ABSTRACT

SCARLATA, CANDACE DAVIS. Relationships among Stress, Reproduction and Housing Conditions in Captive Pygmy Rabbits (*Brachylagus idahoensis*). (Under the direction of John Godwin and Roger Powell.)

Pygmy rabbits (*Brachylagus idahoensis*) are the smallest leporids in the world, the only rabbits in the United States that dig their own burrows and are uniquely adapted to sagebrush-steppe habitats. In 2001, the collapse of the Columbia Basin population prompted the initiation of a captive breeding program to facilitate reintroduction. Unfortunately, attempts to establish a self-sustaining population in captivity have been thwarted by poor reproduction and high mortality. One theory is that chronic stress due to suboptimal housing may be responsible. Chronic stress, measured as the heightened secretion of stress hormones (e.g. glucocorticoids), has been shown to have negative effects on both reproduction and health in numerous species.

The overall goal of this project was to explore the relationships among reproduction, stress and housing conditions in the captive pygmy rabbit. The first objective of this study was to characterize gonadal and adrenal activity in captive pygmy rabbits through the monitoring of fecal progestagens and glucocorticoids. This study represents the first assessment of hormone patterns in a rabbit species other than the domestic rabbit. HPLC analyses verified the presence of both cortisol and progesterone in the excreta of pygmy rabbits and physiological validations demonstrated that changes in glucocorticoids and progestagens were associated with biologically relevant events such as stress or pregnancy. Thus, non-invasive fecal hormone techniques can be used to monitor adrenal and gonadal activity in pygmy rabbits, which can ultimately allow us to

understand hormonal changes associated with copulation, pregnancy, parturition, stress and seasonality.

During pregnancy, fecal hormones profiles in female pygmy rabbits were characterized by a large spike in progestagens shortly after mating, a gradual increase in progestagen and glucocorticoid concentrations throughout gestation and a decrease in hormone concentrations to baseline shortly after birth (Day 24). The spike in progestagens one day after mating is a significant discovery for this species as it provides a quick and reliable way of determining if a successful mating occurred.

Seasonal analyses of hormone excretion found that progestagen baselines did not vary between the non-breeding and breeding seasons and significant elevations in the secretion of progestagens were associated with mating and pregnancy. Fecal glucocorticoid concentrations were highest during the winter (Jan - Mar). Trends within the breeding season revealed a gradual decrease in glucocorticoids towards the end of the season (May, June), which coincides with an increase in breeding success.

The second goal of this study was to identify hormonal correlates of reproductive success. Females that failed to conceive had significantly higher glucocorticoids and lower progestagens during the breeding season than successfully breeding females. In addition, higher progestagen baselines were associated with females that had a greater rate of conception and females that produced more litters and young. During pairings, females that successfully mated showed a large increase in progestagens after copulation and had significantly lower stress levels than females that did not get pregnant. During gestation, females with larger litters showed higher progestagens and glucocorticoids during the second half of pregnancy than smaller litters. Although hormone levels during

gestation were not associated with post-natal litter survival, higher glucocorticoid concentrations during lactation were associated with females whose litters died before emergence from the natal burrow.

The third goal of this study was to determine if aspects of captive housing, specifically enclosure size and soil enrichment, were associated with elevated stress levels. A comparison of pen types used during the non-breeding season found that females' glucocorticoid baselines were negatively associated with pen size and significantly lower in soil-enriched pens. The highest glucocorticoid concentrations were detected in crates, which were both the smallest pens available and lacked soil. In addition, the movement of females from non-soil pens to identical pens half filled with soil resulted in a significant decrease in glucocorticoid baselines. Overall, female pygmy rabbits showed a greater decrease in stress levels in response to the addition of soil than an increase in pen size.

Moreover, this study detected a significant difference in glucocorticoid excretion between the two facilities. A comparison between females housed in identical pens at the two facilities found significantly higher glucocorticoid levels and lower reproductive success at Facility 2, suggesting that some factor other than housing conditions must be influencing adrenal activity. One possibility is that differences in husbandry routines may be responsible for these facility differences. Future studies should explore these husbandry differences to identify which factors contribute to higher stress levels and provide mitigating strategies for improving reproductive success in this captive population.

Relationships among Stress, Reproduction and Housing Conditions in Captive Pygmy Rabbits (*Brachylagus idahoensis*)

by

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DEDICATION

To my husband Vinnie and my bunny Paedomorpheus!

BIOGRAPHY

I was born September 14, 1980 in Thousand Oaks, California, deep in the heart of the Conejo Valley (English translation: “Rabbit” Valley). Little did I know that many decades later I would spend a large chunk of my life investigating the reproduction and evolution of the very family of animals that my hometown had in abundance. In fact, one of the few pets my family had was a wild cottontail named Ruby, which as is turns out did not like captivity.

Despite this obvious exposure to rabbits as a child, my interest in leporids did not start until graduate school. In college, I was exposed to the world of zoo biology through several research projects under the mentorship of Dr. Cheryl Asa from the St. Louis Zoo. The plethora of research opportunities available at the zoo exposed me to the world of conservation and the dire need for further research. It was through these experiences that I was inspired to learn more about wildlife conservation and pursue a career in research rather than veterinary medicine.

In 2002, I entered a graduate program at Duke University with the young innocence of wanting to save the world, specifically endangered species. During my first summer at Duke, I had an opportunity to develop my own independent research project at the Rocky Mountain Biological Laboratory. My objective was to locate various populations of pikas and snowshoe hares in the Gothic area and subsequently determine the amount of sexual dimorphism found in each of these populations. This project led me to my master’s project which tackled a larger question integrating life-history theory and

morphological evolution. The broader purpose of my research was to decipher which evolutionary forces were responsible for the evolution of sexual dimorphism in rabbits. I chose to focus on *Sylvilagus* species because they are one of very few mammalian genera in which females are consistently larger than males.

Several years into my graduate program at Duke, I realized I wasn't following my true passions, conservation biology and animal welfare. Through a connection from my master's research, I was offered an opportunity to design a research project studying the Columbia Basin pygmy rabbit and I jumped at the opportunity. For my dissertation research, I chose to investigate the relationship between physiology and reproductive fitness as they relate to environmental variables in captive pygmy rabbits. While this project provides a good starting point to pursue my passions, my ultimate goal is to generate a more holistic research model for managing small populations of endangered species through the integration of several fields including physiology, reproductive biology, genetics, behavior, nutrition, reproduction and animal health. Who knows what species I will research next, but I am thankful for all the little pygmy rabbits for providing me with hours of cuteness and motivation in the midst of grants and lab work.

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Thank you so much to Nicole Presley for not only teaching me everything about working in a lab and providing a voice of reason when things went awry, but also for your friendship. I could not have accomplished this project without you. Also, thank you to Nicole Abbondanza and Sarah Putnam for providing support and keeping the lab running smoothly, even on your days off. A special thanks to my lab girls, Natalka Melnycky, Kate Archibald, Amy Flaggs and Kate McKinnon for your friendship and company during the long days and nights in the lab!

Funding for this project was provided by a large grant from the Morris Animal Foundation and several supporting grants from the Smithsonian Institution, Friends of the National Zoo, Washington Department of Fish and Wildlife, the Oregon Zoo Future for Wildlife Fund, and Washington State University. Also, North Carolina State University provided me with funding and teaching opportunities that were essential to my development as a professor.

Outside the world of research, many graduate students have also provided encouragement and support, but it was their unwavering friendship that helped me maintain my sanity…most of the time. Thank you to Lara Oliver, Paul Klenk, Kim Rosvall, Briana Vogen, Molly Miller, Elizabeth Irish, Laura Kovacs, Jeff Kovacs, Tanya Kossler, Nick Graf and many others for your kind hearts and comic relief. Thanks for entertaining my engineering side. Particular thanks to those friends who convinced me it was imperative that I own a pet rabbit for the sake of my career as a biologist. Petey (aka. Paedomorpheus) has provided me much stress relief and inspiration during overwhelming work weeks and even taught me much about the nature of rabbits. Petey, thank you for

the many hours of stimulating conversation. Without you, I would have just been talking to myself. Last, I want to express my deepest love and gratitude to my husband Vinnie and my wonderful parents, who have provided endless encouragement and support during this crazy journey. ☺ I love you!

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CHAPTER 1. CHARACTERIZING GONADAL AND ADRENAL ACTIVITY IN PYGMY RABBITS

Introduction

Rabbits are often assumed to be prolific breeders, and thus unlikely to be threatened with extinction. However, nearly half of extant rabbit species are either endangered or threatened in a portion of their natural range (Smith, 2008). Of the 29 rabbit species worldwide, nine are listed as endangered or critically endangered by the International Union for Conservation of Nature, three are being considered for listings as endangered, and two are federally listed as endangered in the U.S. (Chapman and Flux, 1990; Smith, 2008). Because of their use in research and as pets, domestic rabbits have been studied extensively, but surprisingly little is known about the biology of other rabbit species. Research on other taxa (e.g., Felidae (Brown, 2006)and Canidae (Wildt et al., 2010)) suggests that differences in physiology may exist within families such as Leporidae (rabbits and hares), and thus species-specific knowledge may be required to develop effective *in situ* or *ex situ* conservation actions (Wildt et al., 2010).

The plight of one particular species, the pygmy rabbit (*Brachylagus idahoensis*), has been the focus of a U.S. Fish and Wildlife Service (USFWS) recovery effort, and highlights the need for more research into rabbit biology (USFWS, 2004; 2007). Pygmy rabbits are the smallest leporids in the world and the only ones in the United States that dig their own burrows (Oliver, 2004). They are ecological specialists that live in dense stands of sagebrush rangelands (*Artemisia* spp) with deep, loose soil (Dobler and Dixon,

1990; Green and Flinders, 1980; Janson, 2002; Rachlow et al., 2005). They depend on sagebrush for both shelter and a large proportion of their diet, up to 99% in winter (Green and Flinders, 1980; Wilde, 1978). In fact, some consider the pygmy rabbit to be a keystone species in sagebrush habitat because their burrows are used by other animals and they provide an abundant source of food (Flinders, 1999; Oliver, 2004). Pygmy rabbits reach maturity at about 7-11 months of age, and rarely survive longer than three years in captivity (Illig, 2009). The breeding season varies slightly with latitude, but generally begins in late February and ends in mid-June (Fisher, 1979; Gahr, 1993; Hays, 2001). Pygmy rabbits are believed to be induced ovulators, with a gestation period of 24 days and lactation period of 14-21 days (Elias, 2004; USFWS, 2007). Females can breed and conceive within days of giving birth, and in captivity, can produce up to five litters per year (Illig, 2009).

The historical range of pygmy rabbits covered much of the Great Basin, but in recent decades it has decreased substantially likely because of habitat destruction and degradation, especially in the Columbia Basin (Green and Flinders, 1980; Hays, 2001). In 2001, the sudden collapse of the Columbia Basin population prompted the initiation of a captive breeding program to produce offspring for reintroduction. This distinct population segment was listed as federally endangered in 2003 and based on recent field surveys, none may exist in the wild today (USFWS, 2007). Despite the potential for fast population growth, reproductive output has been low and the captive population is not self-sustaining (Illig, 2009). Early field studies suggested that pygmy rabbits can produce 12-18 young per year (Wilde, 1978); in captivity, however, females produced, on

average, fewer than seven young per year and fewer than two young survived to the next breeding season during the years of this study (Elias, 2004; Illig, 2009). Thus, a current priority of the recovery effort is to better understand the biology of pygmy rabbits, including reproductive and adrenal function, and use that information to identify factors associated with low reproductive success (USFWS, 2007).

The objectives of this study were to: 1) develop and validate methods for noninvasive monitoring of key steroid hormones involved in reproduction (progestagens) and stress (glucocorticoids); 2) characterize patterns of hormone excretion during pregnancy and lactation, and 3) examine seasonal patterns of fecal progestagen and glucocorticoid production. As the first study to characterize the reproductive and adrenal endocrinology of this endangered rabbit species, it provides information for developing appropriate management strategies to monitor animal welfare and increase reproduction in pygmy rabbits and perhaps other endangered leporid species.

Methods

Animals and Facilities

Animals used in this study were all captive-born, adult female pygmy rabbits (n = 28) housed at the Small Mammal Research Facility at Washington State University (WSU, Pullman, WA). Rabbits were provided water, grain-forage pellets (produced at the WSU Feed Mill) and a variety of fresh greens (i.e., big sagebrush clippings, lettuce, dandelion, parsley and clover) daily. All animals were housed outdoors in one of three pen types: circular (4.7 m^2), rectangular (4.0 m^2) and oval (1.0 m^2). Circular and oval

pens were constructed from galvanized steel water tanks enclosed by a wire-mesh top and rectangular pens were constructed from wire-mesh siding surrounded by a plastic barrier to retain soil. All pens were filled with 0.5 to 1 m of compacted soil and covered by a corrugated greenhouse roof. Oval pens were used during the non-breeding season only. Each enclosure was enriched with a plastic nest box, artificial burrows (7.6 cm diameter plastic drainage tubes) and sagebrush branches.

Females were housed singly, except during brief 1 - 6 day pairings when a male was introduced into the female's pen. Males were removed after copulation was observed or females became aggressive. Pygmy rabbits experience a post-partum estrus, so most females were re-paired 1 - 9 days after parturition to maximize the number of litters produced per year (Elias et al., 2006). Females were classified as pregnant if they exhibited nest-building behaviors within 24 days of mating (Elias et al., 2006). Not all pregnancies could be verified by the presence of young because mothers give birth underground and possibly bury or consume their young (Elias et al., 2006). Young typically emerge from the natal burrow about 2 weeks after birth and weaning was defined as 21 days after birth or the date young were separated from their mother (Adams et al., 2001; Elias et al., 2006).

Sample Collection and Steroid Extraction

To examine hormone profiles of pygmy rabbits, fecal collections took place between October 2005 and December 2008, spanning three breeding and non-breeding seasons. Fresh fecal samples were collected 4 - 7 times a week during the breeding season (Mar-Jun) and 2 - 4 times a week during the non-breeding season (Oct-Feb).

Samples were collected with gloves at approximately the same time every day and keepers avoided the collection of old or urine-contaminated samples. Each fecal sample consisted of 20-50 fecal pellets that were placed in a re-sealable plastic bag and immediately frozen at -20 ºC until analysis. Samples were lyophilized (Labconco Lyophilizer, Kansas City, MO) and crushed into a fine powder using a rubber mallet. Using a similar extraction method to that described by Brown et al. (1994b), 0.1 g of dried fecal material was added to 5 ml of 90% ethanol, vortexed for 40 minutes and centrifuged at 1300 *g* for 20 minutes. Next, the supernatant extract was poured into a second set of tubes and the remaining fecal material was resuspended in 5 ml of 90% ethanol, re-vortexed for 1 minute and re-centrifuged. Extracts were combined, evaporated to dryness and resuspended in 1 ml phosphate buffer (0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4, 0.15 M NaCl; pH 7.0). Steroid extraction efficiency averaged 91% (range 82% - 99%) as determined by the recovery of tritiated cortisol added to feces before extraction. Samples were further diluted 1:10 and 1:3 in buffer before analysis for progestagen and glucocorticoid metabolites, respectively.

Enzymeimmunoassays

A single-antibody progesterone enzymeimmunoassay (EIA) was used to quantify fecal progestagen metabolites. The EIA used a monoclonal antibody produced against 4-pregene-11-ol-3,20-dione hemisuccinate:BSA (CL425, 1:10,000; C. Munro, University of California, Davis), a horseradish-peroxidase conjugated progesterone label and progesterone standards (Sigma Chemical Co., St. Louis, MO). The sensitivity of the assay was 0.78 pg/well. The cortisol EIA used a polyclonal cortisol antibody (R4866,

1:20,000; C. Munro, University of California, Davis), a horseradish-peroxidase conjugated cortisol label and cortisol standards (Young et al., 2004). Assay sensitivity was 3.90 pg/well. Intra-assay coefficients of variation were less than 10% and inter-assay coefficients of variation were less than 15% and 10% for the progesterone and cortisol assays, respectively.

Each EIA was validated by demonstrating: 1) parallelism between binding inhibition curves of dilutions of pygmy rabbit fecal extracts and the respective standard curve (progesterone or cortisol), and 2) significant recovery of exogenously added steroid (>90%) to fecal extracts before analysis. The progestagen assay was validated by demonstrating an increase over baseline during gestation. Physiological validation of the glucocorticoid assay was demonstrated by showing a significant increase ($p < 0.05$) in glucocorticoid concentrations above baseline within 48 hours after transfer of an animal to a new facility (n = 8 rabbits), a presumed stressful event (See Appendix A).

High-Performance Liquid Chromatography (HPLC)

The number and relative proportions of progestagen and glucocorticoid metabolites in pygmy rabbit fecal extracts were determined by reverse-phase HPLC (Microsorb C-18 Column; Rainen Inc., Woburn, MA) using modifications of the methods described by Monfort et al. (1991). Before HPLC, samples were passed through a C-18 matrix column (Spice Cartridge, Rainin, Inc., Oakland, CA) and eluted with 5 ml of 80% methanol to remove contaminants (sample loss was <10%). Progestagen metabolites were separated using a gradient of 20-32% acetonitrile over 15 min, increasing to 50% over 50 min and then to 100% over 55 min (1 ml/min flow rate, 1 ml fractions).

Glucocorticoid metabolites were separated using a linear gradient of 20-100% methanol in water over 80 min (1 ml/min flow rate, 1 ml fractions). Tritiated steroids (~4,000 dpm) were added to each run to determine co-elution patterns of progestagen (progesterone) and glucocorticoid (cortisol, corticosterone) immunoactivity. HPLC fractions of pygmy rabbit fecal eluates were taken to dryness and reconstituted in assay buffer, and immunoreactivity was quantified by the appropriate EIA.

Data Analysis

Progestagen and glucocorticoid data were averaged over the pre-breeding (1 Jan – 29 Feb) and breeding (1 Mar – 31 Jun) seasons for each female. Overall individual means and baseline means were calculated for each hormone within each female. Baseline means were calculated using an iterative process where all peak values two standard deviations above the mean were excluded and means were recalculated until extreme values were excluded (Brown et al., 1994a). Baseline means provided an estimate of basal hormone secretion that excluded temporary increases in hormone secretion caused by reproductive events or stressful events. All hormone data are reported as mean ± standard error of the mean. Differences in hormone concentrations between the pre-breeding and breeding seasons were assessed using paired t-tests. Assumptions of normality were checked by examining normal probability plots and calculating a Shapiro-Wilks statistic.

For seasonal analyses, data were averaged across 3-month intervals, beginning in January, as follows: winter (Jan - Mar), spring (Apr - Jun) and fall (Oct - Dec). Data were also averaged by month. Monthly and seasonal differences in fecal hormone

concentrations were determined using a repeated-measures general linear model, followed by a post-hoc multiple comparison analysis to determine directionality and magnitude.

To examine age effects on hormone secretion, data were divided into three categories: 1-year old, 2-year old and 3-year old. Age groups included animals that were ± 6 months of the target age. Average and baseline means among individuals across the three age groups during each of the two time periods (pre-breeding and breeding season) were analyzed by one-way ANOVA.

During gestation, maximal hormone concentrations were calculated from the highest value measured between days 21 and 24 post-mating (i.e., the last days of pregnancy). Significant elevations in progestagens during gestation were determined using a paired t-test that compared individual progestagen values and individual progestagen baselines during the breeding season. For each pregnancy, the ratio between maximal hormone concentration and baseline mean was calculated to estimate peak magnitude while controlling for individual variability in hormone secretion. During lactation, days that overlapped with a subsequent pregnancy were removed so data reflected only nursing females.

All statistical analyses were conducted using Microsoft Excel 2003 (Seattle, WA, USA) and SPSS Version 15.0 for Windows (Chicago, IL, USA). For all analyses, significance was assessed at the 0.05 level.

Results

Identification of Hormone Metabolites by HPLC

Based on comparisons between immunoactivity and elution of known radioactive tracers, both cortisol and progesterone were excreted in feces of pygmy rabbits. Progesterone EIA analysis of HPLC fractions identified three peaks, of which only 10% was associated with the tritiated progesterone reference tracer. The majority of progestagen immunoactivity was associated with two more polar, as yet unidentified, metabolite peaks. By contrast, the majority of glucocorticoid immunoactivity (>70%) was associated with native cortisol, as evidenced by co-elution with the tritiated cortisol reference tracer. The cortisol EIA antibody crossreacts less than 0.7% with corticosterone, so patterns detected in this study were primarily those related to cortisol excretion. A corticosterone RIA (MP Biomedicals, Solon, OH), which crossreacts with corticoid metabolites in diverse species (Wasser et al., 2000), also detected glucocorticoid immunoactivity in pygmy rabbit fecal extracts; the cortisol EIA, however, was used because it detected more glucocorticoids in the same samples based on sample dilution curves.

Reproductive Success during the Breeding Season

In 2006, 12 females were monitored and collectively they produced 107 young in 34 litters. Eight of these females had young survive to emergence (n = 46 young) and seven females had young survive to weaning (n = 28 young). In 2007, 10 females were monitored and they produced 88 young in 31 litters. Seven of these females had young survive to emergence (n = 47 young) and six females had young survive to weaning (n =

24 young). In 2008, 10 females were monitored and they produced 79 young and 25 litters. Seven of these females had young survive to emergence (n = 33 young), but only five had young survive to weaning (n = 23 young).

Three of the females were monitored for two breeding seasons and one female was monitored for three breeding seasons. The majority of females (63%) studied produced 3 - 4 litters per year, but two females failed to conceive, nine females had only one or two litters and one female produced five litters in one year. The number of conceptions decreased at the end of the breeding season with 34 out of 90 conceptions in March, 31 conceptions in April, 21 conceptions in May and four conceptions in June. Table 1 provides a summary of the number of litters and number of young born or emerged separated by birth month. The mean duration of gestation for these pygmy rabbits was 23.7 ± 0.11 days (range, 20 - 25). Because the gestation period was less than a month, some litters were born in the same month they were conceived.

