjustment of offspring sex, variation in offspring sex may be non-adaptive. Specifically, the availability of food resources may affect offspring sex ratios through sex-specific mortality. During gestation, female embryos may be more vulnerable to excessive maternal glucose levels, generating male-biased offspring sex ratios when food is abundant (Cameron et al. 2008). Conversely, during lactation, male offspring may be more vulnerable to maternal food stress, generating female-biased offspring sex ratios when food is scarce (McClure 1981). Effects of food availability and diet composition on offspring sex have primarily been investigated in lab populations of rodents (Alexenko et al. 2007; Fountain et al. 2008; Dama et al. 2011), with little work done on mammals in the wild.

In this paper, we test predictions about offspring sex allocation using data from a long-term study of free-living golden-mantled ground squirrels (*Callospermophilus lateralis*). First, at the population level, we ask if offspring sex ratios respond to changes in adult sex ratios (Fisher 1930; Werren & Charnov 1978). Second, we ask if mothers who are able to invest more in offspring, invest more in sons than daughters (Trivers & Willard 1973). Third, we ask if mothers adjust offspring sex ratio in response to local competition (Clark 1978), either with kin or nonkin. Fourth, we ask if changes in food availability for mothers at the time of conception contribute to variation in offspring sex ratio (Cameron 2004).

## **4.3 Methods**

### *4.3.1 Study site and species*

We conducted this study at the Rocky Mountain Biological Laboratory (2900 m), located in the East River Valley, Gunnison County, Colorado (38°58'N, 106°59'W). We studied one

population of golden-mantled ground squirrels at this site over an 18 year period (1995-2012). Golden-mantled ground squirrels, about 200-300 g in size, are considered to be a solitary, asocial species (Armitage 1981; Michener 1983). Adults are thought to establish distinct home ranges (Michener 1983; Ferron 1985; Jesmer et al. 2011), and interactions among adults are primarily agonistic, potentially even among adult kin (Michener 1983, Ferron 1985). At our 13-ha study site, squirrels generally inhabited dry meadows and rocky slopes adjacent to stands of aspen or spruce; habitat characteristics and vegetation are further described in McEachern et al. (2011). Females emerged from hibernation in April or May, mated soon after, and weaned their pups from mid June to mid July; gestation in golden-mantled ground squirrels is 28 days (Cameron 1967) and weaning occurs when pups reach 30 days of age (Phillips 1981). Adults began to immerge for hibernation in early August, and all squirrels remained belowground by October.

We censused this population of ground squirrels each year in May, after adults emerged from hibernation and began mating. We captured squirrels with Tomahawk live traps (Model 201) baited with black oil sunflower seeds and peanut butter, set in areas of squirrel activity and checked at 30-minute intervals. Our methods were conducted according to protocols approved by the Animal Care and Use Committees of the University of California, Davis, and the Rocky Mountain Biological Laboratory, and followed guidelines approved by the American Society of Mammalogists (Sikes et al. 2011). At capture, we recorded sex (anogenital distance), reproductive status (colour and swelling of nipples for females), and body mass to the nearest 1g. We attached a numbered metal tag to each ear for permanent identification, and applied a unique dyemark to the dorsal pelage for individual identification during behavioural observations. Daily trapping continued until all adults were identified and marked, and was used later as necessary to replace eartags when they had been lost, or renew dyemarks when squirrels moulted.

Adult ( $\geq 1$  year old) density varied several-fold in this population over the study period, from a minimum of 0.9 squirrels/ha in 1999 to a maximum of 3.2 squirrels/ha in 2005 (Kneip et al. 2011). Additionally, adult female density was heterogeneously distributed across the study site, with six discrete localities of female activity centred in suitable rocky, dry meadow habitat patches containing burrow systems that are occupied long-term. In the year of highest population density (2005), local density of breeding females ranged from 1-6 females per locality. Competition at high densities seems likely; female golden-mantled ground squirrels maintain exclusive use of a core area around their burrow system within a larger home range (Jesmer et al. 2011), and the reduced survival of females following high-density years is likely due to increased occupancy of poor-quality habitats that are not sufficient to sustain long-term survival (Kneip et al. 2011).

We calculated local breeding female density as the number of reproductively active females in each locality. Females were categorized as reproductive if they exhibited swollen nipples during gestation or chewed nipples during lactation. Rarely, a female was observed to use more than one locality; in these cases, she was counted in the breeding female density of each locality, and the breeding female density assigned to her was the sum of females in both localities.

Beginning in June of each year, we monitored reproductive females for indications that their litters would soon emerge and searched their home ranges daily for the emergence of pups. Litters were trapped as they emerged from the natal burrow at weaning, and emergence date was recorded as the day on which the first pup was seen aboveground. Each pup was weighed, and sexed by ano-genital distance. Maternity was determined through a combination of spatial, temporal, and behavioural data: assignment was suspected when a litter emerged within a female's core area of activity, and confirmed when she directed affiliative behaviours (e.g. nose-

greeting, Ferron 1985) toward the pups in the days following emergence. Mothers were almost always trapped with their pups on the day of weaning. The few litters for which maternity could not be definitively assigned (e.g. when natal burrows were close and pups intermingled before trapping was complete) were excluded from analysis.

