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REPRODUCTIVE DELAY IN FEMALE CAPE GROUND SQUIRRELS

(Xerus inauris)

by

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B.A. College of St. Benedict, 1995

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
in the Department of Biology
in the College of Sciences
at the University of Central Florida
Orlando, Florida

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ABSTRACT

The Cape ground squirrel, *Xerus inauris*, is a highly social cooperative breeder that forms groups containing multiple breeding females. While the distribution of reproduction among group members is fairly even (i.e. exhibits low reproductive skew), previous studies of Cape ground squirrels suggest the reproductive development of sub-adult females is inhibited by the presence of adult breeding female group mates. As reproductive delay is known to be influenced by a number of different parameters, my goal was to determine if other factors affected the timing of sexual maturity, and if so, which factors are the most influential. In this study, I simultaneously test the relative power of seven different social and environmental parameters at explaining the variation in the female age of sexual maturity in two populations of Cape ground squirrels. Field work was conducted at two study sites in southern Africa, where trapping, behavioral and hormonal data were collected to determine the timing of reproductive development. Hormonal data was analyzed through the use of steroid enzyme immunoassay analysis to quantify the concentration of gonadal hormone in fecal samples which indicate the onset of sexual maturity. Prior to the start of the field season, I conducted an initial experiment to determine the best alternative form of fecal storage if freezing was unavailable. I found that drying feces provides a more reliable method for long-term preservation of fecal steroid concentrations when compared to storing fecal samples in alcohol. Data associated with each of the seven parameters was analyzed using model selection to simultaneously measure the ability of different combinations of parameters to explain the observed variation in female age of sexual maturity. I found that an increase in the number of adult breeding female group mates and related adult male group mates resulted in a substantial inhibition of female reproductive

maturity. I concluded that, while female Cape ground squirrels gain many advantages from group living, their sexual maturity is primarily influenced by a tug-of-war among social parameters, with minimal direct influence by environmental ones.

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OVERALL INTRODUCTION

Cooperative breeding refers to a social system in which individuals other than the parents provide care for offspring, delay dispersal and delay reproduction (Solomon and French, 1997). Such groups typically vary in the distribution of reproduction among group members, formally known as reproductive skew (Keller and Reeve, 1994; Vehrencamp, 1983). Recent investigations into reproductive skew have led to the development of multiple theoretical models that attempt to explain the variation in reproductive distribution with most models falling into two categories: transactional and incomplete control models. Transactional models assume someone maintains complete control over the distribution of reproduction within the group, while incomplete control models assume reproduction is shared because no group member is able to completely manipulate the allocation of the group's reproductive output.

Support for these skew models and the underlying mechanisms of reproductive distribution have been provided largely by investigations of animal societies exhibiting high reproductive skew (Lewis and Pusey, 1997) and obligate cooperative breeding (helpers are necessary for successful breeding; Brown, 1987). Such investigations have focused on how ecological constraints and social factors influence reproductive skew through their impact on the suppression of subordinate group members. Further research using facultative cooperative breeders with low skew could provide a better understanding of the relative importance of factors affecting skew as such species afford a different system with which to test the predictions of reproductive skew theories.

The Cape ground squirrel (*Xerus inauris*) is a facultative cooperative breeder that exhibits

low skew and delay in sexual maturity (Waterman, 2002). As such, these squirrels are a good species in which to study the mechanisms underlying reproductive delay and implications for conflicting reproductive skew models. In accord with established theoretical models of reproductive skew, female Cape ground squirrels possess characteristics that commonly found in species with high levels of skew; however, Cape ground squirrel groups almost always contain multiple breeding females with no evidence of a dominance hierarchy (Waterman, 1995) suggesting additional factors may play a role in the distribution of reproduction.

I examined the simultaneous effects of these additional social and environmental factors on the age of sexual maturity to identify the major determinant(s) of reproductive delay and corresponding skew in female Cape ground squirrels. My first objective was to verify inhibition of sexual maturity and potential sexual activity of sub-adults by monitoring the reproductive hormones in pre-reproductive and reproductive female Cape ground squirrels using non-invasive methods. Using fecal progesterone concentrations, I examined whether sub-adult females demonstrated signs of ovarian activity and/or pregnancy. As part of my first objective, I tested the effects of different storage treatments on the hormone concentrations of female Cape ground squirrel fecal samples. I aimed to determine the best alternative storage treatment, if freezing was not a viable option while in the field (Chapter 1). My second objective was to examine the relative effects of the following parameters on the age of sexual maturation: number of adult breeding female group mates, rates of aggression from adult females, number of related adult male group mates, number of exposures to unrelated adult males, body mass, density (group size) and resource levels. My results are discussed in terms of their ability to explain the low levels of reproductive skew found in female Cape ground squirrel social groups (Chapter 2).

CHAPTER 1: EFFECTS OF DIFFERENT STORAGE METHODS ON CAPE GROUND SQUIRREL FECAL STEROID HORMONE CONCENTRATIONS

Abstract

Fecal steroid analysis is an increasingly common non-invasive technique used in both captive and field studies to measure an animal's approximate hormonal levels and corresponding physiological state. Fecal collection in the field necessitates storage and transportation methods that will prevent the degradation of hormonal metabolites by fecal bacteria. To determine the most stable and therefore preferred method of storage, 48 fecal samples were collected from 6 captive female Cape ground squirrels (*Xerus inauris*). Each sample was randomly divided into three sub-samples to be processed for storage through freezing, drying, or preservation in ethanol. Frozen samples were stored at -20° C, dried-treated samples were desiccated in a conventional oven at 40° C for 4 hours, and alcohol-treated samples were preserved in 3 ml of 95% ethanol. Samples were stored for 330 days followed by enzyme immunoassay analysis (EIA) to determine their progesterone and estrone conjugate (E₁C) concentrations. Validations were performed to establish that the progesterone and E₁C assays accurately measure fecal progesterone and estrone conjugate concentrations and were sensitive enough to detect biologically meaningful differences in these steroid metabolite concentrations in female Cape ground squirrels. Validation results showed a significant difference in progesterone concentrations of gravid females compared to sub-adults and non-gravid females. There was no difference in estrone conjugates among the three physiological states. Duration of storage time

did not affect progestogen or estrone metabolite concentrations after being frozen for 3 months.

Storage treatment results showed no significant difference between frozen and dried samples, but a significant difference was found between frozen and ethanol samples in both progestogen and estrone conjugate concentrations demonstrating that drying feces provides a reliable method for long-term preservation of fecal steroid concentrations and is the better alternative when freezing is not a viable option.

Introduction

Fecal steroid analysis is an increasingly popular non-invasive technique due to its enormous potential for answering wide-ranging questions in endocrinology (Schwarzenberger et al., 1997, Whitten et al., 1998, Brown et al., 1997). Combined with behavioral data, endocrine analysis can provide valuable information concerning the physiological mechanisms mediating numerous behaviors that ultimately influence a species' viability, reproduction and life history. Application of fecal-based investigations in monitoring gonadal function has become popular since this method removes the necessity of obtaining blood samples from the study subjects. Assessing reproductive profiles requires repeated sample collection so the appeal of fecal steroid analysis (especially in free-ranging species) is apparent when considering that stress from frequent handling can affect hormone secretion (Hamilton & Weeks 1985, Millspaugh et al., 2002, Wasser et al., 2000). Studies examining reproduction in free-ranging species using fecal steroid analysis have involved taxa as diverse as muriquis, *Brachyteles hypoxanthus* (Strier & Ziegler 2005); tule elk, *Cervus elaphus nannodes* (Stoops et al., 1999); deer mice, *Peromyscus maniculatus* (Harper & Austad 2004); black rhinoceros, *Diceros bicornis minor* (Garnier et al., 1998); and spotted owls, *Strix occidentalis* (Washburn et al., 2004).

Monitoring endocrine function by fecal analysis can be susceptible to problems resulting from high individual variability in reproductive hormone concentration. Steroid hormones are subject to metabolism by the liver as well as specific intestinal bacteria before they are eliminated via feces (Taylor 1971, Palme et al., 1996). High intra-individual variation, possibly due to fluctuations in rates of metabolism and re-absorption of steroid hormone and amount of gut bacteria within an individual across time, may make it difficult to detect slight differences

between individuals when examining patterns and cycles of multiple group members. Researchers must attempt to control for any variation due to methodological techniques, including preservation and storage procedures, which may alter hormone concentrations to avoid misinterpretation of the data (Lynch et al., 2003).

Fecal collection in the field is especially vulnerable to artificial fluctuations in steroid hormone concentration resulting from less than ideal fecal storage and transportation methods. Optimal storage techniques minimize degradation of steroid hormone metabolites by naturally occurring fecal bacteria, bacterial enzymes (Woods 1975), temperature (Schlenker et al., 1999) and ultraviolet light (Matkovics 1972). Microbial transformation of steroid hormone metabolites can occur within hours after defecation (Mostl et al., 1999, Wasser et al., 1988) due to the often high bacterial loads found in fecal matter. Leaving fecal samples untreated can alter fecal steroid concentration in a species specific manner (increased: baboon: Wasser et al., 1988; black rhinoceros: Galama et al., 2004; decreased: cow: Masunda et al., 1999, Schlenker et al., 1999). Such findings suggest that the direction of fecal steroid concentration changes may be influenced, in part, by digestive strategy (carnivore, omnivore, forestomach or caecocolic fermenter). Decreases in temperatures are known to drastically reduce the growth rate of bacteria and Schlenker et al. (1999) found that lower temperatures were found to slow the decline in concentrations of both estrogen and progesterone in cattle feces being stored at 30° C and 5° C. Ultraviolet radiation (e.g. prolonged sun exposure) has also been found to degrade hormones by inducing steroid transformation (Matkovics 1972) resulting in a modified hormone concentration.

In order to minimize degradation of the fecal steroid, it is typically recommended to store

fecal samples at sub-zero temperatures until endocrine analysis can be performed (Whitten et al., 1998). While freezing is the preferred method under controlled laboratory conditions and recent studies have recommended the use of a newly developed field method of fecal steroid hormone extraction (separating steroids from fecal material by solid phase extraction: Ziegler & Wittwer 2005), these techniques require either additional skill and/or equipment in the field making them not readily employable by most field researchers. Thus, efficacy tests of alternative fecal storage methods for a wide variety of taxa are needed. With the exception of Beehner & Whitten (2004), previous studies investigating alternative field preservation or storage methods have focused on short-term effects of storage treatments and are limited to primates, carnivores, artiodactyla, and perissodactyla (baboon: 180 d, Khan et al., 2002; 30 d, Lynch et al., 2003; 400 d, Beehner & Whitten 2004; cheetah: 7 d, Terio et al., 2002; white tailed deer and elk: 7 d, Millspaugh et al., 2003; black rhino: 180 d, Galama et al., 2004; and giraffe, black rhino, dama gazelle, mountain goat: 90 d, Neumann et al., 2002). Terio et al. (2002) recommended ethanol as the best alternative preservation method for maintaining fecal progesterone and estrogen metabolite concentrations in cheetah feces for periods of up to 2 weeks. These results however were inconsistent with previous findings in baboon feces (Khan et al., 2002, Lynch et al., 2003) which were found to have altered steroid concentrations in ethanol-treated fecal samples. While Lynch et al. (2003) suggests freezing fecal samples in 95% ethanol at -20° C for up to 2 weeks as the best alternative preservation method, they also obtained results capable of detecting reproductively significant events (i.e. pregnancy) with samples stored in 95% ethanol in a charcoal refrigerator. Galama et al. (2004) recently found drying feces in a solar box cooker and mixing feces in 80% MeOH are both effective methods to maintain absolute and relative

progestogen concentrations for black rhino fecal samples for up to 180 days. Drying fecal samples can, however, result in a loss of steroid hormone in some species (Ziegler & Wittwer 2005).

Noticeably missing among these studies are long-term fecal storage investigations addressing storage treatment effects in a more diverse array of species and the consideration of storage effects at the steroid hormone profile level. Within Mammalia, rodents are the most diversified order and comprise the greatest number of species. Their diversity provides an invaluable tool for answering a limitless number of endocrine questions. In recent years, fecal steroid analysis has been used to investigate endocrine hormone concentrations in free-living deer mice (*Peromyscus maniculatus*) and southern red-backed voles (*Clethrionomys gapperi*: Harper & Austad 2004), free-living midday gerbils (*Meriones mefidianus pall.*: Kuznetsov et al., 2004), captive oldfield mice (*Peromyscus polionotus*: Good et al., 2003, 2005), Belding's ground squirrels (*Spermophilus beldingi*: Mateo et al., 2005) and laboratory mice and rats (Touma et al., 2004 and Eriksson et al., 2004, respectively). With such analyses becoming more popular with rodents, especially free-living, the necessity of determining alternative fecal storage methods is apparent. Additionally, previous fecal steroid analysis studies have concentrated on the effects of storage techniques on measured hormone concentrations. An analysis of changes in hormone concentrations at the profile level (i.e. both high and low concentrations) due to storage effects is crucial for understanding the effects on cycling gonadal hormones.

My goals in this study were to: 1) validate that progestogen and E₁C enzyme immunoassay analysis (EIA) accurately measure progestogen and estrone conjugate concentrations in feces of the African Cape ground squirrel (*Xerus inauris*, Family Sciuridae,

Tribe Xerini), 2) demonstrate that the fecal progesterone and E₁C assays are sensitive enough to detect biologically meaningful differences in progesterone and estrone conjugate concentrations in sub-adult, non-gravid and gravid adult female Cape ground squirrels, 3) examine the effects of storage of samples stored at sub-zero temperatures on hormonal concentrations and 4) determine the effects of fecal samples stored in alcohol or dried in a drying oven on hormonal concentrations.