The average lifespan of females used in this study was 2.02 ± 0.18 years (n = 27), whereas the average lifespan of females born at this facility between 2005 and 2007 that survived to emergence was only 1.00 ± 0.12 years (n = 52 females) (Illig, 2009).

Longitudinal Hormone Profiles

Based on 90 pregnancies in 26 females, fecal hormone profiles during gestation were characterized by: 1) a large increase in progestagen metabolites shortly after mating (~20-fold increase on Day 1); 2) a gradual increase in progestagen and glucocorticoid concentrations throughout gestation, with maximum concentrations observed on Days 21 - 24; and 3) a decrease in progestagen and glucocorticoid concentrations to baseline

shortly after birth (Day 24) (Figure 1.1). A spike in progestagens was observed consistently one day after copulation and averaged 1163.38 ± 126.86 ng/g (range, 192.94 ng/g - 5367.83 ng/g; n = 72 pregnancies). A significant elevation of progestagens above baseline (62.65 ± 3.18 ng/g; range, 39.46 – 87.58 ng/g) was observed by Day 10 of gestation ($p < 0.05$ for Days 10-24). At the end of gestation, a second peak in progestagen metabolites was observed between Days 21 and 24, and averaged 138.13 ± 8.13 ng/g (range, 51.95 – 449.20 ng/g; n = 79). On average, this peak represented a 2.73 ± 0.18 fold increase in progestagens over baseline as calculated by the ratio between the maximum progestagen value and the female's progestagen baseline (n = 79). The end of gestation progestagen peak was observed on Day 21 in 13.5% of females, Day 22 in 16.2% of females, Day 23 in 29.7% of females and Day 24 in 40.5% of females (for pregnancies where at least two samples where collected during this period). A 2-fold increase in progestagen concentrations over baseline was observed in 66% (52 out of 79) of pregnancies where at least one sample was collected on either Day 23 or 24.

The maximum glucocorticoid concentration at the end of gestation (Days 21 - 24) averaged 181.43 ± 17.27 ng/g (range, 52.71 - 883.38 ng/g; n = 79), which represented an average increase in glucocorticoids of 2.86 ± 0.23 (n = 79) over baseline as calculated by the ratio between the maximum glucocorticoid value and the female's glucocorticoid baseline. Responses were variable, however, and a two-fold increase in glucocorticoids was observed in only 60.7% of females. The exact date of this peak also was variable and observed on Day 21 in 18.2% of females, Day 22 in 19.5% of females, Day 23 in 28.6% of females and Day 24 in 33.8% of females. The day after birth (Day 25), both

progestagen and glucocorticoid concentrations started to decline. During lactation (days 25 - 39), progestagen concentrations remained at baseline (62.31 ± 3.23 ng/g) unless the female became pregnant again, whereas glucocorticoids were more variable with concentrations remaining elevated above baseline in some females (n = 15 pregnancies), while concentrations returned to baseline within a few days in others (n = 46). Glucocorticoid concentrations during lactation averaged 86.77 ± 9.44 ng/g (n = 61).

Progestagen and glucocorticoid profiles in three representative females are presented in Figure 1.2 to demonstrate differences in hormone excretion during pairings, pregnancy and parturition. All three females were transferred to identical circular breeding pens in January or February. In general, females were re-paired and able to conceive within 9 days of giving birth (Figure 1.2a). This female experienced three pregnancies, but did not conceive during a fourth pairing near the end of the breeding season. The first litter died the day after parturition, but this female successfully weaned young from her second and third pregnancies. Glucocorticoid concentrations were more elevated during the nursing period of the first unsuccessful pregnancy, than the following two successful pregnancies.

The female in Figure 1.2b was mated six times, but failed to conceive and was moved into a much larger carport pen (75 m^2) with a male later in the season. Baseline glucocorticoid concentrations in this female were higher on average and more variable than the female in 2a. The female in Figure 1.2c was not paired with a male until April, which was unsuccessful, but she successfully conceived during the second pairing 22 days later. She gave birth in June, but none of the young survived to weaning.

Glucocorticoid concentrations in Figure 1.2c were elevated during unsuccessful pairings and, similar to the first pregnancy in Figure 1.2a, also were elevated during lactation. Except in a few cases (3 of 107), females did not demonstrate either a spike in progestagens or a gradual increase in progestagens over the next month following an unsuccessful mating attempt.

Seasonal Hormone Patterns

Overall progestagen means during the pre-breeding season (51.41 ± 2.45 ng/g, n = 20) were approximately half those observed during the breeding season (107.19 ± 8.92 ng/g; n = 18, t = -6.208, $p < 0.01$, df = 12). Progestagen baselines also were lower during the pre-breeding season (49.96 ± 2.54 ng/g; n = 20), as compared to baseline means during the breeding season (62.65 ± 3.18 ng/g; n = 18; t = -3.396, p = 0.005, df = 12). However, when data during gestation were removed, there was no significant difference in progestagen baselines between the pre-breeding and breeding seasons (51.37 ± 2.47 ng/g; n = 18; t = -0.750, p = 0.467, df = 12). Overall mean progestagen concentrations in the fall were significantly lower than both winter and spring seasons (F = 10.847, $p < 0.001$, n = 21); however, no seasonal differences were detected in progestagen baseline concentrations (F = 2.729, p = 0.077, n = 21, Table 1.2). Progestagens differed among months for overall (F = 10.475, $p < 0.001$, n = 19) and baseline (F = 3.770, p = 0.001, n = 16) means, but not when pregnant females were removed (F = 0.971, p = 0.481, n = 4). A post-hoc analysis found that progestagen concentrations were highest in March, April and May due to pregnancies during the breeding season (Figure 1.3, top panel). When

pregnancies were excluded, progestagen baselines showed no monthly differences and averaged between 50 and 60 ng/g (Figure 1.4).

Glucocorticoid profiles showed the opposite trend to progestagens, with higher overall mean concentrations during the pre-breeding season (93.23 ± 3.76 ng/g, n = 18) than those during the breeding season (72.87 ± 3.01 ng/g, n = 18; t = 2.506, p = 0.0276, df = 12). Baseline concentrations of glucocorticoid metabolites also were higher during the pre-breeding (72.07 ± 2.75 ng/g, n = 18) than the breeding (56.04 ± 1.96 ng/g, n = 18; t = 2.948, p = 0.012, df = 12) season. Overall mean glucocorticoid concentrations were higher during the winter than the spring (F = 5.795, p = 0.006, n = 21, Table 1.2), whereas baseline concentrations were higher during the winter than both the spring and fall (F = 7.966, p = 0.001, n = 21, Table 1.2). Glucocorticoids differed across months for overall (F = 10.475, p < 0.001, n = 19) and baseline (F = 3.770, p = 0.001, n = 16) means. Monthly post-hoc analyses revealed a seasonal pattern in glucocorticoids that was lowest between April and June and highest between January and March for overall means (Figure 1.3, bottom panel) and baseline means (Figure 1.4).

Discussion

This study represents the first assessment of hormone patterns in a rabbit species other than the domestic rabbit (*Oryctolagus cuniculus*) and is one of only three studies that have validated fecal steroid monitoring techniques in a rabbit (Korndorfer et al., 1998; Monclus et al., 2006). HPLC analyses verified the presence of both cortisol and progesterone in the excreta of pygmy rabbits, and changes in glucocorticoids and

progestagens were associated with biologically relevant events such as transfer to a new facility and pen or pregnancy. Thus, our results confirm that non-invasive fecal hormone techniques can be used to monitor adrenal and gonadal activity in pygmy rabbits, which may ultimately allow us to understand hormonal changes associated with copulation, pregnancy, parturition, stress and seasonality.

Next, our analysis revealed a large spike in progestagen excretion that occurred following mating. This marked increase over baseline was observed on Day 1 in all verified pregnancies where samples were collected. By comparison, a 2-fold peak in progestagen concentration at the end of gestation was detected in only 66% of pygmy rabbit pregnancies, indicating that progestagen analysis at the end of gestation is not as reliable a method for identifying pregnancy. Many studies on domestic rabbits have examined progesterone patterns during gestation (Beyer and McDonald, 1973; Browning et al., 1980; Chen et al., 1995; Gonzalez-Mariscal et al., 1994; Gonzalez-Mariscal et al., 2009; Gonzalez-Mariscal et al., 1996; Harrington and Rothermel, 1977; Hilliard and Eaton, 1971; Hilliard et al., 1967; Hoffman and Gonzalez-Mariscal, 2006; Korndorfer et al., 1998; Negatu and McNitt, 2002), but few studies collected samples frequently enough to detect this post-copulatory spike. In those rabbit studies with adequate data, the primary steroids produced after copulation were progestagens, specifically progesterone and 20α-dihydroprogesterone (20α-OHP), with quantities being 1000-fold greater than estradiol and testosterone (Hilliard and Eaton, 1971; Hilliard et al., 1967; Ramirez and Beyer, 1988). 20α-OHP, a metabolite of progesterone, is synthesized and secreted in response to luteinizing hormone (LH) (Dorrington and Kilpatrick, 1966; Hilliard et al.,

1967; Ramirez and Beyer, 1988). Hilliard et al. (1971) reported a 10-fold increase in 20α-OHP 4 - 6 hours after coitus that was followed by a drop below baseline around the time of ovulation (9 - 12 hours after coitus). Progesterone also increased during this period, but concentrations were only one-fifth those of 20α-OHP (Ramirez and Beyer, 1988). These studies provide evidence that a 10-fold spike in progestagens, similar to that observed in pygmy rabbits, also occurs in domestic rabbits and suggests it may be caused by an increase in 20α-OHP, rather than progesterone alone. The spike in pygmy rabbits was detected 1 day after copulation because of the lag time from secretion to excretion. From a management standpoint, this spike may provide a quick and reliable means of determining if a successful mating has, in fact, occurred.

Evidence from domestic rabbits suggests this progestagen surge may be part of a neuroendocrine reflex response that plays a role in ovulation, fertilization and early embryonic development (Chen et al., 1995). In domestic rabbits, copulation stimulates the release of LH, which increases the synthesis and release of progestagens (20α-OHP and progesterone) from ovarian interstitial tissue (Dorrington & Kilpatrick, 1966). This release activates progesterone receptors, which stimulate the production of collagenase in the theca interna cells and a breakdown of follicular connective tissue. A weakening of the follicle wall, coupled with an increase in fluid volume inside the follicle, leads to ovulation (Espey and Lipner, 1994). In addition, an increase in progestagens after mating may serve a behavioral function. In intact females, sexual receptivity and chinning (scent-marking) in domestic rabbits decreases 24 hours after mating, temporarily recovers and then remains inhibited across gestation (Hoffman and Gonzalez-Mariscal, 2007). Several

studies have shown that progestagens inhibit estrous behavior in domestic rabbits (reviewed in (Beyer and McDonald, 1973), although there is some skepticism about whether progestagens actually play a role in the immediate post-mating inhibition (Hoffman and Gonzalez-Mariscal, 2007).

In pygmy rabbits, a late-gestation peak in progestagens was observed primarily on Days 23 and 24 (70.2% of pregnancies), just 0 - 1 days before birth. By comparison, domestic rabbits exhibit a gradual rise in progestagens over the course of pregnancy, with the peak and decline occurring well before parturition. Most studies on New Zealand White rabbits (gestation period = 32 days) have reported that plasma progesterone concentrations increase rapidly in early gestation until day 10, peak between days 11 and 15, decrease starting between days 18 to 25, and reach baseline at birth (day 32) (Browning et al., 1980; Challis et al., 1973; Gonzalez-Mariscal et al., 1994; Harrington and Rothermel, 1977; Negatu and McNitt, 2002). Of relevance to this study are the findings of Korndorfer et al. (1998), which showed that gestational patterns of plasma versus fecal progestagens differ slightly. Fecal data indicated a smaller peak between days 11 - 14 followed by a larger peak around day 23 - 25, six to eight days before birth (Korndorfer et al., 1998). Thus, the fecal progestagen profile in domestic rabbits is more similar to that observed in pygmy rabbits, in that both profiles identified peak progestagen concentrations near the end of gestation, rather than during the middle. Perhaps a different metabolite, detected only by fecal analyses, accounts for the differences observed between plasma and fecal pregnancy profiles.

For pygmy rabbits, the peak in progestagen concentrations at the end of gestation represented a 2- to 3-fold increase over baseline, which is relatively small compared to that observed in other species. For example, using the same assay, fecal progestagen concentrations during gestation increase approximately 100-fold to 400-fold over baseline in the cheetah (Brown et al., 1996), almost 20-fold in the North American river otter (Bateman et al., 2009) and approximately 4-fold in the chinchilla (Busso et al., 2007). In the domestic rabbit, however, Korndorfer et al. (1998) found a similar 2- to 3-fold increase in fecal progestagens during gestation, suggesting that these two rabbit species may share similar a less robust increase in progestagen secretion during pregnancy.

In examining seasonal patterns of hormone excretion, analyses found that overall mean progestagen concentrations were highest during any period that was associated with successful pairings and pregnancy. However, when pregnancies were removed, progestagen baselines did not vary between the non-breeding and breeding seasons, and there were no significant hormonal changes during the transition from the non-breeding season to the beginning of estrus. Thus, progestagen baselines cannot be used as an indicator of readiness to breed. By contrast, there was a clear seasonal effect on fecal glucocorticoid excretion, with concentrations being highest during the winter, specifically January, February and March. This pattern was similar to that reported for European rabbits (*Oryctolagus cuniculus*), in which serum cortisol concentrations peaked during the winter between December and February (Bensaad and Bayle, 1985). Hormone trends within the pygmy rabbit breeding season also revealed a gradual decrease in

glucocorticoids as the season progressed, reaching a low in May and June, which interestingly coincided with an increase in breeding success. In terms of litter survival, March was the month with the lowest reproductive success and the highest glucocorticoid concentrations during the breeding season. Conception rates (% of matings that resulted in a pregnancy) in March were 56%, as compared to 81% and 63% in April and May, respectively. In addition, no litters born in March survived to emergence. In fact, during the 3 years of this study, the earliest surviving litter was born 18 April, while 23 litters born before this date died. Thus, these data suggest that stress levels in females may be inversely related to litter survival during the breeding season, although further studies are needed to verify this relationship.

In summary, this study demonstrated that non-invasive fecal hormone monitoring techniques can be used to assess gonadal and adrenal activity in the pygmy rabbit. A marked spike in progestagen excretion shortly after mating appears to signal a successful copulation, and could be used as a breeding management tool. Gradual increases in progestagen and glucocorticoid concentrations were observed throughout gestation, with a return to baseline shortly after birth. Excretion of fecal glucocorticoids was highest during the pre-breeding winter season and the lowest glucocorticoid concentrations were associated with the highest rates of offspring production and survival towards the end of the breeding season, suggesting a possible link between heightened adrenal activity and lowered reproductive fitness in this species.

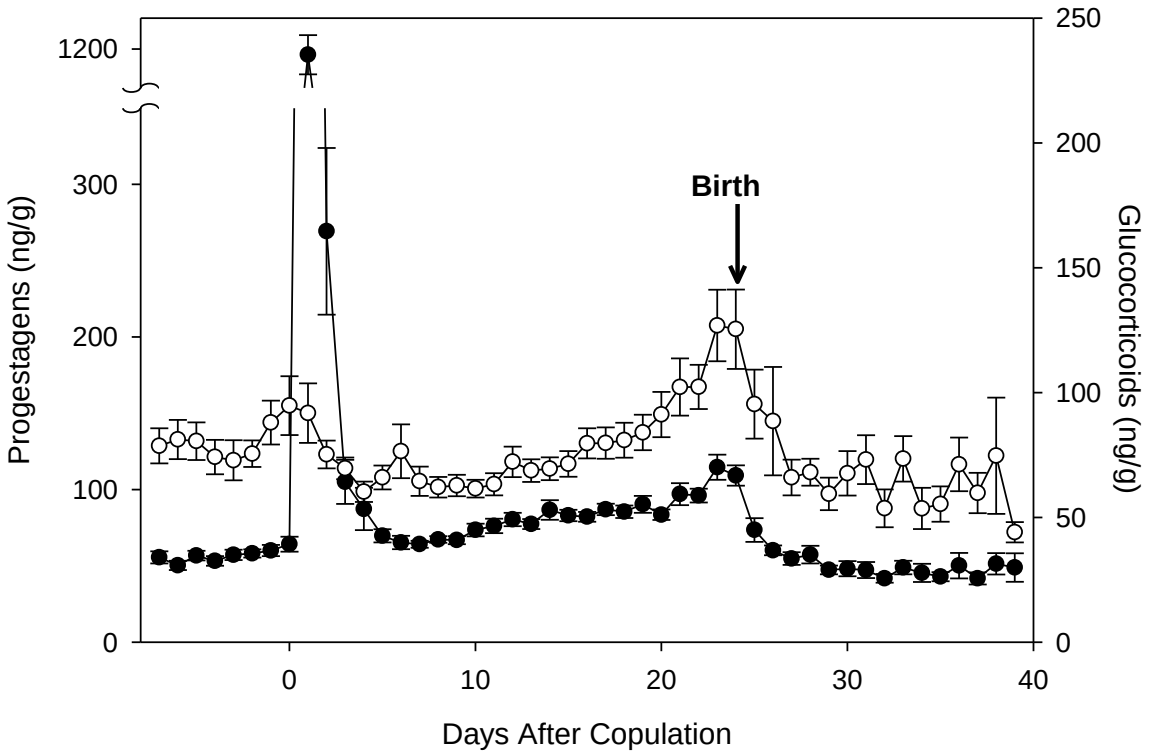
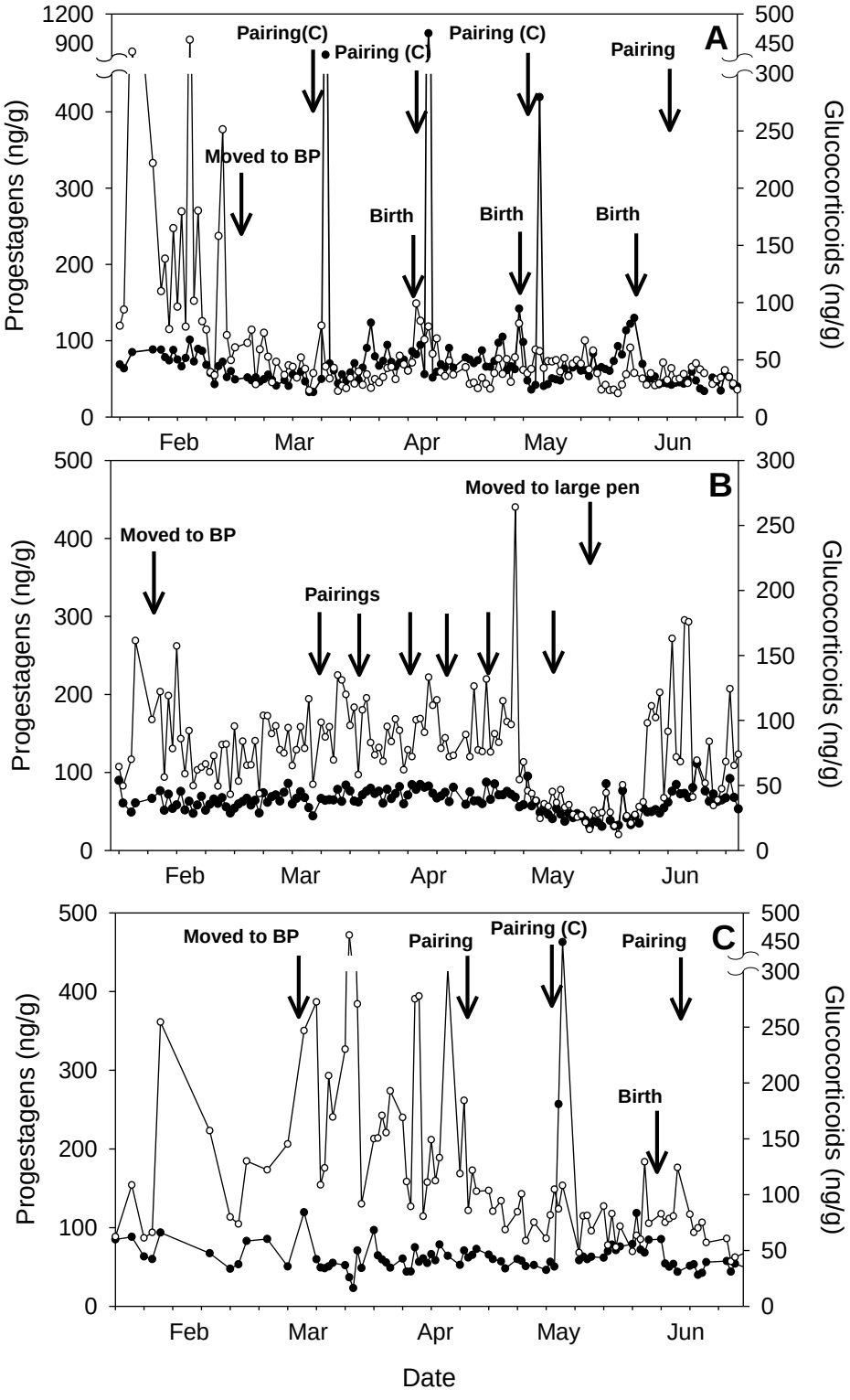


Figure 1.1. Mean (± s.e.m.) concentrations of fecal progestagen (●) and glucocorticoid (○) metabolite concentrations for 7 days before mating, 24 days of gestation and 14 days during lactation from 26 pregnant pygmy rabbit females (n = 90 pregnancies).

Day 0 = copulation. Parturition is indicated by the arrow on Day 24. Hormone concentrations during the lactation period (Days 25-45) were quantified until kits emerged from the natal burrow (Days 38-39). Data were aligned to the progestagen peak (Day 1).

Figure 1.2. Longitudinal patterns of fecal progestagen (●) and glucocorticoid (○) concentrations in A) a successfully breeding female, B) a female that failed to conceive, and C) a female that conceived, but failed to wean offspring.

Note differences in y-axis units. “Moved to BP” indicates transfer of the female to a breeding pen (BP). “Pairing” indicates dates of pairing, and “Pairing (C)” indicates a pairing where conception occurred.