#### *4.3.2 Adult sex ratio and female density*

At the population level, we calculated offspring sex ratio as the percentage of male pups produced out of the total pups each year. Rarely, pups were killed by predators before they were trapped and sex could be determined; these pups were excluded from analysis. To assess whether offspring sex ratios are negatively correlated with adult sex ratios, we fit a linear model of adult sex ratio (adult males divided by adult females) on per cent male pups. Additionally, to assess the effect of female density on offspring sex ratio at the population level, we fit a linear model of breeding female density on per cent male pups.

#### *4.3.3 Maternal investment*

We defined maternal investment in a pup as the pup's weight at weaning (weight measured within 48 hours of litter emergence from the natal burrow). We defined total maternal investment as the litter mass (aggregate mass of pups at weaning, Broussard et al. 2005; Gedir & Michener 2014). We were able to calculate litter mass for a subset (n=32) of litters. We tested relative investment in sons vs. daughters in three ways. First, we tested if male pups weighed more than their sisters at weaning, using a paired t-test of mean male weight versus mean female weight within each litter. Second, we modelled relative investment in male pups versus female pups (aggregate mass of male pups at weaning, divided by aggregate mass of female pups, for each litter) as a function of total maternal investment (litter mass) in a generalized linear mixed model

(GLMM), with maternal identity and year fitted as random effects. Third, we modelled relative investment in male pups vs. female pups (mean mass of male pups at weaning, divided by mean mass of female pups, for each litter) in a boosted generalized linear mixed model (BGLMM, Tutz & Groll 2010) with the same set of predictors.

#### *4.3.4 Local competition with kin and nonkin*

We used maternal identity to reconstruct the relatedness of females born within the study area. Females that immigrated into the study site, whether as juveniles or adults, were assumed to be unrelated to resident females; thus, their only relatives were direct descendants (daughters, grand-daughters). Kin density was calculated as the number of related breeding females (those with coefficient of relatedness  $\geq 0.125$ ) using the same locality as the focal female. Nonkin density was calculated as the number of unrelated breeding females using the same locality as the focal female. Both kin and nonkin densities were treated as categorical variables (1, 2, 3, or 4 female kin; 1, 2, 3, or 4+ female nonkin) to investigate non-linear effects. Maternal age, categorized as yearling, 2, or  $\geq 3$  years old, was known for females born in the study area and was estimated for females that immigrated as juveniles or yearlings based on body mass.

To explore the effect of local density on litter sex ratio, calculated as the proportion of male pups in each litter, we fit linear multi-level models (GLMMs) with a binomial error structure and logit link. We specified kin density and nonkin density as fixed effects. We also included maternal age, and the interaction between maternal age and kin density, as fixed effects because age might confer dominance, hence competitive ability. We incorporated female identity as a random effect, to control for multiple litters produced by individual females. We also included year as a

random effect, to control for annual stochasticity in environmental conditions that may have affected sex ratios. Parameter estimates  $\beta$  ( $\pm$  SE) are reported in the logit form.

#### *4.3.5 Food availability at conception*

Golden-mantled ground squirrels are primarily herbivores (McKeever 1964), and green herbaceous vegetation in our study area becomes available immediately after melting snow exposes bare soil (Van Vuren & Armitage 1991). Hence, we used date of snow melt as the indicator of the onset food availability each year to evaluate glucose-driven effects on offspring sex ratio (Cameron 2004; Helle et al. 2008). We expected females to have low levels of circulating glucose before snow melt and thus gestate litters that were female-biased; litters conceived after snow melt would be male-biased. We estimated the date of conception for each litter by subtracting 58 days (length of gestation + lactation) from the date of litter emergence. We then subtracted date of conception from date of snow melt to yield an estimate of food availability at the time of conception; this estimate of food availability we used as a predictor of litter sex ratio in a GLMM with a binomial error structure and logit link. As before, maternal identity and year were included as random effects.

Data were analysed using R version 3.0.2 (R Core Team 2013). We used the packages lme4 (Bates et al. 2014) and rethinking (McElreath 2014) to fit multi-level models.

## **4.4 Results**

### *4.4.1 Adult sex ratio and female density*

We recorded 127 litters that were weaned within the study area from 1995 through 2012; of these, litter size, sex ratio at weaning, and maternity were known for 101 litters, born to 64

females. Across all litters in all years, the sex ratio of weaned pups was 48.7% male, which was not significantly different from the null hypothesis of 50% males (exact binomial test, two-tailed,  $P = 0.545$ ). By year, the per cent of male pups produced varied from 18.2% (in 2000) to 60.5% (in 1995).

Adult sex ratio of the population did not significantly predict offspring sex ratio ( $F_{1,16}=1.24$ ,  $P=0.283$ ,  $R^2=0.07$ ; Figure 1a). By contrast, the number of breeding females (log-transformed) present in the population was a significant predictor of offspring sex ratio ( $F_{1,16}=17.36$ ,  $P<0.001$ ,  $R^2=0.52$ ; Figure 1b).

