THE EFFECT OF ENVIRONMENT ON THE REPRODUCTIVE POTENTIAL OF *EX SITU* CHEETAHS (*ACINONYX JUBATUS*)

by

Diana C. Koester
A Dissertation
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Doctor of Philosophy
Environmental Science and Public Policy

Committee:

_____	Dr. Larry Rockwood, Dissertation Director
_____	Dr. Geoffrey Birchard, Committee Member
_____	Dr. Adrienne Crosier, Committee Member
_____	Dr. Elizabeth Freeman, Committee Member
_____	Dr. Albert Torzilli, Graduate Program Director
_____	Dr. Robert Jonas, Department Chairperson
_____	Dr. Donna Fox, Associate Dean, Student Affairs & Special Programs, College of Science
_____	Dr. Peggy Agouris, Dean, College of Science
Date: _____	Spring 2014 George Mason University Fairfax, VA

The Effect of Environment on the Reproductive Potential of *Ex Situ* Cheetahs (*Acinonyx jubatus*)

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at George Mason University

by

Diana C. Koester
Bachelor of Science in Zoology
The Ohio State University, 2008

Director: Larry Rockwood, Professor
Environmental Science and Policy

Spring 2014
George Mason University
Fairfax, VA

DEDICATION

This dissertation is dedicated to my incredible parents, Dennis and Patricia Koester, who may not have always agreed with my choices, but have never failed to tell or show me how much I am loved and supported. Although it may have been said over a few glasses of wine that I am an amalgam of each of your worst traits, I can honestly say that it is because of these qualities that I have gotten to where I am today. To my mother, you have given me a fierce determination to succeed and an unwillingness to tolerate less than the best from myself and those around me. You have shown me that no matter the situation, it is always possible to take what is around you and turn it into something extraordinary and beautiful. To my father, you have instilled in me an attention to detail and the strong sense of pride that can be gained from doing things right. Your unfaltering belief in my abilities has always given me the confidence to get through whatever struggle I was facing. All I needed to do was walk out to the shop and hear "don't worry, Diana, you'll get it done" and I knew I would get through it. It is because of you both that I developed a passion for the outdoors and all things feathered and furry. The responsibility and empathy you taught me to take with those things under my care has been the seed for my interest in conservation. Everything that I will achieve in this field will be because of both of you.

Now and always, you have my double, double, double love.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my advisors, Drs. Larry Rockwood and Adrienne Crosier, for seeing potential in the shy girl that came to those first interviews and agreeing to share your expertise with me. You each have provided me with the perfect combination of guidance and independence I needed to develop as a student and a conservation scientist. I will use the excellent example you have provided to mentor others one day they way that you have me. Dr. Elizabeth Freeman, thank you so much for your contagious positivity, your tireless understanding, and for lifting my spirits whenever I needed it. I always look forward to walking into your office because I know that no matter the discussion topic, I will always end up smiling. Dr. Geoffrey Birchard, thank you for your support and for pushing me out of my big-picture comfort zone into understanding the importance of mechanisms. I will whole-heartedly embrace both as I move forward in my career. Thank you to SCBI scientists Drs. Janine Brown, Dave Wildt, Nucharin Songsasen, Buddhan Pukazhenthi, and Pierre Comizzoli for offering so much support and answering my numerous questions over the years. To the endocrine laboratory staff Niki Presley and Sarah Putman, I say thank you very much for being so accommodating and always taking the time to help me in the lab when I had questions. Thank you to the SCBI veterinary and keeper staff, Lisa Ware, Copper Aitken-Palmer, Priscilla Joyner, and Lacey Braun, for contributing your time and effort to helping with this project. Thank you so much to the interns that helped me along the way, Ryan Lefmann, Sean Michael Perry, Kate Archibald, Elyse Persico, and Ryan Freed, you have taught me more about myself than I think I ever taught you, I'm so glad I got to know each of you. Especially to Ryan, I miss seeing you cuttlefish through the hallway more than I can say; you are an amazing person. Thank you to my fellow graduate students and dear friends Morgan Maly, Shawna Cikanek, Remy Schoenemann, Lara Mouttham, Jennifer Nagashima, Ashley Franklin, Ryan Valdez, Jonatan Pribluda, Lauren Henson, Megan Brown, Megan Baker, Pammy Hider, Santiago Gonzalez, and Mike Hallworth. You were all the best support system I could have asked for; I would have been lost without you. Thank you to Dr. Brenda Tondi and Rosanne Jones for putting up with my snark and giving me the ability to keep a roof over my head while struggling to light a fire under those stubborn undergrads. And finally, thank you to the staff at all my participating institutions – White Oak Conservation Center, the Philadelphia Zoo, Dickerson Park Zoo, the Wilds, Wildlife Safari, and the San Diego Zoo Safari Park. Without your hard work and contributions this would have been impossible. A special thank you to Erica Lipanovich and Corienne Pisacane for opening up your homes to me and to all the friends I made while traveling to each facility, thank you for all the amazing experiences. You have given me some of my favorite memories.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5**α**-Dihydrotestosterone ..... DHT
Arginine Vasopressin ..... AVP
Aromatase ..... $P450_{aro}$
Artificial Insemination ..... AI
Association of Zoos and Aquariums ..... AZA
Corpus Luteum CL
Corticotropin-Releasing Hormone ..... CRH
Enzyme Immunoassay ..... EIA
Follicle Stimulating Hormone ..... FSH
Glucocorticoid Receptor ..... GR
Gonadotropin Inhibitory Hormone ..... GnIH
Gonadotropin Releasing Hormone ..... GnRH
High-Performance Liquid Chromatography ..... HPLC
Hypothalamic-Pituitary-Adrenal ..... HPA
Hypothalamic-Pituitary-Gonadal ..... HPG
In Vitro Fertilization ..... IVF
Institutional Animal Care and Use Committee ..... IACUC
Luteinizing Hormone ..... LH
Mineralocorticoid Receptor ..... MR
Principle Component Analysis ..... PCA
Radioimmunoassay ..... RIA
RFamide-Related Peptide ..... RFRP
Smithsonian National Zoological Park ..... NZP
Species Survival Plan ..... SSP
Standard Deviation ..... SD
Standard Error of the Mean ..... SEM
Steriodogenesis-Stimulating Protein ..... STP

ABSTRACT

THE EFFECT OF ENVIRONMENT ON THE REPRODUCTIVE POTENTIAL OF *EX SITU* CHEETAHS (*ACINONYX JUBATUS*)

Diana C. Koester, Ph.D.

George Mason University, 2014

Dissertation Director: Dr. Larry Rockwood

The cheetah (*Acinonyx jubatus*) is recognized as threatened due to extirpation from most of its historic range, with continuing declines resulting in only about 10,000 individuals remaining in nature today. Smithsonian National Zoo scientists have been studying cheetahs for more than 30 years, but the species fails to reproduce consistently in captivity. Association of Zoos and Aquariums Species Survival Plan (SSP) findings reveal that only about 20% of animals in the North American population have ever reproduced, causing reliance upon imported cheetahs from Africa to sustain *ex situ* population numbers. Although the species is known for low genetic variation and the production of 75% malformed spermatozoa per ejaculate, the relatively high fecundity of free-ranging cheetahs indicates that these traits do not ultimately cause poor reproductive success. Rather, it appears that *ex situ* cheetahs require specific husbandry and management techniques to encourage breeding success. Therefore, the main focus of this

research was to investigate the effects of multiple management-related environmental factors on reproductive potential of male and female cheetahs. A secondary focus was to validate a testosterone enzyme immunoassay to assess fecal metabolite concentrations and generate longitudinal gonadal hormone profiles for male cheetahs. Data generated for this purpose revealed support for a lack of reproductive seasonality in male cheetahs and remarkable variation of glucocorticoid concentrations within and between individuals of both sexes. The management practice of housing male cheetahs in groups has significant positive impacts on testicular function over holding males as singletons, and one male within each group exhibits higher reproductive fitness as higher seminal quality and testosterone concentrations than coalition members. This male also initiates the most interactive behaviors with group members. Human exposure, both indirectly through housing in enclosures exposed to the public and directly through interaction with high numbers of keeping staff, has marked negative impacts on reproductive metrics in male cheetahs. Female cheetahs show no relationship between environmental factors and ovarian or adrenal hormone concentrations. However there was a positive relationship between short-term glucocorticoid and estrogen production, perhaps a by-product of increased physical and adrenal activity occurring near times of maximal ovarian activity. Results from this work will be used to recommend changes in management protocols to improve the reproductive potential of cheetahs in managed collections. These changes are crucial for establishing a self-sustaining captive population and ensuring a more stable future for this unique species.

CHAPTER ONE: LITERATURE REVIEW

The Wild Cheetah

The cheetah (*Acinonyx jubatus*) is a medium-sized, highly specialized, cursorial felid endemic to Africa and parts of the Middle East (Leyhausen 1990). The only extant member of its genus, the cheetah is capable of reaching speeds of around 105 km h^{-1} for short distances (Sharp 1997). It is extremely well adapted for high speeds, utilizing a slender body with long legs, enlarged respiratory system, a highly flexible spine, and semi-retractable claws to catch fast prey unavailable to other predators (Eaton 1970, Leyhausen 1990, Marker et al. 2003c, Hayward et al. 2006). Cheetahs are capable of exploiting a surprisingly large variety of prey, but prefer abundant, medium-sized species (27 kg average) such as Thomson's gazelle (*Eudorcas thomsonii*), springbok (*Antidorcas marsupialis*), and impala (*Aepyceros melampus*), perhaps to aid rapid consumption and limit the threat posed by kleptoparasites (Marker et al. 2003c, Hayward et al. 2006). As a medium-sized predator capable of reaching extreme speeds during hunting, cheetahs hold a unique and highly important niche.

Currently, about 10,000 cheetahs are estimated to remain in the wild. Free-ranging populations have decreased by more than 30% since the 1980s and continue to decline (Durant et al. 2008). The continuing decline can be attributed mostly to conflict with local farmers and substantial habitat loss throughout their native range (Marker et al.

2003b). It is estimated that cheetahs have disappeared from about 76% of their historic range on the African continent and in Asia has been reduced to a small, critically endangered population located in Iran (Durant et al. 2008). Protected areas throughout Africa have provided refuge for most large carnivores, supporting lions (*Panthera leo*) and spotted hyena (*Crocuta crocuta*) in high abundances (Marker et al. 2003b). These species steal cheetah kills and prey on adults and cubs, causing cheetahs to avoid most protected areas, particularly in southern Africa (Marker et al. 2003b). Approximately 90% of the largest *in situ* cheetah population is located on game ranches and livestock farmland, where the species is regarded as a pest and actively removed (Marker et al. 2003a, Marker et al. 2003b, Marker et al. 2008a). The continued decline of wild cheetah populations highlights the increasing need for conservation efforts on both free ranging and *ex situ* populations.

Social Structure

In the wild, female cheetahs are solitary, only socializing for extended periods of time with recent offspring prior to dispersal (Caro and Collins 1987). The formation of social groups is exceedingly rare among felids, occurring only in lions, feral domestic cats (*Felis silvestris catus*) and male cheetahs (Leyhausen and Tonkin 1979). A study conducted in the Serengeti found that just over 40% of male cheetahs encountered were living as singletons, while the other 60% lived in groups of usually two or three, but sometimes up to five individuals termed 'coalitions' (Caro and Collins 1987). In the wild, cheetah coalitions consist solely of littermates about 70% of the time, with the remainder formed by unrelated males collaborating and/or or joining existing sibling groups (Caro

1994). Once coalitions are formed, stable and strong social bonds develop among its members (Caro 1994, Ruiz-Miranda et al. 1998).

Coalitions of males are more likely to acquire and hold territories than singleton males, but territories controlled by coalitions are not significantly larger and do not allow access to more females than singleton territories (Caro 1994, Marker et al. 2008a). In southern Africa, where the largest remaining wild populations exist, male and female cheetah home ranges are exceptionally large (averaging 1650 km^2 in size) and frequently overlap with farmland (Marker et al. 2008a). In east Africa, however, limited by protected area boundaries, solitary female cheetahs defend territories averaging 833 km^2 in size (Caro 1994, Gottelli et al. 2007). Smaller territories held by male coalitions average only 37 km^2 and several usually occur within a single female's home range (Caro 1994, Gottelli et al. 2007). Due to the solitary nature of females and unique distribution of individuals, reproductive benefits achieved through group living, such as males lions having access to female prides and extended pride tenure, are not available to coalitions of male cheetahs (Packer and Pusey 1982).

Despite the exceedingly unique social structure of the cheetah, there are few studies on the complex social interactions in this species and their relationship to reproduction. Existing hypotheses concerning social structure are largely speculative, since observation of wild cheetah copulations is rare. Although aspects of male lion groups may be quite different from male cheetah coalitions (e.g. lion coalitions can be much larger), they have been described in greater detail and therefore may be useful to help understand social nuances in male cheetahs.

For instance, in male lion groups containing highly related members, (similar to most cheetah coalitions), just one or two of the members usually dominates reproductive success and the remaining males are classified as non-reproductive 'helpers' (Packer et al. 1991, Caro 1994). Helper males increase the breeding success of their coalition members, and thus achieve greater increased fitness through the matings of the male relatives (Packer et al. 1991). Although male cheetah coalitions are consistently smaller than lion coalitions with related members, it is possible that reproductive success is such that at any given time, one or two males within a coalition do most of the breeding, while other males primarily help in defending territory or in hunting. For example, there is some evidence that female cheetahs mate with only one male from each coalition at each encounter, therefore creating variance in reproductive success among males within a coalition (Gottelli et al. 2007). It has been speculated that male cheetahs may rotate in their role as the 'breeder' such that one male breeds with all encountered females until some point in time when this task is taken over by another coalition member. In this situation, overall mating success would be relatively equal among group members, supported by observations of remarkably low aggression within male coalitions (Caro 1994). To date, however, there is no direct behavioral or genetic data to support this assumption.