Methods

Fecal Sample Collection and Extraction

Physiological Validation

Fecal samples were collected from 11 free-ranging female Cape ground squirrels trapped at the S.A. Lombard Nature Reserve (SALNP) near Bloemhof, South Africa (27°35'S, 25°35'E) between May 26 – Oct 10, 2004. Two samples were collected from sub-adults, five from non-gravid adults and four from gravid adults. Females were considered adult if the nipples were dark and elongated, indicating that they had previously bred (Waterman 1996). Gravid and non-gravid females were classified by examination of changes in the vulva, nipples, and weight as well as behavioral observations of estrus, and maternal isolation (Waterman 1995). As well, pregnancy for each individual gravid adult was confirmed by emergence of viable offspring. All fecal samples were stored in a -20° C freezer until transported back to the United States on dry ice. Collection of these fecal samples was part of a larger study that documented reproductive behaviors as well as pregnancies.

All fecal samples were extracted following Graham et al. (2001). Briefly, each sample was weighed out to exactly 0.50 g and placed in an 8-mL glass evaporation-proof vial (National Scientific, Pittsburgh, PA) into which 5 mL of 80% ethanol was added to extract the hormone. All vials were capped and placed on a shaker (Eberbach Corp., Ann Arbor, MI) overnight. Sub-samples were then centrifuged (2,500 rpm, 30 minutes), and the supernatant was poured off and stored at -20° C until EIA analysis.

Storage Time

Ten fecal samples were collected from ten free-ranging adult female Cape ground squirrels trapped at SALNP between July 25-27, 2005. All fecal samples were stored in a -20° C freezer for 1-3 days before being transported back to the United States on dry ice. Each fecal sample was thawed, mixed thoroughly and divided into four 0.50 g sub-samples. The first set of sub-samples from each of the ten individuals was extracted immediately following the methods outlined above. Subsequent sets of sub-samples were extracted one month and three months after initial extraction.

EIA Validation and Storage Treatment Condition

Forty-eight fecal samples were collected from six captive adult female Cape ground squirrels housed at SALNP. Details of captive conditions are reported in Bouchie et al. (2006). Fecal samples were collected between May 10 – June 22, 2003 from a plastic tray below each individual cage. Each tray was covered with a sheath of 40% shade cloth to keep urine and fecal matter separate. Each fecal sample was randomly subdivided into 3 ~1 g sub-samples prior to being stored in alcohol, dried or frozen. Frozen sub-samples were immediately stored in a -20° C freezer. Ethanol treated sub-samples were preserved in 3 ml of 95% ethanol at room temperature (to mimic storage conditions expected in most field situations). Sub-samples for the dried treatment were wrapped in aluminum foil, pressed flat, and dried in a conventional oven (Kelvinator S2620, Johannesburg, South Africa; 60 x 60 x 65 cm) at 40° C for 4 hours. To determine sufficient drying time, 28 test samples were placed in the conventional oven at 40° C and weighed at 1 hour increments until no more weight was lost. Using this method, I established that a 4 hour drying time was sufficient to eliminate all fecal moisture. Dried treated

sub-samples were weighed before and after drying to control for differences in water content across treatments. Percent dry mass was obtained by dividing dry weight by initial (wet) weight. Percent dry mass was then multiplied by 0.50 g to determine final weight used for extraction. Frozen-treated fecal samples were transported back to the United States on dry ice, while ethanol and dried-treatment samples were transported under their assigned treatment conditions. All sub-samples were stored for 330 days.

Frozen sub-samples were extracted following Graham et al. (2001), with modifications as follows for the alcohol and dried treatments. Frozen aliquots were thawed for 15 minutes, weighed out to exactly 0.50 g and placed in an 8-mL glass evaporation-proof vial (National Scientific, Pittsburgh, PA) into which 5 mL of 80% ethanol was added to extract the hormone. Alcohol treated sub-samples already in 3 ml of 95% ethanol received an additional 2 mL of 57.5% ethanol to make a total of 5 mL of 80% ethanol to match the extraction protocol used for the dried and frozen samples. The exact mass of each sub-sample for alcohol treatment was recorded when each whole sample was sub-divided into aliquots. These weights were used, instead of the 0.50 grams as in the frozen and dried treated aliquots, in calculating progestogen concentrations. Dried treated sub-samples were weighed out using the calculated final weight (see treatment section) and were placed in an 8-mL vial with 5 mL of 80% ethanol. Sub-samples were then extracted following the methods outlined above.

EIA Validations and Assay Protocols

EIA Validations

Serial dilutions of a fecal extract pool from a captive Cape ground squirrel were used to

validate the enzyme immunoassay for fecal progestogen and estrone conjugates (1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512 and 1:1024). Parallelism between serial dilutions of fecal extracts and standard curves was determined by a test of equality of two slopes (Zar, 1996).

Progestogen EIA

Fecal extracts were diluted (1:51 - 1:150) in assay buffer (0.02 M Trizma, 0.30 M NaCl, 0.1% BSA, and 0.1% Tween 80) before analysis. I used a progestogen EIA protocol previously outlined in Graham et al. (2001). In brief, microtiter plates (Nunc; Fisher Scientific; Pittsburgh, PA) were coated with affinity purified goat anti-mouse gamma globulin (Sigma Chemicals, St. Louis, MO) dissolved in coating buffer (0.015M Na₂CO₃, 0.035 M NaHCO₃; pH 9.59) and incubated overnight at room temperature. Wells were emptied and refilled with a second coating buffer containing preservative (0.02M Trizma, 0.30M NaCl, 1.0% BSA, 0.01% NaN₃; pH 7.5) and stored at room temperature. Plates were used within one week of coating.

The progestogen EIA GAMG (affinity purified goat anti-mouse gamma globulin 50 µg/plate; Sigma-Aldrich, St. Louis, MO) coated plates were washed (0.04% Tween 20) three times and 50 µl of diluted samples, standards (progesterone Sigma-Aldrich, St. Louis, MO), and controls were dispensed. Biotinylated progesterone (100 µl; 1:590,000; provided by F. Schwarzenberger, Vienna, Austria) was added to each well prior to the addition of 100 µl of mouse anti-progestogen antibody (1:440,000; Quidel clone #425 supplied by C. Munro, Davis, CA). Plates were sealed and incubated at room temperature overnight. After incubation, plates were washed and 200 µl streptavidin-peroxidase conjugate (1 µl in 24 ml assay buffer; Roche Diagnostics Co. Indianapolis, IN) was added to each well. Following 45 min room temperature incubation with the enzyme, plates were washed and incubated (30 min; room temperature) with

200 μ l substrate solution (500 μ l of 0.016M tetramethylbenzidine in dimethyl-sulphoxide and 100 μ l of .1752M H₂O₂ diluted in 24 ml of substrate buffer (0.01M C₂H₃Na; pH 5.0)). Stop solution (50 μ l of 3M sulfuric acid) was used to stop the enzyme reaction once the desired color was reached and the optical density was measured using an Emax plate reader (Molecular Devices, Sunnyvale, CA) with a test filter of 450 nm and a reference filter of 650 nm. All samples, controls, and standards were assayed in duplicate.

Estrone conjugates EIA

Fecal extracts were diluted (1:6 - 1:100) in phosphate buffer before analysis. I used an estrone conjugate (E₁C) EIA protocol previously outlined in Munro et al. (1991). The antiserum (R522) cross-reacts with estrone-3-glucuronide (100%), estrone-3-sulfate, (66.6%), estrone (238.0%), estradiol-17 β (7.8%), estradiol-3-glucuronide (3.8%) and estradiol-3-sulfate (3.3%). In brief, microtiter plates (Nunc; Fisher Scientific; Pittsburgh, PA) were coated with affinity purified antibody (stock solution, 10-fold dilution) dissolved in coating buffer (50 mmol/L bicarbonate buffer; pH 9.6). I coated the flat-bottom plates with 50 μ l of the antibody coating solution per well, sealed and incubated overnight at room temperature. Plates were used within three days of coating.

The E₁C coated plates were washed (0.04% Tween 20) three times and 50 μ l of phosphate buffer was immediately added to all sample and standard wells and 100 μ l was dispensed into blank rows. Plates were allowed to sit for 15-20 m to equilibrate. I then added 50 μ l of samples, controls and standards, followed by 100 μ l of enzyme conjugate (diluted 100-fold in phosphate buffer) to each well, covered plates tightly and incubated overnight at room temperature. After incubation, plates were washed and blotted dry. Substrate solution (per liter,

50 mmol of citrate, 1.6 mmol of hydrogen peroxide and 0.4 mmol of 2,2'-azino-di-(3-exthylbenzthiazoline sulfonic acid) diammonium salt, pH 4.0) was prepared and 100 μ l was added to each well. Plates were placed on a shaker until the desired color was reached (~50 min) indicative of the enzyme conversion of the substrate. Plates were read on an Emax plate reader (Molecular Devices, Sunnyvale, CA) with a test filter of 405 nm and a reference filter of 650 nm. All samples, controls, and standards were assayed in duplicate.

Statistical Analysis

To compare the mean progesterone and estrone conjugate concentrations between squirrels in different physiological states I used the Kruskal Wallis test. I used a repeated measure analysis of variance to determine the difference in hormone metabolite concentrations at different storage time periods compared to values obtained from immediate assay analysis (3 d following collection). Fecal progesterone and estrone conjugate concentrations among storage treatments were compared using repeated measures analysis of variance (Neter, 1990). I used simple contrasts to compare fecal progesterone and E₁C concentrations between each alternative treatment (alcohol and dried) and frozen treatments when the overall treatment effect was significant. Samples stored at sub-zero temperatures were considered reference values as freezing is a validated method considered to maintain stable steroid hormone concentrations for up to 2 years (Hunt & Wasser, 2003). Linear regression and correlation analyses were performed to evaluate the correspondence between the reference group (frozen) and other treatment group sub-samples when all samples from each treatment were pooled together. To determine similarity between peak patterns of progesterone and estrone conjugate profiles, I

computed peak and baseline concentrations by an iterative process (Graham et al., 2002, Brown et al., 1999). I calculated the mean concentration of all samples and temporarily removed values greater than the mean plus 1.75 standard deviations (SD) from the data set. These values were considered significant elevations. I then recalculated the mean and repeated the removal process until no values were higher than the mean plus 1.75 SD. Baseline concentrations consisted of the remaining fecal progestogen or estrone metabolite values. Percent agreement between timing (day) of peaks from treatment profiles and reference profiles were calculated and compared using a Chi-squared test. When assumptions of statistical tests were not met, data were normalized using a natural log transformation. Analyses were performed with SPSS 11.5.0 for Windows (SPSS, Inc., Chicago, IL). Means are reported with SE unless otherwise noted. Statistical significance was set at $p < 0.05$ for all analyses except for Kruskal-Wallis tests where significance was set at $p < 0.10$.

Results

EIA Validation

Serial dilutions of selected fecal samples produced displacement curves parallel to that of the E₁C and progesterone standard curves. The test of the equality of slopes gave a value of $t = 1.21$, $df = 7$, $p = 0.86$ and $t = -2.52$, $df = 8$, $p = 0.98$ for E₁C and P4 respectively (Fig. 1). The sensitivity of the progestogen assay was 11.3 ± 4.6 pg/well. Inter-assay coefficient of variation was 7.0% (20% binding) and 12.5% (60% binding) ($n = 11$ plates). Intra-assay coefficient of variation was 5.4% and 5.5% for low and high pools, respectively ($n = 11$ plates). The sensitivity of the E₁C assay was 19.7 ± 7.4 pg/well. Inter-assay coefficient of variation was 5.8% (38% binding) and 13.7% (83% binding) ($n = 9$ plates). Intra-assay coefficient of variation was 28.3% and 9.6% for low and high pools, respectively ($n = 9$ plates).

Physiological Validation

Mean fecal progestogen concentrations differed among sub-adult, non-gravid adult and gravid adult female Cape ground squirrels ($\chi^2 = 7.57$, $p = 0.02$; Kruskal-Wallis). Gravid adult females had significantly higher fecal progestogen concentrations than either sub-adult or non-gravid adult females ($\chi^2 = 4.50$, $p = 0.03$ and $\chi^2 = 6.00$, $p = 0.01$ for sub-adult and non-gravid respectively). There was no significant difference between mean fecal progestogen concentration of sub-adult and non-gravid adult females ($\chi^2 = 1.35$, $p = 0.25$). Mean fecal estrone conjugate concentrations differed among sub-adult, non-gravid and gravid adult females ($\chi^2 = 4.66$, $p = 0.10$). Adult females, both gravid and non-gravid, had significantly higher fecal estrone conjugate concentrations than sub-adult females ($\chi^2 = 3.75$, $p = 0.053$ and $\chi^2 = 3.43$, $p =$

0.06 for non-gravid and gravid respectively) (Fig. 2).

Storage Time Experiment

Duration of storage time did not affect absolute amount of progesterone and estrone metabolite concentrations ($F_{2,20}=2.34$, $p=0.122$ and $F_{2,20}=3.181$, $p=0.063$, respectively; repeated measures ANOVA). There was no difference in fecal progesterone concentrations between 0 and 1 or 0 and 3 months of frozen storage time ($F_{1,10}=0.58$, $p=0.46$ and $F_{1,10}=2.82$, $p=0.12$, respectively). There was no difference in fecal estrone conjugate concentrations between 0 and 1 month ($F_{1,10}=0.47$, $p=0.51$), or between 0 and 3 months ($F_{1,10}=4.31$, $p=0.07$).

Alternative Storage Treatment Experiment

Storage treatment affected absolute amount of fecal progesterone and estrone metabolite concentrations ($F_{2,10}=10.74$, $p=0.003$ and $F_{2,10}=5.96$, $p=0.02$, respectively). There was no difference in fecal progesterone concentrations between frozen and dried samples ($F_{1,5}=1.90$, $p=0.23$). While dried-treated fecal samples maintained a similar fecal estrone conjugate concentration compared to frozen samples, there existed a slight, though non-significant trend of decreased values ($F_{1,5}=5.70$, $p=0.06$). Fecal samples stored in 95% ethanol had significantly higher fecal progesterone concentrations than frozen samples ($F_{1,5}=9.84$, $p=0.03$) and significantly lower fecal estrone metabolite concentrations ($F_{1,5}=6.74$, $p=0.048$). The ethanol storage treatment increased fecal progesterone concentrations by greater than 20% in 55% of the samples (Fig. 3).