#### *4.4.2 Maternal investment*

There was no relationship between total litter mass (a proxy for total maternal investment) and litter sex ratio ( $F_{1,30}=0.108$ ,  $P=0.744$ ). Within a litter, sons were not heavier than daughters (paired t-test:  $t_{31}=-0.162$ ,  $P=0.872$ ). After controlling for maternal identity and year, relative investment in sons did not vary with total maternal investment (GLMM effect of litter mass:  $\beta=-0.001\pm0.002$ ,  $t=-0.673$ ,  $P=0.500$ ; Figure 2). The ratio of average mass of sons to average mass of daughters also did not vary with maternal investment (BGLMM effect of litter mass:  $\beta=0.0001\pm0.0002$ ,  $t=0.624$ ,  $P=0.532$ ).

#### *4.4.3 Local competition with kin and nonkin*

At the individual female level, local density of total breeding adult females was not significantly related to litter sex ratio ( $F_{1,99}=2.04$ ,  $P=0.156$ ,  $R^2=0.01$ ). However, litter sex ratio was significantly affected by the density of the type of breeding female. Solitary females, defined as those not in the presence of female kin or nonkin, produced litters that were 40% male (95% CI= 26.5-55.97% male). Density of breeding kin predicted a shift toward production of sons at

densities above 2 kin, although the effect was significant only at the highest density (4 kin,  $\beta = 3.08 \pm 1.5$ ,  $z = 2.03$ ,  $P = 0.042$ ; Fig. 3). By contrast, there was no significant effect of density of nonkin breeding females on litter sex ratio (Figure 3). Additionally, although maternal age did not affect offspring sex ratio ( $\beta = 0.04 \pm 0.09$ ,  $z = 0.48$ ,  $P = 0.635$ ; Figure 3), there was an interaction between maternal age and kin density, where older females ( $\geq 3$  years) surrounded by many breeding kin shifted toward production of daughters ( $\beta = -1.45 \pm 0.73$ ,  $z = -1.96$ ,  $P = 0.050$ ); yearling females surrounded by many breeding kin produced more sons (Figure 4).

#### *4.4.4 Food availability at conception*

At the individual female level, timing of conception in relation to food availability had no significant effect on litter sex ratio ( $\beta = 0.01 \pm 0.01$ ,  $z = 1.05$ ,  $P = 0.293$ ; Figure 5). Females who conceived litters after snow melt did not produce more male-biased litters than females who conceived litters before snow melt. Litter size ranged from 1-8 pups, with an average litter size of  $5.05 \pm 1.39$  pups. Litter size did not vary in relation to conception date (GLMM effect of conception date:  $\beta = -0.02 \pm 0.01$ ,  $z = -1.20$ ,  $P = 0.229$ ). Litter sex ratio did not vary significantly by litter size ( $F_{7,93} = 1.48$ ,  $P = 0.185$ ).

## **4.5 Discussion**

In this population of golden-mantled ground squirrels, variation in offspring sex ratio did not conform to predictions from frequency-dependent (Fisher 1930; Werren & Charnov 1978), maternal condition (Trivers & Willard 1973), or non-adaptive (sex-specific embryonic mortality; e.g. Larson et al. 2001) theories of sex allocation.

Extending Fisher's (1930) theory of equal sex allocation, Werren & Charnov (1978) demonstrated that selection could favor shifts away from 1:1 offspring ratios, towards the

temporary overproduction of the rarer sex. An inverse relationship between adult sex ratio and offspring sex ratio has been found in some mammalian species, such as lions (*Panthera leo*, Creel and Creel 1997), and humans (Lummaa et al. 1998), but not in other species, such as gray-tailed voles (*Microtus canicaudus*, Bond et al. 2003). Among ground squirrels, our study found no support for golden-mantled ground squirrels, and Gedir and Michener (2014) found only weak support for Richardson's ground squirrel (*Urocitellus richardsonii*). Fisher's theory may be most applicable to populations with limited dispersal, where adult sex ratios at the time of conception may accurately predict future mating opportunities for new offspring.

The Trivers-Willard theory has been widely tested across mammalian taxa (Cockburn et al. 2002; West 2009), but evidence from rodents remains equivocal; some studies report results that are consistent with predictions whereas others, including ours, report results that do not support the theory (Sikes 2007). Inconsistent results might have several causes. One is that rodents are typically polytochous; mothers in polytochous species might manipulate litter size instead of sex ratio in response to maternal condition, potentially complicating predictions of sex ratio adjustment (Williams 1979; Sikes 2007). Another is the validity of the assumption that males in better condition enjoy higher reproductive success. Although male body size has been linked to mating success in many mammals (Lindenfors et al. 2007), the assumption is not always verified for species tested under the Trivers-Willard theory, including golden-mantled ground squirrels. A third is that the ability of a female to invest in offspring may reflect extrinsic as opposed to intrinsic condition (Sikes 1996). Ground squirrels appear to combine "capital" and "income" breeding strategies (Stearns 1992), meaning that while maternal condition before conception may influence reproductive investment, the majority of resources invested in reproduction are acquired during the period after parturition (Broussard et al. 2005). Lactation in ground squirrels

is fuelled almost entirely by daily caloric intake (Kenagy et al. 1989); thus, our measure of litter mass at weaning largely represents post-natal maternal condition.