Assuming equal breeding success for coalition males, research on the Serengeti population indicates that singleton males achieve the greatest reproductive payoffs of any grouping strategy (Caro 1994). As 60% of males observed in this population were in coalitions and coalitions do not appear to achieve reproductive benefits over singletons,

the advantage of being in a coalition may instead be related to increased foraging success, greater ease of territory acquisition, and/or decreased vigilance (Caro 1994). In support of this assumption, male cheetahs in coalitions have been observed to pursue larger prey items and spend a larger proportion of the day eating compared to their singleton counterparts (Caro 1994). Additional studies on cheetah social structure will not only serve to increase understanding in a species with surprisingly little existing data on this topic, but will also provide indispensible information to future research on reproduction both in wild and *ex situ* populations.

Cheetahs in Managed Collections

Cheetahs in zoos are popular ambassadors for raising public awareness about the plight of wild counterparts, African habitat and diversity loss. This species has been used in captivity to attract funding to directly support field conservation efforts including pioneering work by zoos (Wildt et al. 1988, Howard et al. 1992, Wildt et al. 1993, Wielebnowski 1999, Wielebnowski et al. 2002b, Crosier et al. 2006), universities (Munson et al. 1999, Munson et al. 2002), and the underpinning of many cheetah-oriented African nongovernmental organizations (Marker et al. 2003b, Marker et al. 2005, Marker et al. 2008b).

In North America, many captive cheetahs are managed by the Association of Zoos & Aquariums' (AZA) Species Survival Plan (SSP) (Wildt and Grisham 1993, 2013). The North American Cheetah SSP focuses on expanding fundamental knowledge of this species by studying *ex situ* populations. Research on the SSP cheetah population has produced substantial knowledge about the species' biology, work that would have

been impossible to conduct in free-living animals, including data on reproduction (Wildt et al. 1988, Howard et al. 1992, Wildt et al. 1993, Roth et al. 1995, Brown and Wildt 1997, Howard et al. 1997, Brown et al. 2001, Crosier et al. 2006, Crosier et al. 2009), behavior (Wielebnowski and Brown 1998, Wielebnowski 1999, Wielebnowski et al. 2002b), nutrition (Dierenfeld 1993, 2009), genetics (O'Brien et al. 1983, O'Brien et al. 1985, O'Brien et al. 1987), and disease (Munson et al. 1999, Munson et al. 2002, Munson et al. 2005).

Although efficient reproduction does not appear to be a problem for wild populations (Caro 1994), captive breeding of the cheetah remains difficult (Marker-Kraus and Grisham 1993). At the beginning of 2013, there were 1661 cheetahs at 250 institutions in managed collections worldwide. However, only 33 of these institutions have successfully bred this species (Marker 2012). At the end of 2012, only 16.5% of the captive population had ever reproduced (Marker 2012). About 80% of captive cheetahs in the North American SSP (2013), including valuable founder animals, have died without ever reproducing. Long-term maintenance of current genetic variation is therefore impossible to achieve within cheetahs in managed collections.

The cheetah is assumed to have suffered at least one severe, ancient population bottleneck that significantly reduced genetic variation (O'Brien et al. 1983, O'Brien et al. 1987). This reduction in genetic diversity has been correlated with several deleterious traits, such as high levels of structurally abnormal spermatozoa (Wildt et al. 1993), high instance of dental anomalies (Marker and Dickman 2004), increased cub mortality (O'Brien et al. 1985), and an increased susceptibility to infectious diseases (O'Brien et al.

1985, Brown et al. 1993, Evermann et al. 1993). Continued decline in genetic diversity would undoubtedly intensify these population survival risks. This makes maintenance of as much existing genetic variation as possible a top priority for cheetah conservation programs. Fortunately, the genetic diversity of the SSP population is high (97.85%), but largely because of continued African imports, with such animals currently comprising approximately 40% of SSP cheetahs (2013). Genetically valuable animals brought into captivity that fail to breed, result in a permanent loss of their genetic contribution to the species.

Captive individuals can serve as an important reservoir population for their wild counterparts, maintaining similar genetic diversity to prevent genomic loss in the event of sudden, unexpected *in situ* population decline. To be useful in this way, however, captive populations need to be self-sustaining, and eliminate their reliance on wild populations to maintain numbers and genetic diversity. To date, the captive cheetah population has never been self-sustaining, and managers have never been able to produce sufficient numbers of cubs each year to even maintain current SSP population numbers (2013). Studies focused on increasing natural reproduction in managed collections are key to achieving a self-sustaining *ex situ* population.

Reproductive Biology of the Cheetah

Seasonality in Females

Although wild female cheetahs can conceive throughout the year, studies on Serengeti and Namibian free-ranging populations investigating reproductive seasonality revealed that more litters (average litter size: 3.5 cubs) were conceived during the wet

season (November to May), resulting in 70% of cubs being born between March and July (Laurenson et al. 1992, Caro 1994, Marker et al. 2003a, Crosier et al. 2007). This is unlikely a product of variation in day length, which changes very little near the equator (Serengeti: 2.3° S; Namibia: 22.5° S), or shortage of males, as females encounter males on their territory year round (Laurenson et al. 1992, Caro 1994). Instead, these areas experience the most rainfall from January through April, creating high quantities of forage grasses used for hiding cubs (Laurenson et al. 1992, Caro 1994, Crosier et al. 2007). The highest densities of newborn ungulate prey species are also observed in the season immediately following the rains (Laurenson et al. 1992, Caro 1994, Crosier et al. 2007). Breeding appears to be timed so cubs are born when their environment is the best suited for their survival, both in terms of concealment from predators and providing an abundant supply of prey, which also facilitates a successful pregnancy (Laurenson et al. 1992, Caro 1994, Crosier et al. 2007).

Studies using captive females, whose ovarian activity is monitored via excreted hormone metabolites in the feces, show that there is no seasonal reproductive cycle (Brown et al. 1996b, Terio et al. 2003). Fluctuations in excreted ovarian hormone concentrations were unrelated to season (Brown et al. 1996b, Terio et al. 2003). Analyzed females were both wild-caught individuals housed in outdoor enclosures in Namibia (Terio et al. 2003), as well as captive bred females housed in institutions across North America at varying latitudes (Brown et al. 1996b). Together, these studies support the notion that there is no known physiological change occurring in female cheetahs preventing reproductive success during certain times of the year. Instead, wild individuals

generally conceive and give birth to offspring when environmental conditions are the most conducive to cub survival (Laurenson et al. 1992, Caro 1994, Crosier et al. 2007). Females held at captive facilities, released from *in situ* survival pressures, can produce litters irrespective of season (Brown et al. 1996b, Terio et al. 2003).

Seasonality in Males

The birth of wild litters throughout the year indicates a lack of a strong seasonal effect, if any, on reproductive physiology of the male cheetah. This is in contrast to seasonal felids such as the Pallas cat (*Otocolobus manul*) (Swanson et al. 1996), sand cat (*Felis margarita*) (Herrick et al. 2010), and snow leopard (*Panthera uncia*) (Johnston et al. 1994). In these species, *ex situ* males demonstrate marked oscillations in androgen and sperm concentrations corresponding to identified reproductive seasons (Johnston et al. 1994, Swanson et al. 1996, Herrick et al. 2010). Interestingly, analysis of reproductive physiology and seminal traits from Namibian males yielded a significant increase in testes volume corresponding to the wet season when most litters are conceived (Crosier et al. 2007). This, however, was not accompanied by an analogous increase in seminal quality (Crosier et al. 2007). This change in testes volume was not observed in males that were maintained *ex situ*, possibly indicating that housing males in captivity has eliminated any seasonal effect exhibited by wild males (Crosier et al. 2007). Although androgen concentrations in circulation have been reported for male cheetahs, these were collected over a one hour period from individual males under anesthesia, only allowing for a basic comparison of hormone concentrations to other known species (Wildt et al.

1993). A longitudinal analysis of long-term androgen patterns has yet to be completed for male cheetahs either in the wild or in managed collections.

Endocrinology of Female Reproductive Function

Reproductive function involves a variety of hormones that are essential for regulating the processes that control ovarian activity. Endocrine secretion from the hypothalamus and the pituitary is primarily responsible for the events of the ovarian cycle (Senger 2005). The cycle consists of two parts: the follicular phase and the luteal phase, named for the dominant structure present on the ovary during each phase (Senger 2005).

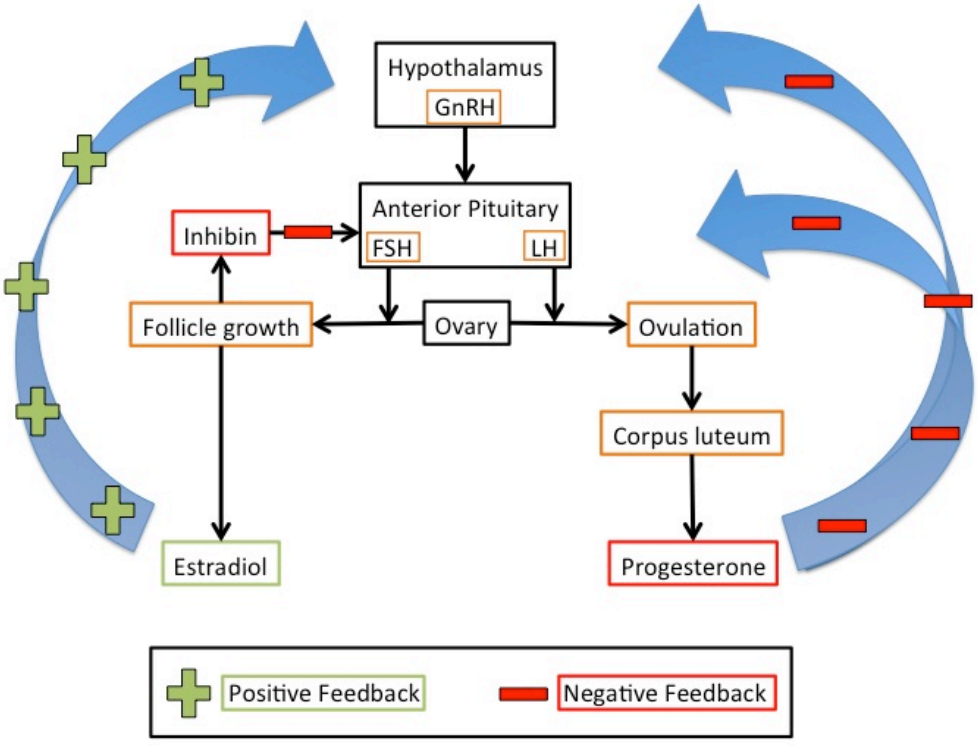


Figure 1.1 Interactions of female reproductive hormones in the HPG axis (Senger 2005)

Hormone secretion and control of the ovarian cycle is regulated by multiple negative and positive feedback mechanisms within the hypothalamic-pituitary-gonadal (HPG) axis (Figure 1.1) (Senger 2005). The hypothalamus produces and releases gonadotropin releasing hormone (GnRH) which stimulates the release of two gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), from the anterior lobe of the pituitary (Senger 2005).

Upon reaching the ovary, FSH stimulates the growth and development of follicles, for which the follicular phase is named, including the contained oocyte as well as the cells surrounding it (Norris 2006). The cells that directly contact the growing oocyte develop into granulosa cells (Norris 2006). Granulosa cells secrete a basement membrane separating themselves and the oocyte from the second layer of developing cells called theca cells (Norris 2006). As follicles grow, the granulosa cells secrete fluid that results in the formation and enlargement of a fluid-filled cavity called the antrum (Norris 2006). During the follicular phase of the ovarian cycle, growing follicles synthesize and release estrogens through a process called the two-cell theory of steriodogenesis (Figure 1.2) involving a cooperative effort between theca and granulosa cells (Norris 2006). Theca cells are stimulated by LH to synthesize androgens, particularly androstenedione, from cholesterol via conversion to pregnenolone and then progression through the Δ^5-pathway of androgen synthesis (Figure 1.3) (Norris 2006). Androgens are then aromatized to form estradiol by FSH stimulated granulosa cells. FSH increases aromatase ($P450_{aro}$) levels in granulosa cells as well as stimulates the cells to produce inhibin, which feeds back on the anterior pituitary to selectively inhibit FSH

release (Norris 2006). Estrogen production by follicles during this phase increases until a threshold level is reached causing an LH surge, shortly followed by ovulation of the oocyte (Senger 2005, Norris 2006).