When fecal samples from all individuals were pooled together, there was no difference in the slopes of the regression lines from a plot of dried versus frozen-treated samples and the

from a plot of alcohol versus frozen-treated samples for either log-transformed progesterone or estrone conjugate concentrations ($t = -0.17$, $df = 90$, $p = 0.57$ and $t = 0.73$, $df = 90$, $p = 0.23$, respectively). The correlation between log-transformed fecal progesterone concentrations from dried-treated and corresponding frozen samples was significantly higher than for ethanol-treated samples ($Z = 2.05$, $df = 47$, $p = 0.04$). The correlation between log-transformed fecal estrone conjugate concentrations from dried-treated and frozen samples tended to be higher than for ethanol-treated samples, although not significantly ($Z = 1.75$, $df = 47$, $p = 0.08$) (Fig. 4).

Graphing progesterone profiles of both treatments and reference samples for each squirrel revealed a stronger similarity in peak pattern between frozen and ethanol-treated samples than frozen and dried-treated samples. In particular, female 17 exhibits parallel rise and fall in frozen and alcohol progesterone profiles (Fig. 6). However, once baseline concentrations were determined, dried-treated samples showed a significantly higher percentage of agreement in timing of peaks with corresponding frozen samples than ethanol-treated samples did with corresponding frozen samples (87.2% versus 66.0% agreement, $\chi^2 = 5.93$, $p = 0.03$; Chi-squared test). Additionally, ethanol-treated samples produced false peaks (ethanol samples peaked when frozen samples did not) 94% of the time when ethanol and frozen samples disagreed, while false peaks occurred 64% of the time when dried-treated samples disagreed with frozen samples (Table 1).

Additionally, female 17 exhibited a progesterone profile possibly indicative of ovarian activity (e.g. ovulation), though captive conditions removed the possibility of pregnancy. While reproductive cycle information is unclear for female Cape ground squirrels, the fecal progesterone profile of female 17 involved an increase above baseline with progesterone

concentrations from frozen reference samples remaining elevated for at least ten days.

Examining EIC profiles of both treatments and reference samples for each squirrel showed little difference in similarity in peak pattern between either treatment with reference samples. There was no significant difference between percent agreement of peak pattern between dried-treated and frozen samples and percent agreement between ethanol-treated and frozen samples (87.2% versus 83% agreement, $\chi^2 = 0.34$, $p = 0.16$). When the timing of peaks did not agree between treatment and reference sample, dried-treated fecal samples showed false peaks 50% of the time, while alcohol-treated fecal samples showed false peaks 62% of the time (Table 1).

Discussion

Assay validation

Both P4 and E₁C enzyme immunoassays measured progesterone and estrone conjugate concentrations accurately and precisely in fecal samples of Cape ground squirrels. Previous studies of rodent endocrinology that used fecal steroid analysis (Harper & Austad 2004, Kuznetsov et al., 2004, Good et al., 2003, Touma et al., 2004, Eriksson et al., 2004, Mateo et al., 2005) have validated both radioimmunoassays and enzyme immunoassays for glucocorticoids, however my study is the first to validate both progesterone and estrone conjugate enzyme immunoassays for fecal samples from a rodent. As a result, these assays have the potential to be utilized as a non-invasive technique to answer a wide variety of reproductive questions of the largest, most diverse and most broadly studied order of mammals.

Physiological assay validation

Although detectable concentrations of progesterone were present in all age groups and all reproductive conditions, they noticeably increased during pregnancy. These results indicated that fecal progesterone steroid analysis can be useful in detecting pregnancy and possibly other reproductive events in Cape ground squirrels. While these samples were collected during peak breeding season, I expect similar results would be obtained as estrus occurs year-round in these ground squirrels (Waterman 1996), eliminating effects of seasonality on hormone concentrations. The low levels of progesterone concentrations in non-gravid females may be influenced by small sample size that would reduce the probability of collecting luteal samples from cycling adults. Additionally, low progesterone concentrations may be indicative of a short luteal phase (Bouchie

et al., 2006) or possibly induced ovulation. Further research into the source and regulation of progesterone secretion would benefit the understanding of progesterone profiles as more information is gained through hormone analysis.

Detectable concentrations of fecal estrone conjugates were also present in fecal samples of all age groups and all reproductive conditions with adult females (gravid and non-gravid) exhibiting significantly higher concentrations compared to sub-adult females. As sub-adults may have not yet developed a fully functioning reproductive system, lower concentrations of estrogen metabolites in feces of sub-adults may be expected. These results demonstrate the potential ability of fecal estrone conjugate analysis to provide evidence of sexual maturation. While this investigation did not reveal a difference in fecal estrone conjugate concentration between gravid and non-gravid adult females, a study with a larger sample size may elucidate such a distinction.

Storage Time

Fecal progestogen and E₁C concentrations were unaffected by storage at -20° C for up to 3 months, suggesting that freezing fecal samples has little impact on fecal progestogen and estrone conjugate measurements. A slight, although insignificant increase in fecal estrone conjugate concentrations did occur between 1 and 3 months of frozen storage suggesting further investigation into effects of freezing fecal samples for more than 3 months (e.g. 6, 9 and 12 months) is needed.

Storage Treatment

My results demonstrate that drying feces provides a reliable method for long-term preservation of progestogen and estrone conjugate concentrations and is the better alternative

when freezing is not a viable option. Fecal progestogen concentrations remained stable in oven-dried feces from Cape ground squirrels after long-term storage. While they tended to be slightly lower, absolute estrone conjugate concentrations from dried feces did not differ significantly from concentrations in frozen feces. These data illustrated that storage treatment effects do not differ across different steroid hormone concentrations (i.e. there are not greater fluctuations due to treatment effects at higher or lower hormone metabolite concentrations). My results were similar to those found in previous storage experiments. Drying black rhinoceros fecal samples in a solar box cooker did not affect the concentration of progestogens compared to control values (180 d, Galama et al., 2004). Similar results were found in a short-term study with drying sifaka fecal samples (3 weeks; Brockman & Whitten, 1996). My results did differ slightly with findings from Terio et al. (2002) where they found the use of a solar or conventional oven to dry cheetah fecal samples resulted in alterations in both progesterone and estrogen metabolite concentrations.

Samples stored in ethanol showed marked alteration in both fecal progestogen and estrone conjugate concentrations suggesting that ethanol does not provide a reliable long-term preservation method in this species. Over half of the ethanol-treated samples showed an increase in progestogen concentrations by over 20% and a decrease in estrone conjugate concentrations by over 30% compared to frozen-treated samples. Due to the common occurrence of such increases in progestogen concentrations found in ethanol-treated fecal samples, I do not recommend the use of ethanol-treated samples for distinguishing reproductive states. Results from the ethanol-treated fecals were not consistent with previous findings in other species. Galama et al. (2004) or Terio et al. (2002) found that fecal samples stored in alcohol did not have

significantly different fecal progesterone or estrone conjugate concentrations from controls.

Lynch et al. (2003) reported that storing fecal samples in 95% ethanol at sub-zero temperatures resulted in no significant change from initial concentrations for both progesterone and estrogen metabolites. They did find a significant increase in fecal progesterone concentrations in samples stored in 95% ethanol in a charcoal refrigerator, however these samples still maintained the ability to distinguish reproductive states (gravid versus non-gravid) (Lynch et al., 2003). A study on baboon fecal steroid hormone storage found that fecal samples stored in ethanol over a six month period showed an increase in estrogen metabolite concentration for ~90 d, followed by a decrease to almost initial concentrations after ~ 180 d (Khan et al., 2002).

The direction of change (increases or decreases) in detectable fecal steroid hormone concentrations during long-term storage may be influenced by a combination of variables including digestive strategy, storage treatment, storage time and antibody specificity. Changes in the amount of hormone metabolites in a fecal sample are due to the influence these variables have on the metabolic transformation of the hormone. Before being excreted in the feces, steroid hormones are typically metabolized by the liver and excreted into the urine and bile as conjugates (via glucuronic acid or sulfate) to inactivate them and to increase their water-solubility (Ziegler and Wittwer 2005, Taylor 1971, Adlercreutz et al., 1976). Once in the gut, the hormone metabolites are deconjugated by intestinal microorganisms and bacteria and, if not reabsorbed, are excreted through the feces (Adlercreutz et al., 1976, Groh et al., 1993, Palme et al., 1996, Matkovics 1972). Hormones are excreted in the feces in both conjugated and unconjugated forms, although most species contain a higher percentage of conjugated than free steroids (Ziegler et al., 1996). As a result of this process, the form in which the metabolite is

excreted influences how it is transformed during storage by the fecal microbes and bacteria (Bokkenheuser & Winter 1980, Jarvenpaa et al., 1980). Route of excretion is also important since a species that excretes a large percentage of hormone metabolite via feces has the potential for larger changes in detectable concentration changes from fecal analysis than a species that excretes most of the hormone metabolite in its urine.

An animal's digestive strategy (i.e., carnivore, omnivore, forestomach or caecocolic fermenter) may influence modification of detectable fecal steroid concentrations as a result of differences in fecal bacteria loads and amount of dietary fiber. Retention time and pH of the digestive tract of an animal influence the amount and type of microorganisms able to be maintained, thus resulting in a higher amount of microorganisms capable of being excreted. Low pH and short retention times limit the number and species of microorganisms, while neutral pH and long retention times are associated with an increase in number and species of microorganisms (Stevens & Hume 1998), suggesting that fermenting herbivores likely have higher numbers of microorganisms in their feces than carnivores. Among herbivores, forestomach fermenters digest microbial cells while caecocolic fermenters typically lose microbial cells in the feces (Hume 1999) resulting in higher microbial loads in caecocolic fermenters. Amount of dietary fiber may also influence changes in steroid hormone concentrations by affecting intestinal microorganism metabolism of the hormone or retention time of the digesta (Wasser et al., 1993, von der Ohe et al., 2004, Klasing 2005).

Previous storage studies collectively suggest that fecal storage treatment (this study included) and storage time affect directional changes in the concentration of steroid hormone metabolites. Storage treatment may indirectly affect the changes in hormone concentrations by

the treatment's effect on the activity of fecal bacteria and microorganisms. Sub-zero temperatures, alcohol and lack of moisture can kill microorganisms or slow down microflora growth and may prevent further transformation of steroid hormones and hormone metabolites. Significant increases in steroid hormone concentrations may be due to the continued extraction of hormone metabolites by ethanol from fecal samples over time (Khan et al., 2002). Hunt & Wasser (2003) present an explanation for fluctuating changes in fecal steroid hormone concentrations over time. They suggest that steroid hormone metabolites may be steadily released from lipid micelles and then taken up again during micelle reformation. This hypothesis would help explain the results obtained by Hunt & Wasser (2003) and Khan et al. (2002) where fecal steroid hormone concentrations steadily inclined and then declined over time.

Antibody cross-reactivity may also influence changes in fecal hormone metabolite concentrations as the metabolites continue to be transformed during storage (Terio et al., 2002). The antibody developed for an immunoassay may be either a highly specific or a group-specific antibody. A highly specific antibody has been defined as cross-reacting with only the parent hormone (Khan et al., 2002), while a group-specific antibody typically has a high affinity for a set of steroid metabolites derive from the parent hormone (Wasser et al., 2000, Khan et al., 2002, Schwarzenberger et al., 1997). An artificial rise in hormone concentration may be a result of the antibody cross-reactivity of newly formed hormonal metabolites created by microbial transformation (Terio et al., 2002, Wasser et al., 2000). To determine affects of antibody specificity on fecal hormone metabolite concentrations over time, the specificity of an antibody should be assessed at a species-specific level using high performance liquid chromatography (HPLC). High performance liquid chromatography can be used to determine immunoreactivity

of the species-specific metabolites (Ziegler & Wittwer, 2005).

In reviewing storage effect studies, there were seven studies that tested the effects of leaving fecal samples untreated at ambient temperature over varying amounts of time in a wide range of species (Table 2). As expected, increases in steroid hormone metabolite concentrations were seen in hindgut (caecocolic) fermenters, while decreases were seen in foregut fermenters and omnivores with two exceptions which may be due to temporal effects (analyses performed after approximately 24 hours of storage). The influences of antibody specificity were also apparent in these seven studies as the investigations using specific antibodies showed only decreases in hormone metabolite concentrations, while group-specific antibody analyses resulted in increases in 57% of the investigations (no effect = three, decreases = three, increases = eight).

Taken together, the above variables provide an explanation for the significant increase seen in the progestogen concentrations in the ethanol treated fecal samples. Cape ground squirrels are herbivores and caecocolic fermenters indicating their feces likely contains high microbial loads. As well, this study involved long-term storage and used a group-specific progestogen-antibody in the EIA analysis. As the high number of fecal bacteria continues to transform the progestogens into newly formed metabolites that cross-react with the group-specific antibody and the ethanol continues to extract these metabolites out over time, the measured concentration of progestogen through EIA analysis will increase.

The above explanation does not clarify however, the decreased concentrations observed in estrone conjugate concentrations of ethanol treated fecal samples. The E₁C antibody used in this investigation cross-reacts with three main estrone conjugates, however it is possible that the bacteria found in Cape ground squirrel feces transforms estrone into metabolites that do not

cross-react with this antibody. Consequently, the high fecal microbial loads may be reducing the number of cross-reacting estrone metabolites resulting in a decrease in the EIA measured estrone conjugate concentrations.