Food availability at conception has been proposed as a non-adaptive cause of biased offspring sex ratios in mammals (Cameron 2004; Cameron & Linklater 2007). Differential susceptibility of female embryos to circulating glucose levels (“glucose toxicity,” Larson et al. 2001) has been documented in rodents, including elevated female embryo mortality (Savchenko et al. 2014) resulting in male-biased litters (Helle et al. 2008). While we did not measure maternal glucose levels in our study, we did not detect changes in sex ratio with timing of conception relative to the vegetation growing season. At snow melt, the consumption of new, green vegetation by pregnant females should trigger a rise in circulating levels of glucose above the fasting levels that are characteristic of winter hibernation, reducing viability of female embryos; yet, litters conceived after snow melt were not male-biased. Further, litter sex ratio did not vary by litter size suggesting that biased sex ratios were not accomplished through selective mortality that reduced overall litter size.

Density of breeding females appeared to influence offspring sex ratio. At the population level, more female offspring were produced in years of low density, and more male offspring were produced in years of high density. This finding is consistent with theories of local resource competition (Clark 1978; Silk 1983), where females facing high competition for resources bias investment away from the philopatric sex (in this case, females). However, this density effect was expressed differentially at the individual level. Litter sex ratio did not vary in response to total density or nonkin density, but responded strongly to kin density. Further, the effect of kin was nonlinear, as females in the presence of kin produced more sons than did solitary females only in particularly dense kin neighbourhoods. If density-dependent competition among breeding

females is also nonlinear, 4 female kin may represent a threshold of habitat saturation beyond which additional daughters are unlikely to be recruited. The role of kinship in the behavioural interactions and spatial organization of golden-mantled ground squirrels has not been studied. However, even though the species is considered solitary and asocial, it possible that female kin are less agonistic toward each other than to nonkin and accordingly share space. Thus, competition with kin is disproportionately intense, especially at high densities. Kin identification might be based on familiarity (Hare 1998), self-referential scent matching (Mateo 2010), or both (Holmes 1995).

Interestingly, the effect of kin density was reversed for older females: in dense kin neighbourhoods, older (3 years or more) females were much more likely to produce daughters than sons. A negative relationship between sex ratio (sons produced) and maternal age is expected when daughters remain philopatric, since daughters born late in a female's life will compete with her for fewer years than daughters born early (Cockburn et al. 1985; Clutton-Brock and Iason 1986). However, in our study offspring sex ratio was not affected by maternal age *per se*; instead, the effect we detected might have been caused by female dominance within the matriline that is conferred by age. Dominant females may be more able to bear the costs that result from competition with additional philopatric daughters than are younger or subordinate females (Clark 1978), or perhaps only dominant females are able to facilitate the recruitment of additional philopatric daughters, who provide greater fitness benefits than would dispersing sons. Female dominance is reliably associated with positive reproductive consequences in social species (Stockley & Bro-Jorgensen 2011; Clutton-Brock and Huchard 2013), and may be in relatively asocial species as well.

Local resource competition has been invoked to explain changes in offspring sex ratio in a variety of mammalian taxa, including primates (Silk 1983; van Schaik & Hrdy 1991; Silk & Brown 2008), ungulates (Kruuk 1999; Hewison & Gaillard 1996; Sheldon & West 2004), and marsupials (Cockburn et al. 1985; Johnson et al. 2001; Schwanz & Roberts 2014). Our study found support for local resource competition was most pronounced when females live in dense neighbourhoods of female kin. In other rodents, evidence for sex-ratio adjustment in response to local density is mixed. The number of breeding females influenced offspring sex ratios in four species of *Acomys* mice, but the direction of the effect (male- or female-bias) differed by species (Nováková et al. 2010). Local female density increased the proportion of males born to Damaraland mole-rats (*Fukomys damarensis*, Lutermann et al. 2014), but did not affect offspring sex ratios of Richardson's ground squirrels (Gedir and Michener 2014). Few studies have considered relatedness in calculations of local density. Local density of females did not affect offspring sex ratios of Townsend's voles (*Microtus townsendii*), nor did presence or absence of a matriline member (Lambin 1994). Yellow-bellied marmots (*Marmota flaviventris*) did not alter offspring sex ratios according to local density, but young females produced female-biased litters in the presence of kin (Armitage 1987). Inconsistent findings among species may result in part because the role of kinship was not considered in all studies. Alternatively, different findings among species may truly represent different effects of female density, resulting from differing social systems that alter the costs and benefits of producing sex ratios biased toward sons or daughters (Silk & Brown 2008).