A
Pairing(C)
Pairing (C)
Pairing (C)
Pairing
Moved to BP
Birth
Birth
Birth
B
Moved to large pen
Moved to BP
Pairings
C
Moved to BP
Pairing
Pairing (C)
Pairing
Birth
Progestagens (ng/g)
Glucocorticoids (ng/g)
Feb
Mar
Apr
May
Jun
Date

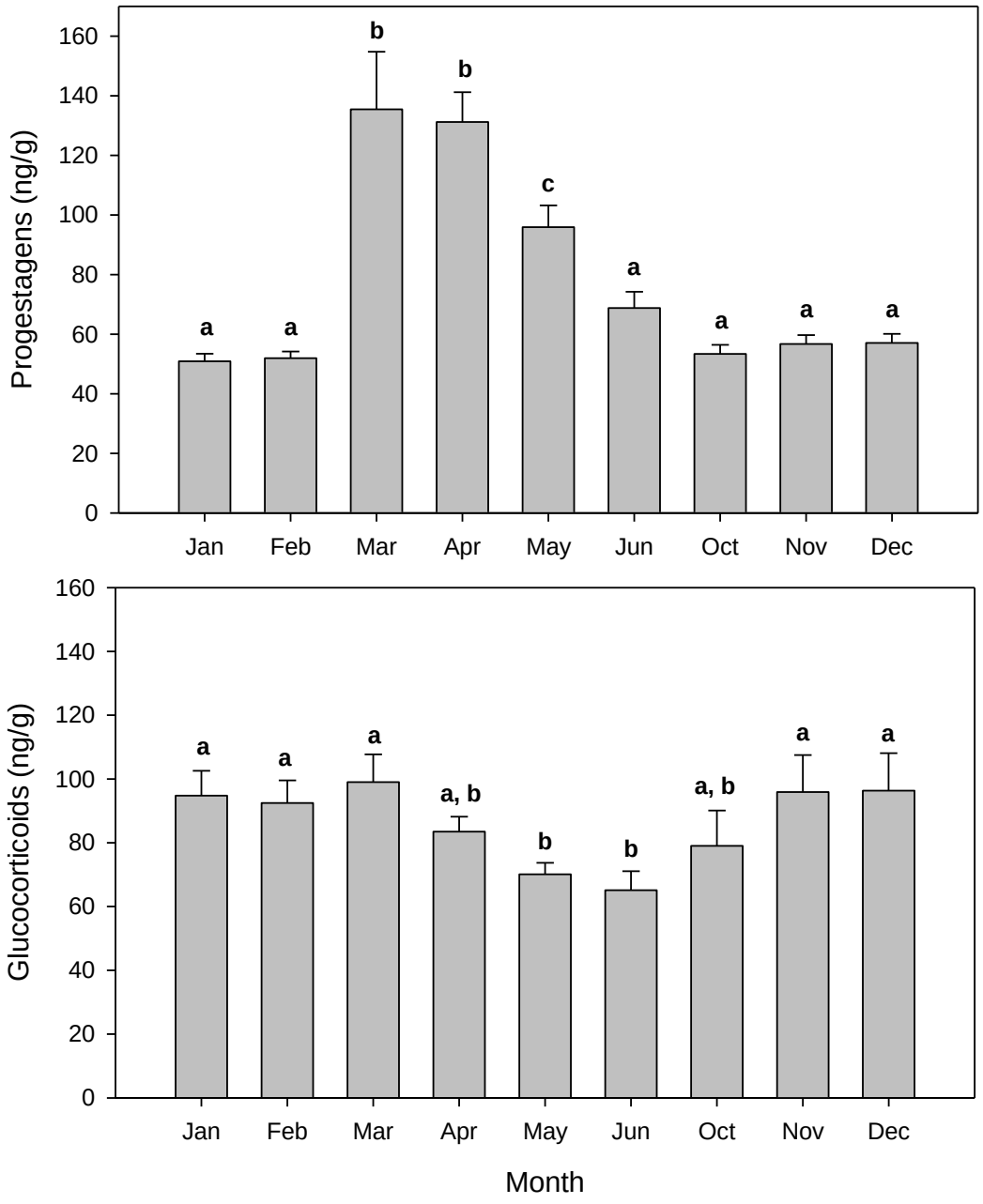


Figure 1.3. Overall monthly mean (± s.e.m.) concentrations of fecal progestagens (top panel) and glucocorticoids (bottom panel) in adult pygmy rabbits (n = 32 females).

[a,b,c]Means with the same letter are not significantly different (P > 0.05).

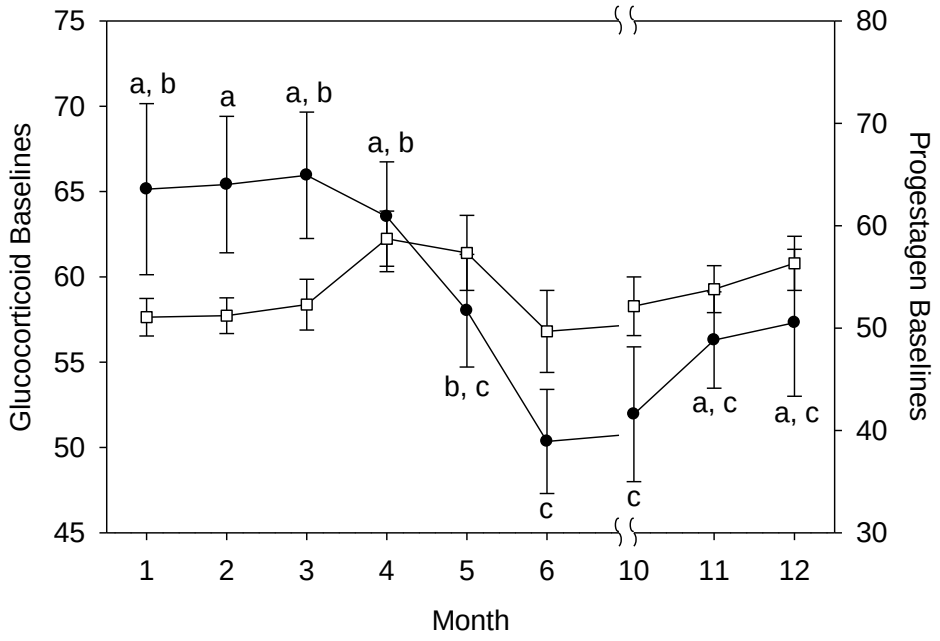


Figure 1.4. Monthly changes in baseline means (± s.e.m.) for fecal glucocorticoids (●) and progestagens (□) in captive female pygmy rabbits.

Monthly progestagen baseline means excluded pregnancies and did not show significant monthly differences. [a,b,c]Glucocorticoid baseline means with the same letter are not significantly different ($P > 0.05$).

Table 1.1. Reproductive data for study females during the breeding season, indicating the number of litters conceived and pups born each month and the percentage of litters and young that survived to emergence from the burrow.

Month	Number of Litters Born	Number of Kits Born	Litter Emergence (%)[1]	Young Emergence (%)[1]
March	11	36	0% (0/11)	0% (0/36)
April	34	112	38% (13/34)	43% (48/112)
May	24	61	47% (11/24)	51% (31/61)
June	20	61	50% (10/20)	62% (38/61)
Total	90	273	39% (35/90)	44% (120/273)

[1]Litter and young emergence was based on the month they were born, not the month they were conceived or emerged. Also, litter emergence represents % of litters that had at least one young survive to emergence.

Table 1.2. Seasonal differences in fecal progestagen and glucocorticoid concentrations in captive female pygmy rabbits.

	Progestagens (ng/g)		Glucocorticoids (ng/g)	
Season	Overall Mean	Baseline	Overall Mean	Baseline
Winter	90.41 ±12.41[a]	54.98 ± 2.38[a]	88.37 ± 6.31[a]	65.52 ± 3.84[a]
Spring	101.93 ± 7.56[a]	59.34 ± 3.34[a]	67.20 ± 3.76[b]	54.16 ± 3.26[b]
Fall	56.22 ± 3.42[b]	53.50 ± 2.83[a]	84.04 ± 7.68b[a, b]	54.03 ± 2.91[b]

Values are means ± s.e.m.
[a, b, c]Within each column, values with different superscripts differ ($p < 0.05$)

CHAPTER 2. HORMONAL CORRELATES OF REPRODUCTIVE SUCCESS IN CAPTIVE PYGMY RABBITS

Introduction

For many threatened or endangered species, captive breeding programs are an important tool for protecting, maintaining and supplementing declining populations (Gibbons et al., 1995; Mallinson, 1995). Captive environments can provide animals with unlimited food, protection from predators, and access to genetically-matched mating partners, but the success of these programs hinges on the establishment of self-sustaining populations. Unfortunately, failure to breed in captivity and high rates of neonatal mortality have hindered the success of many captive breeding programs (Snyder et al., 1996). Understanding why some programs succeed and others do not requires an in-depth understanding of the factors that affect reproduction and welfare.

One species in need of improved management is the pygmy rabbit (*Brachylagus idahoensis).* The captive breeding program began in 2001 after the sudden collapse of the population in the Columbia Basin from five locations to one location (Hays, 2001). This population was federally listed as endangered in 2003, but may be extinct in the wild today (USFWS, 2004; 2007). In domestic rabbits, females do not exhibit clear estrous cycles, as follicles develop continuously, and ovulation is induced 9-12 hours after mating (Batra and Kallstrand, 1979; Ramirez and Beyer, 1988). Pygmy rabbits are also believed to be induced ovulators and can conceive within days after parturition, allowing females to be pregnant and lactating at the same time. The reproductive potential of wild

pygmy rabbits during the breeding season, which lasts from late February to early June, is estimated to be 18 young per year (three litters of six offspring each) (Elias et al., 2006). By contrast, individual pygmy rabbit females in captivity rarely produce more than two surviving offspring per season.

Early in the captive breeding program (2001-2003), low conception rates were the greatest problem. The U.S. Fish and Wildlife Service (2007) estimated that only 44% of all wild-caught Columbia Basin pygmy rabbits conceived in captivity and only 20% of pairings resulted in confirmed pregnancies (conception rate). In 2004, Idaho and Columbia Basin pygmy rabbits were intercrossed to increase allelic diversity and counteract a suspected inbreeding depression, and to increase reproductive fitness (USFWS, 2007). Recent estimates of reproductive success in captivity report that 74% of females (Columbia Basin, Idaho and intercross rabbits) conceived at least once during the breeding season, but only 37% of pairings resulted in confirmed pregnancies (Elias et al., 2006). Considering that the average conception rate of domestic rabbits varies from 52% to 89% depending on the month and the interval between parturition and re-mating, there is still room for improvement (Lopez et al., 1994; Mmereole, 2009; Sittmann et al., 1964; Yamani et al., 1992).

In more recent years (2004-2008), high infant mortality was the limiting factor on population growth, with the majority of neonatal deaths (96%) occurring within the first 4 days of birth due to unknown causes (Illig, 2009). Between 2006 and 2008, over 59% of litters and 55% of individual rabbit young died before emergence from natal burrows, i.e., within the first two weeks after birth (Elias, 2004; Illig, 2009). In addition, fewer

than half of the females in the captive population produced young that survived to emergence. Despite the potential to have 2 - 7 young per litter and up to five litters per year in captivity, during this study, captive females averaged fewer than three emerged young per year, with only two surviving to the next breeding season (Elias et al., 2006; Illig, 2009). Thus, a priority of the recovery effort was to characterize the reproductive and stress physiology of pygmy rabbits and identify what factors were associated with low reproductive success (USFWS, 2007).

One way to monitor reproduction and welfare in wildlife species is to measure gonadal and adrenal steroids excreted in urine or feces (Lasley and Kirkpatrick, 1991; Millspaugh and Washburn, 2004; Wasser et al., 2000; Young et al., 2004). The non-invasive nature of this approach allows frequent collection without the need to handle the animals. For many species, fecal analyses are more practical, as urine is difficult to collect in most captive enclosures. In addition, fecal data provide a pooled estimate of hormone production from the previous 12-24 hours, dampening the influence of acute fluctuations in secretion on estimates (Millspaugh and Washburn, 2004). Non-invasive methods are now commonly used to monitor reproductive status, ovulation, pregnancy, parturition, seasonal fluctuations in reproductive activity, as well as animal health and welfare (Brown, 2006; Brown et al., 1994b; Durrant, 1995; Lane, 2006; Shepherdson et al., 2004; Young et al., 2004).

Several studies have shown that stress due to inadequate housing conditions, inappropriate social interactions or other stressors in captivity can negatively influence reproductive function (Carlstead and Shepherdson, 1994; Morgan and Tromborg, 2007;

Tilbrook et al., 2002). Stress, as assessed by the heightened secretion of glucocorticoids, can disrupt reproduction during four key reproductive events: mating, conception, gestation, and lactation. The overall goal of this study was to investigate patterns of fecal progestagen and glucocorticoid excretion during these key reproductive events and identify hormonal correlates of reproductive success. The specific objectives were to determine if there are hormonal differences between: 1) females that did and did not conceive; 2) successful and unsuccessful pairings; and 3) pregnancies that did and did not produce young that survived to emergence.

Methods

Animals and Facilities

Animals used in this study (n = 41) were captive-born, adult females (aged 1-3 years) housed at two facilities: Washington State University, Pullman, WA (Facility 1) and the Oregon Zoo, Portland, OR (Facility 2). All animals were housed off-exhibit and were exposed to natural fluctuations in photoperiod and temperature. Pygmy rabbits were provided water, grain-forage pellets (produced by the WSU Feed Mill) and a variety of fresh greens (big sagebrush clippings, lettuce, dandelion, parsley and clover) daily.

During the breeding season (March – June), females were housed in one of four pen types: 1) Circular pens, 2) Rectangular pens, 3) Half-soil pens, and 4) Carport pens. Circular, rectangular and carport pens were used at Facility 1, while only circular and half-soil pens were used at Facility 2. Circular pens (2.4 m diameter, 4.7 m^2) were constructed from galvanized steel water tanks filled with 0.5 to 1.0 m of compacted soil.

Rectangular pens were 4.0 m^2 (~2.5 x 1.5 m) surrounded by wire-mesh siding with a plastic barrier to keep soil in. Half-soil pens were rectangular 1.8 x 3.7 m (6.7 m^2) pens with a concrete floor covered in wood shavings on one half and about 0.5 m of soil on the other half. Each carport pen (75 m^2) was constructed from a carport enclosed by wire-mesh that contained several large mounds of soil (~1 meter tall). Each pen was enriched with a plastic nest box, artificial burrows (7.6 cm diameter plastic drainage tubes) and sagebrush branches. All pens were covered by a corrugated greenhouse roof or carport roof.

In the carport pens, one male and one female were housed together throughout the breeding season. In all other pens, females were housed as singletons, except during brief 1 - 6 day pairings when males were introduced into the females' pens**.** Males were removed after copulation was observed or females became aggressive. Females were paired 1 - 6 times during the breeding season with genetically matched males and re-bred if they showed no behavioral signs of pregnancy. Females were classified as pregnant if they exhibited nest-building behaviors within 24 days of mating. Not all pregnancies could be verified by the presence of young because mothers give birth in underground burrows and sometimes bury or consume their young. Young typically emerged from the natal burrow about 2 weeks (defined as 14 days) after birth; the end of the weaning period was defined as 21 days after birth or the date young were separated from their mother (Adams et al., 2001; Elias et al., 2006).

During the breeding season, keepers maintained detailed records of reproductive behaviors including observed copulations, digging of natal burrows, nest-building, and

birth. Each female was categorized as a "conceiving female" if she conceived and produced offspring or a "non-conceiving female" if she was mated, but never conceived. Each pairing was categorized as either "successful" if the female conceived during the specific mating period or "unsuccessful" if she did not. Each pregnancy was categorized as either a "surviving litter" is the female produced offspring that survived to emergence from the natal burrow or a "non-surviving litter" if she gave birth, but none of her offspring survived to emergence.

Fecal Sample Collection and Processing

Feces were collected regularly from January 2006 through December 2008, spanning three breeding seasons. Fresh fecal samples were collected 4 - 7 times a week during the breeding season (Mar - Jun). Samples were collected at approximately the same time each day and keepers avoided the collection of old or urine-contaminated samples. Each fecal sample consisted of ~20 - 50 fecal pellets that were placed in a re-sealable plastic bag and immediately frozen at -20º C until analysis. Samples were lyophilized (Labconco Lyophilizer, Kansas City, MO) and crushed to a fine powder using a rubber mallet. Fecal glucocorticoid and progestagen metabolites were extracted from samples as described by Brown et al. (1994b) with minor modifications. Briefly, 0.1 g of dried fecal material was added to 5 ml of 90% ethanol, vortexed on a multi-tube vortexer for 40 minutes and centrifuged at 1300 *g* for 20 minutes. Next, the supernatant extract was poured into a second set of tubes and the remaining fecal pellet was resuspended in 5 ml of 90% ethanol, re-vortexed for 1 minute and re-centrifuged at 1300 *g* for 20 minutes. Extracts were combined, evaporated to dryness and re-suspended in 1 ml phosphate

buffer (0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4, 0.15 M NaCl; pH 7.0) using a sonicator. Steroid extraction efficiency averaged 91% (range 82% - 99%) as determined by recovery of 100 ul of tritiated cortisol added to feces before extraction. Samples were further diluted 1:10 and 1:3 in buffer before analysis for progestagen and glucocorticoid metabolites, respectively.

Enzymeimmunoassays

Fecal progestagen and glucocorticoid concentrations were quantified using assays validated for pygmy rabbits (See Appendix A). A single-antibody progesterone enzymeimmunoassay (EIA) used a monoclonal antibody produced against 4-pregene-11-ol-3,20-dione hemisuccinate:BSA (CL425, 1:10,000; C. Munro, University of California, Davis), a horseradish-peroxidase conjugated progesterone label and progesterone standards (Sigma Chemical Co., St. Louis, MO). Assay sensitivity, based on 95% of maximum binding, was 0.78 pg/well. The cortisol EIA used a polyclonal cortisol antibody (R4866, 1:20,000; C. Munro, University of California, Davis), a horseradish-peroxidase conjugated cortisol label and cortisol standards (Young et al., 2004). The assay sensitivity was 3.90 pg/well. Intra-assay coefficients were less than 10% and inter-assay coefficients of variation were less than 15% and 10% for the progesterone and cortisol assays, respectively.

Data Analysis

For each female, individual overall and baseline means were calculated for both progestagens and glucocorticoid concentrations from longitudinal hormone data collected during the breeding season (March - June). Individual baseline means were calculated

using an iterative process where all values two standard deviations above the mean were excluded and means were recalculated until all extreme values were excluded (Brown et al., 1994a). Baseline means provide an estimate of basal hormone secretion that excludes temporary increases in hormone secretion due to reproductive or stressful events.

For comparisons among females, a general linear model, blocked by facility, was used to compare overall and baseline means (progestagens or glucocorticoids) between non-conceiving and conceiving females. Next, linear regression analyses were used to test whether means during the breeding season were related to annual reproductive success. For each female, annual reproductive success was quantified using two absolute measures (number of litters produced and number of young produced in one breeding season) and two relative measures (number of litters produced per mating opportunity (rate of conception) and number of young produced per mating opportunity (rate of offspring production)).

For each pairing (1 - 6 day periods), an average glucocorticoid and progestagen value was calculated from samples collected during this period. A general linear model, blocked by facility, was used to compare average hormone concentrations during pairings between successful and unsuccessful pairings.

For each pregnancy, three hormone estimates were calculated: 1) late gestation mean, 2) end of gestation peak, and 3) lactation mean. To standardize hormone profiles for each pregnancy, hormone concentrations were aligned to the day of copulation (Day 0) or if copulation was not recorded, the day of an initial spike in progestagens (Day 1). The late gestation mean represents average hormone concentrations during the last third

of gestation (Days 17-24). The end of gestation peak was estimated as the maximum hormone value from the final days of gestation (Days 21-24) and the lactation mean was calculated using all samples collected during lactation (Days 25-39). Lactation days that overlapped with a subsequent pregnancy were removed so means reflected only nursing females.

For comparisons among pregnancies, these three hormonal values were compared between surviving and non-surviving litters (litter survival) and to number of young born (litter size). A general linear model, blocked by facility, was used to compare hormone values and litter survival and a multiple linear regression analysis was used to compare hormone values to litter size. Litter loss is highest during the first 4 days of lactation, so daily hormone values during this period were also compared to litter survival using a general linear model, blocked by facility.

All data are reported as mean ± standard error of the mean. For all analyses, an alpha level of 0.05 was used. Statistical analyses were carried out using SPSS Version 15.0 for Windows (SPSS Inc., Chicago, IL).

Results

Hormone Excretion during the Breeding Season and Pregnancy

During the breeding season, overall concentrations of fecal progestagens averaged 104.9 ± 6.2 ng/g (n = 41) and fecal glucocorticoids averaged 129.2 ± 12.2 ng/g (n = 41). In addition, Table 2.1 summarizes mean fecal progestagen and glucocorticoid concentrations during different reproductive periods (n = 123 pregnancies from 41

females). There were no significant age differences in progestagen or glucocorticoid results ($p > 0.05$), so data were pooled for subsequent evaluations. Rabbits at the two facilities did not differ in fecal progestagen concentrations ($p > 0.05$), but they did differ for fecal glucocorticoid concentrations. During the breeding season, overall glucocorticoid means were higher at Facility 2 (175.1 ± 15.9 ng/g, $n = 23$) as compared to Facility 1 (70.5 ± 4.9 ng/g, $n = 18$, $t = 5.65$, $p < 0.001$). In addition, baseline glucocorticoids of females at Facility 1 averaged 56.61 ± 3.82 ng/g ($n = 18$), whereas baseline glucocorticoids at Facility 2 averaged 124.48 ± 9.07 ng/g ($n = 23$) ($t = -6.27$, $p < 0.001$, $df = 40$; Figure 2.1). This represents a two-fold difference in glucocorticoid excretion, with higher concentrations in females at Facility 2. To control for this difference, a facility effect was added into all subsequent statistical models.