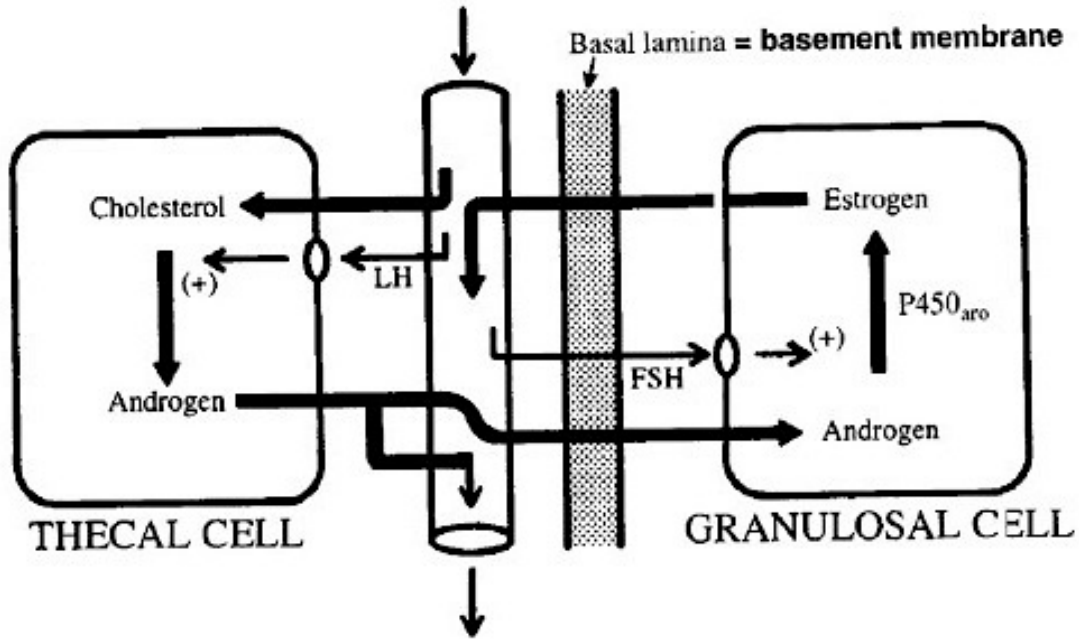


Figure 1.2 Two-cell theory of steriodogenesis (Norris 2006)

Ovulation marks the end of the follicular and the beginning of the luteal phase of the ovary. This step cannot be achieved without a progressive increase in circulating estradiol concentrations from growing follicles (Norris 2006). Increased estradiol also serves to initiate estrous behavior in females of most species and enhance attractiveness of the female to the male (Norris 2006). Once a threshold level of estradiol is reached, the surge center of the hypothalamus is triggered to release a large pulse of GnRH that results in the LH surge and subsequent ovulation (Senger 2005, Norris 2006). In spontaneously

ovulating species, this threshold estradiol level combined with low circulating progesterone concentration is enough to achieve ovulation (Senger 2005). In induced ovulators, physical stimulation of nerve endings involved in reproduction or exposure to appropriate male pheromones is also needed to provoke the hypothalamus surge center to release its pulse of GnRH (Senger 2005).

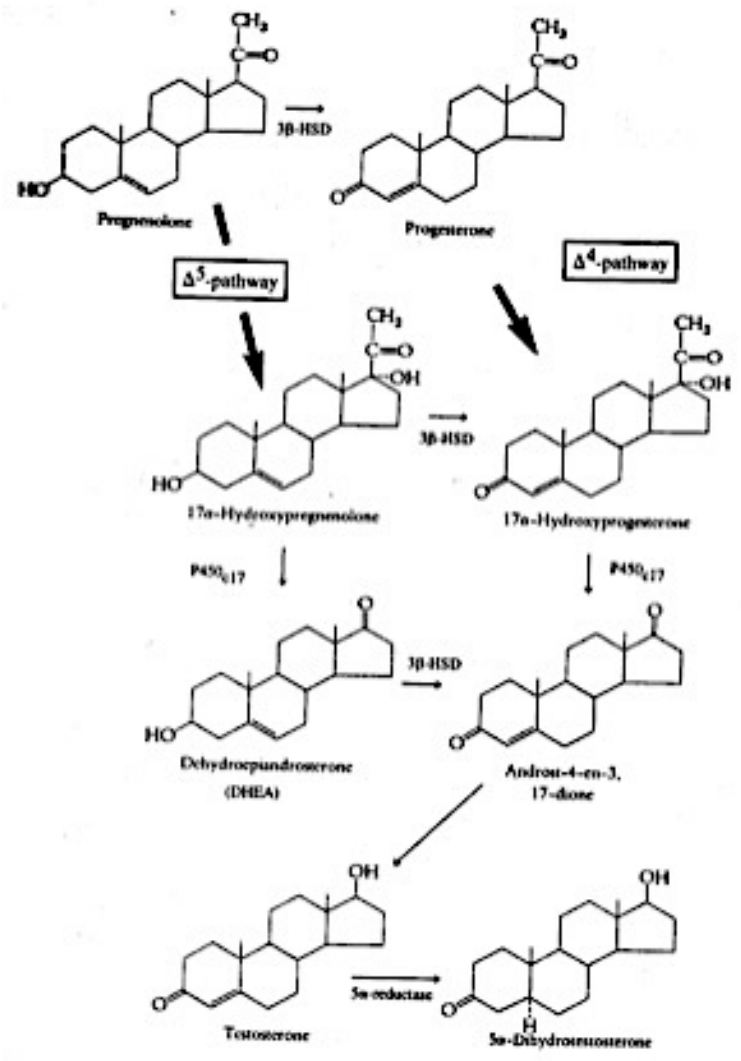


Figure 1.3 Pathways for androgen synthesis (Norris 2006)

The luteal phase is so named for the formation of the corpus luteum (CL) from the granulosa and some theca cells after ovulation. This process, called luteinization, is induced by the LH surge and results in a temporary endocrine gland that secretes mostly progesterone, along with other progestogens and estrogens into general circulation (Norris 2006). Progesterone from the CL inhibits release of GnRH from the hypothalamus so additional follicular development cannot occur during this phase (Norris 2006). The life span of the CL is dependent upon species, as is the relative importance of the CL in maintaining pregnancy. Upon its degeneration, steroid production declines and the female's body will begin to reset for another follicular phase unless pregnancy has occurred (Senger 2005, Norris 2006). Once degeneration and production of steroid hormones by the CL is complete, the HPG axis is free from the inhibitory action of progesterone and follicular development is again resumed (Norris 2006).

The Estrous Cycle of the Cheetah

An estrous cycle consists of the physiological events that occur between one period of sexual receptivity (estrus) and the next (Senger 2005). Similar to other felids, the cheetah is polyestrous and an induced ovulator, meaning that copulation is required for induction of a LH surge and ovulation (Brown et al. 1996b, Senger 2005). When copulation does not occur there is no ovulation, therefore no CL form and the cheetah may remain in a constant follicular phase (Senger 2005). The female will experience waves of follicular development during proestrus and estrus periods, but no true luteal phase will occur until stimulation from copulation (Senger 2005). Although incidences of spontaneous ovulations have been noted in several other felid species such as the lion

(Schramm et al. 1994) and the clouded leopard (*Neofelis nebulosa*) (Brown et al. 1995, Howard et al. 1997), occurrence in the cheetah is extremely rare, causing less than 2% of ovulation instances (Brown et al. 1996b). Mean estrous cycle length of the cheetah is 13.6 days with estrus (elevated estradiol concentrations and receptivity to males) lasting an average of 4.1 days (Brown et al. 1996b). There is considerable variation both within and among individuals for each of these values, however (Asa et al. 1992, Brown et al. 1996b, Brown et al. 2001).

Estradiol concentrations measured in feces are 25–60 ng g^{-1} at baseline and range from 100–750 ng n^{-1} at peak concentrations (Brown et al. 1996b). Progestogen metabolite concentrations remain at baseline levels (0.7–6.0 μg g^{-1}) unless mating and ovulation occur (Brown et al. 1996b). One to ten days later, progestogen levels increase 100 to 400-fold and remain elevated until parturition (about 94 days from mating) or CL regression at the end of the non-pregnant luteal phase (38 to 59 days from mating) (Brown et al. 1996b). In females that fail to conceive after copulation, progestogen concentrations during a non-pregnant luteal phase or 'pseudopregnancy', typical for felids are not different from progestogen levels observed in pregnant females (Wildt et al. 1981a, Brown et al. 1995, Graham et al. 1995, Brown et al. 1996b).

Based on studies utilizing laparoscopic observation of ovaries and measurements of serum and fecal estradiol concentrations, it is known that female cheetahs experience variable periods of unexplained ovarian quiescence (acyclicity) depicted by the absence of estradiol peaks (Figure 1.4) (Wildt et al. 1993, Brown et al. 1996b, Brown et al. 2001). All females monitored for more than a year (n = 7) experienced acyclic periods two to

five months in duration that were neither synchronous among females nor associated with season (Brown et al. 1996b). The etiology of these acyclic periods is not understood, but current dogma holds that it is related to suboptimal husbandry or management practices (Brown et al. 2001).

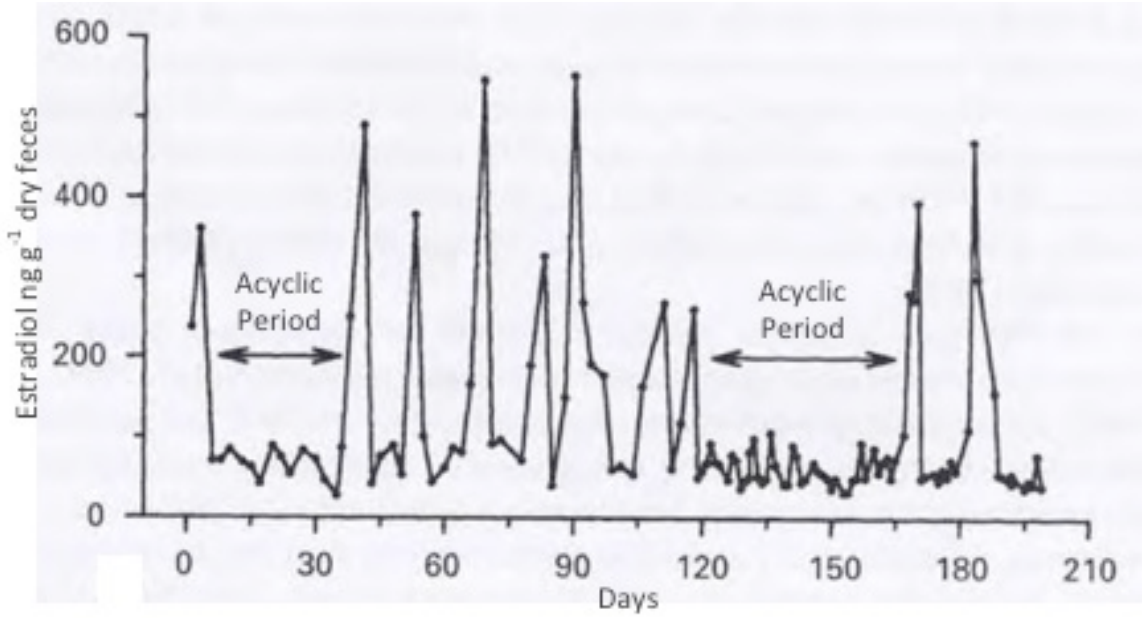


Figure 1.4 Female cheetah estradiol profile depicting periods of acyclicity (Brown 2006)

Behaviors indicative of estrus are generally known to be relatively constant across threatened felid species and similar to those displayed by the domestic cat (Michael 1961). These behaviors include: rolling, object rubbing, vocalizing, pacing, increased locomotor activity, self-grooming, urination, urine spraying and marking, investigative activity, and lordosis (Michael 1961). Female cheetahs, however, have historically been known to display an extreme amount of individual variation in expression and occurrence

of typical felid estrous behaviors, leading to belief that estrus may even be 'silent' in some individuals (Wildt et al. 1981b).

Specific research utilizing captive individuals has found that increases in estradiol concentrations measured from feces were significantly correlated with changes in the occurrence of five specific behaviors; rolling, object rubbing, investigative activity, vocalizing, and urine spraying (Wielebnowski and Brown 1998). Unlike other felids, in which types of behaviors change in a predictable manner during estrus, the number and types of specific correlated behaviors varied between individual cheetahs (Wielebnowski and Brown 1998). This means that identification of changes in behavior indicative of estrus in the cheetah requires intricate knowledge of each individual female's standard behavioral repertoire for comparison (Wielebnowski and Brown 1998). This is a difficult and time-consuming endeavor that most institutions that want to breed cheetahs are incapable of accomplishing in sufficient detail. Inability to identify or predict the occurrence of estrus is just one of many reproductive hurdles presented by this species while attempts are continually made to further conservation efforts.

Endocrinology of Spermatogenesis

Endocrine control of sperm production includes several mechanisms that are analogous to hormonal regulation within females, but unlike the female system, males lack a surge center in the hypothalamus causing GnRH to be continually released in regular waves (Senger 2005). As in the female, GnRH causes release of the gonadotropins LH and FSH from the anterior pituitary, which stimulate action in Leydig cells, the male equivalent of theca cells, and Sertoli cells, the male equivalent of

granulosa cells, respectively (Figure 1.5) (Senger 2005, Norris 2006). These two cell types interact with each other through steriodogenesis remarkably similar the two-cell theory reported for females (Figure 1.2) (Norris 2006). Although it is known that LH, FSH, androgens and other paracrine factors control sperm production, many specific endocrine mechanisms involved in this process are still not clear, especially for less commonly studied species (Norris 2006). The generalized progression of events is understood, however, and will be described here.