One of the prevailing hypotheses for the physiological mechanism underlying the adjustment of offspring sex ratios centres on stress hormone (corticosteroid) levels; indeed, by influencing the levels of other important hormones, the corticoadrenal stress response may be the basis for

multiple mechanisms of sex bias in mammals (Linklater 2007). Negative social interactions are known to affect stress hormone secretion in rodents (reviewed in Tamashiro et al. 2005), including in pregnant females (Stefansky et al. 2005), and sex ratio studies that have measured stress levels find that stressed females tend to produce more sons (reviewed in Cameron 2004). In ground squirrels, offspring sex ratios appear linked to social status and stress hormone levels: female yellow-bellied marmots who were socially-avoidant produced more sons than daughters (Armitage 1987), as did female Richardson's ground squirrels with higher corticosterone levels during gestation (Ryan et al. 2012; Ryan et al. 2014). Interestingly, litter sex ratio in our study was more sensitive to the density of related than to unrelated females. If corticosterone levels do play a causal role in biasing offspring sex ratios in ground squirrels, this finding suggests that social interactions with female kin are more stressful than interactions with nonkin. Stressful social interactions are often implicated in the reproductive inhibition of subordinate females (Clutton-Brock & Huchard 2013). If dominant female ground squirrels are able to induce shifts in the offspring sex ratios of subordinate kin (toward production of sons), instead of suppressing reproduction completely, they may be able to reduce competition for their own daughters without sacrificing gains to inclusive fitness.

The relative roles of cooperation and competition with kin have not been empirically determined for most species, despite their importance in the evolution of sociality (West et al. 2002). Members of the ground squirrel clade span the range of social organization (Michener 1983), and tension between competition and cooperation with female kin, variably expressed at different densities, may in some species constrain the evolution of complex social systems. The extent to which biasing offspring sex ratios effectively alters the competitive/cooperative landscape for

females is yet to be understood in this and other relatively asocial species, and warrants further study.

#### **4.6 Conclusion**

We found that golden-mantled ground squirrels adjusted offspring sex ratios in response to female density, at both the population and the individual scale. Surprisingly, the local density of female kin had the most pronounced effect on offspring sex ratio, which adds to a growing body of literature (reviewed in Hatchwell 2010) suggesting that social interactions with kin may be important in both the spatial organization and population dynamics of relatively asocial species.

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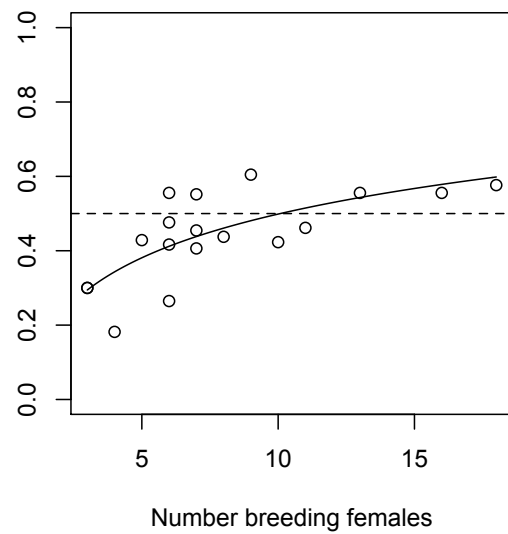
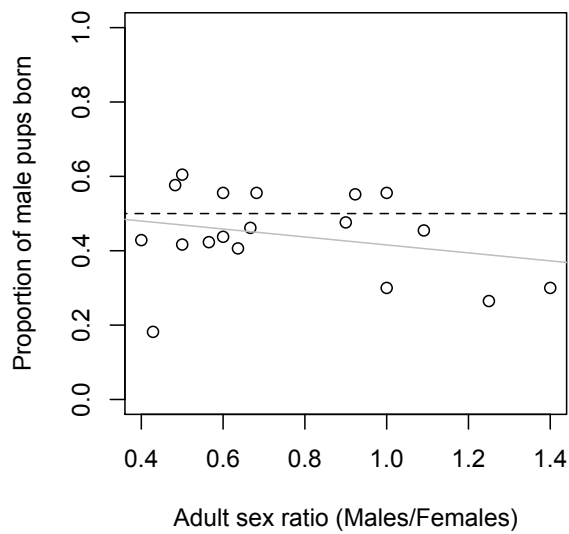
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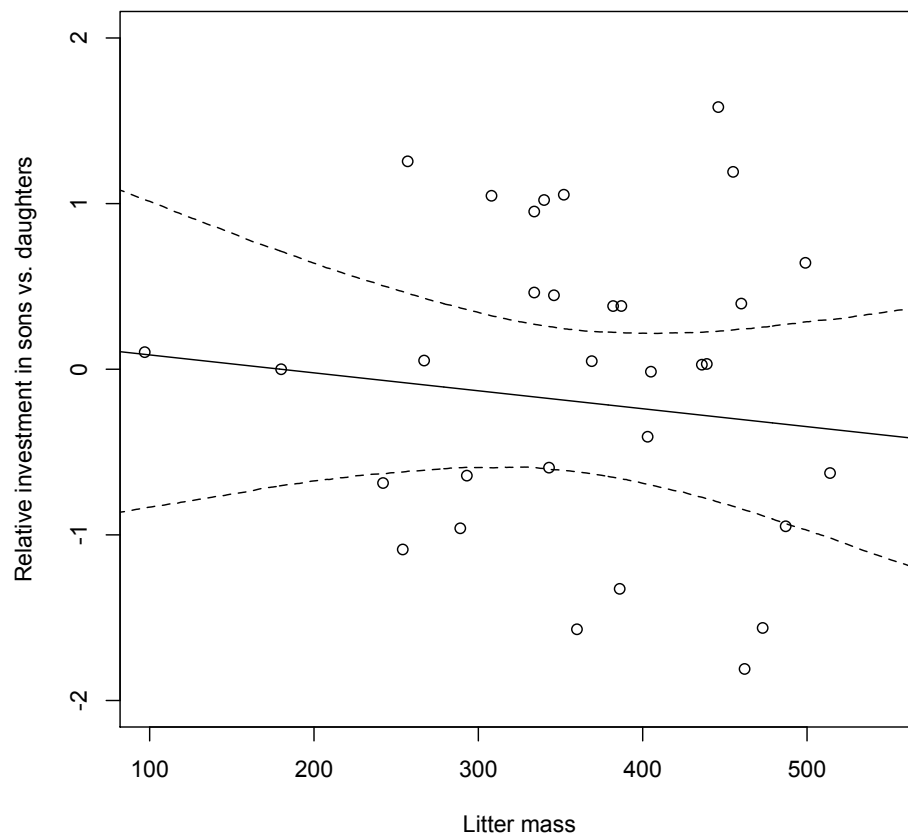
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## 4.8 Figures