My examination of relative hormone metabolite concentrations (temporal relationships as hormone concentrations naturally fluctuate) emphasizes the relevance of assessing efficacy of alternative storage methods at the profile level and the importance of determining a baseline for each treatment in order to identify similarity in peak pattern. While a visual inspection of peak patterns suggests that storage in ethanol provides an adequate alternative to freezing fecal samples, results from my baseline analysis revealed that the timing of peaks (i.e. potential reproductive events) from dried-treated samples more closely matched the peak pattern of frozen-treated samples. As well, ethanol-treated fecal samples are more likely to give false peaks than dried-treated samples. I encourage this type of comparison as part of the assay validation since studies addressing relative hormone concentrations are able to determine a storage treatment's effects on different concentrations of fecal hormone metabolite; a necessity when assessing biologically relevant events via rises and falls in fecal hormone metabolite concentrations. Examining hormone profiles as well as mean steroid hormone concentrations will provide a more comprehensive evaluation of the utility of possible alternative fecal preservation methods.

A consensus on the most appropriate alternative fecal sample preservation method may never be determined as the steroid hormone response to different storage methods appears to be species-specific as well as hormone-specific (Khan et al., 2002). Accordingly, determining the ideal storage method for the specific hormone/hormone metabolite, species, length of storage

time before analysis, and the questions being addressed should be considered an essential part of the assay validation procedure. In the end, multiple “ideal” storage treatments may exist depending upon the question being asked. If relative hormone concentrations are of main concern, as is common in reproductive studies, several methods may be suitable assuming all samples are treated using the same method. My investigation strongly encourages field researcher to consider each of the above variables to ensure limited problems in the field and accuracy in the analyses.

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CHAPTER 2 REPRODUCTIVE DELAY IN THE FEMALE CAPE

GROUND SQUIRREL (*Xerus inauris*)

Abstract

Many social animals are cooperative breeders and are characterized by group members that exhibit delayed dispersal from the natal group, reproductive suppression, and alloparental care. In such groups, non-breeding helpers raise young produced by dominant breeders producing a skew in the distribution of reproduction. Reproductive suppression (e.g. reproductive delay) has been shown to be a primary mechanism underlying reproductive skew and influenced by factors such as the presence of breeding female group mates, exposure to related and unrelated males and/or resource levels. Amount of resources may influence the age of sexual maturity in sub-adult females directly through effects on body condition and indirectly through effects on social structure. Initial field studies of Cape ground squirrels (*Xerus inauris*) indicated a delay of reproduction as a delay of first estrus in sub-adult females differed with number of adult breeding female group members. I investigated reproductive suppression in free-ranging female Cape ground squirrels by examining social group structure, behavior and hormone profiles of pre-reproductive and reproductive females to verify inhibition of sexual maturity and to determine the mechanisms involved. Fieldwork was conducted at two study sites in southern Africa where trapping, behavioral and hormonal data were collected to determine the timing of reproductive development. I found that an increase in the number of adult breeding female group mates and related adult male group mates resulted in a substantial inhibition of female reproductive maturity. I concluded that, while female Cape ground squirrels gain many

advantages from group living, their sexual maturity is primarily influenced by the adult breeding female group mates' ability for reproductive suppression and their own capacity to maximize lifetime reproductive success while minimizing inbreeding.

Introduction

Cooperative breeding is found in many social mammals and is defined by characteristics of group members, including delayed dispersal from the natal group, reproductive suppression, and care for others' offspring (Solomon and French, 1997). Such groups typically vary in the distribution of reproduction among group members, formally known as reproductive skew (Keller and Reeve, 1994; Vehrencamp, 1983). Reproductive skew exists as a continuum ranging from high skew (i.e., singular breeders: one dominant female produces most or all of the young) to low skew (i.e., plural breeders: groups with multiple breeding females) (Brown, 1987; Creel and Macdonald, 1995). Such variability has led to the development of multiple theoretical models that attempt to explain this variation with most models falling into two categories: transactional and incomplete control models. Transactional models maintain that the power of partitioning the group's reproductive output is completely controlled by either the dominant individuals ("concession" models) or the subordinates ("restraint" models) (Reeve and Ratnieks, 1993; Vehrencamp, 1983). Concession models explain the minimum amount of reproduction given to subordinates to maintain group stability ("staying incentives") and reduce fights for additional reproductive opportunities ("peace incentives"), while restraint models explain the maximum amount of reproduction subordinates can obtain before they are evicted from the group. Recent consideration has begun to question the degree of control any member can be assumed to maintain, resulting in a second class of reproductive skew models termed "incomplete control" or "tug-of-war" (Clutton-Brock, 1998). These models assume that reproduction is shared because no group member is able to completely manipulate the allocation of the group's reproductive output.

Concession and restraint models generate opposite predictions as the same factors that make grouping beneficial for the subordinate (decreasing incentives needed for remaining in the group) also make grouping beneficial for the dominant (increasing the amount of reproduction the dominant is willing to share) (Johnstone, 2000). Accordingly, when relatedness between dominants and subordinates is high and ecological constraints are high, the concession model predicts that skew will be high; while the restraint model predicts low skew (Johnstone, 2000). The incomplete control model predicts reproductive skew to decrease with, or be insensitive to, increases in relatedness and be insensitive to ecological constraints (Reeve et al., 1998).

Empirical support for skew models and the underlying mechanisms of reproductive distribution have been provided primarily by investigations of animal societies exhibiting high reproductive skew (Lewis and Pusey, 1997) and obligate cooperative breeding (helpers are necessary for successful breeding; (Brown, 1987). High skew species are suggested to be more common as the selective pressure on any one factor that promotes skew may increase the monopolization of reproduction, while low skew requires the reduction of selective pressure on all factors that may influence the distribution of reproduction (Gilchrist et al., 2004). Further investigations using facultative cooperative breeders with low skew could provide a better understanding of the relative importance of factors affecting skew as such species afford a different system with which to test the predictions of reproductive skew theories.

Prior studies of low skew species have involved the banded mongoose (*Mungos mungo*), lions (*Felis leo*) and rodents (*Marmota flaviventris*, *Ctenomys sociabilis*). Of these species, the banded mongoose and lions both have similar features including: low resource constraint, reduced inbreeding pressure, low reproductive suppression, minimal to non-existent dominance

hierarchy and high relatedness among group members (De Luca and Ginsberg, 2001; Packer et al., 2001). In accord with skew theory, there is no evidence of dominants controlling reproduction in either of these species, most likely because they derive many benefits from communal living and lack any incentive to control group mate's reproductive output. Conversely, explaining low skew in cooperative breeding rodents has been more difficult as they often exhibit high resource constraint (i.e. high costs of natal dispersal) and reduced individual direct fitness in larger groups (yellow-bellied marmot, *Marmota flaviventris*, Armitage and Schwartz, 2000; tuco-tuco, *Ctenomys sociabilis*, Lacey, 2004). Unlike lions and banded mongoose, some cooperatively breeding rodents maintain such characteristics that should predispose them to high skew, yet they exhibit very limited monopolization of reproduction.

Determining the proximate and ultimate causes of skew in the distribution of reproduction within groups is crucial for understanding why cooperative breeders exist. Skew in female mammals results primarily from reproductive suppression of sexually mature group members and/or reproductive delay in sub-adults. While reproductive suppression is typically characterized by a lack of reproductive activity in already mature females, reproductive delay (temporary postponement in sexual development that controls normal ovarian function) is a commonly overlooked form of suppression (O'Riain et al., 2000). The onset of puberty (first ovulation) is the culmination of an extended, tightly-regulated developmental process of the neuroendocrine system that is vulnerable to both stimulating and inhibiting influences (Bronson and Rissman, 1986; Ojeda et al., 1980). Because an individual's developmental process and social grouping are multifaceted and dynamic, one might expect to find considerable complexity in the ways in which reproductive systems are affected. Mechanisms by which puberty is

delayed can be mediated by a combination of social and environmental factors, including presence of and/or aggression by adult breeding females, presence of related males, absence of unrelated males, body condition, density, and resource levels (Wasser and Barash, 1983).

Adult breeding females may delay the first ovulation in sub-adult females pheromonally through chemical signals from urine or feces of group mates (Carter and Roberts, 1997; Drickamer, 1977), and/or behaviorally as agonistic attacks resulting in increased physiological stress and decreased gonadal activity (Abbott, 1984; Abbott et al., 2003). Mechanisms of inbreeding avoidance due to exposure to only related males has also been shown to inhibit reproductive activity (Bennett et al., 1997), as well as an absence of appropriate stimuli from unrelated males (Carter et al., 1980; Carter and Roberts, 1997). Environmental factors including rainfall and primary productivity may indirectly affect the timing of reproductive maturation. Primary productivity can influence reproductive maturation as a result of its effects on population density and female body condition, both of which have been shown to have a significant impact on the timing of sexual maturity (Drickamer, 1977; Schneider and Wade, 2000).

The Cape ground squirrel (*Xerus inauris*) is a facultative cooperative breeder that exhibits low skew and delay in sexual maturity (Waterman, 2002). As such, these squirrels are a good species in which to study the mechanisms underlying reproductive delay and implications for conflicting reproductive skew models. Following established theoretical models of reproductive skew, female Cape ground squirrels maintain characteristics that should predispose them to high levels of skew: group mates (female and male) are closely related, group-living females maintain higher per capita reproduction than solitary individuals, high environmental constraints on

dispersal, and sub-adults appear to be reproductively suppressed by adult breeding female group mates (Waterman, 2002, unpublished data). However, Cape ground squirrel groups almost always contain multiple breeding females with no evidence of a dominance hierarchy (Waterman, 1995) suggesting additional factors may play a role in the distribution of reproduction.

I examined the simultaneous effects of these additional social and environmental factors on the age of sexual maturity to identify the major determinant(s) of reproductive delay and corresponding skew in female Cape ground squirrels. My first objective was to verify inhibition of sexual maturity and potential sexual activity of sub-adults by monitoring the reproductive hormones in pre-reproductive and reproductive female Cape ground squirrels using non-invasive methods. Using fecal progesterone concentrations, I examined whether sub-adult females demonstrated signs of ovarian activity and/or pregnancy. My second objective was to examine the relative effects of the following parameters on the age of sexual maturation: number of adult breeding female group mates, rates of aggression from adult females, number of related adult male group mates, number of exposures to unrelated adult males, body mass, density (group size) and resource levels. Based on female Cape ground squirrels' associated biological and ecological traits, I predicted all parameters to be influential on age of sexual maturity, except for agonistic behaviors from adult females and exposure to unrelated males (Table 3). Addressing these questions in the field with naturally congregated groups, where dispersal is freely available, is critical to understanding the mechanisms of suppression.

Methods

Biology of the study organism

The Cape ground squirrel is a highly social, non-hibernating cooperative breeder found in arid regions of southern Africa. This species maintains a very unique social structure with male social groups living separately from female groups; the stability of both types of groups being primarily maintained by the benefits they receive from enhanced predator detection and deterrence (Waterman, 1995; Waterman, 1997). Female groups are made up of one to three breeding adult females and related offspring and are notable by their lack of aggression and the lack of a dominance hierarchy (Waterman, 1995). Males periodically visit female groups to ascertain the reproductive condition of breeding females; however they are typically chased out of the group. Adult female Cape ground squirrels breed year round with a distinct birth peak in late winter (early dry season) and can successfully breed up to four litters a year (Waterman, 1996). Cape ground squirrels appear to be spontaneous ovulators (Bouchie et al., 2006) with males showing scramble competition (Waterman, 1998). Estruses last approximately 3 hours with an operational sex ratio of 11:1 males to females (Waterman, 1998). Related (from the same natal group) males do participate in mating attempts, however they generally gain late access to the estrus female (i.e. after she has copulated with more dominant, older males; Waterman, unpublished data). Days of estrus of an adult female group mate appear to be the only time sub-adults are exposed to unrelated adult males for an extended period of time (Waterman, 1996; Waterman, 1998), while a sub-adult's exposure to related adult males can vary greatly as some males delay dispersal and remain in their natal group well beyond sexual

maturity (Waterman, 1995).

Field studies of Cape ground squirrels indicate evidence for a delay in female sexual maturity; sub-adults in social groups with more breeding adult females matured later than sub-adults in groups with fewer adult females. Additionally, 8 out of 10 subordinates became sexually mature within 1 month of not being in the presence of any adult females from their social group (Waterman, 2002). Severe environmental conditions frequently result in lost litters causing many females to cycle more frequently. Females that do not lose their litters isolate from the social group between parturition and juvenile emergence (45-50 days), locating themselves and their offspring in a different sleeping burrow (Waterman, 1996). Consequently, sub-adult females that are members of a social group with only one adult breeding female are more likely to find themselves in the absence of reproductively mature females who could potentially suppress their sexual maturity. Sub-adult members of social groups with 2 or 3 breeding females are unlikely to ever find themselves alone because of the squirrel's asynchrony of breeding, high pregnancy loss and infant mortality (Waterman, 2002). As a result, sub-adults in these large groups must choose between dispersal or remaining in their natal group and experiencing a likely delay in sexual maturity from their constant exposure to adult breeding females. Following the onset of sexual maturity, there appears to be no suppression of reproductive activity (Waterman, 1995; Waterman, 2002).

Study Sites

Fieldwork was conducted at two study sites in southern Africa that differ in rainfall and resource availability. My low resource site was on a private 3500-ha farm 185 km southeast of

Windhoek, Namibia (23°25'S, 18°00' E) in the Kalahari-bushveld region (Waterman, 1995). My high resource site was at the S.A. Lombard Nature Reserve (SALNP), South Africa (27°35'S, 25°35' E), a 3,660 hectare reserve consisting of Cymbopogon-Themeda veld and Kalahari grassland on a flood plain (van Zyl, 1965). The South African study site receives an average annual rainfall of 504 mm (range: 203-934) compared to 199 mm (range: 60-374) at the Namibian site. Precipitation is confined mainly to the period November to April at both sites. The Namibia field season was conducted from July to October, 2004, while the South African field season was from May to November, 2004. The Namibian study site experienced a drought year during the Nov 2002- Apr 2003 rainy season, receiving only 66 mm of rain. Number of juveniles trapped in 2003 was low, however the Nov 2003 – Apr 2004 rainy season was normal (209 mm) causing a recommencement in reproduction (juveniles trapped: 2002: 16; 2003: 27; 2004: 59). The South African study site received normal rainfall during both 2002-2003 and 2003-2004 rainy seasons (474 mm, 510 mm; respectively).