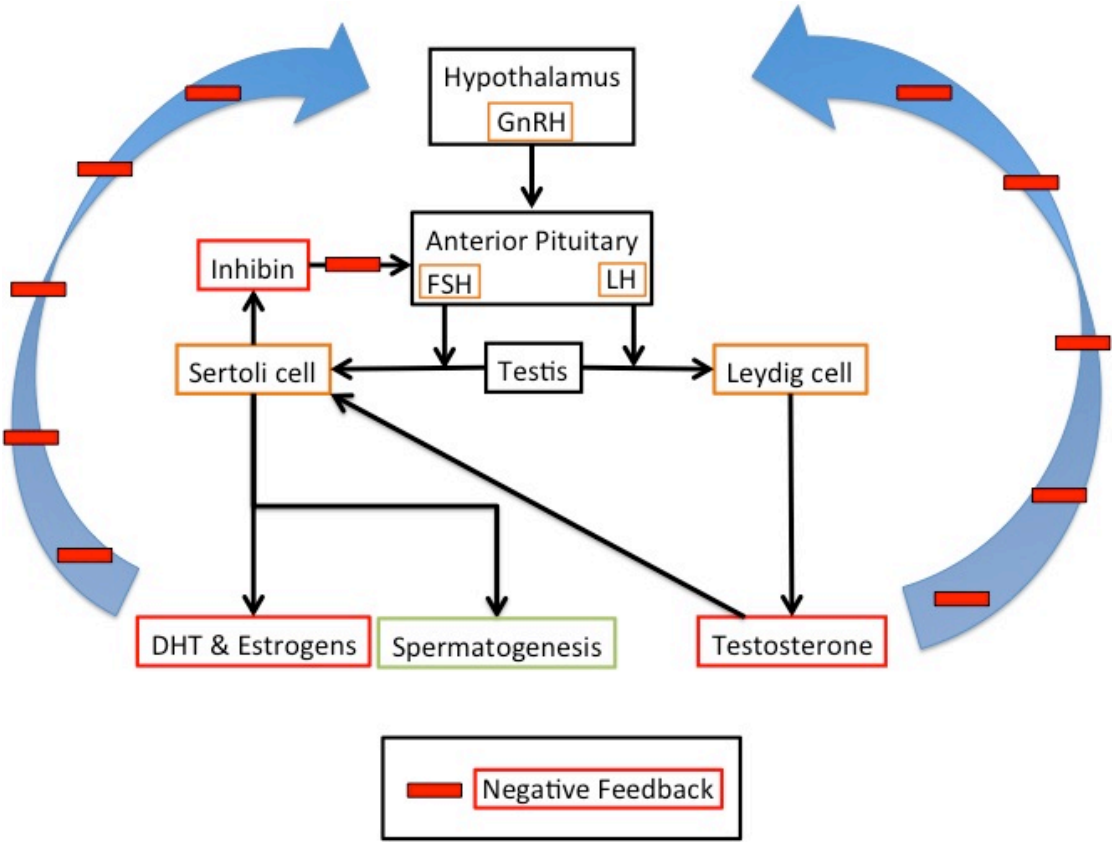


Figure 1.5 Interactions of male reproductive hormones in the HPG axis (Senger 2005, Norris 2006)

Luteinizing hormone that is released from the anterior pituitary binds to receptors on the membrane of Leydig cells and stimulates cholesterol to be converted to progestogens and then to androgens such as testosterone via the Δ^4-pathway (Figure 1.3) (Norris 2006). Steriodogenesis of androgens after Leydig cell receptors bind LH is enhanced by a paracrine regulator called steriodogenesis-stimulating protein (STP) which is produced by FSH-stimulated Sertoli cells (Norris 2006). Androgens, namely testosterone, regulate the functions of Sertoli cells and their role in spermatogenesis (Senger 2005, Norris 2006). Testosterone is also believed to stimulate meiosis in primary spermatocytes which will eventually become spermatids that are released into the lumen of the seminiferous tubules of the testes (Senger 2005, Norris 2006). Aromatase synthesis is stimulated in Sertoli cells by FSH (Senger 2005). Aromatase converts testosterone produced by Leydig cells into estradiol, a major hormone involved in negative feedback control of hypothalamic release of GnRH (Senger 2005). Estradiol also appears to have direct effects on spermatogenesis as estrogen receptors are present on germ cells in much higher numbers than testosterone receptors (Norris 2006). This indicates that testosterone acts through important paracrine mechanisms to effect the process of sperm production (Norris 2006). Although testosterone is likely the most important androgen influencing sperm production, other androgens such as androstenedione or 5**α-**dihydrotestosterone (DHT) may also be present in significant amounts depending on species (Norris 2006).

Reproductive Physiology of the Male Cheetah

Studies on the reproductive physiology of the male cheetah have examined animals from wild populations including those in the Serengeti (Wildt et al. 1987b) and

Namibian ecosystems (Crosier et al. 2007), as well as those from *ex situ* managed collections both in South Africa (Wildt et al. 1983) and North America (Donoghue et al. 1992, Wildt et al. 1993, Roth et al. 1995). The common conclusion across all studies is the extraordinarily high proportion (about 75%) of structurally abnormal spermatozoa per ejaculate that cannot be attributed to age, season, diet, or disease. Although this percentage of malformed spermatozoa is higher than many other felids, it is similar to genetically compromised felid species such as the Florida panther (*Puma concolor coryi*) (Roelke et al. 1993) and Asiatic lion (*Panthera leo persica*) (Wildt et al. 1987a). This leads to the conclusion that teratospermia in cheetahs, or the production of ≥60% structurally abnormal spermatozoa per ejaculate, is at least partially due to the well-known lack of genetic diversity in this species (O'Brien et al. 1983, O'Brien et al. 1985, O'Brien et al. 1987). The observation of teratospermia in cheetahs both in managed collections as well as in the wild indicates that current difficulties breeding cheetah in captivity is not related to physiological deficiencies in males (Wildt et al. 1993, Crosier et al. 2007).

Comparisons of ejaculate characteristics of cheetahs to normospermic populations of other felid species have indicated that even normal spermatozoa from teratospermic cheetahs are likely compromised in their ability to penetrate the zona pellucida of the oocyte and achieve embryo cleavage (Wildt et al. 1993). It is known that sperm motility plays an important role in regulating gamete interaction in males of this species (Donoghue et al. 1992, Roth et al. 1995). There is some evidence that circulating testosterone concentrations are lower in cheetah than in other assessed felid species,

leading to the conclusion that there may be a correlation between consistently low testosterone and poor overall semen quality in the cheetah (Wildt et al. 1988, Wildt et al. 1993). There has also been speculation that individual male cheetahs with especially low testosterone would not experience a hormonal incentive to mate (Wildt et al. 1993). However, testosterone concentration and its relation to ejaculate quality and behavior have yet to be fully examined in male cheetahs.

Reproductive Technologies

The use and development of reproductive technologies for conservation strategies have been pivotal in increasing scholarly knowledge of wild species, improving management of *ex situ* populations, and even restoring species to nature (Pukazhenthi and Wildt 2004). Research on a range of carnivore species has revealed an astonishing species specificity of reproductive mechanisms, emphasizing the need for baseline studies on animals of marked conservation concern (Comizzoli et al. 2009). Reproductive technologies applied to cheetahs to be discussed in the following include non-invasive monitoring of reproductive hormones, electroejaculation and cryopreservation of spermatozoa, artificial insemination (AI), and to some extent, *in vitro* fertilization (IVF).

With the development of the radioimmunoassay (RIA) and enzyme immunoassay (EIA) for specific hormones, plotting of hormone concentrations over time from serial blood samples became possible (Pukazhenthi and Wildt 2004). Longitudinal collection of blood samples from wild carnivore species, however, is impossible and dangerous, leading to work revealing the ability to measure voided steroid hormone metabolites in the feces of felids (Brown et al. 1994, Brown and Wildt 1997). Employing this technique

on cheetahs, a great deal of reproductive knowledge has been generated such as: 1) basic information regarding the ovarian cycle (Wildt et al. 1993, Brown et al. 1994, Brown et al. 1996b, Brown and Wildt 1997, Wielebnowski and Brown 1998, Wielebnowski 1999, Brown et al. 2001, Adachi et al. 2011, Brown 2011) and induction of ovulation (Brown et al. 1996b, Brown 2006, 2011); 2) influence of age (Crosier et al. 2011), stress (Terio et al. 1999, Young et al. 2004, Ludwig et al. 2013) and group housing on ovarian function (Wielebnowski et al. 2002b, Kinoshita et al. 2011); and, 3) development of ovarian control protocols for AI (Howard et al. 1992, Howard et al. 1997, Pelican et al. 2006). Although much has been learned utilizing this technique, there are still significant reproduction knowledge gaps existing for this species. Non-invasive hormone monitoring remains the methodological cornerstone of research aimed at improving conservation protocols.

Due the difficulty associated with achieving successful natural breeding of cheetahs in captivity, AI techniques began to be developed for this species in the early 1980s to circumvent issues such as mate incompatibility (Pukazhenthi and Wildt 2004). Paired with species-specific research determining appropriate exogenous gonadotropin regimes to induce ovulation, some success of developed AI protocols has been achieved (Howard et al. 1992, Howard et al. 1997, Wildt and Roth 1997, Howard 1999, Howard and Wildt 2009). Future utility, however, requires consistent offspring production using fresh as well as frozen-thawed sperm. Improvements have been made in recent years to cheetah sperm processing and cryopreservation protocols in order to maintain sufficient viability for AI, but successful offspring production using this method has not been

accomplished within the last decade (Crosier et al. 2006, Crosier et al. 2009, Terrell et al. 2010, 2011a, b, 2012).

Cryopreservation of electroejaculated spermatozoa has also been an important tool for the banking of genetic material from individual males living in zoos in North America as well as from more than 150 wild born males obtained through a partnership with the Cheetah Conservation Fund (CCF) in Namibia (Wildt et al. 1993, Crosier et al. 2007, Comizzoli et al. 2009). Samples from *in situ* males could only be obtained using 'field friendly' cryopreservation techniques which have provided the ability to infuse the captive population with new genetic material without continued removed of animals from the wild (Wildt et al. 1997). Cryorepositories of systematically collected gametes, embryos, blood products, tissue, and DNA for defined conservation programs, termed genome resource banks, provide a genetic insurance policy in the event that a local catastrophe severely reduces wild populations (Wildt et al. 1997). Therefore, both genome resource banks and a healthy, self-sustaining captive population can protect the species against extinction due to unforeseen circumstances.

Combined with previously mentioned techniques, IVF has been explored in the cheetah by adapting the system developed in the domestic cat for exotic felids (Johnston et al. 1991, Donoghue et al. 1992, Roth et al. 1995, Pelican et al. 2006, Crosier et al. 2011). Using this system, as high as 68% of inseminated cheetah oocytes fertilize and 34% of fertilized embryos progress to at least the 8-cell stage of development (Donoghue et al. 1992, Crosier et al. 2011). IVF technology is in its early stages, so it is clearly not capable of being used as a genetic management tool for reliable offspring production.

Combined with *in vitro* oocyte maturation, however, it has the potential to be a rescue strategy for genetically valuable females that unexpectedly die or require ovariohysterectomy (Pukazhenthi and Wildt 2004, Pelican et al. 2006).

The Stress Response

The Hypothalamic-Pituitary-Adrenal Axis

In order to survive, organisms must have mechanisms in place to cope with energetically demanding conditions or perturbations to homeostasis termed 'stressors' in order to return to a their baseline steady state (Nelson 2005). A stress response is a suite of physiological and behavioral responses that help to reestablish homeostasis (Nelson 2005). Within seconds after the body perceives a stressor, the catecholamines epinephrine and norepinephrine are released from the adrenal medulla as well as nerve fibers of the sympathetic nervous system (Nelson 2005). This immediate reaction, termed the 'fight or flight response', is responsible for changing cardiovascular tone, respiration rate, and blood flow to the muscles, but it is only a part of the body's reaction to a stressor (Nelson 2005). Immediately after the occurrence of a stressor, the hypothalamic-pituitary-adrenal (HPA) axis is also stimulated (Figure 1.6), but full effect of this response takes a few minutes to be achieved (Nelson 2005). Due to the fact that catecholamines are unable to cross the blood-brain barrier, mediation of stress by the HPA axis appears to be more versatile and complex and is the subject of a multitude of studies, both past and present, attempting to fully understand its effects and mechanistic relationships (Nelson 2005).