Comparisons among Females

Statistical analyses found significant relationships between hormone concentrations and a female's ability to conceive, which was categorized as either a non-conceiving or conceiving female. Specifically, non-conceiving females had higher glucocorticoid overall ($F = 2.92$, $p = 0.096$, $n = 41$) and baseline ($F = 4.95$, $p = 0.032$, $n = 41$) means and lower progestagen overall ($F = 10.46$, $p = 0.003$, $n = 41$) and baseline ($F = 4.49$, $p = 0.041$, $n = 41$, Figure 2.2) means than conceiving females.

Figure 2.3 depicts representative hormonal profiles for a female that conceived and another that did not conceive after mating. In the conceiving female (Figure 2.3a), progestagen concentrations spiked during successful pairings and increased during pregnancy, while glucocorticoid concentrations increased at the end of gestation, but

remained below 100 ng/g during most of the breeding season. In the non-conceiving female (Figure 2.3b), progestagen concentrations were consistently low and did not surge after pairings, while glucocorticoid concentrations were elevated and variable throughout most of the breeding season.

Next, analyses found significant relationships between baseline hormone concentrations and several measures of annual reproductive success. Progestagen overall and baseline means correlated positively with the number of litters, number of young produced, and the rate of conception ($p < 0.05$, Table 2.2). Rate of offspring production showed a positive trend with progestagen overall ($p = 0.052$) and baseline ($p = 0.091$) means, but was not significant. Glucocorticoid baselines correlated negatively with the number of litters produced per female, but not with the number of young, conception rate or rate of offspring production (Table 2.2). Overall glucocorticoid means did not correlate with any measures of annual reproductive success ($p > 0.05$).

Comparisons among Pairings

Mean hormone concentrations were calculated for 208 pairings, 118 of which resulted in confirmed pregnancies. Mean glucocorticoid concentrations were significantly higher in females during unsuccessful pairings than successful pairings ($F = 5.28$, $p = 0.023$, $n = 208$, Figure 2.4). In addition, females had significantly higher mean concentrations of progestagens during successful pairings than unsuccessful pairings ($F = 56.07$, $p < 0.001$, $n = 208$, Figure 2.4).

For 89 of the 118 successful pairings, fecal samples were collected the day after copulation and all of these samples showed an increase in progestagens above baseline.

On average, the spike in progestagens on Day 1 was 17 times greater than the female's progestagen baseline (ratio of spike to baseline concentration: 17.22 ± 1.83, $n = 89$, see Figure 2.3a).

Comparisons among Pregnancies

Hormone concentrations were assessed in females during 123 pregnancies, 72 (59%) of which did not have any offspring survive to emergence. In total, 69% of litters (83 of 123 litters) experienced death of at least one young before emergence. Litter size was explored because analyses found that litter size correlated positively with litter survival ($F = 13.92$, $p < 0.001$, $n = 123$). For example, young born in litters with fewer that 3 young had a 77% mortality rate, whereas young born in litters larger than 4 had a 45% mortality rate ($p = 0.01$). Average infant mortality rate before emergence was 55% (228 of 416 young). In addition, survival of either a single or all young was more likely to occur in larger litters with 59% of litters greater than 4 surviving and only 27% of litters less than 3 surviving to emergence. And in litters that survived to emergence, on average, 91% of young born survived to emergence ($n = 51$).

Mean progestagen concentrations during late gestation (Days 17 - 24) were positively and significantly related to litter size, but not litter survival (Table 2.3). Mean glucocorticoid concentrations during late gestation were not related to litter size, but females with surviving litters showed a trend towards increased glucocorticoid means during the late gestation period ($p = 0.056$). Peak progestagen and glucocorticoid concentrations at the end of gestation (Days 21 - 24) correlated positively with litter size, but not litter survival (Table 2.3).

During lactation (Days 25 - 39), females with non-surviving litters tended to have higher mean glucocorticoid and progestagen concentrations during lactation than did females that had surviving litters ($p < 0.10$, Figure 2.5). Progestagen and glucocorticoid means during lactation showed no relationship to litter size. During the first four days of lactation, females with non-surviving litters had higher glucocorticoid concentrations than females with surviving litters on Lactation Day 1 (Day 25, $F = 3.57$, $p = 0.064$, $n = 65$), Lactation Day 3 (Day 27, $F = 9.75$, $p = 0.004$, $n = 31$) and Lactation Day 4 (Day 28, $F = 75.09$, $p < 0.001$, $n = 25$).

Discussion

The results of this study suggest that increased adrenal glucocorticoid activity during the breeding season negatively affects reproductive success of pygmy rabbits in captivity. Females that had trouble conceiving showed higher glucocorticoid and lower progestagen concentrations during the breeding season and specifically during pairings. For the females that conceived, glucocorticoid and progestagen concentrations during late gestation were positively correlated to litter size, but not litter survival, whereas glucocorticoid concentrations during lactation were higher in females with litters that died before emergence from the natal burrow.

Analyses of longitudinal glucocorticoid excretion over an entire breeding season found an inverse relationship between glucocorticoid baselines and the number of conceptions per female each breeding season. Specifically, females that failed to conceive exhibited significantly higher glucocorticoid and lower progestagen baselines

than reproductively successful females, which indicates that chronic glucocorticoid production compromises overall fecundity on an individual basis. This finding is consistent with other studies that found that animals with low glucocorticoid baselines had higher annual reproductive success (reviewed in Busch and Hayward, 2009). Glucocorticoid and progestagen overall means showed similar trends, but these data may not be as useful as baselines for identifying females that are chronically stressed or exhibit compromised reproductive potential because they include peak hormone values associated with the end of gestation.

Stress can affect overall fecundity in several ways, thus, this study explored hormones during key reproductive phases including mating, gestation and lactation. During mating, stress can reduce conception rates through either altered mating behaviors or the suppression of reproductive hormones necessary for stimulating folliculogenesis and ovulation (Brann and Mahesh, 1991; Dobson and Smith, 2000; Reeder and Kramer, 2005; Tilbrook et al., 2002). In pygmy rabbits, females during unsuccessful pairings were characterized by significantly higher glucocorticoid and lower progestagen concentrations compared to females during successful pairings, suggesting that females that were stressed during pairing were less likely to become pregnant.

In addition, a large spike in progestagen excretion during mating periods reliably predicted whether a successful pairing occurred. Because parturition and subsequent pairings can occur within days of each other, a threshold value can be useful in distinguishing between successful pairings, unsuccessful pairings and parturition. Based on an empirical threshold value of 225 ng/g (calculated as 1.5 SD above the average

maximum progestagen concentrations detected at the end of gestation), a spike in progestagens was detected in 93% of pairings that resulted in a litter (7% chance of a false negatives), but only 2% of pairings where a litter was not observed (false positives). False negatives may have been caused by an accidental collection of male feces or old feces during the time of mate introductions. False positives may have been caused by instances where a mating occurred but either ovulation or fertilization failed, or a pregnancy occurred but young were reabsorbed or never found by keepers.

The consistency of this spike in progestagens one day after mating is a significant finding for this species because previous methods of confirming pregnancy were time-consuming, delayed several weeks and not very reliable. Copulation is brief (< 1 second) in this species, so actual intromission is difficult to observe and thus pygmy rabbit keepers typically rely on nest-building behaviors to confirm pregnancies (Elias, 2004; Elias et al., 2006). These behaviors begin about 2 - 3 weeks after mating, sometimes within 3 - 4 days of parturition, and require time-consuming monitoring of pygmy rabbit activity. Moreover, nest-building is not the most reliable method of confirming a pregnancy. Between 2006 and 2008, only 70% of pregnant females at WSU were observed building a nest 2 weeks after mating (unpublished logbook data). By comparison, our progestagen mating test can be conducted within a few days of pairing and provides a more reliable way of determining if a successful pairing occurred. Ultimately, the monitoring of progestagens during pairing can assist in achieving greater reproductive efficiency by allowing keepers to re-breed females that did not get pregnant and preventing keepers from re-breeding females that are already pregnant. In addition,

the monitoring of glucocorticoids during mating can help identify stressors that may be affecting conception so that mitigating strategies can be developed to maximize success during pairings.

Next, we looked at hormone concentrations during gestation to see if they correlated with litter size or litter survival. During gestation, maternal glucocorticoids can cross placental barriers and interfere with the behavioral, motor and neuroendocrine development of offspring, ultimately affecting offspring survival (Carlstead, 1996; Carlstead and Shepherdson, 1994; Seckl, 2004; Zarrow et al., 1970). In addition, high levels of stress during gestation can result in embryonic absorption or fetal abortion, ultimately affecting litter size (Glaubach et al., 1951; Seckl, 2004; von Borell et al., 2007). In pygmy rabbits, we found that progestagen and glucocorticoid concentrations at the end of gestation were positively correlated with litter size, but not litter survival. This suggests that, although maternal hormones during gestation are not directly related to litter survival, they may provide insight into the number of fetuses present.

In most mammals, high concentrations of progestagens during gestation are maintained by the corpus luteum and/or placenta. And in many species, placental mass and the number of corpora lutea increase with litter size, suggesting a possible explanation for higher progestagen levels in female rabbits carrying larger litters (Alexander, 1964; van der Lende and Schoenmaker, 1990). Similar to pygmy rabbits, studies in sheep and goats found that progesterone concentrations during gestation were positively correlated to number of corpus lutea and/or fetuses (Butler et al., 1981; Gadsby et al., 1972; Jarrell and Dziuk, 1991).

Glucocorticoids, on the other hand, play a more complex role during gestation and thus may not be indicative of maternal stress. Glucocorticoids during most of gestation are of maternal origin, but at the end of gestation, large amounts of glucocorticoids are secreted from the fetal adrenal gland (reviewed in Fowden, 1995). These glucocorticoids are essential for preparing many of the fetal organs for postpartum functions such as breathing, feeding and glucose synthesis and storage. For example, in sheep, the pre-partum increase in glucocorticoids affects the maturation of the lungs, liver and gut, all of which are vital to immediate neonatal survival (Fowden, 1995). In addition, fetal glucocorticoids affect the placenta by promoting the conversion of progesterone to estradiol. This conversion removes the "progesterone block" and triggers the endocrine cascade that leads to parturition (Senger, 1999). Because late-term glucocorticoids are produced by the fetus, more fetuses should result in the production of more fetal glucocorticoids, and this could explain why glucocorticoid concentrations were positively correlated to litter size in pygmy rabbits.

Although progestagen and glucocorticoid concentrations at the end of the gestation in pygmy rabbits did not directly correlate to litter survival to emergence, it did correlate to litter size, which is one of many factors that can influence infant survival. In domestic rabbits, litter size has been identified as one of the important predictors of infant mortality with medium-sized litters showing the lowest mortality (Roedel et al., 2009). In the case of pygmy rabbits, larger litters had the lowest infant mortality rate and highest rate of litter survival. One theory is that larger litters possess a greater collective ability to thermoregulate through huddling. Rabbit young are atricial and have a limited ability to

thermoregulate when born, so huddling prevents exposure to cold temperatures and allows young to conserve energy for growth and survival (Bautista et al., 2003; Gilbert et al., 2007; Roedel et al., 2009). In summary, large litter size in pygmy rabbits is not just a way to increase the probability that at least one young will survive, but rather it increases the likelihood that the entire litter will survive.

Last, we examined hormones during lactation. In many species, increased maternal glucocorticoids during lactation can alter nursing behavior and milk output of the mother, which can negatively influence postnatal development and survival of offspring (Fowden, 1995; Hemsworth, 2003; Hofer and East, 1998; Reeder and Kramer, 2005; von Borell et al., 2007). In pygmy rabbits, glucocorticoid excretion correlated negatively with litter survival during early lactation, but not during gestation, suggesting that high concentrations of glucocorticoids may have a larger influence on litter survival during the post-natal period than the pre-natal period. Similar to domestic rabbits, the first four days of lactation showed the highest infant mortality, suggesting that this is a critical period in pygmy rabbit development that determines whether the young survive or die before emergence (Roedel et al., 2009; Zeoli et al., 2008). Out of 123 litters born during this study, 69 litters (56%) died within the first four days, three more litters (2%) died within nine days, whereas the remaining 51 (42%) litters had at least one offspring survive to emergence. Glucocorticoid analyses found that females that lost their litters had higher glucocorticoid concentrations during the first 4 days of lactation, suggesting a hormonal link to litter survival. Future studies should focus on this critical period to

determine whether glucocorticoids affect litter survival negatively or whether higher glucocorticoids are a stress response to the loss of a litter.

In summary, stress can disrupt reproduction in a number of ways, but an understanding of the hormonal factors that influence reproduction is essential to maximizing breeding success. Overall, these results suggest that chronic elevation of glucocorticoids during the breeding season not only affected whether females could conceive, but also how often they conceived during pairings and the survival of their litters. Specifically, elevated glucocorticoid concentrations during mating and lactation appears to negatively impact reproductive success and thus these reproductive periods should be targeted in future studies. Ultimately, the identification of factors that cause elevated glucocorticoids in these rabbits may allow us to alleviate these stressors and increase the reproductive output of the captive population.

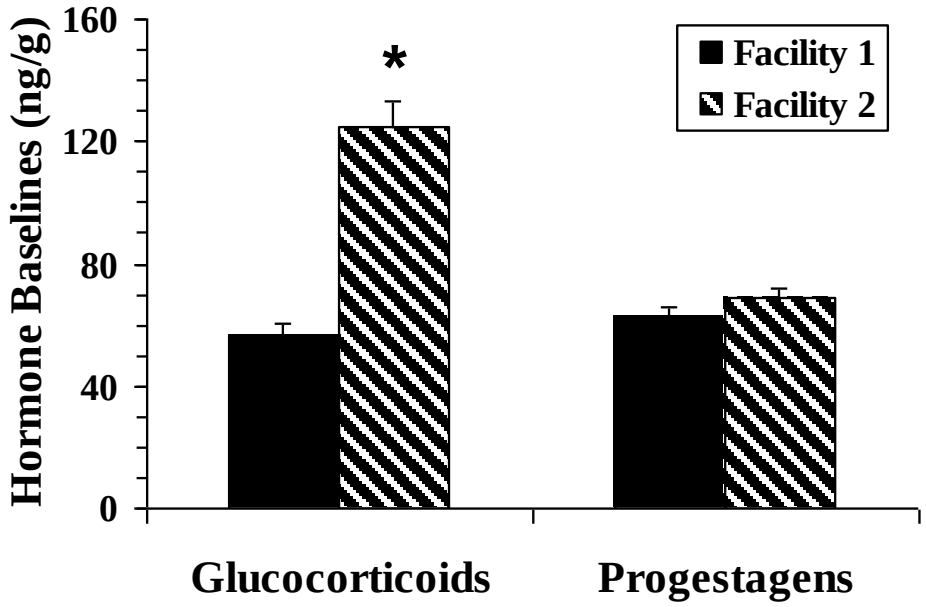


Figure 2.1. Facility differences in hormone baselines (mean ± s.e.m.).
* represents significant differences between facilities ($p < 0.05$)

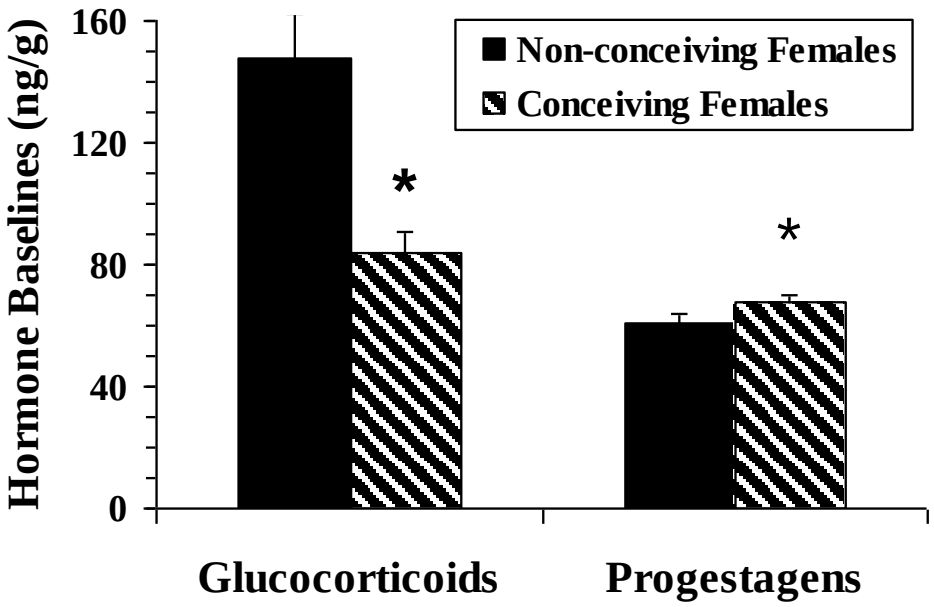


Figure 2.2. Hormone baselines of conceiving and non-conceiving female pygmy rabbits (mean ± s.e.m.).

* represents significant differences between conceiving and non-conceiving females ($p < 0.05$)

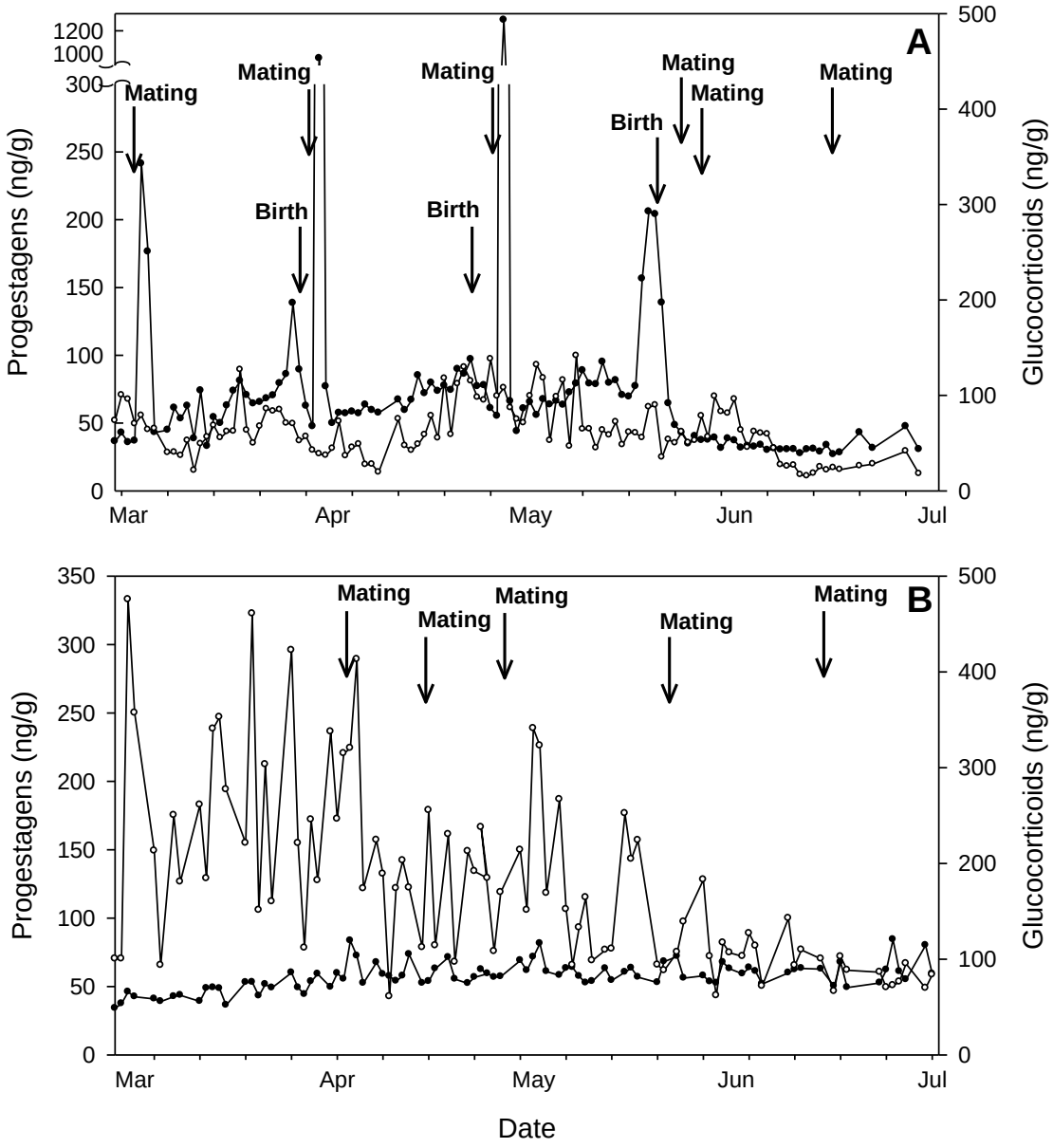


Figure 2.3. Longitudinal profiles of progestagen (●) and glucocorticoid (○) concentrations in: A) a successfully conceiving female that had 3 pregnancies followed by 3 unsuccessful pairings, and B) a non-conceiving female that was paired 5 times, but failed to conceive.

Dates of pairings are indicated by arrows labeled with "Mating" and dates of parturition are indicated by arrows labeled with "Birth."