Age of Sexual Maturity Determination

I operationally defined the age of sexual maturity as the day of first estrus determined through a combination of behavioral, hormonal and trapping data. Behaviors specific to day of estrus (e.g. increased male activity including sniffing, chasing and copulating with estrus female) provided a direct indication of the age of sexual maturity. However, if estrus behaviors were missed, I was able to backdate from maternal isolation (parturition) and juvenile emergence using a growth curve established for each site as well as data on gestation and lactation length (Namibia: Waterman, 1996; South Africa: this study). For South African squirrels, I fit growth data (mass in grams versus days after emergence) to a power model function using non-linear

regression analysis (Fig. 6). Progesterone concentrations were obtained via enzyme immunoassay analysis (EIA) of fecal samples from adult breeding females to establish the characteristics of a “typical” progesterone profile of a reproductively active individual. Progesterone profiles from sub-adults were then compared to these reference profiles to verify reproductive activity. As well, sub-adult progesterone concentrations were analyzed to establish a baseline concentration and sexual maturity was assumed if an individual’s initial rise in progesterone concentrations above the baseline was maintained from more than 30 days. I used body mass, nipple length and vulva characteristics to supplement my behavioral and hormonal data. Prior to a female Cape ground squirrel’s first pregnancy, her nipples are tiny and unremarkable, but become long, dark and swollen after her first parturition (Waterman, 1996). Characteristics of a female’s vulva can be used to estimate day of estrus, as the vulva swells greatly a few days before and on the day of estrus (Waterman, 1996) and can contain sperm plugs following estrus (Pettitt, unpublished data).

Behavioral Observations

Behavioral observations of female social groups (7 at SALNP; 6 at Namibian site) were carried out throughout the field season to assess reproductive status, burrow mates, locations of sleeping burrows, aggressive interactions between adult and sub-adult females and interactions between unrelated males and sub-adult females. Behavioral data were collected using all-occurrences, focal (during day of estrus) and scan sampling (Altmann 1974). Observations were made from trees, hides, and vehicles using 10X50 binoculars and a 15-45X60 spotting scope. Individuals were marked for permanent identification using PIT tags (AVID U.S.A.) and freeze-branding (Quick Freeze; Rood & Nellis 1980). Squirrels were dye-marked on both sides of the

body (Rodol D; Melchior & Iwen 1965) for identification during behavioral observations. Reproductive behaviors collected included interactions with unrelated and related males, female proceptive behavior (spatial cohesion, between female and male), and mating behaviors (copulation attempts, aboveground copulations, female and male entering/exiting burrow simultaneously). Estrus was confirmed when behaviors were consistent with reproductive activity as described in Waterman (1998). Antagonistic behaviors between adult and sub-adult females collected included spatial displacements, chases, and fights.

Trapping and fecal collection

Squirrels were trapped using the procedures outlined in Waterman (1995). I collected feces every three days by trapping pre-reproductive and newly reproductive females. Cape ground squirrels readily defecate immediately after being trapped (Waterman, per com). A total of 14 social groups (Namibia: 6; South Africa: 8) were studied resulting in a collection of 20-40 samples collected weekly. Efforts were made to trap and collect most fecal samples in the morning (between 6:00am and 10:00am) to avoid heat stress during mid-day and because in many species studied thus far, reproductive and stress hormones have diurnal patterns of synthesis and release (Sousa and Ziegler, 1998). Individuals observed to be near estrus (i.e. receiving increased attention from males, swollen vulva, multiple males arrive into the area; Waterman 1995, 1998) were trapped daily unless trapping efforts began to interfere with reproductive behaviors. Fecal samples were collected within 30 minutes of trapping. Samples were frozen immediately and returned to the United States on dry ice for fecal steroid analysis. This research project took place with authorization from the University of Central Florida

Institutional Animal Care and Use Committee (IACUC), approval #02-18.

Time of Day Validation

Diurnal variation in fecal progesterone metabolite excretion appears to be species-specific (high morning concentrations in marmosets, *Callithrix jacchus*, (Sousa and Ziegler, 1998) and tamarins, *Saguinus mystax*, (Lottker et al., 2004); no diurnal variation in baboons, *Papio hamadryas*, (Beehner and Whitten, 2004). As a result, I tested whether time of day affected progesterone metabolite concentrations by collecting 14 paired morning (8:00-11:00) and afternoon (11:00 – 15:00) fecal samples from 9 female Cape ground squirrels. Progesterone concentration of each sample was determined by enzyme immunoassay analysis. Paired comparisons showed that there was no diurnal variation in fecal excretion of progesterone in female Cape ground squirrels in samples collected in the early morning versus early afternoon. ($Z = -1.036$, $p = 0.30$; Wilcoxon signed ranks test), indicating fecal samples from all time periods could be used.

Progesterone EIA

The progesterone EIA was previously validated for Cape ground squirrel fecal samples indicating that the enzyme immunoassay analysis measures progesterone concentrations accurately and precisely (Chapter 1).

Fecal extracts were diluted (1:51 - 1:150) in assay buffer (0.02 M Trizma, 0.30 M NaCl, 0.1% BSA, and 0.1% Tween 80) before analysis. I used a progesterone EIA protocol previously outlined in Graham et al. (2001). In brief, microtiter plates (Nunc; Fisher Scientific; Pittsburgh, PA) were coated with affinity purified goat anti-mouse gamma globulin (Sigma Chemicals, St.

Louis, MO) dissolved in coating buffer (0.015M Na₂CO₃, 0.035 M NaHCO₃; pH 9.59) and incubated overnight at room temperature. Wells were emptied and refilled with a second coating buffer containing preservative (0.02M Trizma, 0.30M NaCl, 1.0% BSA, 0.01% NaN₃; pH 7.5) and stored at room temperature. Plates were used within one week of coating.

The progestogen EIA GAMG coated plates were washed (0.04% Tween 20) three times and 50 µl of diluted samples, standards, and controls were dispensed. Biotinylated progesterone (100 µl; 1:590,000; provided by F. Schwarzenberger, Vienna, Austria) was added to each well prior to the addition of 100 µl of anti-progestogen antibody (1:440,000; Quidel clone #425 supplied by C. Munro, Davis, CA). Plates were sealed and incubated at room temperature overnight. After incubation, plates were washed and the enzyme streptavidin-peroxidase conjugate (Roche Molecular Biochemicals, Indianapolis, IN) was added to each well. Following 45 min room temperature incubation with the enzyme, plates were washed and incubated (30 min; room temperature) with substrate solution (500 µl of 0.016M tetramethylbenzidine in dimethyl-sulphoxide and 100 µl of 0.1752M H₂O₂ diluted in 24 ml of substrate buffer (0.01M C₂H₃Na; pH 5.0)). Stop solution (50 µl of 3M sulfuric acid) was used to stop the enzyme reaction once the desired color was reached and the optical density was measured using an Emax platereader (Molecular Devices, Sunnyvale, CA) with a test filter of 450 nm and a reference filter of 650 nm. All samples, controls, and standards were assayed in duplicate.

Longitudinal Hormone Profiles

To determine progestogen profiles, I computed peak and baseline concentrations by an iterative process (Brown et al., 1999; Graham et al., 2002). I calculated the mean concentration

of all samples from each site and temporarily removed values greater than the mean plus 1.75 standard deviations (SD) from the data set. These values were considered significant elevations. I then recalculated the mean and repeated the removal process until no values were higher than the mean plus 1.75 SD. Baseline concentrations consisted of the remaining fecal progesterone values.

Progesterone concentrations from eight adult breeding females were used to establish the characteristics of a normal progesterone profile of a reproductively active individual (for representative profile see Fig. 7a). The pattern of fecal progesterone concentrations from these adults synchronized with behaviors indicative of estrus, parturition and lactation. Progesterone concentrations increased from baseline and continued to rise from the day of estrus, peaking just before parturition, after which concentrations dropped to almost baseline levels. After parturition, progesterone concentrations rose and peaked again during lactation. Following lactation, concentrations of progesterones dropped markedly and remained consistently low and showing no signs of cyclic ovarian activity until the subsequent estrus.

Fecal progesterone profiles of sub-adult female Cape ground squirrels were compared to the established profile of a typical sexually mature female to assist in determining their age of sexual maturity. Sub-adult females did not demonstrate signs of ovarian activity until the day of their first estrus and showed limited success in their first reproductive attempts. Successful pregnancy in sub-adults was associated with a clear rise in progesterone concentrations starting on the day of estrus, followed by a short decline and then a rapid increase in progesterone concentrations around the time of parturition (Fig. 7). I used any rise in progesterone concentrations above baseline with a maintained elevation for a minimum of 20 days as

indicative of reproductive activity and therefore, sexual maturity.

Data Analysis

In my initial analysis I considered a number of potential variables that have previously been shown to influence the timing of female sexual maturity, including: number of adult female group mates (AdFem), number of adult related male group mates (RelMal), number of exposures to adult unrelated males (UnrelMal), rates of aggression experienced by sub-adults (initiated by adult female group mates) (Agg), body mass at earliest known age of sexual maturity (Mass), density/group size (Density) and resource level/site (Resource). The number of exposures to adult unrelated males was calculated using data collected during the time period between the minimum age of sexual maturity and the observed age of sexual maturity for each individual. Rates of aggression from adult female group mates were calculated throughout the time period between two months prior to earliest known age of sexual maturity through two month following observed age of sexual maturity for each individual. All other variables used data collected at the time of minimum age of sexual maturity.

I used a model selection approach, rather than hypothesis testing, due to its superiority in explaining an ecological pattern with a series of independent parameters (Burnham and Anderson, 2002). Model comparison allows an arbitrary number of competing hypotheses to be tested at the same time, each of which competes on equal footing to explain the observed data. Because the number of sub-adult females sampled ($n = 22$) was small relative to the number of parameters ($K = 7$) in most models (i.e., $n/K > 40$), I used Akaike Information Criterion corrected for small sample size (AIC_c) to compare the extent to which my data supported each

model (Burnham & Anderson, 1998). First, I calculated residual sum of squares using multiple linear regression for each model. I then calculated maximum log-likelihood using the following equation: $\ln(L_{\max}) = -q/2(\ln(2\pi V_r + 1))$, where q is the sample size and V_r is equivalent to the residual sum of squares obtained from the multiple linear regressions. Last, I calculated $AIC_c = -2*\ln(L_{\max}) + (2pq/q-p-1)$, where $\ln(L_{\max})$ is the value of the maximized log-likelihood over the unknown parameters, given the model and the data, p is the number of model parameters and q is the sample size (Quinn & Keough, 2002, Burnham & Anderson, 1998). I considered the model with the smallest AIC_c to be the most parsimonious. I calculated the AIC_c differences between the best model and the other alternative models ($\Delta_i = AIC_{c_i} - \text{minimum } AIC_c$) to determine the relative ranking of each model; models for which $\Delta_i \leq 2$ have substantial support and were considered for biological importance (Burnham and Anderson, 2002). Models with Δ_i values between three and seven indicate that the model has considerably less support. Additionally, I calculated Akaike weights ($w_i = \exp[-\Delta_i/2] / \sum \exp[-\Delta_i/2]$) to assess the weight of evidence that the selected model was the actual Kullback-Leibler best model in the set of models considered (Burnham and Anderson, 2002). Only the six top-ranked candidate models are reported.

Statistical outliers were identified and removed if Cook's distance was > 1 (Field, 2000). Data distributions were analyzed for normality by Shapiro-Wilk tests. Non-normally distributed samples were subjected to a natural log transformation and retested for normality. When data could not be transformed to achieve normality, nonparametric statistics were used. All analyses were performed in SPSS 11.5 software (SPSS Inc., Chicago, Illinois). Unless otherwise indicated, means ± 1 S.E. are reported. Level of significance was set at 0.05 for each analysis. Steroid concentrations of fecal extracts are reported as mass equivalent per gram of wet feces.

Results

Endocrine Analysis

The progestogen EIA was validated as serial dilutions of selected fecal samples produced displacement curve parallel to that of the progestogen standard curve. The test of the equality of slopes gave a value of $t = -2.52$, $df = 8$, $p=0.98$ (Fig. 1). The sensitivity of the P4 assay was 11.3 ± 4.6 pg/well. Inter-assay coefficient of variation was 7.8% (20% binding) and 13.6% (60% binding) ($n = 42$ plates). Intra-assay coefficient of variation was 6.5% and 4.2% for low and high pools, respectively ($n = 42$ plates).

The patterns of fecal progestogen concentrations synchronized with estrus, isolation and emergence behavior data. As estruses were almost always followed by pregnancy, I was unable to obtain any information regarding reproductive cycling patterns. Both adult and sub-adults maintained low concentrations of progestogen until the day of estrus (Fig. 7).

Site Differences

As a result of resource level differences, my two study sites differed in average body mass, individual density, male dispersal and minimum age of sexual maturity. As expected, average adult body mass was significantly higher in female Cape ground squirrels in the high resource South African site than in the low resource Namibian site (640.3 ± 6.4 g, $n=100$ and 581.4 ± 8.2 g, $n=62$, respectively; $t=-5.65$, $df=160$, $p<0.001$). Density of individuals was higher in Namibia (3.5 adults/ m^2 vs. 1.6 adults/ m^2), while percentage of male Cape ground squirrels that fail to disperse by 16 months of age was higher in South Africa (63% vs 25%; (Waterman, 1996). Minimum age of sexual maturity was earlier in Namibia (7 m vs. 9 m; Waterman,

unpublished data), which was unexpected due to the higher average adult body mass found in South Africa.

Mechanisms of Reproductive Delay

As regression can be seriously affected by influential points, I examined the overall influence of outliers on the models. One outlier (18.5 m at sexual maturity) was removed from the data set (Cook's distance = 1.71), leaving 22 sub-adult female Cape ground squirrels remaining. In the present study, the overall mean age of sexual maturity was found to be 10.7 m \pm 0.34 (range 7.8 – 14.1) after the outlier was removed.