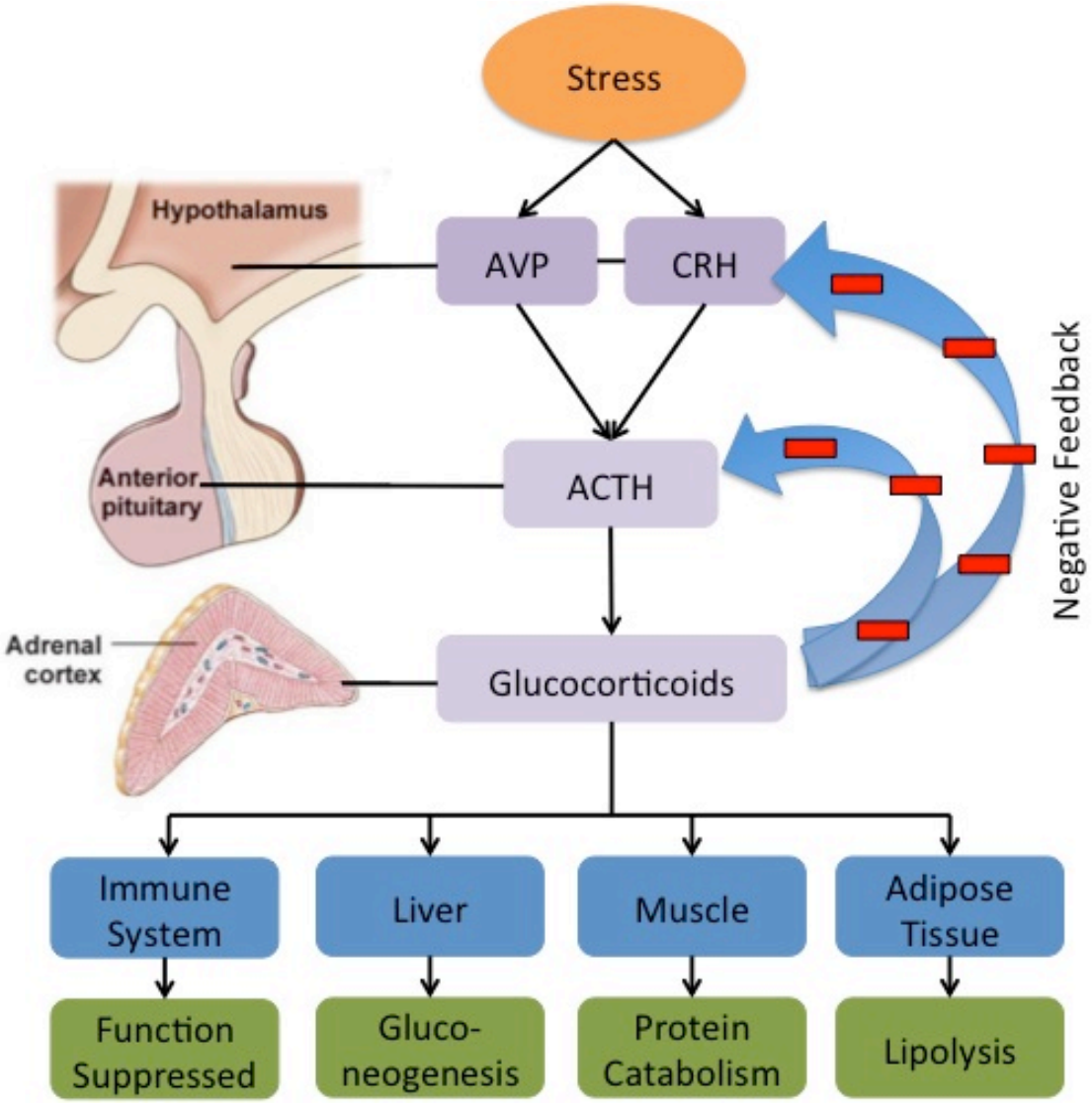


Figure 1.6 The stress response mediated by the HPA axis (Nelson 2005, Norris 2006)

When a mammal perceives an energetically demanding situation, the stress response mediated by the HPA axis begins in the hypothalamus and results largely in the increased availability of energy reserves to aid the body in dealing with the stressor (Nelson 2005). Initially, the stressor stimulates synthesis and release of the neuropeptides corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) mainly from the

paraventricular nucleus of the hypothalamus (Norris 2006). CRH and AVP themselves stimulate the corticotropes of the anterior pituitary to release adrenocorticotropic hormone (ACTH) which acts on the adrenal cortex (Nelson 2005, Norris 2006). The zona fasciculata and the zona reticularis of the adrenal cortex respond to ACTH stimulation by synthesizing and releasing glucocorticoids such as cortisol and corticosterone (Nelson 2005, Norris 2006). Glucocorticoids have actions throughout the body that largely affect energy metabolism to increase and maintain circulating glucose concentrations (Norris 2006). These changes increase the immediate availability of energy, but glucocorticoids also inhibit glucose utilization by peripheral tissues, allowing the brain to have a preferential source of glucose to enhance alertness and function (Nelson 2005, Norris 2006). In addition to this, in response to a stressful event, the HPA axis can also inhibit energetically expensive processes that are not related to immediate survival, such as growth, digestion, immune function, and reproduction (Nelson 2005).

Glucocorticoids are released into circulation in lower concentrations following an episodic circadian rhythm since very little corticosteroid is stored in the adrenals and they are released as they are synthesized (Norris 2006). In diurnal mammals, circulating glucocorticoids are naturally higher in the morning, dropping in the afternoon, and then are lowest at night; this pattern is reversed in nocturnal mammals (Norris 2006). Prior to excretion, most glucocorticoids are metabolized in the liver, where they are conjugated with sulfates or glucuronides to make them more soluble in water. They are then excreted primarily either in urine or feces, depending on species (Norris 2006).

After short-term stressors, shutoff of the HPA axis is achieved through negative feedback effects of glucocorticoids on ACTH excretion from the pituitary as well as CRH release from the hypothalamus (Figure 1.6) (Norris 2006). During instances of chronic stress, however, glucocorticoid secretion can remain elevated due to the actions of AVP, which is not affected by negative feedback caused by high circulating levels of glucocorticoids (Norris 2006). At these times, AVP release increases the sensitivity of the corticotropes in the pituitary to CRH, allowing ACTH release to continue despite exposure to lowered CRH concentrations (Norris 2006). The zona fasciculata and zona reticularis regions of the adrenal cortex are known to exhibit hypertrophy and hyperplasia in response to chronically elevated ACTH in cases of prolonged stress (Norris 2006).

Glucocorticoid actions in the body are mediated by two types of receptors, type II or glucocorticoid receptors (GRs), and type I or mineralocorticoid receptors (MRs) (Funder 1997, De Kloet et al. 1998, Berardelli et al. 2013). In regions of the brain, MRs lose their selectivity to mineralocorticoids and bind glucocorticoids with an affinity 10-fold higher than GRs (Reul and Dekloet 1985, De Kloet et al. 1998, Berardelli et al. 2013). It has been found that MRs binding glucocorticoids are responsible for inhibition of the HPA axis by negative feedback, particularly during periods of low glucocorticoid concentration associated with circadian rhythm (Reul and Dekloet 1985, Dallman et al. 1989, De Kloet et al. 1998, Berardelli et al. 2013). During a stressful event, however, rising glucocorticoid concentrations recruit GRs as the major mechanism controlling negative feedback inhibition of the HPA axis (Berardelli et al. 2013).

The HPA Axis and Reproduction

When the duration of the stress response is acute, or short-term in nature, its effects on reproduction are rather variable based on reports both within and across different species. For example, exposure to an acute stressor or brief administration of stress hormones has been found to cause short-lived increases in circulating testosterone concentrations in rodents (Mann et al. 1986), humans (Rivier and Rivest 1991), and domestic bulls (Thibier and Rolland 1976). Stressors or stress hormones also cause decreased basal LH in domestic rams (Matteri et al. 1984) and have prevented the preovulatory LH surge in some dairy heifers (Stoebel and Moberg 1982). Despite the enigmatic nature of the relationship between acute stress response and reproduction, chronic stress is known to result in suppressed gonadotropin secretion and inhibition of reproduction across species (Tilbrook et al. 2000). The primary mechanism by which this occurs is still in debate, but it is likely due to a complicated combination of mechanisms with actions at various levels of the HPG axis (Tilbrook et al. 2000).

There are growing numbers of reports providing evidence for HPA-HPG axis inhibition within the central nervous system, and some at the level of the gonad, but few indicating HPA axis hormone alteration of pituitary responsiveness to GnRH. At the bottom tier of the HPG axis, receptors for HPA axis hormones have been found in rodent gonads indicating direct negative impacts on Leydig cell function and spermatogenesis (Mor et al. 2001). Within the central nervous system, GRs have been found on RFamide-related peptide (RFRP) cells, the mammalian equivalent of gonadotropin inhibitory hormone (GnIH) (Kirby et al. 2009). Chronic immobilization stress in rats led to an up-

regulation of RFRP expression in the hypothalamus and this was also associated with inhibition of HPG activity (Figure 1.7) (Kirby et al. 2009). Additionally, direct connections have been found between CRH axon terminals and dendrites of GnRH-secreting neurons in the hypothalamus, with reports in primates showing decreased circulating LH concentrations after injection of CRH (Maclusky et al. 1988, Xiao et al. 1989). Although there are likely various other mechanisms involved, these discovered connections between the HPA and the HPG axes are undoubted major participants in the inhibition of reproduction during activation of the HPA axis due to a chronic stressor.

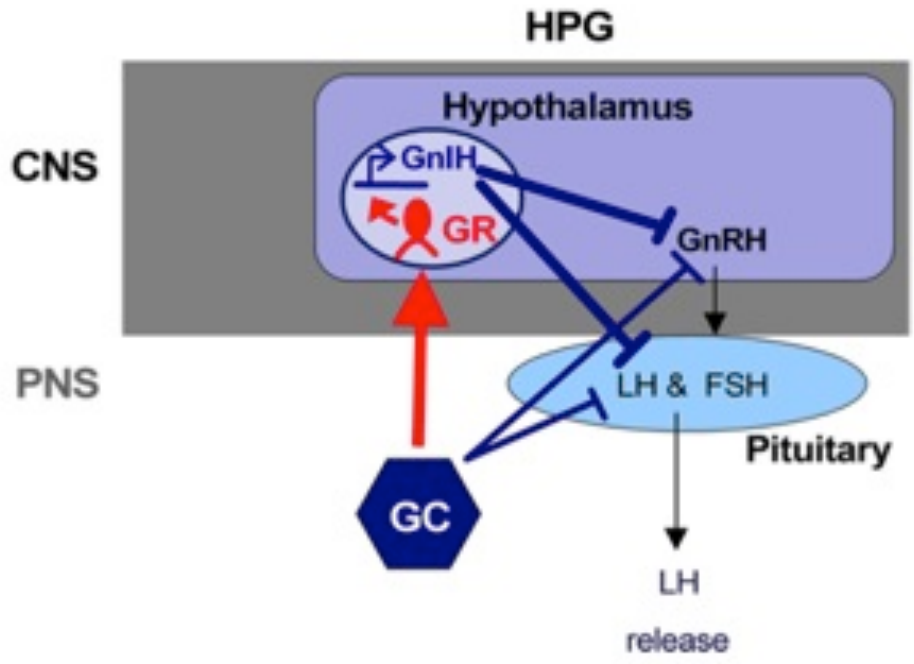


Figure 1.7 Glucocorticoid inhibition of the HPG axis through GnIH (Kirby et al. 2009)

Stress and Reproduction in Cheetahs

It is well documented that *ex situ* management has been found to affect adrenal glucocorticoid concentrations in many felid species including domestic cats (Carlstead et al. 1992, Carlstead et al. 1993b), leopard cats (*Prionailurus bengalensis*) (Carlstead et al. 1992, Carlstead et al. 1993a), pumas (*Puma concolor*) (Carlstead et al. 1992), Geoffrey's cats (*Leopardus geoffroyi*) (Carlstead et al. 1992), and clouded leopards (Wielebnowski et al. 2002a). Specific husbandry-related practices that induce increased adrenal activity in these species include relocation, irregular feeding times, exposure to a high number of different people, physical restraint, and lack of enrichment or appropriate enclosure space. In cheetahs, fecal glucocorticoid levels can be quantified and used as a measure of an individual's stress response to environmental changes (Jurke et al. 1997, Brown et al. 2001, Wells et al. 2004, Young et al. 2004). A study examining glucocorticoid concentrations in 15 adult cheetahs before and after relocation between on and off exhibit enclosures concluded that moving cheetahs to on exhibit areas with high public exposure caused a higher risk of having increased glucocorticoids after movement (Wells et al. 2004). Particularly if the individual was held off exhibit previously (Wells et al. 2004). Together, these studies provide foundation for the assertion that management procedures often act as environmental stressors that can increase glucocorticoid concentrations in cheetahs kept in managed collections.

The link between environmental stressors and decreased reproductive activity has been previously documented in felids (Mellen 1991, Moreira et al. 2007) as well as in a variety of other species, such as Syrian hamsters (*Mesocricetus auratus*) (Jasnow et al.

2001), multiple rhinoceros species (Carlstead and Brown 2005, Metrione and Harder 2011), and tree lizards (*Urosaurus ornatus*) (French et al. 2007). Examination of adrenal cortices and glucocorticoid concentrations in captive and free-ranging cheetahs revealed evidence of chronic stress in captive animals (Terio et al. 2004). This discovery has made investigation of the effect of high glucocorticoid concentrations on the reproductive functioning of *ex situ* cheetahs critical. It has been found that 75% of pair-housed female cheetahs experience prolonged periods of acyclicity that can be rapidly reversed by separation of paired females (Wielebnowski et al. 2002b). This indicates a possible link between environmental stressors and reproductive suppression in this species. In support of this, a study that analyzed fecal glucocorticoid concentrations in female cheetahs classified as reproductive or non-reproductive (based on behavioral indicators of estrus and male interaction) found that non-reproductive females exhibited elevated glucocorticoid concentrations over reproductive counterparts (Jurke et al. 1997).

Although chronic, elevated levels of glucocorticoids are known to have negative impacts on reproductive potential, short-term stressors that only elicit a brief increase in glucocorticoids are a necessary part of an individual's adaptation to novel stimuli and can have positive effects on reproduction and pregnancy (Nelson 2005). To date, however, a possible relationship between acute stress responses and reproductive physiology and behavior has not been explored in cheetahs. It is unknown if cheetahs in managed collections, possibly experiencing chronic stress (Terio et al. 2004), are able to produce predictable adrenal responses to short-term stressors and if this ability, or lack thereof, can be used as an indicator of health and/or reproductive potential.