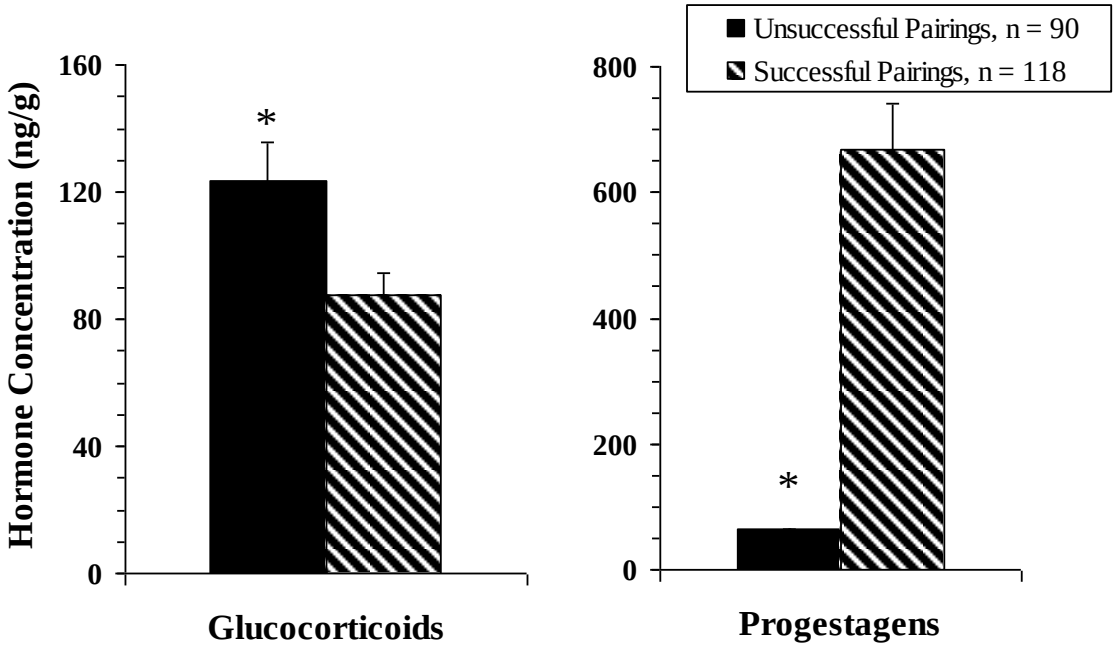


Figure 2.4. Mean hormone concentrations (± s.e.m) of females during successful and unsuccessful pairings.
* represents significant differences between successful & unsuccessful pairings ($p < 0.05$)

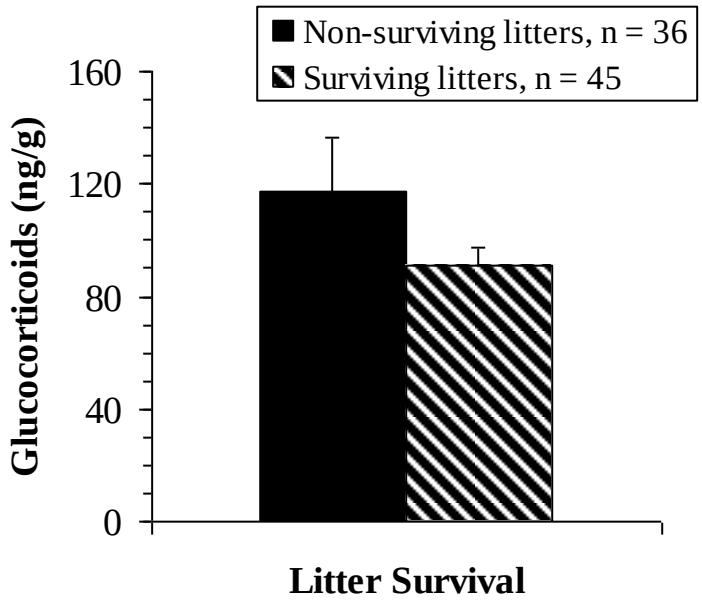


Figure 2.5. Mean fecal glucocorticoid concentrations (± s.e.m) of females during lactation based on survival of litter to emergence.

Table 2.1. Summary of the means, standard errors and sample sizes (n) for progestagen and glucocorticoid concentrations during different reproductive phases in female pygmy rabbits.

Reproductive Event (Time Period)	Progestagens mean ± s.e.m. (range) ng/g	Glucocorticoids mean ± s.e.m. (range) ng/g	N
Breeding Season Baseline (Mar-Jun)	66.3 ± 2.1 (39.5, 98.2)	94.7 ± 7.5 (30.7, 219)	41
Post-Mating Peak (Day 1)[1]	1231.6 ± 135.2 (192.9, 7755.1)	116.6 ± 14.1 (20.2, 883.4)	89
Late Gestation Mean (Days 17 - 24)	98.3 ± 3.5 (30.4, 255.2)	130.9 ± 10.6 (27.4, 939.3)	120
End of Gestation Peak (Days 21 - 24)	129.7 ± 6.3 (30.3, 449.2)	227.5 ± 29.1 (38.2, 2694.2)	120
Lactation Mean (Days 25 - 39)	64.1 ± 2.5 (33.8, 161.2)	104.2 ± 9.2 (19.9, 519.1)	78

[1] Post-Mating peak based on only successful pairings

Table 2.2. Regression analyses comparing progestagen and glucocorticoid baselines to four measures of reproductive success among females.

	Progestagens			Glucocorticoids		
Reproductive Success Measure	t-stat	p-value	n	t-stat	p-value	n
Number of Litters	3.901	<0.001	41	-2.093	0.043	41
Number of Young	2.158	0.037	41	-1.239	0.223	41
Rate of Conception	2.841	0.007	41	-0.999	0.324	41
Rate of Offspring Production	1.734	0.091	41	-0.559	0.579	41

Table 2.3. Results comparing hormones during gestation and lactation to litter size and litter survival

	Progestagens		Glucocorticoids	
	Litter Size	Litter Survival	Litter Size	Litter Survival
Hormone Estimate	t-stat	F-stat	t-stat	F-stat
Late gestation mean[a]	2.639**	1.057	2.154**	3.077*
End of gestation peak[b]	2.617**	0.536	1.477	3.714*
Lactation mean[c]	1.152	2.885*	1.546	1.765*

[a]Average hormone value during the last week of gestation (Days 17-24)
[b]Maximum value observed during Days 21-24 of gestation
[c]Calculated as the average hormone value from Days 25-39
* represents $p < 0.10$, ** represents $p < 0.05$

CHAPTER 3. ENVIRONMENTAL INFLUENCES ON FECAL GLUCOCORTICOID CONCENTRATIONS AND REPRODUCTIVE SUCCESS IN CAPTIVE PYGMY RABBITS

Introduction

For zoo animals, the number of potential stressors in the captive environment are numerous, and the effects often are species-specific. Identifying what captive conditions are associated with high levels of stress or poor reproduction is critically important for effective population management and ultimate success of captive breeding programs. The biological stress response is defined as a physiological reaction to an animal's perception of threat or uncertainty in its environment (Sapolsky, 2002; Seyle, 1976). One of the main components of the stress response is activation of the hypothalamic-pituitary-adrenal axis, which results in the release of glucocorticoids (e.g., stress hormones). These steroids cause the mobilization of energy and the temporary suppression of non-essential functions, such as the reproductive and immune systems, so that an animal can respond adaptively to a threat. Long-term exposure to a stressor, however, can turn temporary suppression into chronic inhibition, which has negative consequences for reproduction and health (Boonstra, 2005; Sapolsky, 2002; Sapolsky et al., 2000; Young et al., 2004). In many species, temporary increases in glucocorticoids can be used to identify acute stressors, while long-term elevations of glucocorticoids are more likely to indicate the existence of a chronic stressor (Reeder and Kramer, 2005; Young et al., 2004).

For endangered species management, an optimal way to evaluate stress is to collect fecal samples and quantify the amount of glucocorticoids excreted under various conditions. The non-invasive nature of this approach allows evaluation of stress without handling the animals, which can compromise the accurate assessment of stress levels (Millspaugh and Washburn, 2004). In addition, fecal data provide a pooled estimate of hormone production from the previous 12-24 hours, thus, dampening the influence of acute fluctuations in secretion (Millspaugh and Washburn, 2004). Using non-invasive hormone monitoring, this study explored the relationship between fecal glucocorticoid concentrations and two potential sources of captivity-induced stress, enclosure size and soil availability, in the pygmy rabbit (*Brachylagus idahoensis*).

The pygmy rabbit is the smallest rabbit species in the world and is uniquely adapted to living in shrub-steppe habitat with deep soil for burrowing (Oliver, 2004). The historical range of pygmy rabbits once covered most of the Great Basin and intermountain regions of the western United States, but in recent decades the range has shrunk significantly, due primarily to habitat destruction (USFWS, 2007). In 2001, four out of five Columbia Basin populations had disappeared, prompting the initiation of a captive breeding program to facilitate reintroduction (USFWS, 2004). The single remaining, distinct population was listed as federally endangered in 2003 and based on recent surveys, none may exist in the wild today (USFWS, 2007). Despite the potential for fast population growth in captivity, reproductive success has been minimal (Elias, 2004). Wild pygmy rabbits can produce up to 18 offspring per year, but captive females

produce, on average, fewer than five offspring per year, of which fewer than two survive to the next breeding season (Elias, 2004; Illig, 2009; Wilde, 1978).

One hypothesis for low reproductive success is that suboptimal housing and husbandry conditions may lead to chronic stress. In Chapter 1, I found that female pygmy rabbits that did not conceive had significantly higher concentrations of fecal glucocorticoids than those that produced surviving offspring, suggesting that stressful conditions in captivity may be negatively affecting reproduction and welfare. Such an effect would be consistent with findings in other species where low reproductive success has been linked to stress ensuing from poor housing conditions, such as inadequate enclosure size and lack of proper environmental enrichment (Carlstead and Shepherdson, 1994; Mellen, 1991; Wielebnowski et al., 2002). The main goal of this study was to determine if housing conditions, specifically enclosure size and soil enrichment, had an effect on fecal glucocorticoid concentrations and reproductive success.

Advocates of animal welfare commonly cite pen size as a potential captive stressor due to its limiting effect on natural behaviors such as exploring, foraging, hiding and mating (Clubb and Mason, 2007; Morgan and Tromborg, 2007). Animal welfare is defined as the "state of an individual in relation to its environment" and failure to cope with the environment is often an indicator of poor welfare (Broom, 1991). Home range size in the wild may play a role in how well an animal adapts to enclosures of different sizes (Clubb and Mason, 2007). In the wild, pygmy rabbits spend the majority of their time within approximately 30 m of their burrows (Gahr, 1993; Janson, 2002; Wilde, 1978). Although some females travel up to 300 m away from their burrow, most females

use about 3000 m^2 as their core home range during the breeding season (Gahr, 1993). Captive enclosures for pygmy rabbits typically provide 3 to 5 m^2 of space, thereby limiting the range to only 1/1000 of that in the wild. In 2004, large pens of about 75 m^2 were built at Washington State University. An initial breeding trial suggested that females produced more litters and more young in these large pens (Elias et al., 2006). Thus, one objective of this study was to evaluate stress hormone levels of pygmy rabbits housed in enclosures of different sizes, with the prediction that females would have lower concentrations of fecal glucocorticoids and higher reproductive success when housed in larger pens.

Another factor that can affect stress and reproduction in captivity is environmental enrichment. Specifically, this study explored the effect of soil enrichment, a housing element that can provide additional space for movement as well as favorable microenvironments. Pygmy rabbits dig their own burrows and give birth underground, so deep, loose soil is important in their natural habitat (Rachlow et al., 2005). But in captivity, soil can transmit diseases among animals so pens must be sterilized and refilled with soil each year, which can be costly. In an effort to increase survival and stop the spread of coccidiosis and mycobacteriosis, concrete pens without soil were built at one facility (USFWS, 2007). During the breeding season, these pens were half-filled with soil to allow females to dig natal burrow, but there was not enough soil to create deep, natural tunnel systems. Thus, the second objective of this study was to evaluate how soil enrichment affected glucocorticoid concentrations and reproductive activity in captive pygmy rabbits. The potential benefit of this research is to identify factors associated with

low reproduction so that mitigating management strategies can be developed to promote animal welfare and increase success of the captive breeding program.

Methods

Animals and Facilities

Animals in this study (n = 50) were all captive-born, adult females (aged 1-3 years) housed at one of two facilities: Washington State University, Pullman, WA (WSU, Facility 1) or Oregon Zoo, Portland, OR (Facility 2). Pygmy rabbits were provided water, grain-forage pellets (produced at the WSU Feed Mill) and a variety of fresh greens (big sagebrush clippings, lettuce, dandelion, parsley and clover) daily. The pre-breeding season was defined as January - February, the breeding season as March - June, and post-breeding season as October - December. For this study, the non-breeding season included only samples collected between October and February.

Together, the two facilities had seven pen types: 1) Circular pens, 2) Oval pens, 3) Rectangular pens, 4) Non-soil pens, 5) Half-soil pens, 6) Carport pens, and 7) Crates (Table 3.1). Circular pens and oval pens were constructed from galvanized steel water tanks filled with 0.5 to 1.0 m of compacted soil. Rectangular pens were also filled with 0.5 – 1.0 m of soil, but were surrounded by wire-mesh siding and a plastic barrier to keep soil in. Non-soil and half-soil pens were rectangular pens with a concrete floor covered in wood shavings. During the breeding season, the non-soil pens were half-filled with about 0.5 m of soil that sloped down to the center of the pen; these are referred to as half-soil pens during this period. Each carport pen was constructed from a carport surrounded by

wire-mesh and contained several large mounds of soil (~1 m tall). Crates were 60 x 60 x 45 cm stainless steel cages surrounded by hardware cloth. All pens were exposed to natural fluctuations in temperature and photoperiod. In addition, all pens were covered by a corrugated greenhouse roof or carport roof and so they were partially shielded from changes in precipitation. Oval pens and crates were used only during the non-breeding season, while carport pens were used only during the breeding season.

With the exception of crates and carports, all pens were enriched with one plastic nest box, one or two artificial burrows (7.6 cm diameter plastic drainage tubes) and sagebrush branches, so the number of potential hiding places was relatively consistent among pens. Carport pens each had two nestboxes and three or four artificial burrows, while each crate contained a plastic nest box and plastic grid mat that allowed feces to fall into the tray below. Carport pens housed one female and one male throughout the breeding season. For the other enclosures, females were housed singly to avoid aggressive interactions, except during 1 - 6 day pairings when a male was introduced into the female's pen. During the breeding season, keepers maintained detailed records of pairings, births and emergence of young from the natal burrows, which occurred approximately 14 days after birth.

For each female, six estimates of reproductive success were calculated: 1) number of litters produced, 2) number of young born , 3) number of young that survived to emergence (> 14 days), 4) % mating success (# of litters per mating opportunity), 5) mean number of young born per mating opportunity and 6) mean number of young that

emerged per mating opportunity. In addition, % emergence (# of young that emerged/ # of young born) was calculated as an estimate of post-natal survival in different pen types.

Fecal sample collection and processing

Fecal collections took place between October 2005 and December 2008, spanning three breeding seasons. During the non-breeding season (Oct - Feb), fresh fecal samples were collected 2 - 4 times a week for 2 - 3 months from five housing groups: crates (n = 7), oval (n = 5), rectangular (n = 6), circular (n = 23), and non-soil pens (n = 14). During the breeding season (Mar - Jun), samples were collected 4 - 7 times a week for 3 - 4 months from four housing groups: rectangular (n = 4), circular (n = 28), half-soil (n = 10) and carport pens (n = 3). Samples were collected at approximately the same time every day (1000 hr) and keepers avoided the collection of old or urine-contaminated samples.

Each fecal sample consisted of ~20 - 50 fecal pellets that were placed in a re-sealable plastic bag and immediately frozen at -20ºC until processed. Samples were lyophilized (Labconco Lyophilizer, Kansas City, MO) and crushed into a fine powder. For steroid extraction, 0.1 g of dried fecal material was added to 5 ml of 90% ethanol using a method similar to that described by Brown et al. (1994b) except that vortexing for 40 minutes was used instead of boiling. Samples were centrifuged for 20 minutes at 1300 *g* and the supernatant extract was poured into a second set of tubes. The remaining fecal pellet was resuspended in 5 ml of 90% ethanol, re-vortexed for 1 minute and re-centrifuged at 1300 *g*. Extracts were combined, evaporated to dryness and resuspended in 1 ml phosphate buffer (0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4, 0.15 M NaCl; pH 7.0). Steroid extraction efficiency averaged 91% (range 82% - 99%) as determined by recovery of

tritiated cortisol added to feces before extraction. Samples were diluted 1:3 in buffer and glucocorticoid metabolites were quantified using a cortisol enzymeimmunoassay (EIA) validated for pygmy rabbits (Scarlata et al., in review).

The cortisol EIA used a polyclonal cortisol antibody (R4866, 1:20,000 dilution; C. Munro, University of California, Davis), a horseradish-peroxidase conjugated cortisol label and cortisol standards (Young et al., 2004). The sensitivity of the assay was 3.90 pg/well and intra- and inter-assay coefficients of variation were less than 10%.

Experimental Design and Analysis

Fecal glucocorticoid concentrations are reported as the overall and baseline mean ± standard error of the mean. Overall means were calculated for each female and represent all samples collected during the specified time period. Individual baseline means were calculated for each female using an iterative process where all peak values two standard deviations above the mean were excluded and means were recalculated until extreme values were excluded (Brown et al., 1994a). Baseline means provide an estimate of basal hormone secretion which excludes temporary increases in hormone secretion due to reproductive or stressful events. Assumptions of normality were checked by examining normal probability plots and calculating a Shapiro-Wilks statistic. For all analyses, significance was assessed at the 0.05 level. All statistical analyses were conducted using Microsoft Excel 2003 (Seattle, WA, USA) and SPSS Version 15.0 for Windows (SPSS Inc, Chicago, IL, USA).

Housing Factors Analysis

The first step of this study was to perform an initial analysis that tested the effects of pen size and soil enrichment, plus several possible confounding factors including facility, age and individual animal. A general linear model with fixed effects was used to investigate the effects of these factors on baseline glucocorticoid concentrations among females housed in different pen types during the non-breeding season. Data from the non-breeding season were used because they included the most variety of pens in terms of soil enrichment and pen size. Because this GLM test was significant, a post-hoc LSD multiple comparison analysis was conducted to determine what housing groups differed significantly and in what direction.

In subsequent tests, each significant factor was analyzed using both an "among-females" and "within-females" comparative framework that minimized the effects of the other significant factors. For example, pen size and soil enrichment comparisons were conducted using only females housed at one of the facilities, while facility comparisons used only females housed in identical pens.

Pen Size Analyses

Objective 1 was designed to test the hypothesis that rabbits housed in larger pens, which more closely mimic pygmy rabbit home ranges, show lower fecal glucocorticoid concentrations. During the non-breeding season, fecal samples were collected for 2-3 months from four housing groups at Facility 1: crates (n = 7 animals), oval pens (n = 7), rectangular pens (n = 6), and circular pens (n = 16). During the breeding season, samples were collected for 4 months from three housing groups at Facility 1: rectangular (n = 6),

circular (n = 18) and carport pens (n = 5). For the "among-females" analysis of pen size, glucocorticoid overall and baseline means were compared among females housed in different sized pens at Facility 1 during the non-breeding season and breeding season using a general linear model.

For the "within-females" analysis, glucocorticoid means were estimated for 29 females before and after the transition to a new pen, and comparisons between pen sizes were conducted using a paired one-tailed t-test. During January and February, several females at Facility 1 were housed in crates (n = 6) or oval pens (n = 7) for several months to allow workers to empty and refill future breeding pens with sterile soil. Then in late February and early March, these females were moved to larger breeding pens at Facility 1. To compare glucocorticoid concentrations between pen sizes, samples were collected for at least 1 month from females housed in the smaller pens (crates & oval pens) and for 3-4 months after being moved to a larger pen (> 3 m^2). In addition, a control group of females (n = 16) that were moved in late February from circular pens to identically sized circular pens were monitored before and after the move. Post-movement fecal collections were longer to allow acclimation to new pen environments and samples during pregnancies were excluded.

Soil Enrichment Analyses

Objective 2 was to test the hypothesis that soil-enriched pens are less stressful than non-soil pens presumably because they allow the animal to cope with stressors and exhibit natural behaviors, such as burrowing. At Facility 2, 10 females were housed in non-soil pens for at least 1 month during January and February. Then during late

February or early March, females were moved into identically sized pens that were half-filled with soil for the breeding season. Fecal samples were collected for 1 - 2 months in the non-soil pens and for 3 months in the half-soil pens. As a control, seven females housed in circular soil pens at Facility 2 were moved to identical circular soil pens and fecal samples were collected before and after being moved as described above. Glucocorticoid overall and baseline means were calculated for each female before and after being moved to a new pen and comparisons between the two pen types were conducted using a paired one-tailed t-test.

For the "among-females" analysis of soil enrichment, glucocorticoid means and four measures of reproductive success (number of litters, number of young born, number of young that survived to emergence, and % mating success) were compared between females housed in either circular pens or half-soil pens at Facility 2 using a two-tailed student's t-test. Because all pen types during the breeding season contained soil, this analysis explored whether differences in the quantity of soil provided had an effect on adrenal activity or reproduction during the breeding season.

Facility Differences

A student's t-test was used to compare fecal glucocorticoid concentrations and six measures of reproductive success (defined earlier) among females housed in circular pens at the two facilities. To account for possible seasonal effects, glucocorticoid data were compared during three time periods: 1) pre-breeding season, 2) breeding season, and 3) post-breeding season. One female was transferred from one facility to another and

monitored during consecutive breeding seasons, so a two-tailed t-test was used to compare daily glucocorticoid concentrations between the two facilities.

Results

Effect of Housing Factors

An initial exploration of factors that could affect adrenal activity found that pen size, soil enrichment and facility, but not age or animal, had significant effects on glucocorticoid concentrations. Glucocorticoid baselines were lower in soil-enriched pens ($t = -3.04$, $p = 0.004$, $n = 53$), showed a negative relationship with pen size ($t = -2.54$, $p = 0.015$, $n = 53$), and were higher at Facility 2 ($t = 2.93$, $p = 0.005$, $n = 53$). A post-hoc analysis determined that females housed in oval, circular and rectangular pens had significantly lower glucocorticoid baselines than females housed in crates and non-soil pens (Figure 3.1). Females housed in crates showed the highest glucocorticoid baselines and because crates were the smallest pens available and lacked soil enrichment, both of these factors were explored in more depth using a comparative framework.