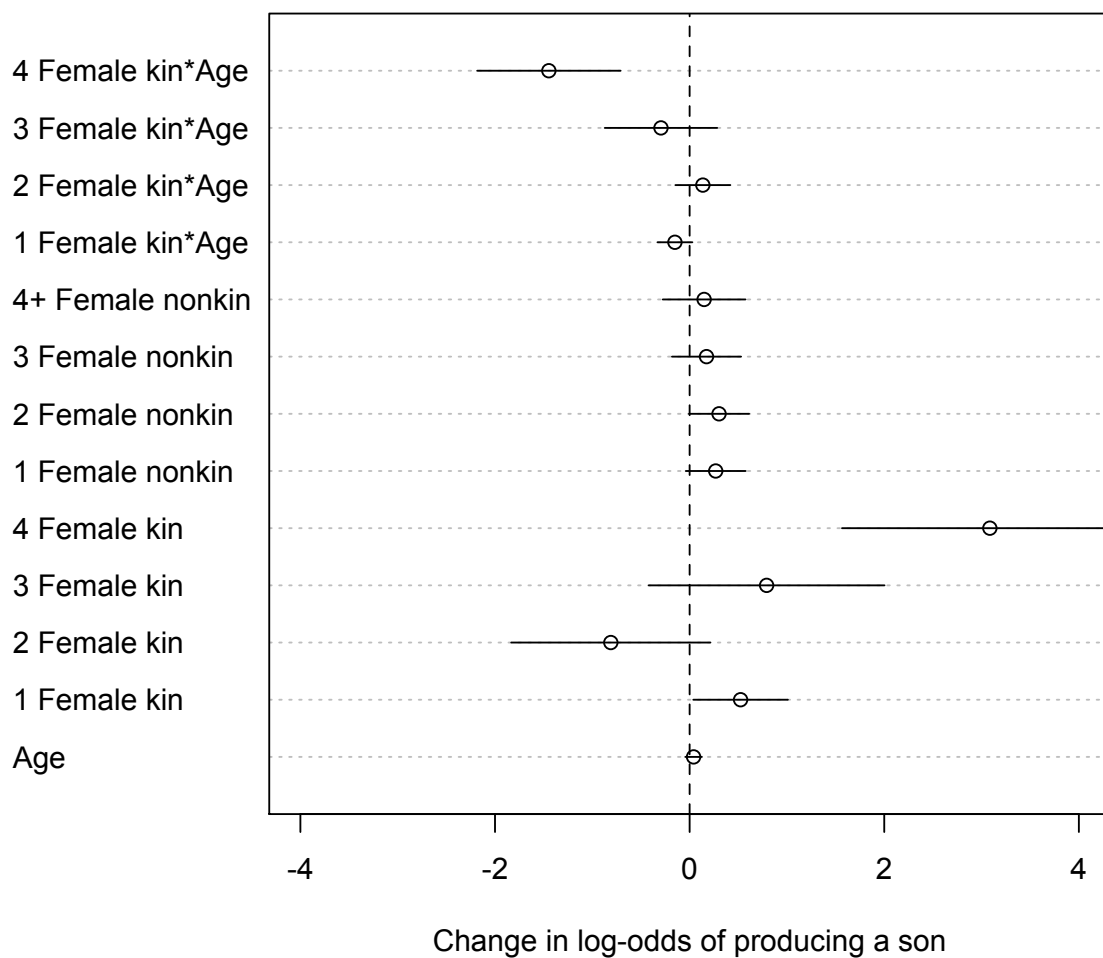
**4.8.1** Proportion of male pups out of total pups produced each year, predicted by (a) adult sex ratio or (b) number of breeding females present in the population that year. Dashed line represents null expectation of 50% males.