CHAPTER TWO: THE EFFECT OF GROUP HOUSING ON THE REPRODUCTIVE FUNCTION OF MALE CHEETAHS (*ACINONYX JUBATUS*)

Abstract

Cheetahs in *ex situ* collections are charismatic 'ambassadors' that help to educate the public while providing unique research opportunities. Most of what is known about the physiology and veterinary/nutritional requirements of the species has been learned from studying *ex situ* collections. Such information is impossible to collect from elusive, free-living animals. Despite the value of captive cheetahs, these populations are not self-sustaining (i.e., more animals die every year than are born). Only ~20% of cheetahs in North American zoos have ever reproduced, and this poor fecundity appears mostly related to suboptimal management. Female cheetahs demonstrate a high degree of reproductive sensitivity to some management-related environmental conditions such as group housing, yet currently, little is known about factors regulating male cheetah reproductive function and success. In the current study, I examined ejaculate quality, adrenal and gonadal hormone metabolite concentrations, and behavior of male cheetahs in managed collections for influences of group housing. I tested the hypotheses that: 1) reproductive fitness is enhanced in males that are singly housed compared to those housed in coalitions, and 2) males initiating the most behavioral interactions with coalition members exhibit increased reproductive fitness than males initiating fewer behaviors. For the first time, this study generated longitudinal hormone profiles

indicative of adrenal and gonadal function in male cheetahs, supporting an absence of seasonality and a large amount of variation within and between glucocorticoid metabolite concentrations among individuals. Overall, testosterone concentrations and ejaculate characteristics were increased for coalition males over those held as singletons. This effect was mostly due to fecal testosterone metabolite concentrations and sperm quality being greater in males initiating the most behavioral interactions with coalition members. Results from my study have direct implications for *ex situ* management because, as data show, increased breeding success may be achieved through long-term group housing of male cheetahs. Future studies are needed to fully understand the nature of the mechanism behind the observed effect and to continue to work to increase reproductive success of cheetahs *ex situ* to ensure a sustainable future for this species.

Introduction

The cheetah (*Acinonyx jubatus*) is recognized as threatened due to extirpation from most of its historic range, with continuing declines resulting in only about 10,000 individuals remaining in nature today (Durant et al. 2008). Wild cheetahs are notoriously difficult to study due to inherent elusiveness. Those in *ex situ* collections are useful for educating the public, understanding basic species biology (e.g., behavior, reproduction, genetics, veterinary, and nutritional requirements), and in developing methods applicable to wild counterparts (e.g., developing anesthetic protocols and monitoring/understanding disease sensitivity). Although scientists have been examining cheetahs for more than 30 years, this species fails to consistently reproduce in captivity (Lindburg et al. 1993, Marker-Kraus and Grisham 1993). Under the Association of Zoos and Aquariums Species Survival Plan (SSP), findings reveal that only about 20% of animals in the North American population have ever reproduced, causing reliance upon imported cheetahs from Africa to sustain *ex situ* population numbers (Marker 2012, 2013).

Although the cheetah is known for low genetic variation (O'Brien et al. 1983, O'Brien et al. 1987) and the production of 75% malformed spermatozoa per ejaculate (Wildt et al. 1993), the relatively high fecundity of free-ranging cheetahs indicates that these traits do not ultimately cause poor reproductive success (Caro 1994). Rather, it appears that *ex situ* cheetahs require specific husbandry and management techniques to encourage breeding success (Lindburg et al. 1993, Caro 1994, Brown et al. 1996b). There is evidence of management-related 'stress sensitivity'; housing two females in the same enclosure causes mutual ovarian shutdown and acyclicity occurs (Wielebnowski et al.

2002b). This effect is reversed within days by placing the females in adjacent pens. Regardless of management, most females in managed collections display sporadic ovarian activity and periodically protracted acyclicity. That is, periods of time with an absence of episodic increases in estradiol concentration indicative of waves of follicular development (Brown et al. 1996b).

To date, few studies have examined environmental factors regulating male cheetah reproductive function and success. Instead, recent male studies have predominantly centered on the sensitivity of sperm to freezing, development of functional cryopreservation protocols, and metabolic function *in vitro* (Crosier et al. 2006, Crosier et al. 2009, Terrell et al. 2011a, 2012). Longitudinal hormone profiles depicting daily fluctuations and long-term patterns of adrenal and gonadal function have not yet been generated for males of this species. Knowing the effects of environmental conditions on reproduction is crucial for guiding best-practice protocols. For example, the official Cheetah SSP Husbandry Guidelines recommend males be housed together in coalitions when possible (2009). This strategy is appealing to zoos because it mimics wild social structure (Caro 1994) and allows an institution to house more animals, creating exciting, multi-individual exhibits. In reality, the impact of this management tactic on captive male reproductive function is unknown, but is potentially significant as females are known to experience intense social suppression of reproduction (Wielebnowski et al. 2002b). Although it is known that the majority of wild males associate in coalitions, it remains undetermined if this tactic represents the best management strategy for housing males in the captive environment, particularly in terms of promoting successful breeding.

A survey of wild cheetahs in the Serengeti determined that despite increased group hunting success and territorial defense, approximately 40% of male cheetahs were living as singletons (Caro and Collins 1987). This may be due in part to limited breeding opportunity for some males within coalitions. It has been found that 94% of wild male cheetah coalitions contain littermates (Caro 1994). The genetic contribution of full-sibling males to reproduction would be very similar and there is likely little benefit for each male in such a cohort to reproduce (Caro 1994). Within coalitions of highly related male lions, for example, just one or two of the members usually dominate reproductive success (Packer et al. 1991, Caro 1994). Thus, wild singleton male cheetahs may experience increased reproductive benefits over some males in coalitions and therefore, group housing of males in managed collections may be contributing to diminished reproductive performance in some individuals.

If male cheetah coalitions are not egalitarian in terms of mating opportunities, it follows that one male within a group must establish himself as the recipient of these reproductive benefits either by behavioral dominance or by winning disputes in the presence of a female. Observations of males in coalitions have reported extremely low frequencies of aggressive behaviors, even over small food sources or females (Caro 1993). In many primate species, a positive relationship has been found between male dominance rank and mating success among animals of the same age class within a group (Cowlishaw and Dunbar 1991, Rodriguez-Llanes et al. 2009, Muniz et al. 2010, Newton-Fisher et al. 2010, Surbeck et al. 2011). Behavioral hierarchy determined between male cheetahs within a coalition may be indicative of a variance in mating success. This may

also be reflected in physiological differences among males within a coalition such as in gonadal or adrenal hormone concentrations or in seminal quality. Although notably different from cheetahs in overall social structure, males of other group living carnivores such as African wild dogs (*Lycaon pictus*) (Creel et al. 1997, Spiering et al. 2010), gray wolves (*Canis lupus*) (Sands and Creel 2004), and dwarf mongooses (*Helogale parvula*) (Creel and Waser 1991) exhibit physiological differences that affect reproductive potential among pack mates according to dominance rank.

In the current study, I examined ejaculate quality, adrenal and gonadal hormone metabolite concentrations, and behavior of male cheetahs in managed collections for influences of group housing. I tested the hypotheses that: 1) reproductive fitness is enhanced in males that are singly housed compared to those housed in coalitions, and 2) males initiating the most behavioral interactions with coalition members exhibit increased reproductive fitness than males initiating fewer behaviors. I believed these effects would be reflected in parallel decreases in excreted glucocorticoids, increased androgens, and better quality ejaculates. A secondary priority was, for the first time, to generate longitudinal hormone profiles of adrenal and gonadal function in male cheetahs. Semen collections throughout the year have provided evidence of a lack of reproductive seasonality in this species (Crosier et al. 2007), but there is currently no information on correlating testicular androgen output. Besides filling a knowledge gap, the information generated by this study will provide indispensible information on the nuances of male cheetah social structure and will allow the continued improvement of management and husbandry protocols in order to achieve a self-sustaining *ex situ* population.

Materials and Methods

Animals

Twenty-nine captive-born, adult male cheetahs housed in seven Association of Zoos and Aquariums (AZA) accredited institutions across the United States were included in this study (Table 2.1).

Table 2.1 Demographic information about male cheetahs (n = 29) used in this study

Facility and Location	Facility Abbreviation	Number of Males (Coalition.Singleton)
Dickerson Park Zoo, MO	DPZ	0.1
Philadelphia Zoo, PA	PHL	3.0
San Diego Safari Park, CA	SD	5.1
Smithsonian Conservation Biology Institute, VA	SCBI	6.1
White Oak Conservation Center, FL	WOCC	4.0
Wildlife Safari, OR	WS	2.2
The Wilds, OH	Wilds	3.1

Animals were housed and managed according to the Cheetah SSP and approved husbandry guidelines for the species (2009, 2013). The Institutional Animal Care and Use Committees (IACUC) of all involved facilities approved animal procedures. Cheetahs were considered to be reproductively mature at two years of age (Crosier et al. 2007). All males were of reproductive age (Crosier et al. 2007) and there were no significant differences ($t_5 = 0.69$; $P = 0.52$) between mean ages of coalition and singleton males (Table 2.2).

Table 2.2 Mean (± SEM) and range of ages of male cheetahs used in this study

Males	Mean Group Age (± SEM)	Age Range
All (n = 29)	6.9 ± 0.5	2.6 – 12.4
Coalition (n = 23)	6.7 ± 0.5	3.2 – 9.6
Singleton (n = 6)	7.9 ± 1.6	2.6 – 12.4

Fecal Collection and Preparation

Freshly voided feces were collected three to four days per week for a period of seven to thirteen months for each male into clean plastic bags, labeled with animal number and date. Samples were then stored or shipped frozen (-20°C) to the SCBI for processing. A non-digestible and harmless marker (e.g., glitter, birdseed, lentils, corn) was added to the diet of coalition males to identify individual fecal samples. All samples (n = 4,653) were then freeze-dried (Lyophilizer; Labconco) to remove water, pulverized using a rubber mallet, and stored in labeled plastic tubes at -20°C until processing.

Steroid hormone metabolites were extracted from fecal samples using previously described methods (Graham and Brown 1996). After 0.2 g of well-mixed fecal powder from each sample was boiled in 5 ml of 90% ethanol for 20 minutes, extracts were centrifuged to remove particulates and the supernatant was transferred into a glass tube. The pellet was resuspended in an additional 5 ml of 90% ethanol, vortexed for 30 seconds, and recentrifuged. Combined ethanol supernatants were dried under air and then resuspended in 1 ml of 100% methanol. Methanol extracts were dried under air and then resuspended in 1 ml of BSA-free phosphate buffer ($NaH_2PO_4 + Na_2HPO_4 + NaCl + H_2O$; pH = 7.0). Extracts were sonicated (15 minutes) and vortexed (30 seconds) prior to

decanting into a plastic tube for freeze storage (-20°C) until evaluation for hormone metabolite concentration.

The efficiency of steroid extraction from feces was evaluated by adding radiolabeled hormone (^{3}H-cortisol or ^{3}H-testosterone; 4000-8000 dpm) to each fecal sample prior to boiling extraction. The minimum recovery of radiolabeled hormone from fecal extracts acceptable for enzyme immunoassay (EIA) was 60% and the mean recovery across males was ~75%. Fecal extracts were diluted 1:20 and 1:200 concentration in BSA-free phosphate buffer for cortisol and testosterone EIA respectively. Samples considered too dilute (binding > 80% of maximum binding) were run at a higher concentration (1:10 – 1:100), and samples too concentrated (binding < 20%) were run at a lower concentration (1:200 – 1:2000).

Fecal Glucocorticoid Metabolite Analysis

Glucocorticoid metabolite concentrations in diluted fecal extracts were determined using a cortisol EIA validated for use in cheetahs (Young et al. 2004). This cortisol EIA employed a polyclonal antibody (1:8500; R4866; C. Munro, University of California, Davis, CA) raised in rabbits against cortisol-3-carboxymethyloxime linked to bovine serum albumin and cross-reacts with cortisol 100%, prednisolone 9.9%, prednisone 6.3%, cortisone 5%, and < 1% with corticosterone, desoxycorticosterone, 21-desoxycortisone, testosterone, androstenedione, androsterone, and 11-desoxycortisol (Young et al. 2004). The antibody was added to 96-well microtiter plates (Nunc-Immuno, Maxisorp; Fisher Scientific) and allowed to equilibrate for 12 – 48 hours (4°C). Unbound antibody was removed with wash solution and diluted samples, in duplicate, and

standards, in triplicate, (0.05 ml; 78 – 20,000 pg/ml; Sigma Diagnostics) were added to the plate. A peroxidase enzyme-conjugated cortisol (1:20,000; 0.05 ml; C. Munro, University of California, Davis, CA) was then added to each well containing sample or standard and the plate was allowed to incubate for one hour (23°C) before unbound components were removed. A chromagen solution was added (0.1 ml) to each well and incubated ~15 minutes before optical densities were determined using a microplate reader (Dynex MRX, reading filter 405 nm, reference filter 540nm).

Sensitivity of the cortisol assay at maximum binding was 3.9 pg/well. The inter-assay coefficient of variation for two internal controls was 8.36% (mean binding – 27.88%) and 4.84% (mean binding – 68.12%) and intra-assay coefficient of variation was < 10% (n = 364 assays). Serially diluted pooled fecal extracts demonstrated displacement curves parallel to those of standard hormone preparations. Recovery of added standard to fecal extract ($y = 0.90x – 18.02$, $r = 0.99$) demonstrated significant recovery ($P < 0.05$). Several measures were calculated to summarize fecal glucocorticoid values during the longitudinal steroid evaluation of each male; 1) an overall mean of all samples for the collection period, 2) a mean baseline that excludes all values greater than the overall mean plus 1.5 standard deviations (SD) (Graham and Brown 1996), and 3) a peak frequency that divides the number of samples greater than three times baseline by total number of samples collected for that male (Young et al. 2004).