Effect of Pen Size

Glucocorticoid concentrations and reproductive success were compared among females that were housed in different pen sizes at Facility 1. Comparisons among the four pen sizes used during the non-breeding season showed that pen size had an effect on glucocorticoid overall means ($F = 6.08$, $p = 0.002$, $n = 35$) and baseline means ($F = 6.67$, $p = 0.001$, $n = 35$, Figure 3.1). However, when the lack of soil enrichment in the crates was taken into account, the pen size effect disappeared for overall means ($F = 0.11$, $p =$

0.90, n = 28) and baseline means (F = 0.82, p = 0.45, n = 28). Comparisons among the three pen sizes used during the breeding season at Facility 1 also revealed no effect of pen size on glucocorticoid overall means (F = 1.66, p = 0.21, n = 33) or baseline means (F = 1.38, p = 0.27, n = 33, Figure 3.2). There were no differences in the number of litters, number of young born, number of young that emerged or conception rate (p > 0.05, Table 3.2) among the pen sizes at Facility 1. Although our sample was limited, all measures of reproductive output for females housed in the carport pens tended to be lower than females housed in smaller breeding pens at Facility 1.

In addition, glucocorticoid excretion was evaluated in several females that were moved from smaller to larger pens. For the control group, no changes in glucocorticoid overall or baseline means were observed after moving individuals to an identical circular pen (overall mean: t = 1.30, p = 0.11, n = 16; baseline: t = 1.22, p = 0.12, n = 16, Figure 3.3). For the crate group, higher glucocorticoid overall mean and baseline concentrations were identified in females while housed in crates as compared to subsequently being moved to the larger pen types (overall mean: t = 4.94, p = 0.002, n = 6; baseline: t = 4.405, p = 0.003, n = 6, Figure 3.3). For the oval pen group, no differences were found in glucocorticoid overall or baseline means between females that were moved from oval pens to larger breeding pens (overall mean: t = 0.298, p = 0.39, n = 5; baseline: t = 0.266, p = 0.40, n = 5, Figure 3.3). In addition, two females moved from oval pens to large carport pens were monitored. Although the sample size was too small for a powerful statistical test, glucocorticoid concentrations were elevated in the large carport pens as compared to the oval pens (t = 125.5, p = 0.002, n = 2).

Effect of Soil Enrichment

A comparison between females housed in half-soil and circular pens at Facility 2 revealed that the amount of soil enrichment did not influence glucocorticoid overall means ($t = 1.75$, $p = 0.09$, $n = 24$), baseline concentrations ($t = 0.568$, $p = 0.58$, $n = 24$, Figure 3.2) or reproductive success ($p > 0.05$ for all measures of reproductive output, Table 3.2) during the breeding season. In contrast, comparisons within females at Facility 2 showed that glucocorticoid concentrations were higher in females while they were housed in non-soil pens as compared to half-soil pens (overall mean: $t = 1.98$, $p = 0.040$, $n = 10$; baseline: $t = 2.25$, $p = 0.026$, $n = 10$), but there were no changes in glucocorticoid means within females in the control group (overall mean: $t = 0.379$, $p = 0.36$, $n = 7$; baseline: $t = 0.118$, $p = 0.45$, $n = 7$, Figure 3.4).

Facility Differences

An analysis of differences among females housed at the two facilities but in identical circular pens found that glucocorticoid overall and baseline means were higher at Facility 2 as compared to Facility 1 during all three time periods (Figure 3.5, Table 3.3). In fact, during the breeding season, glucocorticoid baselines of females at Facility 2 (124.89 ± 9.42 ng/g, $n = 15$) were twice as high as baselines at Facility 1 (61.28 ± 3.57 ng/g, $n = 20$).

In addition, reproductive success differed significantly between the two facilities (Table 3.4). Females at Facility 1 produced a higher number of litters, number of young, number of young that survived to emergence and % mating success. Facility 1 also had greater reproductive success for the number of young born and emerged when the

number of pairing opportunities was taken into account, but the differences were not significant at the individual level (Table 3.4). Since these measures of reproductive success are not independent, a Bonferroni correction for multiple comparisons would require at least one comparison to attain a $p < 0.008$ (= 0.05 / 6) for significance (Rice, 1989). After application of a sequential Bonferroni test, each of the p-values reported as $p < 0.05$ would remain significant, thereby reducing the chance of a type I error. In addition, Table 3.5 lists a number of husbandry differences between the facilities.

To illustrate the difference in glucocorticoid excretion between the two facilities, Figure 3.6 displays the glucocorticoid profile of a female that was transferred from Facility 1 to Facility 2 and was monitored during two consecutive breeding seasons. For this female, glucocorticoid concentrations at Facility 1 (overall mean: 118.85 ± 14.09 ng/g; baseline mean: 78.65 ± 3.06 ng/g) were consistently lower than glucocorticoid concentrations at Facility 2 (overall mean: 367.64 ± 32.13 ng/g; baseline mean: 178.58 + 7.39 ng/g; $t = -14.14$, $p < 0.001$, $df = 84$). This female successfully produced three litters at Facility 1, but failed to produce any litters at Facility 2, despite numerous pairings. This trend towards decreased reproductive success at Facility 2 also was observed in three other females that failed to conceive while housed at Facility 2, but successfully conceived and reared several offspring after being moved to another facility.

Discussion

This was the first study to examine the impact of captive environmental factors on adrenal stress status and reproductive success in rabbits of an endangered species.

Overall, we found that enrichment of a female's enclosure with soil had a positive impact on welfare, as evidenced by lower fecal glucocorticoid concentrations. This effect of soil was more significant than increases in pen size alone. In general, environmental enrichment is aimed at improving animal welfare, specifically, the ability of an animal to cope with the challenges of captivity (Carlstead and Shepherdson, 1994; Shepherdson et al., 2004). Many studies have explored the effects of environmental enrichment such as the addition of hiding structures, novel objects, nesting materials or new foraging opportunities (Carlstead and Shepherdson, 1994), but few have looked specifically at soil as a possible source of enrichment. Given the fossorial nature of pygmy rabbits, soil is an important component of their environment because it provides opportunities for natural behaviors such as digging, exploring and hiding. For black-footed ferrets (*Mustela nigripes*), another fossorial mammal, animals reared in large, seminatural pens where they could dig their own burrows had higher survival after reintroduction than ferrets reared in indoor cages with artificial burrows (Vargas and Anderson, 1999). This suggests that providing pygmy rabbits with soil may not only increase animal welfare, but may also encourage the development of skills needed to survive after release into the wild.

Soil also plays an important role in pygmy rabbit reproduction because females give birth in underground natal burrows. Comparative analyses during the breeding season showed that the quantity of soil in the pen (half-soil vs. full-soil) had little effect on either glucocorticoid excretion or reproductive success, suggesting that the mere presence of soil was the more important factor for animal welfare. Even though females

housed in half-soil pens tended to have a greater rate of conception and produced more offspring than females in full soil pens (only compared at Facility 2), they had a lower success rate of offspring emergence from the natal burrows. This suggests that some factor associated with half-soil pens may be affecting post-natal survival of the young**.** One possibility is that half-soil pens do not provide adequate soil for natal burrows, which can alter the microenvironment in the nests and cause young to be exposed to cold temperatures before the development of adequate thermoregulation (unpublished data, Rachel Lamson & Becky Elias). Future studies should investigate if temperature of the natal burrow plays a role in neonatal survival.

Comparisons of pen types used during the non-breeding season (size range: 0.37 m^2 to 4.67 m^2) showed that small pen size was associated with elevated glucocorticoid concentrations, but this relationship did not hold during the breeding season when the pen size range was greater (size range: 4.0 m^2 to 75m^2). This is because the two smallest pens (crates and oval pens) were only used during the non-breeding season. Confinement in small pens is commonly associated with increases in glucocorticoid excretion and other behavioral indicators of stress in other species (Moriera et al. 2007; Shepherdson et al. 2004; Carlstead et al. 1993, Line et al. 1987, Cassinello & Peters 2000). For example, Line et al. (1987) found that confinement of a rhesus monkey in a small transfer box for 5 minutes significantly increased cortisol concentrations, whereas physical restraint in a larger pen did not elicit such a response. In other species, limited pen size has been associated with increased stereotypies, reduced activity levels, retarded growth, decreased incidence of mating behavior, and an increase in aggression (reviewed in Morgan &

Tromborg, 2007), all of which are considered indicators of stress. In dama gazelles, Cassinello & Pieters (2000) showed that smaller enclosures were associated with an increase in aggression in dominant animals and were indicative of a more stressful environment. By contrast, the transfer of pygmy rabbits between small oval pens to larger pens failed to induce a significant change in glucocorticoid concentrations. Consistent with these findings, Crockett and coworkers (1993; 2000) found that moderate changes in cage size for female longtailed and pigtailed macaques were not significantly related to glucocorticoid excretion or behavioral profiles.

Because the effect of pen size was only detectable among the smallest pen sizes, there may be a point where limited space severely limits the ability to perform natural behaviors and cope with stressors, but further increases in pen size do not result in a detectable increase in animal welfare in pygmy rabbits. In fact, moving females from small oval pens to much larger carport pens unexpectedly was associated with an increase in glucocorticoid concentrations. The carport pens were the largest pens available and represented the closest approximation to the natural home range of pygmy rabbits. Although a species' natural home range has been shown to be a good predictor of how an animal responds to different enclosure sizes, these analyses were only conducted on carnivore species (Clubb and Mason, 2003; Clubb and Mason, 2007). The pygmy rabbit is a prey species and may have a different response to pen size, especially if increases in pen size are not associated with additional hiding opportunities. Large carport pens in our experiment had only two nestboxes, one for the female and one for the male, so the number of hiding opportunities did not actually increase with size. For this as in other

species, it may not be the quantity of space available, but rather the quality of space, such as the availability of hiding and burrowing opportunities, which influences animal welfare (reviewed in Morgan and Tromborg, 2007). Another factor to consider is that large carport pens housed both a male and female together at all times, so social interactions may have influenced glucocorticoid concentrations in these pens. In a number of species, social variables such as aggression, dominance rank, proximity to conspecifics and abnormal social groups can alter glucocorticoid excretion, so future studies should consider exploring the effect of social interactions on adrenal activity in pygmy rabbits (Creel et al., 1996; Creel, 2005; Morgan and Tromborg, 2007).

By far, the highest baseline glucocorticoid concentrations were observed in rabbits housed in the crates and moving these rabbits to larger pens resulted in a significant decrease in glucocorticoid excretion. Crates lacked soil enrichment and were clearly the smallest pens used, representing approximately 8% of the space provided in the average enclosure. In addition, factors such as flooring, cage height or temperature may be negatively impacting welfare in crates. The floor of the metal crates consisted of a stainless steel mesh covered by a rubber grid mat, which prevented waste from building up and kept animals dry. The uneven nature of this flooring can increase the chance of slipping and several studies have shown that animals prefer flooring that provides softer or more solid footing (Morgan and Tromborg, 2007). For example, golden hamsters and rats showed a behavioral preference towards cages with solid floors and bedding over stainless steel cages with wire mesh flooring (Arnold and Estep, 1994; Manser et al., 1995), while pigs showed preference towards soil-like substances such as peat, compost

or sawdust, as compared to woodbark, straw and concrete (Beattie et al., 1998). Also, the height of crates in this study was restricted to 43 cm which could limit movement or behaviors such as periscoping (standing upright on the hindlimbs to perform visual scanning of the surrounding area or to reach food) or perching on top of their nestboxes. One study in domestic rabbits showed that increases in cage height were associated with decreased behavioral signs of stress such as bar gnawing, restlessness and excessive grooming (Hansen and Berthelsen 2000), so future studies should consider cage height as a potential source of stress.

Another factor to consider is the effect of captive environments on an animal's ability to behaviorally thermoregulate. In the wild, pygmy rabbits are able to thermoregulate by seeking shelter in microclimates such as within a burrow or under a bush. However, in captivity, the number of appropriate microclimates is limited by what is available in their pens. In most of the pens, a sealed nestbox was provided for each animal allowing it an enclosed location where it could hide and stay warm during the winter. In the crates, however, a plastic nestbox was placed upside-down on the wire mesh flooring to provide a hiding location, but no lid was attached to the nest box to allow feces to fall through the wire flooring. In colder months, the lack of a closed environment could expose the animal to temperatures outside of the optimal range while denying it an appropriate mechanism to cope with this stressor. Additional studies on the effects of temperature on pygmy rabbit welfare should be conducted to see if this factor is influencing stress levels and reproductive success. Overall, several factors associated with crates such as small pen size, absence of soil, limitations in cage height and lack of

appropriate locations for thermoregulation may all be influencing the expression of natural behaviors in pygmy rabbits and thus may be contributing to elevated concentrations of glucocorticoids.

Lastly, the discovery of a significant facility effect on reproductive rates and glucocorticoid excretion was an unexpected finding. Although the facilities differed somewhat in the availability of different pen types, a comparison among females housed in identical pens at the two facilities still found higher glucocorticoid concentrations and lower reproductive rates at Facility 2. These differences were apparent in evaluations at the population level and also in individual females that were transferred between facilities. Thus, some factor other than housing may also influence adrenal activity and reproductive success in pygmy rabbits. One hypothesis is that differences in husbandry routines may be responsible. Compared to Facility 1, Facility 2 had a more intensive cleaning schedule that included almost daily changing of nestboxes and sweeping of feces from the pens. Routine cage cleaning removes odors associated with marking a territory or indicating reproductive status. Cleaning can also introduce novel stimuli, such as new nestboxes or nesting materials, which may be perceived as stressors. Among group-housed male mice, the removal of scent marks increases aggression, but this aggression can be reduced if some of an animal's scent-marked nesting materials are transferred to the newly cleaned cages (van Loo et al. 2000). Additionally, husbandry routines such as cage cleaning or feeding can result in a forced proximity between animals and their keepers, which can be particularly stressful for prey species (Morgan & Tromborg 2007). In several species, exposure to the public, increased numbers of visitors

or keepers, and/or time spent in proximity to humans can increase physiological and behavioral indicators of stress (Carlstead & Brown 2005; Wielebnowski et al 2002). For example, Wielebnowski et al (2002) found that fecal glucocorticoid concentrations in clouded leopards were positively associated with the number of keepers per facility.

Although the pygmy rabbits at both facilities were housed off-exhibit and away from visitors and predators, the keepers' proximities and time spent near animals during routine husbandry events was much higher at Facility 2. At Facility 1, the preparation of food, the cleaning of nestboxes and behavioral observations using video cameras were performed in a separate room to minimize time spent near the pygmy rabbit pens. In addition, keepers at Facility 1 swept up old feces while leaning into the pen, whereas keepers at Facility 2 were physically inside the pens on top of the rabbit burrows during cleaning. These were only a few of the differences between the two facilities (see Table 3.5), but they suggest possible husbandry factors that may play a role in modulating stress levels in pygmy rabbits and should be explored in future studies.

In conclusion, results suggest that pygmy rabbits exhibit lower reproductive success and higher stress hormone levels when housed in small enclosures, and in pens that lack soil. The worst outcomes were associated with females housed in crates, where the small pen size, absence of soil and limitations in cage height may all contribute to elevated concentrations of glucocorticoids. Several management recommendations for improving the captive breeding program for pygmy rabbits are proposed. First, keepers should make every attempt to provide soil to rabbits, even during the non-breeding season. Although the quantity of soil provided did not have a significant effect on adrenal

activity, rate of conception or pregnancy, deeper soil pens did appear to increase post-natal survival, presumably because that allow for the digging of more naturalistic natal burrows. Second, there was no reproductive health advantage to housing rabbits in large carport enclosures, so space allocated for rabbit housing should be focused on providing more medium-sized pens. In fact, large carport areas could be divided into several medium-sized pens, thus avoiding the need to use crates during the non-breeding season. Last, future studies should explore the effect of different husbandry routines on the stress levels of pygmy rabbits so that mitigating strategies can be developed to promote animal welfare.

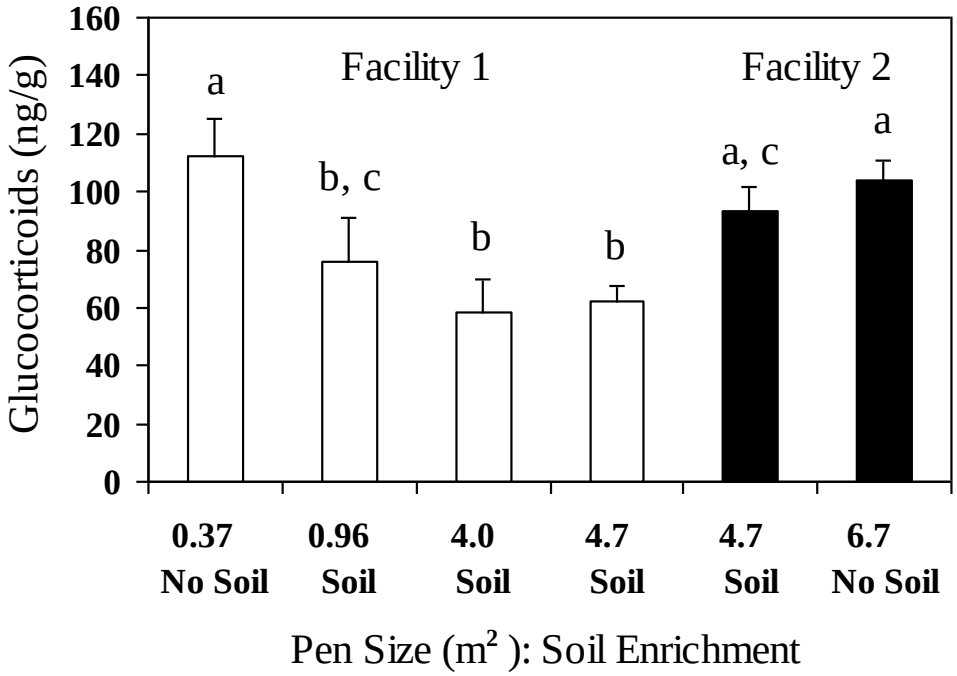


Figure 3.1. Mean fecal glucocorticoid baseline concentrations (± s.e.m.) of females housed in different pen types during the non-breeding season, ordered by increasing pen size (m^2).
White bars = Facility 1; Black Bars = Facility 2; [a,b,c] Means with the same letter are not significantly different ($p > 0.05$).

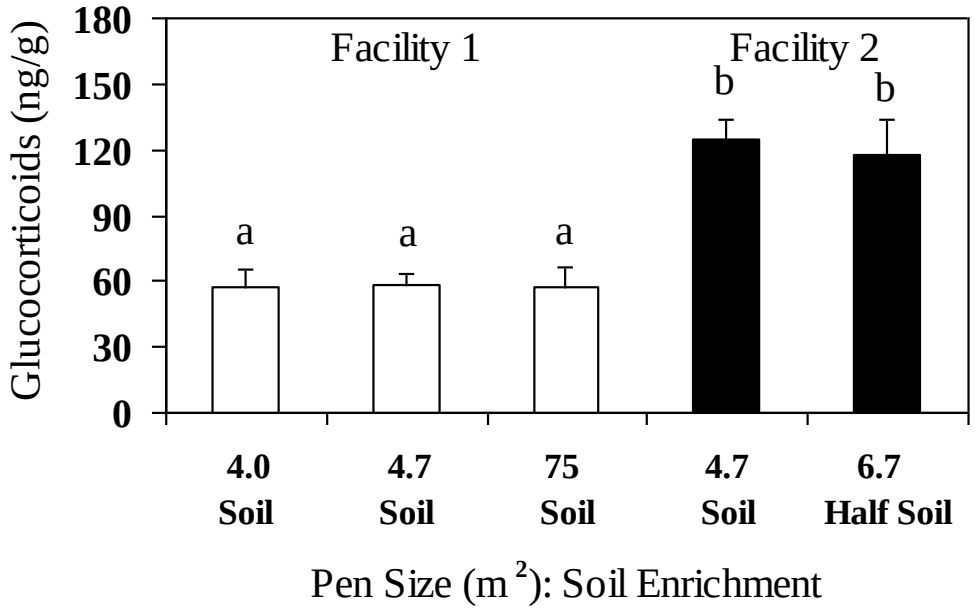


Figure 3.2. Mean fecal glucocorticoid baseline concentrations (± s.e.m.) of females housed in different pen types during the breeding season, ordered in increasing pen size (m^2) and separated by facility.
White bars = Facility 1; Black Bars = Facility 2; ^{a,b} Means with the same letter are not significantly different ($p > 0.05$) .

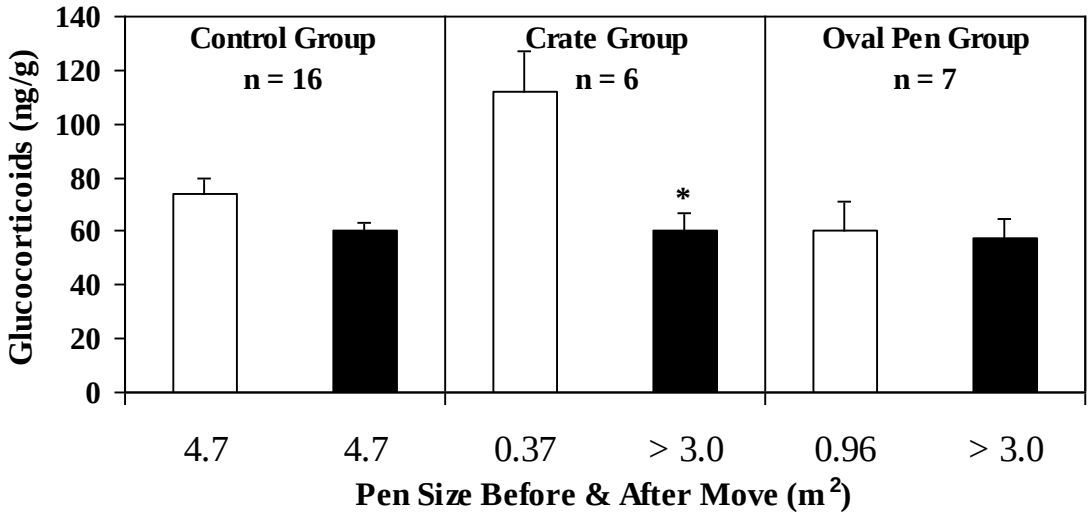


Figure 3.3. Mean fecal glucocorticoid baseline concentrations (± s.e.m.) of females before and after being moved from one pen size to another.
* indicates significant differences within females between glucocorticoid baselines before and after move ($p < 0.05$). For the control group, 16 females were moved from circular pens to identical circular pens.