Timing of female sexual maturity was primarily influenced by social structure, with age at day of first estrus increasing with both number of adult breeding females and adult related males (multiple linear regression; standardized β = 0.41, p = 0.02 and standardized β = 0.52, p = 0.003, respectively). The model consisting of only these two parameters was my most parsimonious model and was 3.7 times better supported than the next five ranked models according to AICc weights (Table 4). It is important to note that these two parameters were included in all of the six top-ranked models, providing strong evidence for the influence of group structure on sexual maturity. Each of these next five ranked models included the number of adult breeding females and related males plus one of the remaining parameters: rates of aggression from adult female group mates, density, resource level, body mass at minimum age of sexual maturity and number of exposures to unrelated adult males (Table 2). These models all had the same Δ AICc value of three signifying they have considerably less support for being able to explain the variation found in age of sexual maturity (Burnham and Anderson, 2002)

compared to the parameters in the top-ranked model. As well, their exceptionally similar AICc weights, indicate that the data cannot adequately differentiate the potential influence each of these parameters has on the age of female sexual maturity.

Number of adult breeding females in Cape ground squirrel social groups influenced the age of sexual maturity of sub-adult female group mates (Mann-Whitney U; $N = 22$, $U = -2.29$, $p = 0.02$). Sub-adult females in social groups with zero or one adult breeding females became sexually mature significantly earlier (10.2 ± 0.37 m, $n=15$, range 8 -13) than similar-aged females in social groups with two or more adult breeding females (11.9 ± 0.49 m, $n=7$, range 10-14; Fig. 8).

Number of adult related males in Cape ground squirrel social groups influenced the age of sexual maturity of sub-adult female group mates ($N = 22$, $U = -2.86$, $p = 0.004$). Sub-adult females in social groups with less than two related adult male group mates became sexually mature significantly earlier (10.0 ± 0.30 m, $n=15$, range 8 -12) than similar-aged females in social groups with two or more adult related males (12.2 ± 0.51 m, $n=7$, range 10 -14; Fig. 9).

While the relative influence the remaining parameters may hold on age of sexual maturity is yet unknown, my data do elucidate certain trends. Rates of aggression between adult and sub-adult female group mates were extremely low (mean = 0.02 aggressive interactions/h ± 0.004 ; range: 0.01-0.04/h) indicating that agonistic behaviors are highly unlikely to influence the timing of female sexual maturity. Data on density, resource level and body mass suggest the reverse of their expected effect on reproductive development. Lower densities, higher resource levels, and greater body mass are normally associated with sub-adults becoming sexual mature earlier; however, my data suggest the opposite. Sub-adults at the Namibian site, with higher density,

lower resources (rainfall) and lower adult body size, became sexually mature significantly earlier than the sub-adults at the South African site (t-test, $t = -2.53$, $df = 20$, $p = 0.02$; South Africa: 11.5 ± 0.49 m, range: 9.5 – 14 and Namibia: 10.0 ± 0.37 m, range: 7.8 – 12.2).

Discussion

Female Cape ground squirrels conceive as early as seven or nine months of age (depending on resource level; Waterman, 1996; unpublished data), indicating that female Cape ground squirrels are physiologically capable of sexual maturation earlier than the majority of the females in the present study. As well, hormonal data confirm the absence of gonadal activity prior to the day of first estrus. These findings indicate that reproductive maturation in most sub-adult female Cape ground squirrels is delayed.

Results of my examination of the potential mechanisms of reproductive delay show that two main features of sub-adult female Cape ground squirrels' social environment have a profound influence on their age of sexual maturation. The simultaneous effects of both adult breeding female group mates and related adult male group mates result in a substantial inhibition of female reproductive maturity. Female age of sexual maturity is primarily a result of the interaction between adult breeding females' capacity for reproductive suppression and sub-adults' ability to maximize lifetime reproductive success while minimizing inbreeding.

A delay in the sexual maturation of sub-adult females by the presence of adult breeding female group mates is quite common in cooperatively breeding rodents, including voles (*Microtus ochrogaster*, (Getz et al., 1983), mice (*Mus musculus*, (Drickamer, 1977), hamsters (*Phodopus campbelli*, Gudermuth et al., 1992), and gerbils (*Meriones unguiculatus*, (Clark and Galef, 2001). Female rodents are known to produce a urinary pheromone that is associated with puberty inhibition and often transferred via soiled bedding (Drickamer, 1977). As Cape ground squirrel social groups readily share sleeping burrows, the means by which this underlying

mechanism could occur is available for exploitation. With such a mechanism set in place, one would expect to find a significant increase in the age of sexual maturity in social groups with more adult females. My data not only support this prediction but are also consistent with results obtained from a separate study of female Cape ground squirrel reproductive maturity (Waterman, 1996). Both the present analysis and Waterman (2002) found sub-adult females in social groups with zero or one adult breeding female became sexually mature significantly earlier than similar-aged females in social groups with two or more adult breeding females.

The effects of adult female Cape ground squirrels on sexual maturity are augmented by inbreeding avoidance mechanisms that appear to act on sub-adult reproductive development simultaneously. My results identify the number of related adult male group mates to be a second major determinant of reproductive delay in female Cape ground squirrels, with sub-adult females in social groups with two or more related adult male group mates becoming sexually mature significantly later than those in groups with fewer related males. Avoidance of incestuous matings resulting in reproductive inhibition has been reported in other rodents (prairie voles, *Microtus pinetorum*: Schadler, 1983; Mashona mole-rats, *Cryptomys darlingi*: Greeff & Bennett, 2000; blacktailed prairie dogs, *Cynomys ludovicianus*: Hoogland, 1995). Mechanisms of inbreeding avoidance are often maintained due to selective pressure against increased homozygosity and the expression of deleterious alleles (Ralls & Ballou, 1982). Greeff & Bennett (2000) suggest that the consideration of inbreeding avoidance as a mechanism of reproductive suppression has been underemphasized in previous suppression studies because the costs of inbreeding are most likely low and alternative mating opportunities appear to be commonly available. While inbreeding costs have been shown to vary significantly (Ralls et al.,

1988, Hoogland, 1992), I expect Cape ground squirrels to maintain a low inbreeding coefficient similar to other species of rodents. Conversely, while sub-adult female Cape ground squirrels are periodically exposed to unrelated adult males, especially on days of group mates' estruses, these males are quickly chased off by the social groups' adult females, limiting the alternative mating opportunities of sub-adults (Waterman, 1995).

As noted earlier, many factors may directly stimulate or inhibit an individual's reproductive development process, ultimately causing a change in the final activation of maturity (a pulsative secretion of gonadotropin releasing hormone (GnRH) by the hypothalamus) (Becker et al., 2002). Some species use aggression in the form of agonistic encounters that cause an increase in glucocorticoids which can suppress reproductive function (wolves, *Canis lupus*: Packard et al., 1985; birds: Silverin, 1986; pine voles, *Microtus pinetorum*: Brant et al., 1998). Many male rodents produce pheromones under the control of androgens present in their urine and associated with female puberty acceleration (Vandenbergh, 1969; Pandey & Pandey, 1988). As well, certain visual and tactile contact of young females by unfamiliar males can play an important role in stimulating reproductive activity in female rodents (mound-builder mouse, *Mus spicilegus*: Feron & Gheusi, 2003; montane voles: Gray et al., 1974; *Microtus ochrogaster*: Richmond & Conaway 1969; Whitten, 1956), especially in induced ovulators. Body condition, density and resource levels have all been implicated in prior suppression studies as possible influences on sexual maturity, sometimes as a result of indirect effects on each other. Availability of food affects reproductive success in mice as the cost of foraging increases with food scarcity (Bronson & Marsteller, 1985; Perrigo & Bronson, 1983; Cassaing, 1984). A review of 14 species of marmots found that environmental harshness increases age of sexual

maturity and can cause delayed dispersal even after reproductive maturity is attained (Blumstein and Armitage, 1999). Hacklander & Arnold (1999) found that only female alpine marmots, *Marmota marmota*, emerging from hibernation in adequate body condition were capable of successfully reproducing.

While my data provided strong support for the influence of social structure on reproductive delay, the remaining parameters have a lesser impact on reproductive development. The limited ability of aggression from adult females and exposure to unrelated males in explaining variation in age of sexual maturity was not unexpected. The extremely low rates of agonistic behaviors seen among all squirrels and specifically between adult and sub-adult female group mates are consistent with our findings that aggressive-related inhibition of reproduction does not hold a strong influence over the timing of sexual maturity in Cape ground squirrels. The limited influence of unrelated males was also not surprising as Cape ground squirrels are spontaneous ovulators (Bouchie et al., 2006) and as such, they do not require stimulation from unfamiliar adult males to initiate their reproductive development. Explaining the limited effects of density, resource level and body mass on age of sexual maturity is less straight-forward. With a higher level of productivity, lower density and better body condition found in the South African site, I expected sub-adult females to become sexually mature at an earlier age than females at the Namibian site. However, I found the reverse. I suggest that the high productivity site in South Africa may indirectly influence the age of sexual maturity through direct effects on the squirrel's social structure. Social groups at the South African site contained significantly higher number adult related males than the Namibian social groups (Waterman, unpublished data). This difference in social structure may be due to a large percent of South African males

responding to high predation risk and low resource competition near their natal burrow that allows them to remain in their natal social group and delay dispersal. This delayed dispersal then appears to increase the age of female sexual maturity as a means to avoid inbreeding.

As previously mentioned, characteristics of female Cape ground squirrels predisposes them to high levels of skew. Cape ground squirrels obtain large anti-predatory benefits from grouping, with the highest levels of mortality possibly occurring during times of dispersal and isolation (Waterman 1996, unpublished data). As female survival rates increase when part of a group, so does her lifetime reproductive success. Female social groups contain mainly close genetic relatives, creating the opportunity for maximizing indirect reproductive success. As well, adult females do suppress the sexual maturity of sub-adults and mechanisms of inbreeding avoidance are probably in place indicated by the significant effects of related males on female sexual maturity. So, what can possibly counter-balance all of these characteristics in order to produce the net result of low reproductive skew?

I suggest four reasons for the low skew exhibited by female Cape ground squirrel groups. First, low levels of skew may be a result of the adult females' inability to control reproduction once sub-adults have gained their maturity (Waterman, 2002). Second, sub-adults can maximize reproductive success associated with group-living only to a certain group size, with juvenile survival decreasing with increasing group size (Waterman, 2002). In response to this limitation of reproductive success, female Cape ground squirrel groups subsequently split into smaller groups when there are four or more adult breeding females in the group (Waterman, 2002), thus preserving lower levels of skew. Third, while sexual maturity is significantly influenced by the presence of related males, the cost of inbreeding may be reduced due to features of their mating

strategy, thus decreasing the need for sub-adults to restrain their own reproductive development. Female Cape ground squirrels show first male advantage with minimal mating guarding and generally mate with older, unrelated males earlier in estrus, copulating with younger, most likely related males later (Waterman, 1998). Fourth, delaying sexual maturity can lead to a decrease in lifetime reproductive success indicating that the age of first reproduction is under considerable directional selection favoring early maturity (Oli and Armitage, 2003) and therefore lower skew.

My results are consistent with the predictions of the incomplete control model of skew. Control over reproduction is not complete by any group member, as adult breeding females do not maintain complete control over the group's reproductive output, even though they do have some influence over the timing of sub-adult reproductive development. Sub-adult females retain some degree of power to limit reproductive delay and are able to restrain their own development to avoid costs of inbreeding. The incomplete control model predicts reproductive skew to decrease with, or be insensitive to, increases in relatedness and be insensitive to ecological constraints (Reeve et al., 1998). Female Cape ground squirrel groups are highly related and exhibit very low levels of skew. As well, our results show that such ecological constraints as rainfall and resource levels have a limited effect on reproductive delay, one of the main mechanisms of skew. The findings of a prior study of a low skew cooperative breeder, the banded mongoose, supported the predictions of the concession model (De Luca and Ginsberg, 2001). There was no evidence for any form of reproductive control in this species, however they suggested adult females could control the distribution of reproduction, but that weak ecological constraints allowed for low costs of dispersal and therefore provided dominants with little leverage (De Luca and Ginsberg, 2001).

Conclusion

Female Cape ground squirrels gain many advantages from group living, but unlike the majority of cooperative breeders, they exhibit low reproductive skew and their sexual maturity is primarily influenced by a tug-of-war among social parameters, with minimal direct influence by environmental ones. Using a facultative cooperative breeder with low skew to investigate the relative importance of parameters affecting reproductive skew provides a unique situation to tease out effects that may be overlooked in species with more common mating and reproductive systems.

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OVERALL CONCLUSION

Determining why cooperative breeders exist and the mechanisms underlying the maintenance of this type of association is essential for understanding the social systems of many animals. The idea that an individual would delay dispersal and reproductive maturity while caring for other's offspring is counter-intuitive to basic Darwinian theory. In order to discern why such circumstances exist, it is necessary to understand the costs and benefits facing each individual group member in terms of environmental constraints, relatedness and their ability to maximize their own reproductive output while controlling the reproductive activity of others in the group.

Controlling reproductive activity is often accomplished at the early developmental stages. The onset of sexual maturity is the culmination of a tightly-regulated developmental process that is vulnerable to both social and environmental influences that are capable of both stimulation and inhibition of this development. My study of reproductive delay in the Cape ground squirrel found that female sexual maturity is delayed due primarily to social factors consisting of the number of adult breeding female and adult related male group mates. In other words, female age of sexual maturity is principally a result of the interaction between adult breeding females' capacity for reproductive suppression and sub-adults' ability to maximize lifetime reproductive success while minimizing inbreeding. The overall reproductive dynamics of each social group results from the tug-of-war between the adult and sub-adult female group mates to control breeding within the group.