Fecal Androgen Metabolite Analysis

There is currently no published EIA to quantify fecal androgen metabolites in the cheetah; therefore, the assay used in this study was first physiologically and hormonally

validated. The testosterone EIA relied on a polyclonal anti-androgen (1:7500) antibody (R156/7; C. Munro, University of California, Davis, CA) used to measure testosterone metabolites from fecal extracts in other species such as the giant panda (Aitken-Palmer et al. 2012). This antibody cross-reacts with testosterone 100%, 5α-dihydrotestosterone 57.37%, < 1% with androstenedione, androsterone, androsteneolone, cholesterol, and β-estradiol, and < 0.02% with progesterone, pregnenolone, and hydrocortisone. Microtiter plates were run in the same way as for glucocorticoid analysis; diluted samples in duplicate, standards in triplicate (0.05 ml; 46 – 12,000 pg/ml; 17β-hydroxy-4-androstein-3-one; Steraloids), and a peroxidase enzyme-conjugated testosterone (1:15,000; 0.05 ml; C. Munro) was added to the plate. The plate was then allowed to incubate for two hours (23°C) before unbound components were removed, a chromagen solution added, and optical densities read.

Physiological validation of the testosterone EIA was completed comparing mean androgen concentrations yielded by the assay between 29 adult male cheetahs (> 2 years in age), 7 young males (< 2 years in age), and 6 adult females. Adult males (0.692 ± 0.036 μg/g dry feces) exhibited a higher ($F_{2,39} = 19.37$; $P < 0.01$) mean androgen concentration than both young males (0.388 ± 0.024 μg/g) and females (0.294 ± 0.027 μg/g). The young male concentration was significantly higher than the female concentration determined by a student's t-test ($t_{10} = 2.59$; $P = 0.02$). Hormonal validation also supported the utility of this assay as fecal samples collected and assayed from a male cheetah implanted with a strong GnRH agonist (Deslorelin) showed a distinct spike in

androgen concentrations before a decline in baseline to a level similar to that observed in females (Figure 2.1).

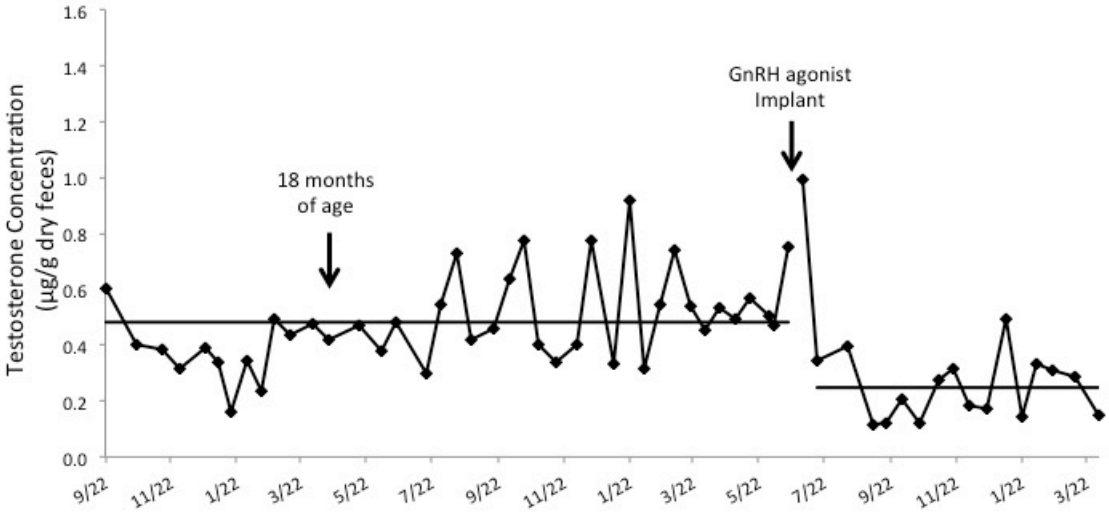


Figure 2.1 Testosterone profile of a male cheetah receiving a GnRH agonist implant, solid horizontal lines indicate baseline testosterone concentration before and after GnRH challenge event

The number and relative proportions of testosterone metabolites in cheetah fecal extracts were determined by reverse-phase high-performance liquid chromatography (HPLC; Microsorb C-18 Column, Rainin, Woburn, MA) (Brown et al. 1996a). Prior to HPLC, samples were reconstituted in 0.5 ml buffer, passed through a C-18 matrix column (Spice Cartridge, Rainin), and eluted with 5 ml of 80% methanol to remove contaminants (sample loss was ~10%). The filtered fecal extract was evaporated to dryness, reconstituted in 60 µl of acetonitrile, and eluted using a gradient of 45% acetonitrile isocratic over 80 – 120 minutes (1 ml/minute flow rate, 333µl fractions). Fractions were

dried down under air, reconstituted in BSA-free phosphate buffer, and testosterone metabolite immunoreactivity was quantified by EIA as described above. Co-elution profiles of ^{3}H-testosterone, ^{3}H-androstenedione, and ^{3}H-dihydrotestosterone were also determined and used as reference tracers.

HPLC analysis detected several fecal metabolites, however, relatively little corresponded with the ^{3}H-labelled androgen reference tracers. Immunoreactivity was primarily associated with presumably conjugated androgens eluting earlier at fractions 8 – 16, similar to what has been published previously in domestic cats and Eurasian and Iberian lynx (Brown et al. 1996a, Jewgenow et al. 2006). From this, to determine the proportion of water-soluble and ether-soluble steroid forms, as well as the proportion of water-soluble forms that were enzyme hydrolyzable, ether extractions and enzyme hydrolysis were performed (Brown et al. 1994). Fecal extracts in BSA-free phosphate buffer were extracted with 10 volumes of diethyl ether to separate water-soluble from ether-soluble forms. Residual aqueous samples were enzymatically hydrolyzed with 50 µl β-glucuronidase/aryl sulfatase (20,000 Fishman U/40,000 Roy U, respectively; Boehringer Mannheim Corp., Indianapolis, IN) at 37°C for 24 hours, and then extracted again with 10 volumes of diethyl ether to separate enzyme-hydrolyzable (organic phase) from non-hydrolyzable (aqueous phase) forms. Fecal metabolites were primarily water-soluble (57.5%), with 34.2% of the immunoreactivity found in the ether fraction after extraction. Of the water-soluble (presumably conjugated) forms, <5% were enzyme-hydrolyzable, similar to what is known in domestic cats (Brown et al. 1996a).

Sensitivity of the testosterone EIA at maximum binding was 2.3 pg/well. The inter-assay coefficient of variation for two internal controls was 10.64% (mean binding – 24.14%) and 5.01% (mean binding – 66.18%) and intra-assay coefficient of variation was < 10% (n = 326 assays). Serially diluted pooled fecal extracts demonstrated displacement curves parallel to those of standard hormone preparations. Recovery of added standard to fecal extract (y = 1.08x – 3.80, $r = 0.99$) demonstrated significant recovery ($P < 0.05$). Several measures were calculated to summarize fecal testosterone values during the longitudinal steroid evaluation of each male, 1) an overall mean of all samples for the collection period and 2) a mean baseline that excludes all values greater than the overall mean plus 1.5 standard deviations (SD).

Semen Collection, Evaluation, and Processing

At least one semen collection was performed on each male within the period of fecal collection, but at least two months after commencement. Methods utilized for anesthesia and semen collection and evaluation were similar to previous studies on cheetah (Wildt et al. 1983, Wildt et al. 1988, Wildt et al. 1993, Crosier et al. 2006). To induce a surgical plane of anesthesia, medetomidine hydrochloride, midazolam, and butorphanol (Domitor®, Pfizer Inc., La Jolla, CA; 22 – 25 µg/kg, 0.2 mg/kg, and 0.3 mg/kg respectively) were injected intramuscularly by hand using a restraint-cage technique or using a pole-syringe or air-pressured darting system if necessary according to protocols described in the Cheetah Husbandry Manual (2009). If needed, propofol (0.5 – 4 mg/kg, intravenously) was administered during the electroejaculation to maintain the appropriate level of anesthesia. Laboratory calipers were used to measure testicular

length and width, which was then used to estimate total testicular volume for each male (Howard et al. 1990). A rectal probe of either 1.6 of 1.9 cm in diameter with three longitudinal electrodes and an electrostimulator (P.T. Electonics, Boring, OR) were used to administer 80 stimuli (2 – 5 V) over a 30-minute interval (Wildt et al. 1983, Wildt et al. 1993).

Semen was collected in pre-warmed, sterile collection vials, and an aliquot (3 µl) was immediately assessed for sperm percent motility and forward progressive status (scale: 0 – 5, five rating equivalent to rapid, straightforward progress (Howard et al. 1990)). A 15 – 20 µl aliquot of raw semen was fixed in 100 µl of 0.3% glutaraldehyde in phosphate buffered saline (pH, 7.4) for assessing sperm morphology (1000x). Spermatozoa were classified as normal or as having one of the following abnormalities: 1) head abnormalities including micro-, macro-, and bi-cephalic; 2) acrosomal abnormalities including missing or damaged acrosomal membranes; 3) midpiece abnormalities including abnormal or missing midpiece, and a bent midpiece with or without retained cytoplasmic droplet; 4) flagellar abnormalities including tightly coiled flagellum, bent flagellum with or without retained cytoplasmic droplet, bi-flagellate, retained proximal or distal droplet; and 5) other abnormalities including spermatid and bent neck (Crosier et al. 2007). Total volume of the ejaculate was measured using a pipette and the remainder, after motility and morphology aliquots were removed, was immediately diluted with an equal volume of sterile Ham's F10 culture medium (HF10; Irvine Scientific, Santa Ana, CA) supplemented with 20 mM Hepes, 5% (v:v) fetal calf serum (Irvine Scientific), pyruvate (1 mM), glutamine (2 mM), 10,000 IU/ml penicillin,

10 mg/ml streptomycin, and 20 mg/ml neomycin (Sigma Chemical, St. Louis, MO). Sperm concentration was determined using a hemocytometer (Wildt et al. 1983). Diluted samples were centrifuged (Eppendorf Mini-Spin, Hamburg, Germany) for 8 minutes at 100*g* and the supernatant was aspirated from the sperm pellet. The pellet was then resuspended in cryodiluents and slow-cooled before being loaded into 0.25 ml straws and cryopreserved over liquid nitrogen using a previously determined protocol (Crosier et al. 2006) for genome banking and utilization by additional studies.

Behavioral Data Collection

Behavioral evaluations were conducted over a total of eight successive days during the period of fecal collection and within six months of electroejaculation to reflect coalition interaction and potential hierarchy during the time of measured testicular functionality. Coalitions of males were observed for a one-hour session each day in their home enclosure to establish interaction behavior frequencies. One-hour sessions were completed in the morning between the hours of 0600 and 1000 and before feeding whenever possible, as preparatory observations for this study revealed this to be a time of high activity during the day. Observations were recorded using methods and definitions of observable behaviors described in the Cheetah Husbandry Manual (2009), and consistent with previously published ethograms for the cheetah (Wielebnowski and Brown 1998, Wielebnowski et al. 2002b). Instantaneous sampling (two minute time intervals) was used to estimate time spent in any behavioral state, and continuous scan sampling was used to measure behavioral event frequencies (Martin and Bateson 2007).

Behaviors collected and respective categories are listed in Table 2.3. To maintain consistency, a single researcher conducted all behavior observations.

Table 2.3 Descriptions of behaviors collected from male cheetahs in coalitions

Behavioral States	
Laying	Side of body in contact with ground, legs to side, eyes open
Standing	As implied
Sleeping	Laying with eyes closed
Crouching	Legs under body like standing, but legs bent, body close to ground
Sitting	As implied
Walking	Forward locomotion at relxed speed, includes trot
Pacing	Walking back and forth across same area repeatedly
Running	Rapid forward locomotion with full extension of limbs and tail
Out of Sight	Cheetah is unable to be seen by observer to assess state
Behavioral Events – Agonistic Behaviors	
Swipe	Strikes at another cheetah with forelimb
Grab	Grasping of part of another cheetah's body with both forelimbs
Bite	As implied, usually follows grabbing behavior
Approach	Walking up to another cheetah within 3 body lengths and focusing
Displace	Approach followed by forcible assumption of other's location
Back away/leave	Avoidance of proximity of approaching cheetah by walking
Chase/follow	Approach and pursuit of retreating individual within 1 body length
Charge/attack	Sudden run or jump towards another cheetah, growling or hissing
Stalk	Body low, advance with eyes focused on prey object
Fight	Follows attack, wrestling and biting each other aggressively
Behavioral Events – Affiliative Behaviors	
Rub	Rubs face, head, or neck on object or conspecific
Groom	Lick, chew, or otherwise clean self or conspecific
Sniff	Olfactory examination of ground, object, or conspecific
Behavioral Events – Individualized	
Roll	Rolls from one side to another, back on ground, paws in the air
Tread	Scraping ground with rear legs without forward locomotion
Urinate	Excretion of urine from a sitting or squat position
Urine Spray	Sprays urine directly behind from standing position with tail raised
Defecate	As implied
Flehmen	Grimace with open mouth, retracted lips, wrinkled nose

Behavioral Events – Vocalizations	
Stutter	Repetitive short throat calls
Meow	Soft call, similar to that of domestic cat
Chirp	High pitched, short call, corners of mouth drawn back
Growl	Low, drawn out "snarling" sound, mouth open showing teeth
Hiss	Silent, drawn out expulsion of air with mouth and eyes wide open
Purr	Low rumbling in throat, similar to domestic cat but louder

In order to record behavioral event data regarding individual male initiator and receiver during interaction behaviors (categories: agonistic and affiliative) and provide information on dominance status within coalitions, an adaptation of the traditional cheetah ethogram recording method was utilized (Figure 2.2; Appendix). By assigning males within a coalition arbitrary numbers 1, 2, or 3, tally marks can be placed in the appropriate box to signify the initiator and receiver of each behavioral event observed. Number of tally marks for each initiated and received behavior was totaled for each male over all hour-long behavioral observation periods (n = 8) and then divided by eight to determine frequency of each initiated/received behavior per hour. Behavior frequencies per hour were used to identify males in each coalition that initiated the most and the fewest behaviors. Males in coalitions of three that were behaviorally ranked in the middle were excluded from analysis.