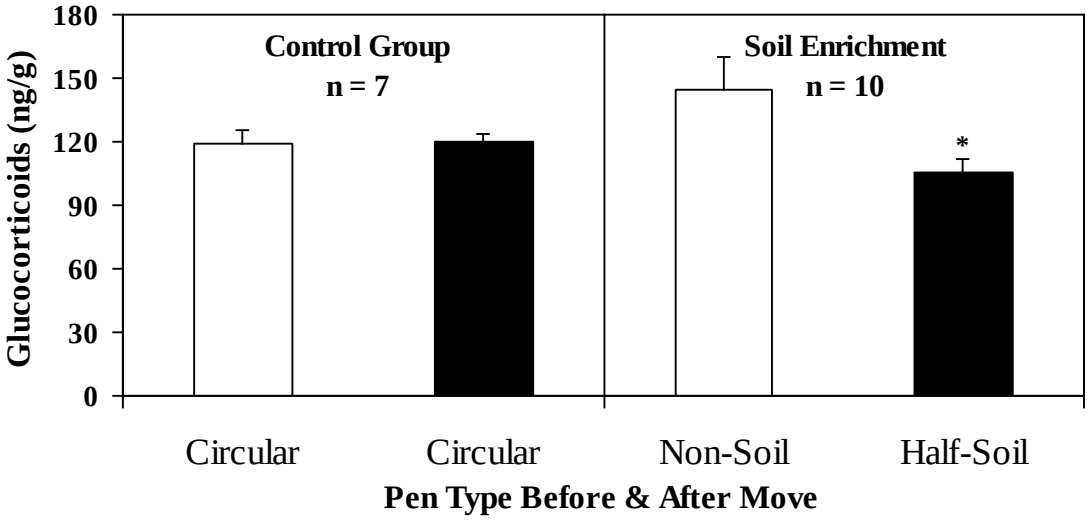


Figure 3.4. Mean fecal glucocorticoid baseline concentrations (± s.e.m.) of females before and after being moved between non-soil and soil-enriched pens.
* indicates significant differences within females between glucocorticoid baselines before and after move ($p < 0.05$). For the control group, 7 females at Facility 2 were moved from circular pens to identical circular pens.

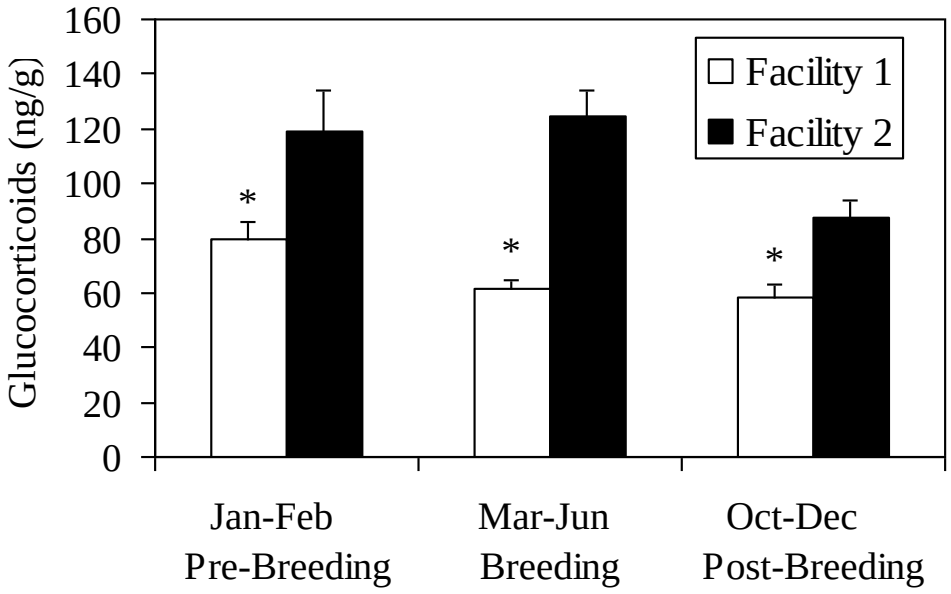


Figure 3.5. Facility differences in mean fecal glucocorticoid baseline concentrations (± s.e.m.) by season.
* Indicates significant differences between facilities ($p < 0.05$)

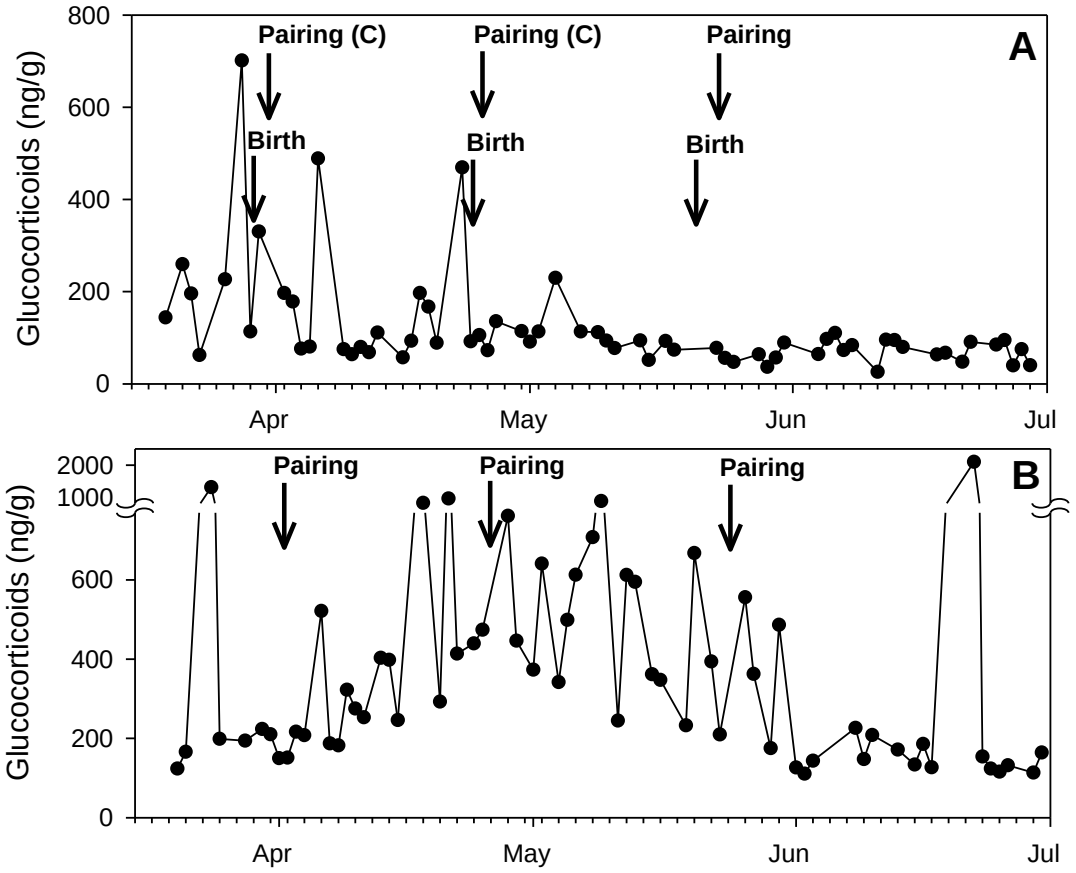


Figure 3.6. Fecal glucocorticoid profiles of a female while housed at (A) Facility 1 and (B) Facility 2 during subsequent breeding seasons.
(Note: Y-axes are different) Dates of pairings, conceptions (C) and births are indicated by arrows and pairings that resulted in a pregnancy are indicated as "Pairing (C)."

Table 3.1. Information on pen types for captive pygmy rabbits housed at two facilities

Pen Type	Size (m^2)	Soil	Facility	Season When Used
Crate	0.37	None	1	Non-breeding
Oval	0.96	0.5 – 1.0 m	1	Non-breeding
Rectangular	4.0	0.5 – 1.0 m	1	Breeding & Non-breeding
Circular	4.7	0.5 – 1.0 m	1 & 2	Breeding & Non-breeding
Non-Soil	6.7	None	2	Non-breeding
Half-Soil	6.7	0.5 m in half of pen	2	Breeding
Carport	75	1.0 m mounds	1	Breeding

Table 3.2. Mean estimates of reproductive success (± s.e.m.) for pen types used during the breeding season at each facility.

Pen Type	Fac	Pen Size	N	Litters[a]	Young[b]	Emerge[c]	% mating	% emerge
Rectangular	1	4.0 m^2	4	3.3 ± 0.8	8.8 ± 2.2	4.3 ± 2.1	92.9 %	48.6 %
Circular	1	4.7 m^2	20	3.3 ± 0.2	9.9 ± 1.3	4.7 ± 1.0	67.0 %	47.7 %
Carport	1	75 m^2	3	2.3 ± 0.3	6.0 ± 1.5	2.0 ± 1.5	87.5 %	33.3 %
Circular	2	4.7 m^2	14	1.3 ± 0.4	4.9 ± 1.5	3.2 ± 1.1	35.3 %	66.2 %
Half-Soil	2	6.7 m^2	11	1.9 ± 0.3	6.7 ± 1.1	2.6 ± 0.8	63.6 %	37.8 %

[a] Litters = average number of litters conceived by females housed in each pen type
[b] Young = average number of young born per female in each pen type
[c] Emerge = average number of young that survived to emergence per female
[d] % mating success = percentage of pairings that resulted in a pregnancy (overall rate of conception)
[e] % emerge = percentage of young born that survived to emergence
Note: There were no significant differences in reproductive success among pen types

Table 3.3. Differences in glucocorticoid means (± s.e.m.) between females housed in circular pens at the two facilities.

		Overall Mean (ng/g)			Baseline Mean
Season	N	Facility 1	Facility 2	t-stat	t-stat
Pre-breeding	16	96.23 ± 10.50	160.23 ± 21.49	2.87*	3.12*
Breeding	28	56.22 ± 3.55	185.52 ± 19.81	6.88**	5.97**
Post-breeding	16	83.18 ± 8.63	211.27 ± 56.96	4.29**	3.95**

* represents $p = 0.01$, ** represents $p < 0.001$
Note: Baseline means presented in Figure 3.5.

Table 3.4. Differences in reproductive success (mean ± s.e.m.) between the two facilities

Reproductive Measure	Facility 1	Facility 2	t-stat	p-value
# litters / female	2.80 ± 0.21	1.25 ± 0.19	5.58	< 0.001
# young / female	8.40 ± 0.90	4.35 ± 0.72	3.56	0.001
# emerge / female	3.71 ± 0.63	2.08 ± 0.50	2.07	0.042
% mating success	64.6 ± 5.12	42.8 ± 6.20	2.66	0.010
# young / mating	1.93 ± 0.22	1.54 ± 0.26	1.11	0.269
# emerge / mating	0.79 ± 0.14	0.71 ± 0.18	0.35	0.729

Note: The first 3 calculations take into account the number of breeding females and the remaining calculations take into account the number of pairing opportunities per female.

Table 3.5. Husbandry differences between the two facilities

Facility 1	Facility 2
* Weekly Nestbox Cleaning	* Daily Nestbox Cleaning
* Pens swept of feces 2 - 3 times a week	* Pens swept of feces 5 - 7 times a week
* Keepers lean into cage to clean	* Keepers stand inside cage to clean
* Proximity to cages: 1 - 2 hrs/day	* Proximity to cages: 3 - 4 hrs/day
* Keepers visit pens for shorter time periods	* Keepers work near pens for several hours
* Trapping Frequency: < once per month	* Trapping Frequency: 1 - 3 times / month
* Animals trapped in nestbox or live-trap	* Animals trapped by hand or in nestbox
* Seasonal enrichment of pens with sagebrush bushes; barren in the winter	* Cages enriched with pots of fresh sagebrush clippings year-round

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APPENDICES

Appendix A. Validation

Parallelism

To assess parallelism, pooled fecal extracts from pygmy rabbit samples were serially diluted and analyzed by EIA for comparison with the standard curves for each hormone. Pooled fecal extracts demonstrated parallelism with the standard curve for both the progestagen and cortisol EIA (Figure A.1.).

Recovery

Both EIAs demonstrated significant recovery of exogenous steroid added to fecal extracts (Cortisol EIA: 90% recovery; Progestagen EIA: 87% recovery).

Extraction Efficiency

Steroid extraction efficiency averaged 91.0% +/- 1.4% (range 0.82, 0.99) as determined by recovery of 3H-cortisol added to feces before extraction.

HPLC

For the corticoid HPLC-purified fractions, one fraction accounted for greater than 70% of metabolites detected by the cortisol EIA. Since this fraction was associated with the cortisol reference tracer, this suggests that the cortisol EIA had extremely low cross-reactivity with other corticoid metabolites present in the sample. For the progestogen HPLC-purified fractions, three peaks were detected by the progestagen EIA. The fraction containing progesterone accounted for only 10% of the metabolites detected, suggesting that the EIA may be detecting other progestagen metabolites such as 5b-pregnane-3a-20a-diol glucuronide, 5a-pregnane-3a-ol-20-one or possibly 20a-hydroxypregnenone (a progestagen found to be released in large quantities in domestic rabbits post-coitum).

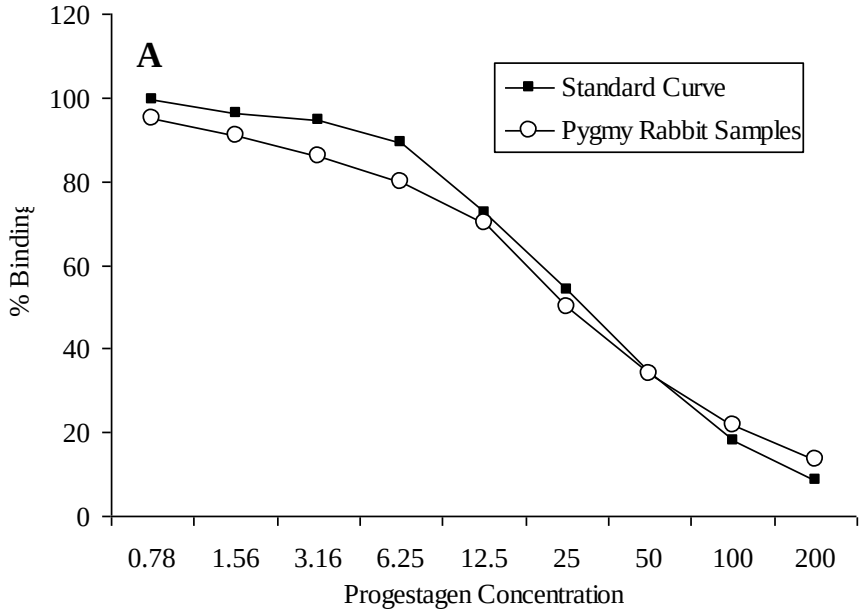
Physiological Validation

In general, physiological validations demonstrated an increase in glucocorticoid excretion within 48 hours of stressful events such as movement to a new pen, mating or birth and an increase in progestagens in association with reproductive events such as copulation and birth.

For physiological validation of the glucocorticoid assay, eight females were monitored for 1 - 2 months after being transferred to Washington State University (WSU) from another facility to determine the glucocorticoid response to transport stress and acclimation to a new pen. Mean glucocorticoid concentrations from samples collected 24 to 48 hours post-transfer were compared to post-transfer glucocorticoid baselines using a one-tailed paired t-test. Samples collected 24 - 48 hours post-transfer were used because they provided an estimate of the glucocorticoid response to transport stress and acclimation to a new pen, while accounting for the time lag in fecal excretion of glucocorticoids. Pre-transfer glucocorticoid baselines were not used because samples were not collected during this period and because post-transfer baselines provided a better estimate of glucocorticoid output while housed at this facility in a particular pen.

Physiological validation of the glucocorticoid assay revealed that all females had elevated fecal glucocorticoid concentrations within 48 hours after being transferred to WSU ($t = 2.17$, $p = 0.033$, $n = 8$). Mean glucocorticoid concentrations within 48 hours post-transfer (244.74 ± 93.60 ng/g, $n = 8$) were approximately five times higher than baseline glucocorticoid concentrations in the same pens at WSU (47.96 ± 11.13 ng/g, $n = 8$).

In addition, the glucocorticoid assay was able to detect long-term elevations in glucocorticoids through the use of longitudinal sampling. Figures A.3 and Figure A.4 show the longitudinal glucocorticoid profiles of 2 females that were moved from one pen type to another. The female in Figure A.3 showed higher glucocorticoid excretion in the smaller and potentially more stressful crate and a drop in glucocorticoid excretion after being moved. The female in Figure A.4 showed higher glucocorticoid excretion while housed in the non-soil pen as compared to a half-soil pen. Overall, both females showed elevated glucocorticoid excretion while housed in the first pen, suggesting differential glucocorticoid excretion in response to housing conditions.



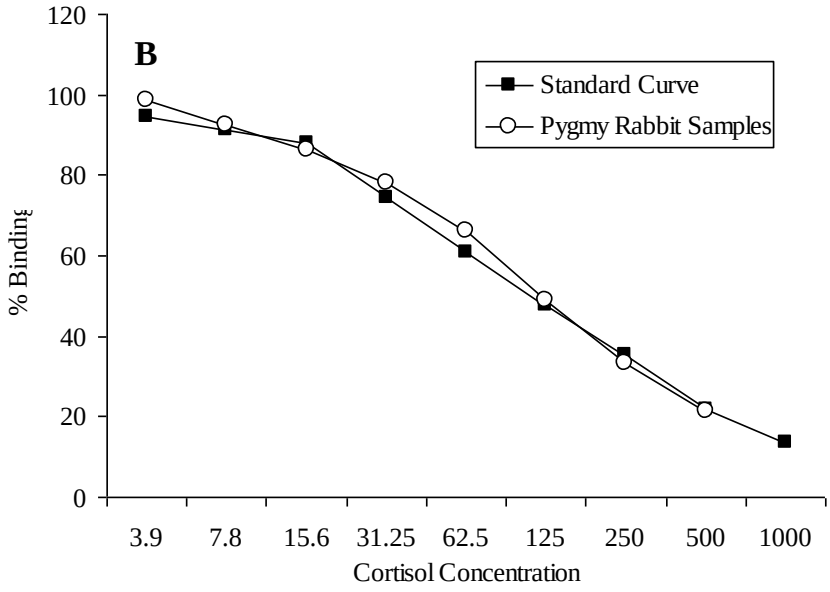


Figure A.1. Parallelisms showing relationships between % binding and hormone concentrations for progestagens (A) and glucocorticoids (B) detected in pygmy rabbit feces as compared to the standard hormone used in their respective enzymeimmunoassays.

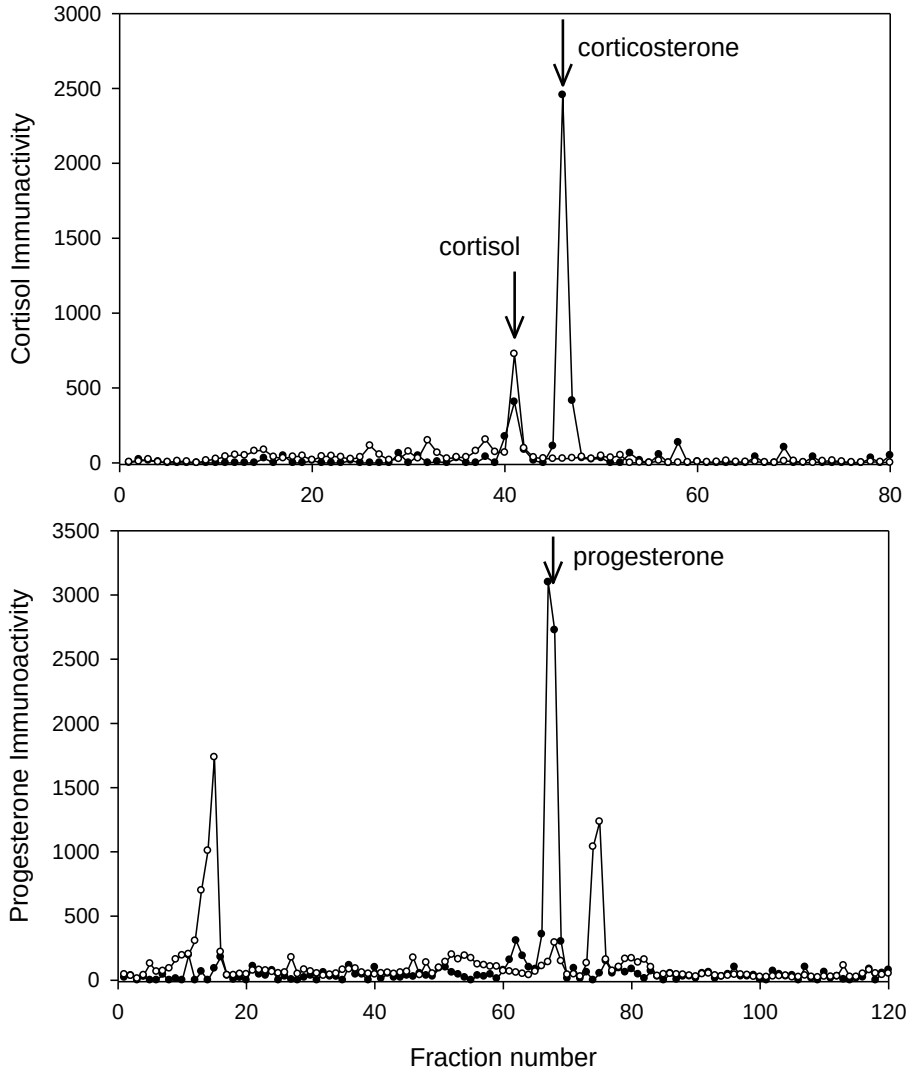


Figure A.2. HPLC co-elution profiles of: A) ^{3}H-cortisol and corticosterone (●) (dpm/fraction) and immunoactive glucocorticoids (○) (pg/fraction) and B) ^{14}C-progesterone (●) (dpm/fraction) and immunoactive progestagens (○) (pg/fraction)

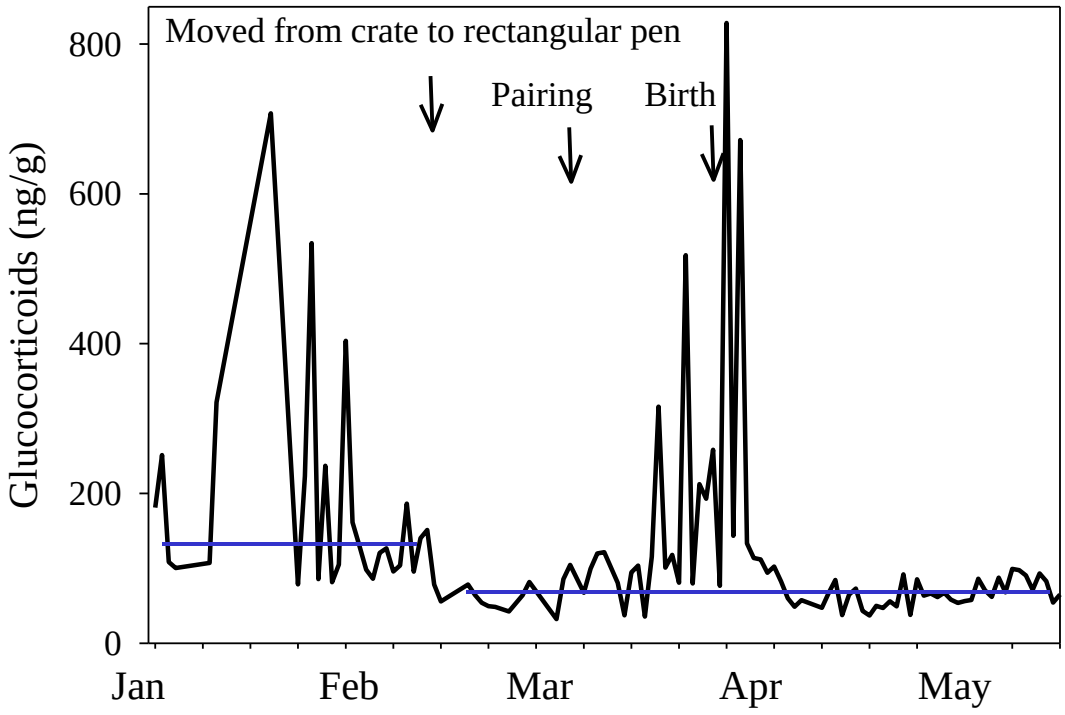


Figure A.3. Longitudinal glucocorticoid profile of a female moved from a crate to a rectangular pen at Facility 1.