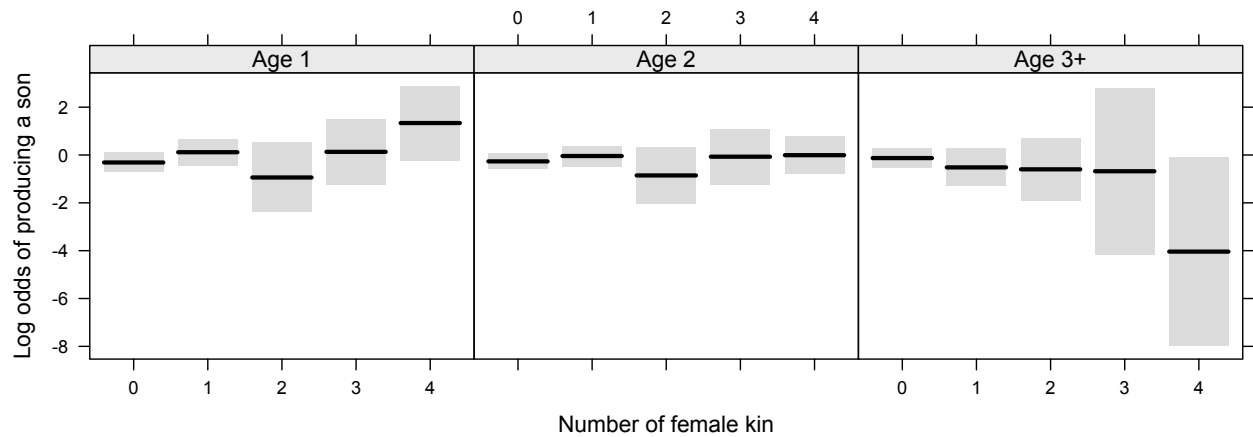
**4.8.2** Relative investment in sons versus daughters [ $\log(\text{total mass of sons}/\text{total mass of daughters})$ ] within a litter, as a function of maternal investment (litter mass). Positive values represent increased investment in sons; negative values represent increased investment in daughters. Points indicate observed values. The solid line represents the maximum likelihood estimate of the mean relative investment by litter mass, after controlling for maternal identity and year; the dashed lines represent the 95% prediction interval.



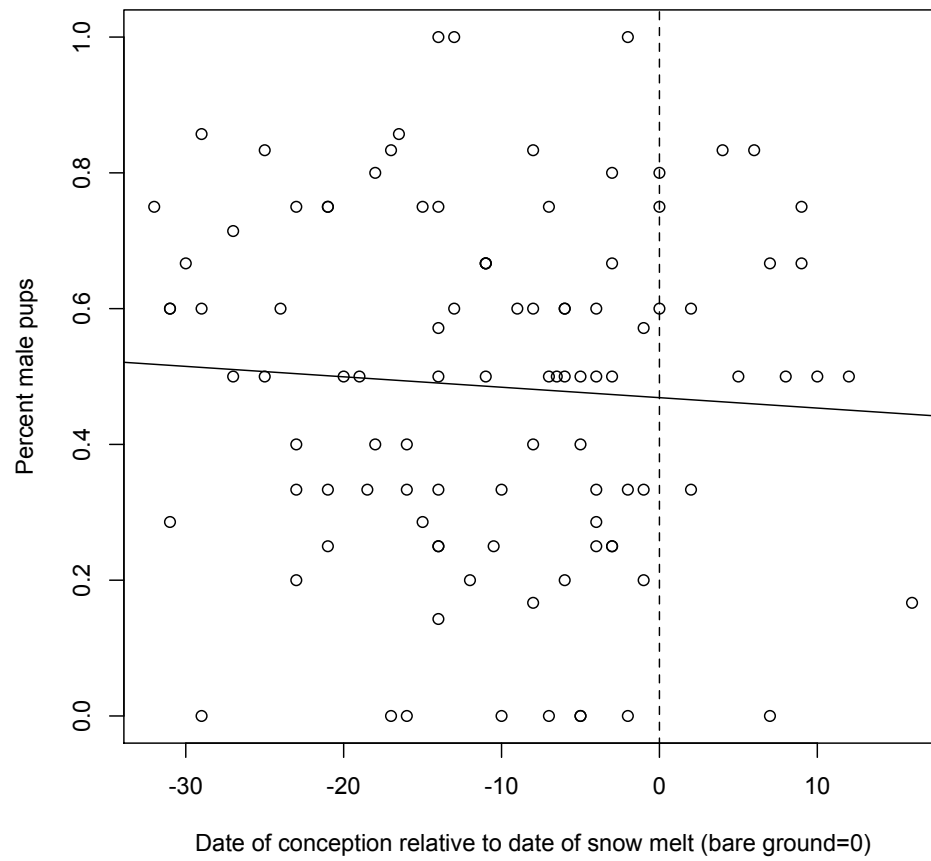
**4.8.3** Coefficients from a GLMM of maternal age, density of related females, density of unrelated females, and an interaction between density of related females and maternal age; coefficients represent change in parameter compared to a solitary female (female kin=0, female nonkin=0). Density of related females (“Female kin”) and unrelated females (“Female nonkin”) were modelled as factors to explore non-linear effects, and Female identity was included as a random effect. Positive coefficients represent increased likelihood of producing a son; negative coefficients represent reduced likelihood of producing a son.