These results are consistent with the predictions of the incomplete control model of skew

as control over reproduction is not complete by any group member. While adult breeding females do have some influence over the timing of sub-adult reproductive development, they do not maintain complete control over the group's reproductive output. Sub-adult females also maintain some measure of power to limit reproductive delay and are able to restrain their own development to avoid costs of inbreeding. Further work on estimating relatedness and reproductive success, as well as determining the fitness advantages of association should be addressed in cooperative breeders, including the Cape ground squirrel. Combined with studies into how ecological constraints inhibit dispersal and independent reproduction, these investigations can provide quantitative tests of reproductive skew models.

Future studies on mammalian cooperative breeding need to continue to address the simultaneous selective pressures that cause the inhibition of sexual maturity. Field studies involving populations that differ in group structure and resource levels may provide further insight into the effects of social and environmental factors on the formation of cooperative breeders exhibiting reproductive delay. The use of fecal steroid analysis in these studies should be considered a reliable method for determining gonadal activity and reproductive maturity. Prior to such analyses, the ideal fecal storage method should be determined while considering the specific hormone/hormone metabolite, species, length of storage time before analysis and the questions being addressed. As well, the simultaneous manipulation of multiple factors, such as social structure, resources, and density, although difficult to implement, is needed to determine the combination of factors that may cause reproductive delay and ultimate, reproductive skew. Determining the causes of skew in the distribution of reproduction within a group will bring much insight into how and why cooperative breeders exist.

APPENDIX A:
FIGURES AND TABLES FOR CHAPTER 1

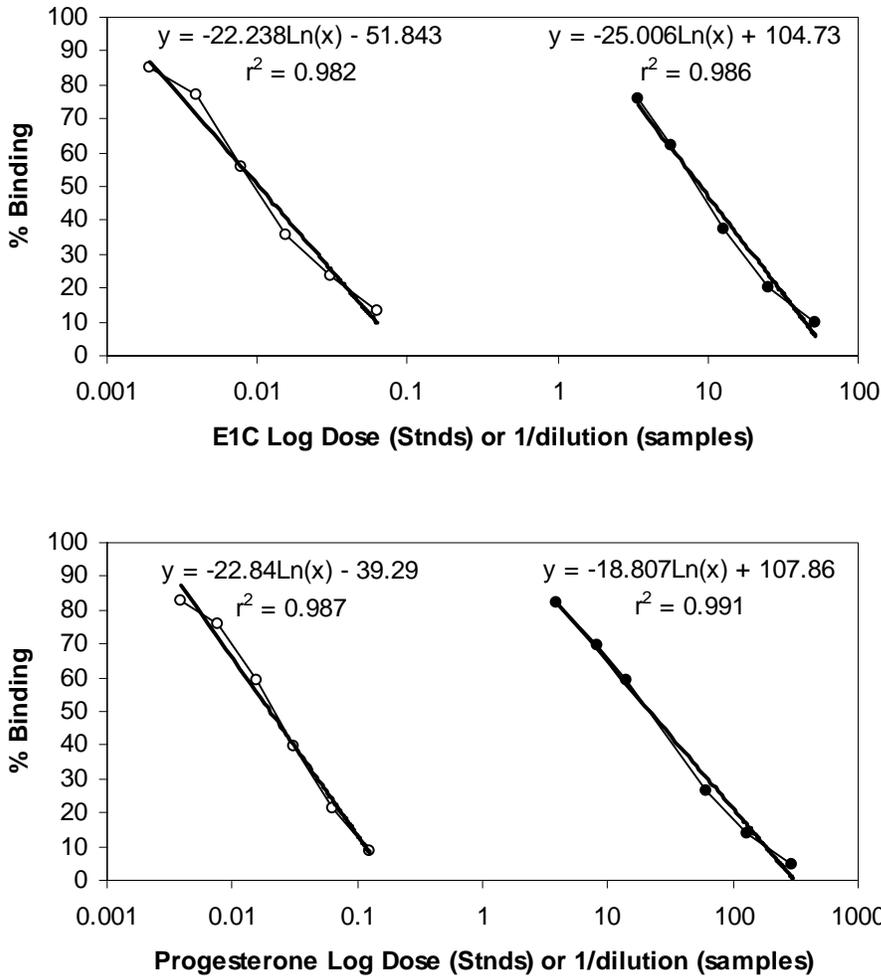


Figure 1: Validation results from a serial dilution of female Cape ground squirrel fecal extracts compared to a standard.

The sample (open symbol) was diluted 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512 and 1:1024 in assay buffer and tested for binding with the progestogen or estrone conjugate antibody in parallel with serially diluted standards (closed symbol) ranging from 1,000 to 15.6 pg/50µl. For progesterone, regression equations derived from the linear portion of the curves produced similar r^2 values for the samples and standards (0.987 and 0.991, respectively). For E₁C, regression equations derived from the linear portion of the curves produced almost identical r^2 values for the samples and standards (0.982 and 0.986, respectively).

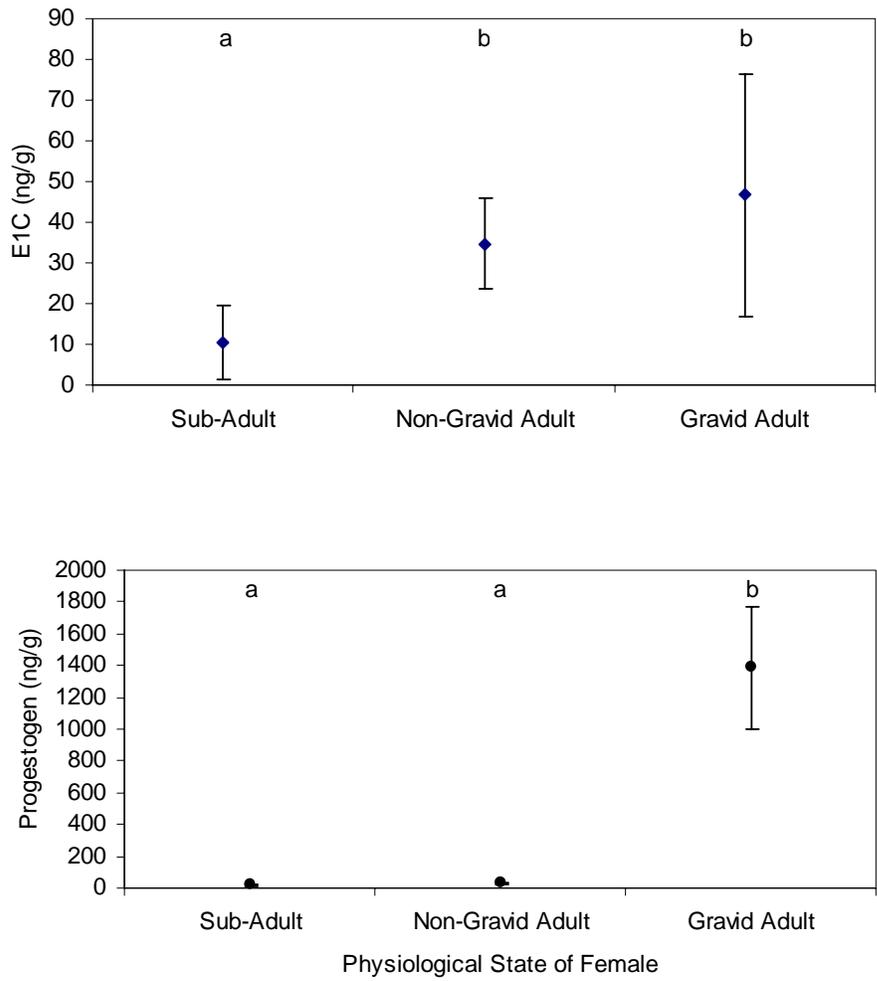


Figure 2: Physiological validation of fecal estrone conjugate and progesterone concentrations.

Fecal samples obtained from sub-adult (n = 2), non-gravid adult (n = 5) and gravid (n = 4) female Cape ground squirrels. Mean concentration ± SE.

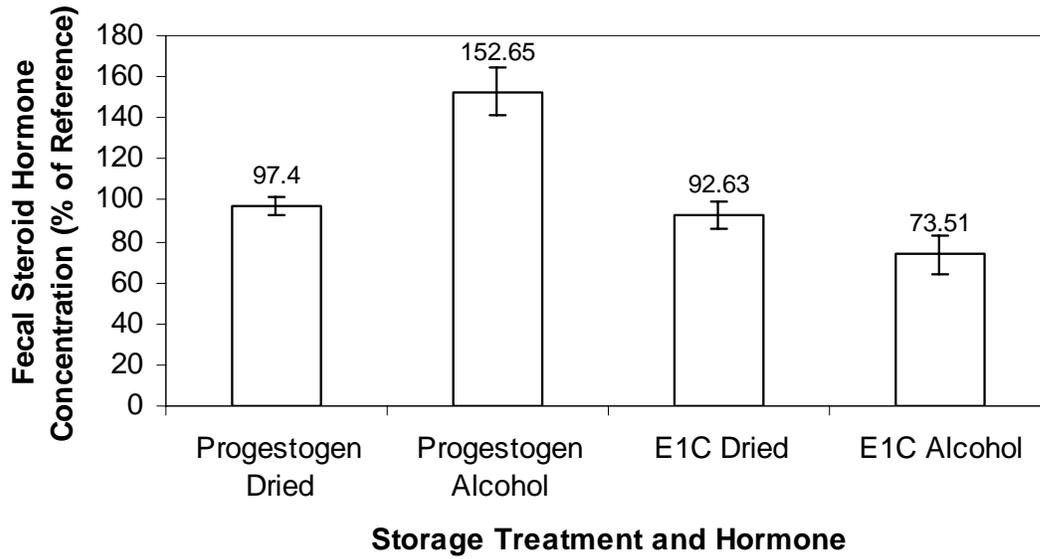


Figure 3: Mean concentration \pm SE for progestogen and estrone conjugate concentrations as percent of frozen reference samples for Cape ground squirrel fecal samples (n=48).

Samples subjected to 330 days of storage following oven-drying or 95% ethanol.

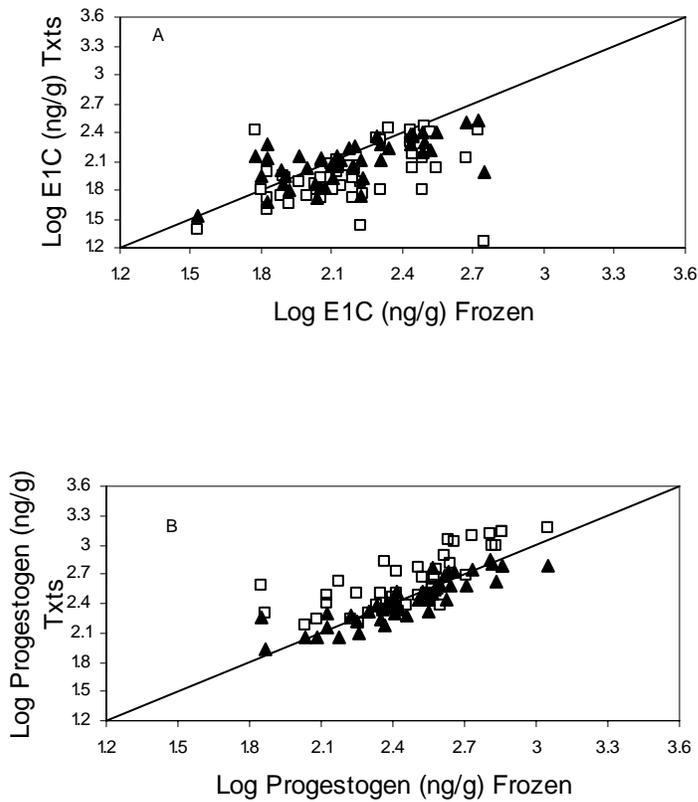


Figure 4: Scatter-plot graphs of progesterone and estrone conjugate concentrations of Cape ground squirrel fecal samples.

Graphs of pooled, log transformed fecal progesterone (A) and estrone conjugate (B) concentrations of fecal samples from captive *X. inauris*. Open boxes (\square) indicate log-transformed fecal hormone metabolite concentrations for alcohol-treated samples and filled triangles (\blacktriangle) indicate log-transformed fecal hormone metabolite concentrations for dried-treated samples. The solid line indicates a slope of 1. (A) P4 (\square) $y = 0.830x + 0.557$, $R^2 = 0.751$; (\blacktriangle) $y = 0.809x + 0.443$, $R^2 = 0.89$; (B) E₁C (\square) $y = 0.435x + 1.003$, $R^2 = 0.41$; (\blacktriangle) $y = 0.559x + 0.880$, $R^2 = 0.67$.

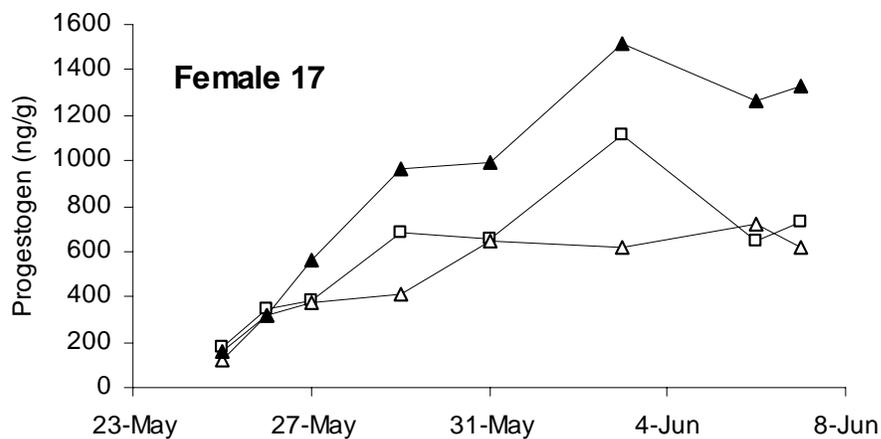


Figure 5: Progesterone profiles of an adult female Cape ground squirrel fecal samples subject to alternative storage treatments.