Note: Straight lines represent baseline approximations

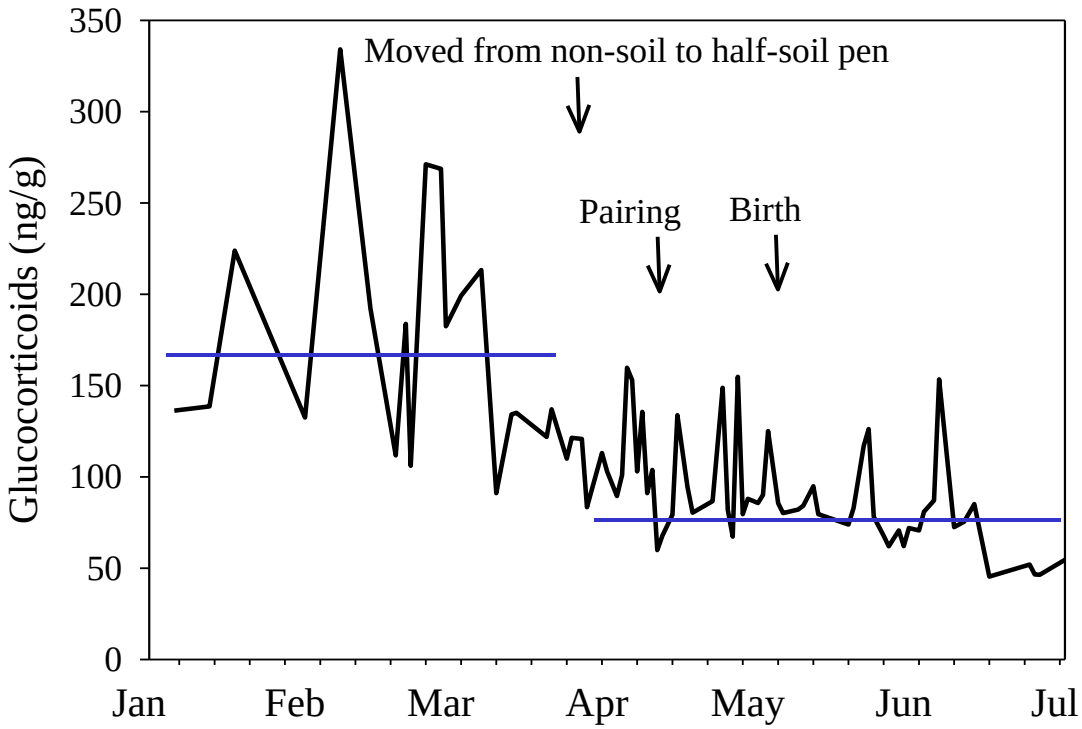


Figure A.4.Longitudinal glucocorticoid profile of a female moved from a non-soil pen to half-soil pen at Facility 2.

Note: Straight lines represent baseline approximations

Appendix B. Age Differences

To examine the effect of age on hormone secretion, data from Facility 1 were initially divided into three age categories: 1-year old, 2-year old and 3-year old. Age groups included animals that were plus or minus six months from the target age. For the "among-females" analysis, a one-way ANOVA was used to compare overall and baseline means among individuals across the three age groups during each of the three time periods (pre-breeding, breeding and post-breeding season). Next, t-tests were used to compare overall and baseline means between 1-year old females and all older females. Sample sizes were limited for older females (n = 13), so data from 2- and 3-year old females were combined. For the "within-females" analysis, four females were monitored for more than one year to determine if aging had an influence on hormone baselines and patterns within females. A two-tailed paired t-test was used to compare hormone concentrations between years, but within individuals.

Among-females analysis

An analysis of variance found no significant differences in progestagen or glucocorticoid concentrations between females of different age groups ($p > 0.05$) during the pre-breeding, breeding and post-breeding seasons. Baseline hormone concentrations for these 3 age groups across seasons are presented in Figure B.1 and B.2. In addition, there were no differences in glucocorticoid or progestagen concentrations between 1-yr olds and older females ($p > 0.05$)

Within-females analysis

Glucocorticoid and progestagen concentrations did not differ among years within indivduals. For example, we had one female (Shasta) that survived and bred for all three years of the study. The hormone profiles from her first, second and third breeding season are practically identical, suggesting that these two hormones are not influenced by age (Figure B.3).

In summary, no significant differences in progestagen or glucocorticoid concentrations were found among or within females of different ages, so we were able to combine all age groups for this study.

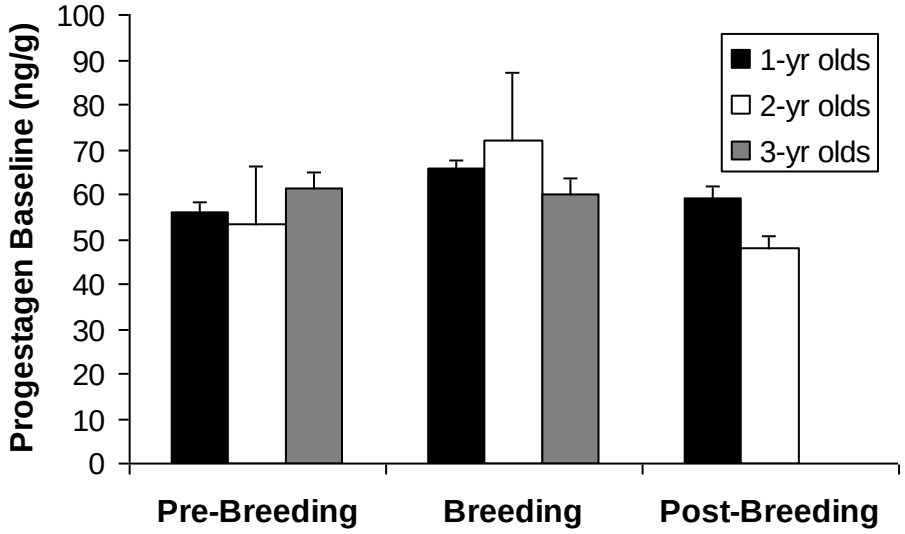


Figure B.1 Age differences in progestagen baselines by season.

Bars represent mean ± s.e.m. for each age group.

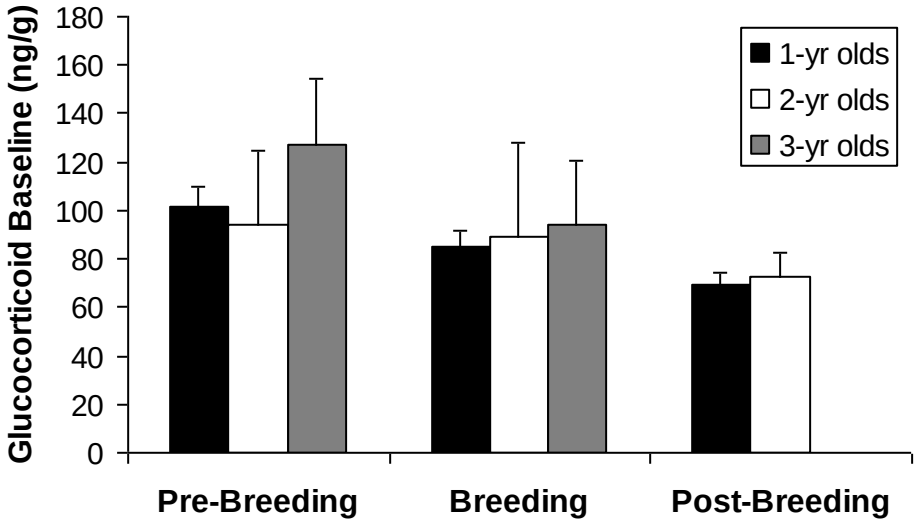


Figure B.2. Age differences in glucocorticoid baselines by season

Bars represent mean ± s.e.m. for each age group.

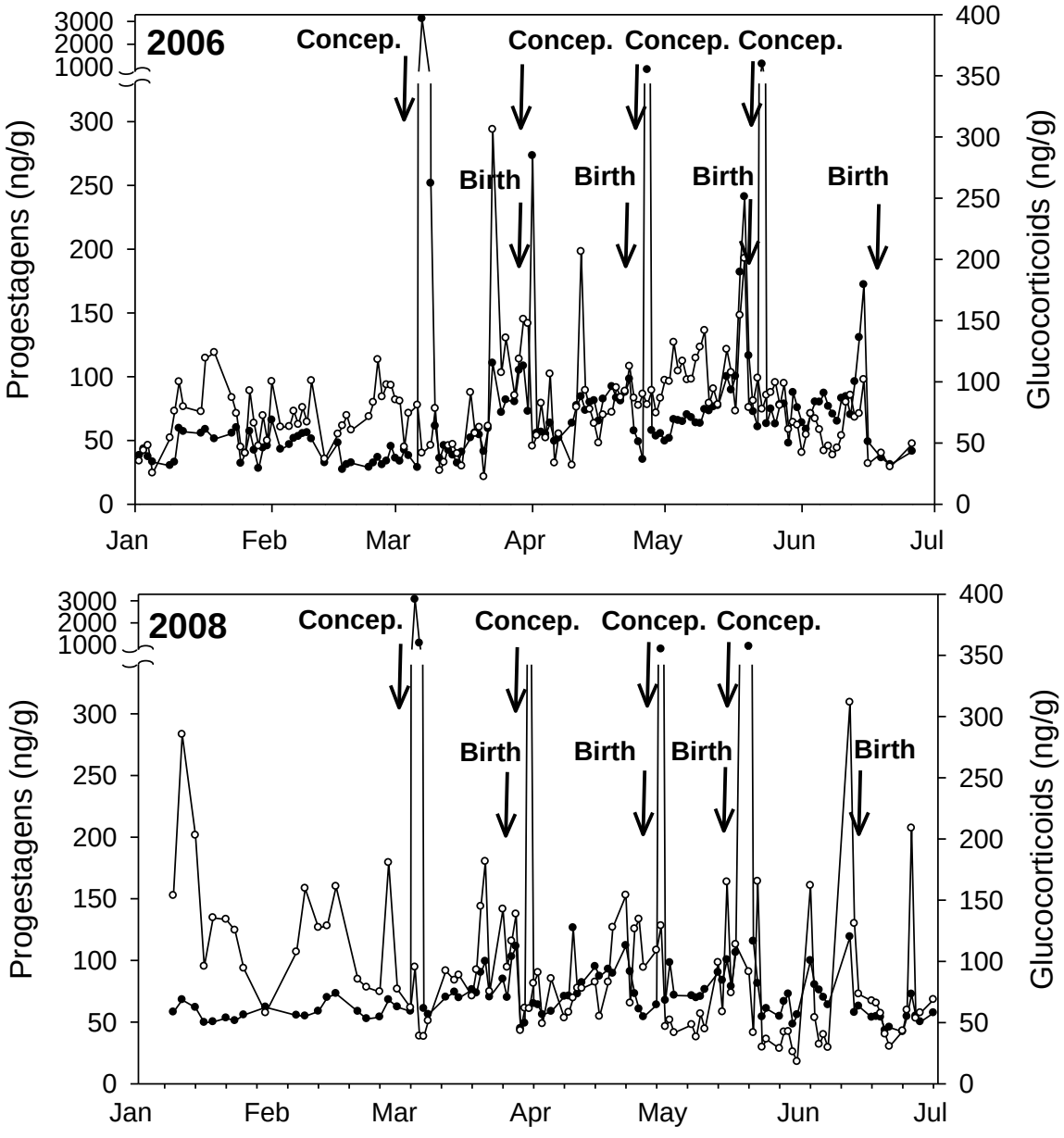


Figure B.3. Longitudinal profiles of fecal progestagen (●) and glucocorticoid (○) concentrations in the same female during the 2006 breeding season (top panel) and 2008 breeding season (bottom panel).

"Concep." arrows = date of conception; "Birth" arrows = date of birth.

Appendix C. Effects of Temperature

Introduction

For this study, soil temperature was explored to determine if this factor had an effect on stress levels and reproductive success of females in captivity. Exposure to temperatures outside a species' adaptive range has been known to increase glucocorticoid secretion and alter behavior in many species (Harper and Austad, 2000). In the wild, animals protect themselves from thermal extremes by seeking out protective microenvironments that are created from the interaction between ambient temperature and substances in the environment such as soil or vegetation. For example, Katzer & Parker (1997) found that pygmy rabbits preferred areas of dense sagebrush bushes because they trap blowing snow, which can create thermally favorable microenvironments during the winter. A greater depth of snow is adaptive to the pygmy rabbit because it provides protection from predators and allows the creation of extensive subnivean burrow systems that provide access to sagebrush. Captive enclosures for pygmy rabbits may not provide the same type of protection, and thus animals may be unable to avoid temperatures outside their adaptive range.

Pygmy rabbit keepers believe that low soil temperatures in captivity may play a role in infant mortality, either through changes in maternal behavior or by exposure of young to cold temperatures before the development of adequate thermoregulation (unpublished data, Rachel Lamson & Becky Elias). This theory is supported by data in pygmy rabbits showing that infant mortality is highest in March, the coldest month of the

breeding season, and that larger litters, which possess a greater collective ability to thermoregulate through huddling, are more likely to survive to emergence from the natal burrow (Illig, 2009). Similar to this, a study conducted on domestic rabbits found that soil temperature was one of the best predictors of nest mortalityThus, this study compared glucocorticoid concentrations in female rabbits between thermoregulated, indoor enclosures and similar outdoor pens exposed to natural fluctuations in temperature during the early months of the breeding season.

Methods

In 2008, several new indoor soil pens (3.0 m^2) were built at Facility 1 in a greenhouse where soil temperatures could be maintained at 60º F. Four females were housed in these temperature-controlled pens and samples were collected from mid-February to mid-May. In addition, samples were collected from six females housed outdoors in circular soil pens (4.0 m^2) at Facility 1 during the same time period. Since this study was only conducted for one year, sample sizes were limited. The "among-females" analyses compared glucocorticoid baselines and reproductive success between females housed in outdoor and indoor pens using a two-tailed t-test. A within-female analysis was not conducted because all females that were moved to indoor pens were previously housed in pens smaller than $1m^2$ during the pre-breeding season, so the comparison would have been confounded by changes in pen size and soil enrichment.

Results

There were no significant differences in glucocorticoid baselines between females housed outdoors and females housed in temperature-controlled pens ($t = -0.055$, $p = 0.96$,

n = 17). Longitudinal profiles of glucocorticoid metabolites for two of these females are presented in Figure C.1. Reproductive success of females housed in greenhouse pens was slightly lower than females housed outdoors, but this difference was not significant (Table C.1)

Discussion

Captive environments can have a negative effect on animal thermoregulation. In the wild, pygmy rabbits are able to thermoregulate by seeking shelter in microclimates such as within a burrow or under a bush. However, in captivity, the number of appropriate microclimates is limited by what is available in their pens. Results from this study did not detect any significant differences in glucocorticoid baselines or reproductive success between females housed in outdoor and indoor pens, but since this study was conducted for only one year, sample size may have limited this analysis. Although reproductive success of females housed in indoor pens appears lower, this may have been influenced by the unexpected infertility of one of the four males used for mating in the indoor pens. On a positive note, the indoor pens did provide us with encouraging reproductive results with the emergence of a litter born in March. During the 3 years of this study, the earliest surviving kit born in an outdoor pen was born 18 April, despite the birth of 23 litters before this date. Although the survival of one litter born in March does not represent a trend, it does provide hope that temperature-regulation of enclosures may be able to have a positive impact on post-natal survival during the early months of the breeding season. Future studies should consider performing a more rigorous experiment to test the effect of soil temperature on the survival of litters.

Additional studies of temperature in pygmy rabbits should consider if other factors associated with the greenhouse, such as loud noises or constant, rather than cyclic temperature changes, have an effect on animal welfare.

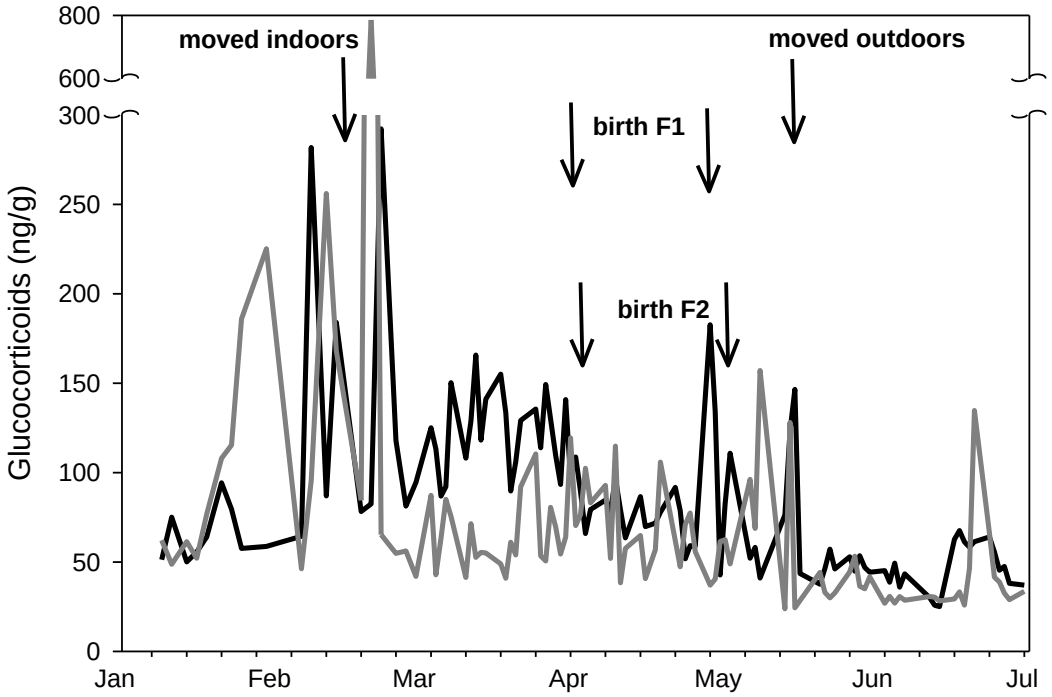


Figure C.1. Glucocorticoid profiles of 2 females that were moved to indoor temperature-controlled pens on 14 Feb.

Female 1 (F1) in grey had 2 litters, the first of which was the first litter born in March to survive to emergence. Female 2 (F2) in black had 2 litters but neither survived to emergence. Both females were later moved to similar outdoor rectangular soil pens on 13 May & 16 May, respectively.

Table C.1. Glucocorticoid concentrations and reproductive data for females housed indoors and outdoors during the first 3 months of the 2008 breeding season

		Glucocorticoids (ng/g)					Unsucc.
Animal	Pen Type	Baseline	Overall	Litters	Young	Emerge	Matings
Blossom	Indoor	66.61	75.78	0	0	0	4
Bubbles	Indoor	56.33	80.04	2	6	3	1
Gouda	Indoor	82.88	102.44	2	9	0	3
Vienna	Indoor	86.80	113.01	3	9	6	3
Brie	Circ. Soil	97.05	108.09	3	5	0	1
Buttercup	Circ. Soil	67.84	90.85	4	10	8	0
Sparrow	Circ. Soil	92.91	99.70	3	10	6	0
Shasta	Circ. Soil	68.63	86.97	4	18	5	0
Olympia	Circ. Soil	84.17	123.00	4	10	4	0
Gretchen	Circ. Soil	107.80	148.27	1	2	1	2