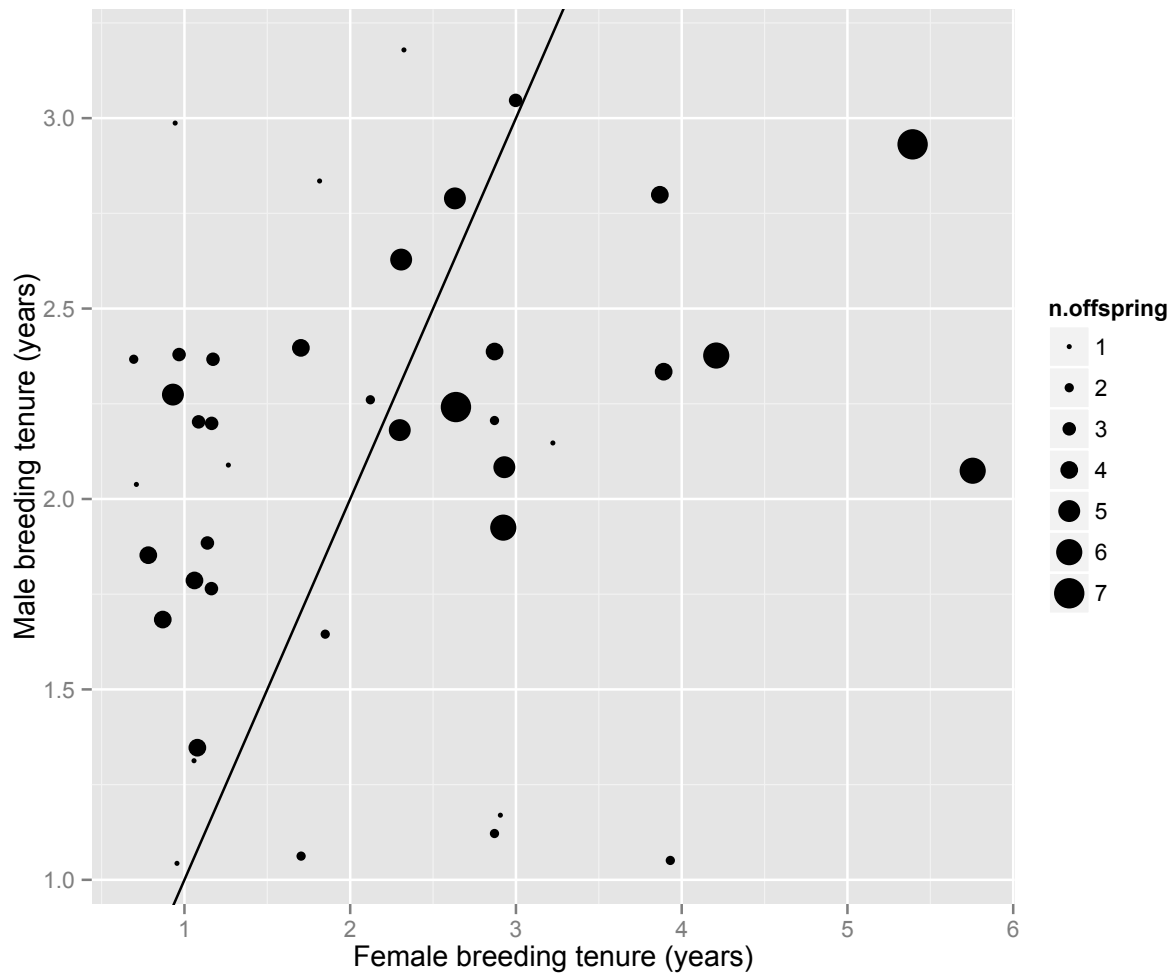
**4.8.4** Modelled relationships between kin density and odds of producing a son for each birth, by maternal age. Effects were plotted with the additional factor (nonkin density) held constant at the mode (0). Black lines are estimates with shaded 95% confidence interval. Zero log odds indicates equal likelihood of producing a son or daughter; positive estimates represent increased likelihood of producing a son, and negative estimates represent reduced likelihood of producing a son.



**4.8.5** Litter sex ratio in relation to conception date, 1995-2012. Vertical dashed line at zero represents date of snow melt.



**Appendix A.** Assortative mating by length of tenure between male and female golden-mantled ground squirrels. Size of points indicates the number of offspring produced by a male-female pair.



**Appendix B.** Individual variation in the benefits of multiple paternity for female golden-mantled ground squirrels.

The slope of the relationship between number of mates and offspring produced per litter is also known as the Bateman gradient: steeper slopes indicate a greater fitness benefit of multiple mating (Bateman 1948). Bateman gradients are usually constructed at the population level for each sex. But Bateman gradients can be constructed for individual females when they produce multiple litters over their lifetime, some of which are singly-sired and some of which are multiply sired. To identify individual variation in Bateman gradient, we fit a generalized linear mixed model (with a Markov Chain in JAGS, using the same 2008-2015 litters used above in the Bateman gradient analysis) with number of offspring in the litter as the outcome variable, number of sires as a fixed effect, and individual female as a random effect with varying slope. Then, because inbreeding may impair fertility, we evaluated the relationship between each female's varying slope and her level of inbreeding. In golden mantled ground squirrels, the extent to which females benefit from multiple mates (measured as the slope of their individual Bateman gradient) is positively correlated with level of inbreeding (internal relatedness, Amos 2001). Our sample size is small (n=14 females), but level of inbreeding explained 47% of the variation in individual Bateman gradient.