Profile of an adult captive female *X. inauris* (Female 17) for fecal samples subjected to freezing, drying and preservation in alcohol (n = 8 for each treatment) collected during early summer 2003 in Bloemhof, South Africa. Profile is shown from frozen samples (□), alcohol treated samples (▲) and dried treated samples (Δ).

Table 1: Percent agreement of timing of peaks between frozen and dried or alcohol treated samples

	Dried				Alcohol			
	% Agree	% Disagree	False Peak	Missed Peak	% Agree	% Disagree	False Peak	Missed Peak
P4	87.2 (41/46)	12.8 (6/46)	67% 4/6	33% 2/6	66.0 (31/46)	34.0 (16/46)	94% 15/16	6% 1/16
E1C	87.2 (41/46)	12.8 (6/46)	50% 3/6	50% 3/6	83.0 (39/46)	17.0 (8/46)	62.5% 5/8	37.5% 3/8

Table 2: Fecal storage experiments testing the effects of leaving fecal samples untreated at ambient temperatures for varying amounts of time and in a wide range of species

Common	Species	Gut	Storage Time	Hormone	Antibody	Direction	Reference
White-tailed deer	<i>Odocoileus virginianus</i>	Foregut	7 d	GC	G	N	Washburn & Millspaugh 2002
White-tailed deer	<i>Odocoileus virginianus</i>	Foregut	7 d	GC	G	N	Millspaugh et al., 2003
White-tailed deer	<i>Odocoileus virginianus</i>	Foregut	6 d	GC	G	D	Millspaugh et al., 2003
Elk	<i>Cervus elaphus</i>	Foregut	6 d	GC	G	D	Millspaugh et al., 2003
Cow	<i>Bos taurus-indicus</i>	Foregut	48 h	P	S	D	Masunda et al., 1999
Cow	<i>Bos taurus</i>	Foregut	24 h	GC	G	I	Moestl et al., 1999
African Elephant	<i>Loxodonta africana</i>	Hindgut	14 d - 730 d	GC	G	I	Hunt & Wasser 2003
Black rhino	<i>Diceros bicornis</i>	Hindgut	30 d	P	G	N	Galama et al., 2004
Black rhino	<i>Diceros bicornis</i>	Hindgut	90 d	P	G	I	Galama et al., 2004
Black rhino	<i>Diceros bicornis</i>	Hindgut	180 d	P	G	I	Galama et al., 2004
Black rhino	<i>Diceros bicornis</i>	Hindgut	24, 48 h	P	G	I	Galama et al., 2004
Horse	<i>Equus caballus</i>	Hindgut	24 h	GC	G	I	Moestl et al., 1999
Pig	<i>Sus scrofa domesticus</i>	Hindgut	24 h	GC	G	I	Moestl et al., 1999
Grizzly Bear	<i>Ursus arctos horribilis</i>	Omnivore	14 d – 730 d	GC	G	D	Hunt & Wasser 2003
Baboon	<i>Papio cynocephalus</i>	Omnivore	21 h	P	G	I	Wasser et al., 1988
Baboon	<i>Papio cynocephalus</i>	Omnivore	21 h	E	S	D	Wasser et al., 1988

Hormone: GC = glucocorticoids, P = progesterone metabolites, E = estrogen metabolites. Antibody: S = highly specific antibody, G = group-specific antibody. Direction: I = increases, D = decreases.

APPENDIX B:
FIGURES AND TABLES FOR CHAPTER 2

Table 3: List of hypotheses and predictions tested to identify the major determinant(s) of reproductive delay in female Cape ground squirrels (*X. inauris*).

Hypotheses	Predictions	Biological Reason for Prediction
Presence of adult breeding females inhibits sexual maturity	Sub-adults will become sexually mature earlier in groups containing fewer adult breeding females	Prior research shows sub-adults in groups with more females become sexually mature later (Waterman, 2002)
Presence of related non-dispersed adult males inhibits sexual maturity	Sub-adults will become sexually mature earlier in groups containing fewer related adult males	Some males delay dispersal, remaining in their natal group years after reaching sexual maturity
Density of individuals inhibits sexual maturity	Sub-adults will become sexually mature earlier in smaller groups	Effects of density on age of sexual maturity is common among rodents
Lower body condition inhibits sexual maturity	Heavier sub-adults will become sexually mature earlier than lighter sub-adults	Arid-living squirrels especially require adequate body conditioning due to the costs of lactation
Lower resource levels inhibits sexual maturity	Sub-adults in higher resource areas will become sexually mature earlier than sub-adults in lower resource areas	Effects of resource levels on age of sexual maturity is common among rodents
Agonistic behaviors of adult breeding females inhibits sexual maturity	Agonistic behaviors by adult breeding females will have no effect on the timing of sexual maturity in sub-adult females	Cape ground squirrels have very low rates of aggression and agonistic interactions can be very costly
Absence of unrelated adult breeding males inhibits sexual maturity	Number of exposures to unrelated adult breeding males will have no effect on the timing of sexual maturity in sub-adult females	Cape grounds squirrels are spontaneous ovulators (exposure to unrelated males is not needed to stimulate sexual maturity) (Bouchie et al., 2006)

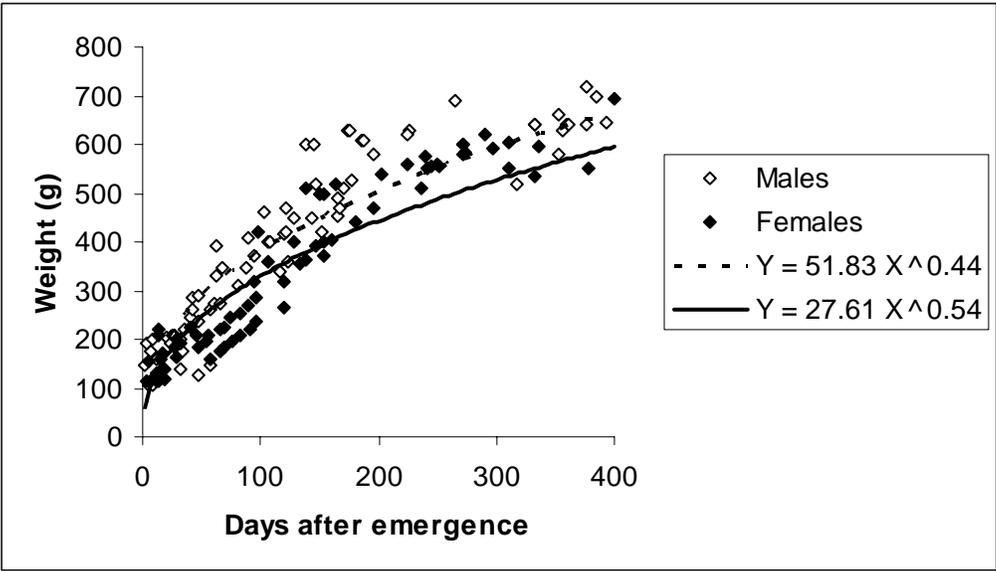


Figure 6: Growth rate of male and female South African Cape ground squirrels.

Growth rate for the first 400 days following emergence; males – dashed line, $Y = 51.83 X^{0.44}$, $r = 0.94$ (n = 75 observations, 10 individuals); females – solid line, $Y = 27.61 X^{0.54}$, $r = 0.95$ (n = 70 observations, 10 individuals).

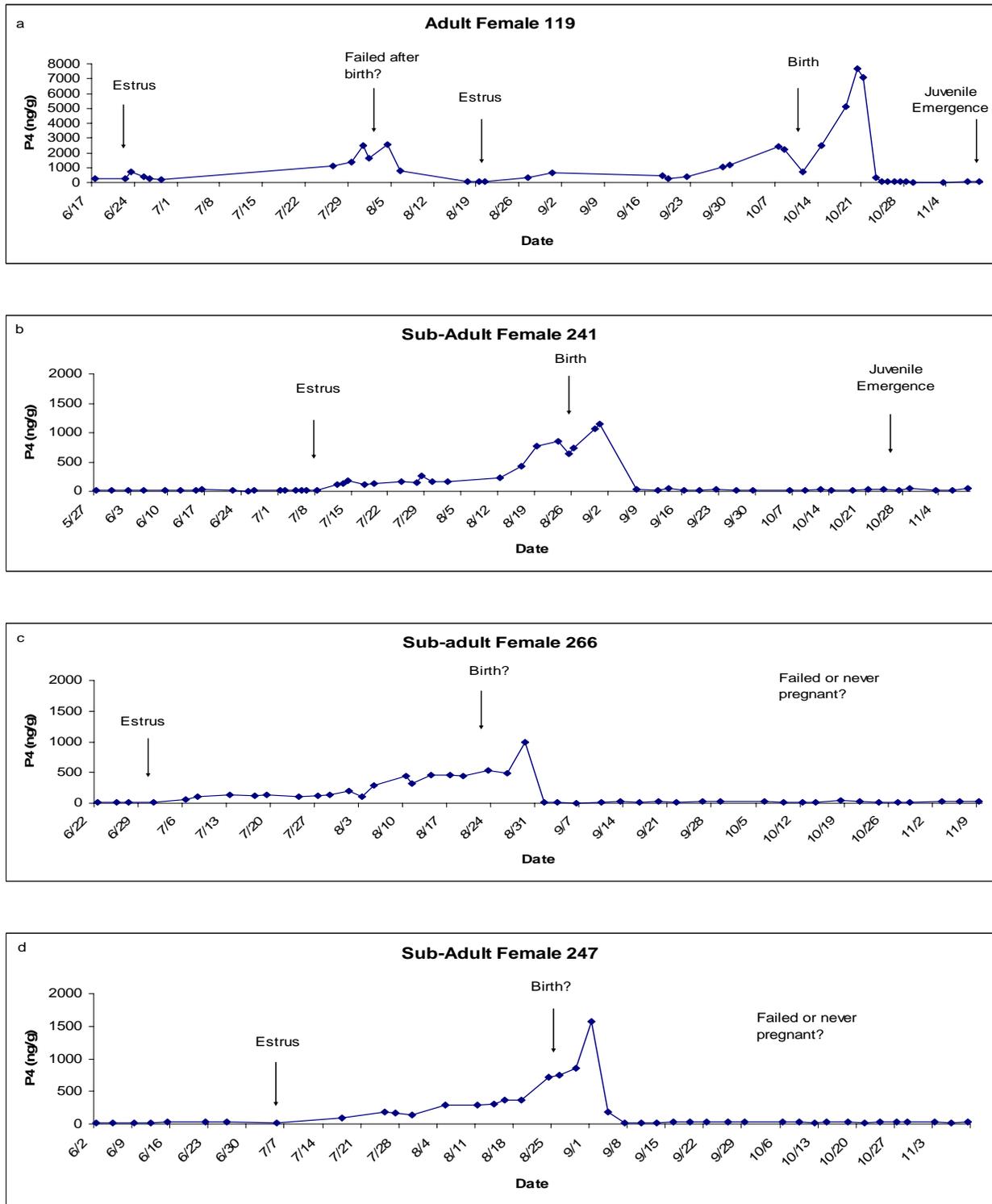


Figure 7: Progesterone profiles from female Cape ground squirrels.

Profiles include 1 adult breeding female who had 1 unsuccessful and 1 successful pregnancy (a), 1 sub-adult female who had 1 successful pregnancy (b), and 2 sub-adult females, neither of whom had a successful pregnancy (c, d).

Table 4: Multiple linear regression models showing the effects of parameters on the age of sexual maturity in sub-adult female Cape ground squirrels.

Model	Parameters (K)	AICc	Δ AICc	<i>w</i>
Adfem*Relmal	3	138	0	0.291
Adfem*Relmal*Agg	4	141	3	0.078
Adfem*Relmal*Density	4	141	3	0.076
Adfem*Relmal*Resource	4	141	3	0.074
Adfem*Relmal*Mass	4	141	3	0.068
Adfem*Relmal*UnrelMal	4	141	3	0.065



Figure 8: Effects of adult breeding female group mates on female age of sexual maturity.

Difference in the age of sexual maturity of female Cape ground squirrels in social groups with zero or one adult breeding females ($n = 15$) compared to sub-adults in social groups with two or more adult breeding females ($n = 7$) ($p = 0.02$).

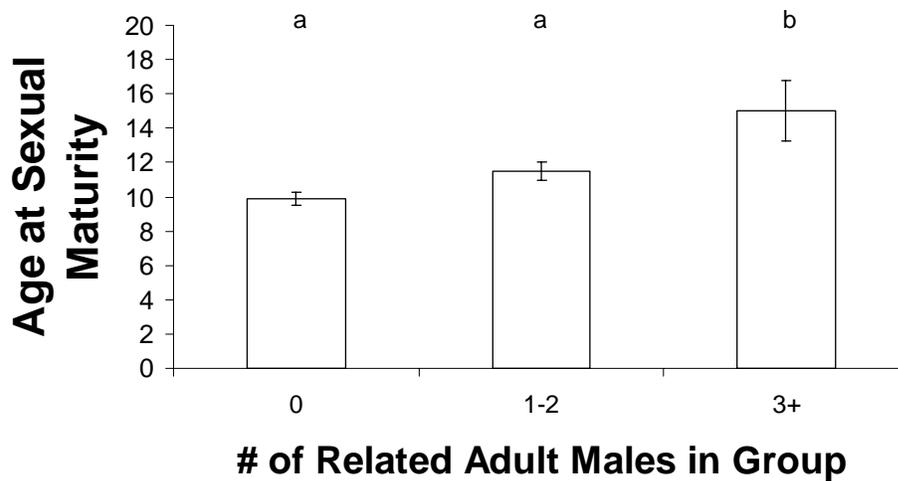


Figure 9: Effects of related adult male group mates on female age of sexual maturity.

Difference in the age of sexual maturity of female Cape ground squirrels in social groups with zero to two adult related males ($n = 15$) compared to sub-adults in social groups with three or more adult related males ($n = 7$) ($p = 0.004$).