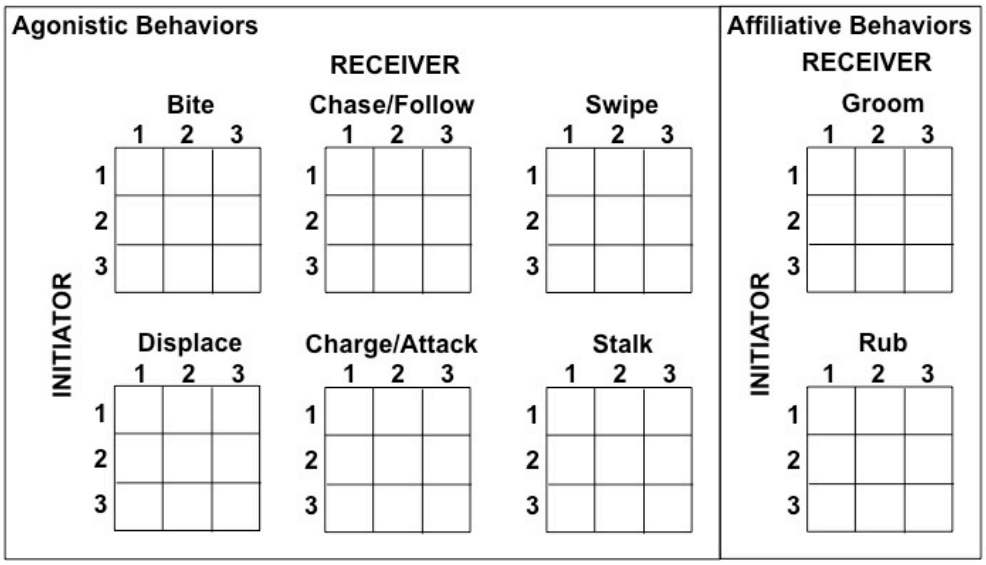


Figure 2.2 Male cheetah behavioral checksheet example, used to record specific interaction behavior within coalitions; for full behavioral checksheet, see Appendix

Statistical Analysis

Prior to analysis, statistical outliers were determined (Hoaglin and Iglewicz 1987) and replaced with the mean for all variables (Sokal and Rohlf 2012). Normality and homoscedasticity were verified using an Anderson-Darling and a Levene's test, respectively (Grafen and Hails 2002). Three sperm metrics, total sperm, total motile sperm, and sperm concentration were log transformed to achieve normality. To maintain statistical integrity, these three sperm metric means were back transformed for visual representation. A one-way analysis of variance followed by the Tukey-Kramer post-hoc comparison was used to examine average monthly glucocorticoid and testosterone concentrations across all males for evidence of seasonality in fecal hormone metabolites (Grafen and Hails 2002). To verify seasonality results, males were grouped by institution

and graphical representations of mean monthly testosterone concentrations were visually investigated for discernable patterns specific to geographical latitude of institution.

The effect of grouping on hormonal and sperm metrics was analyzed using a nested, multivariate mixed model that held exhibit status (on or off exhibit to the public), and grouping (coalition or singleton) as fixed factors, while controlling for the effects of institution, age, number of conspecifics present, the three-way interaction between grouping, exhibit status, and institution, and the interaction between exhibit status and institution (Grafen and Hails 2002). Hormonal metrics analyzed included overall mean and mean baseline concentration for both glucocorticoid and testosterone metabolite excretion, and peak frequency of glucocorticoid metabolite excretion. Sperm metrics analyzed included total testicular volume, ejaculate volume, sperm concentration, total sperm per ejaculate, percent motile sperm per ejaculate, progressive status, total motile sperm per ejaculate, and percent normal sperm per ejaculate (Crosier et al. 2007). The effect of institution on the data was significant. Therefore, in order to appropriately control for this within the statistical model, only institutions that housed both coalition and singleton males were included to examine the effect of grouping on each metric. Following this rule, three institutions were excluded, leaving 21 males total, five singletons and 16 coalition males (n = 6 coalitions of 2 – 3 males each) for analysis (Table 2.1).

Relationships between hormone and sperm metrics mentioned above were evaluated using a Pearson's correlation coefficient (Grafen and Hails 2002). Sperm and hormone metrics for males within coalitions that initiated the most interactive behaviors

with coalition members were compared to the coalition members that initiated the least behaviors using a paired *t*-test (Grafen and Hails 2002). Sperm and hormone metrics for each of these two behavioral groupings of males were compared to singleton males using a student's *t*-test (Grafen and Hails 2002). All statistical procedures were carried out using SAS software (version 9.2, Cary, NC). Effects were considered significant at $P < 0.05$ and data are reported as mean ± standard error of the mean (SEM).

Results

Longitudinal Hormone Characteristics

Population mean and range for fecal testosterone and glucocorticoid metabolite concentrations in male cheetahs monitored for this study are depicted in Table 2.4.

Table 2.4 Population mean and range of fecal testosterone and glucocorticoid metabolite concentrations of male cheetahs in North American zoological institutions (n = 29)

Metric	Mean (± SEM)	Range (Min- Max)
Testosterone metabolite concentration (μg/g)		
Average	0.71 ± 0.05	0.30 – 1.10
Baseline	0.50 ± 0.03	0.21 – 0.82
Glucocorticoid metabolite concentration (μg/g)		
Average	0.54 ± 0.05	0.21 – 1.08
Baseline	0.26 ± 0.03	0.12 – 0.52

Within individual males, testosterone concentrations were relatively stable over time, exhibiting few instances of highly elevated values, while glucocorticoid concentrations displayed widely variable or 'noisy' profiles with frequent peak values more than six times baseline concentrations (Figure 2.3). No significant correlation was

found ($P > 0.05$) between testosterone concentrations (i.e., mean and baseline), glucocorticoid concentrations (i.e., mean, baseline, and peak frequency), and testicular/ejaculate metrics (i.e., total testicular volume, ejaculate volume, sperm concentration, total sperm per ejaculate, percent motile sperm per ejaculate, progressive status, total motile sperm per ejaculate, and percent normal sperm per ejaculate).

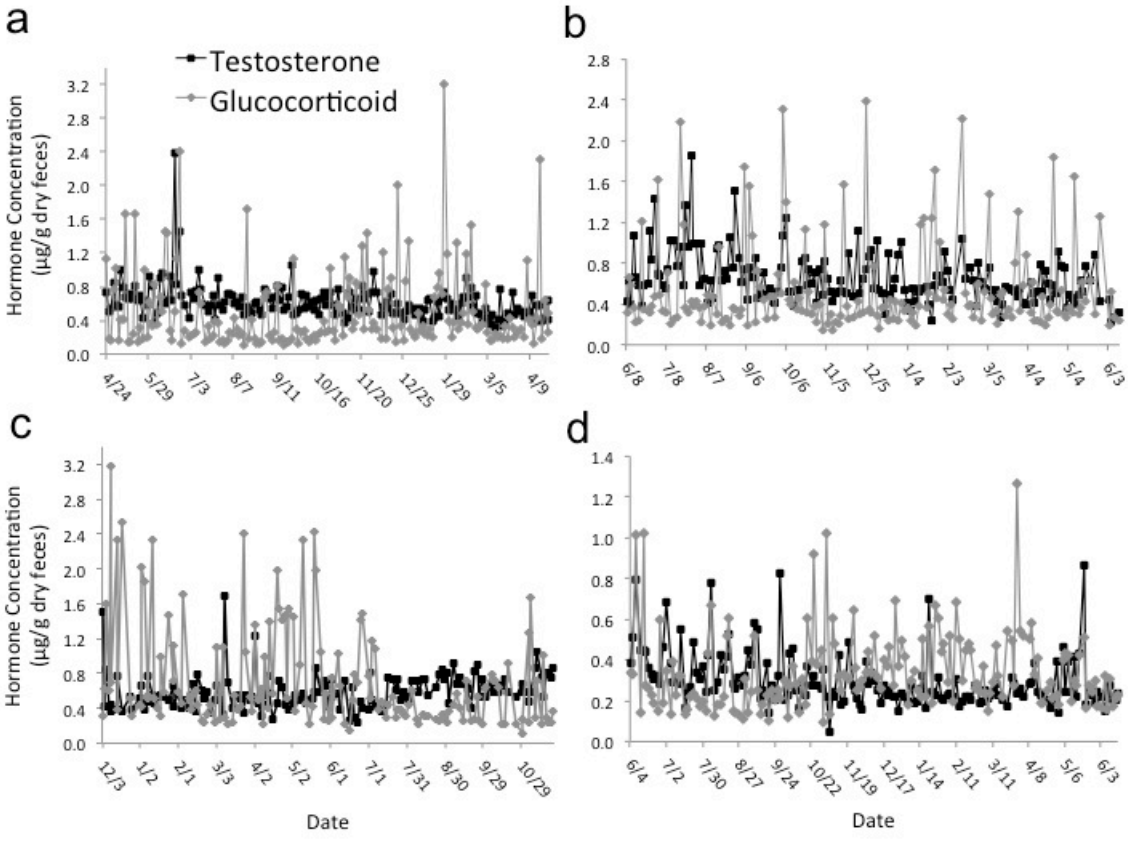


Figure 2.3 Representative fecal hormone metabolite profiles from four male cheetahs in North American zoological institutions

There was no evidence of seasonality in male cheetah longitudinal testosterone ($F_{11,336} = 1.76$; $P = 0.06$) or glucocorticoid ($F_{11,336} = 1.63$; $P = 0.09$) metabolite profiles (Figure 2.4). Visual comparison of males grouped by geographical location of institution showed no discernable differences in patterns of testosterone concentrations due to differences in latitude, also indicative of a lack of seasonality in this species (Figure 2.5).

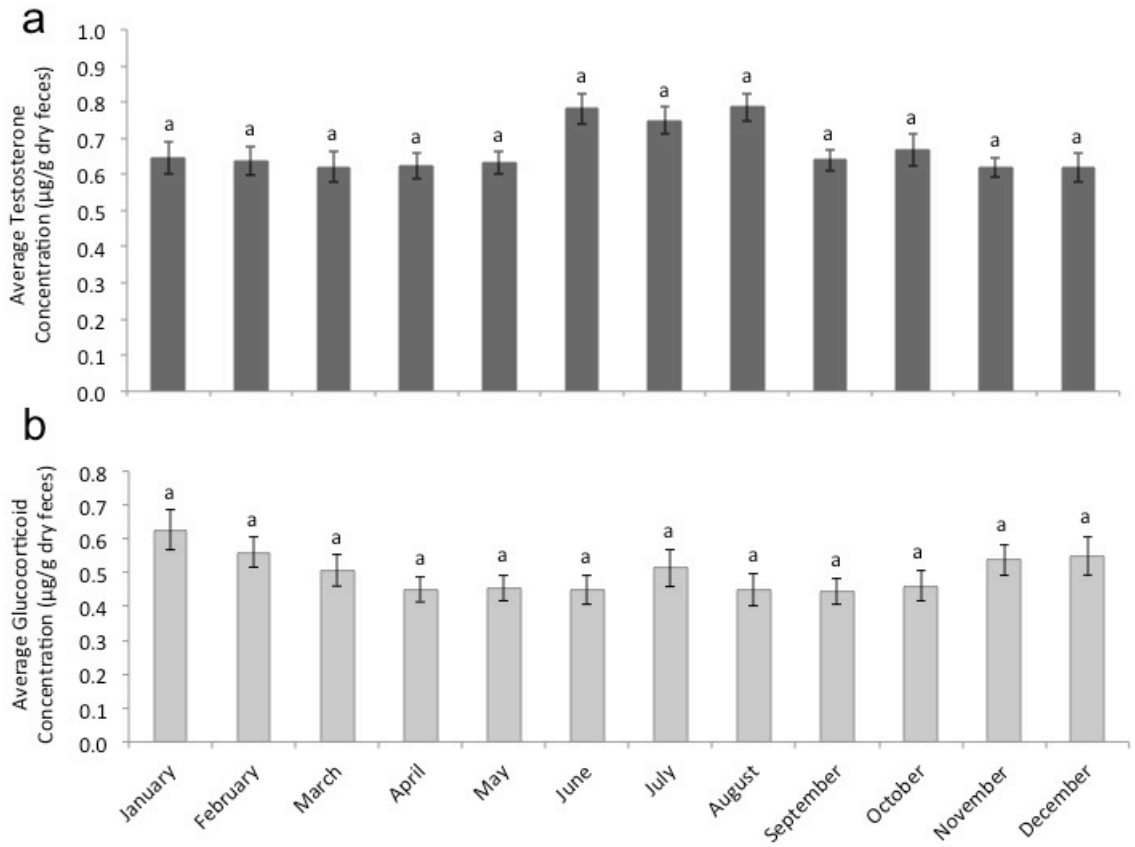


Figure 2.4 Average monthly fecal testosterone (a) and glucocorticoid (b) metabolite concentrations among male cheetahs (n = 29) housed in North American zoological institutions

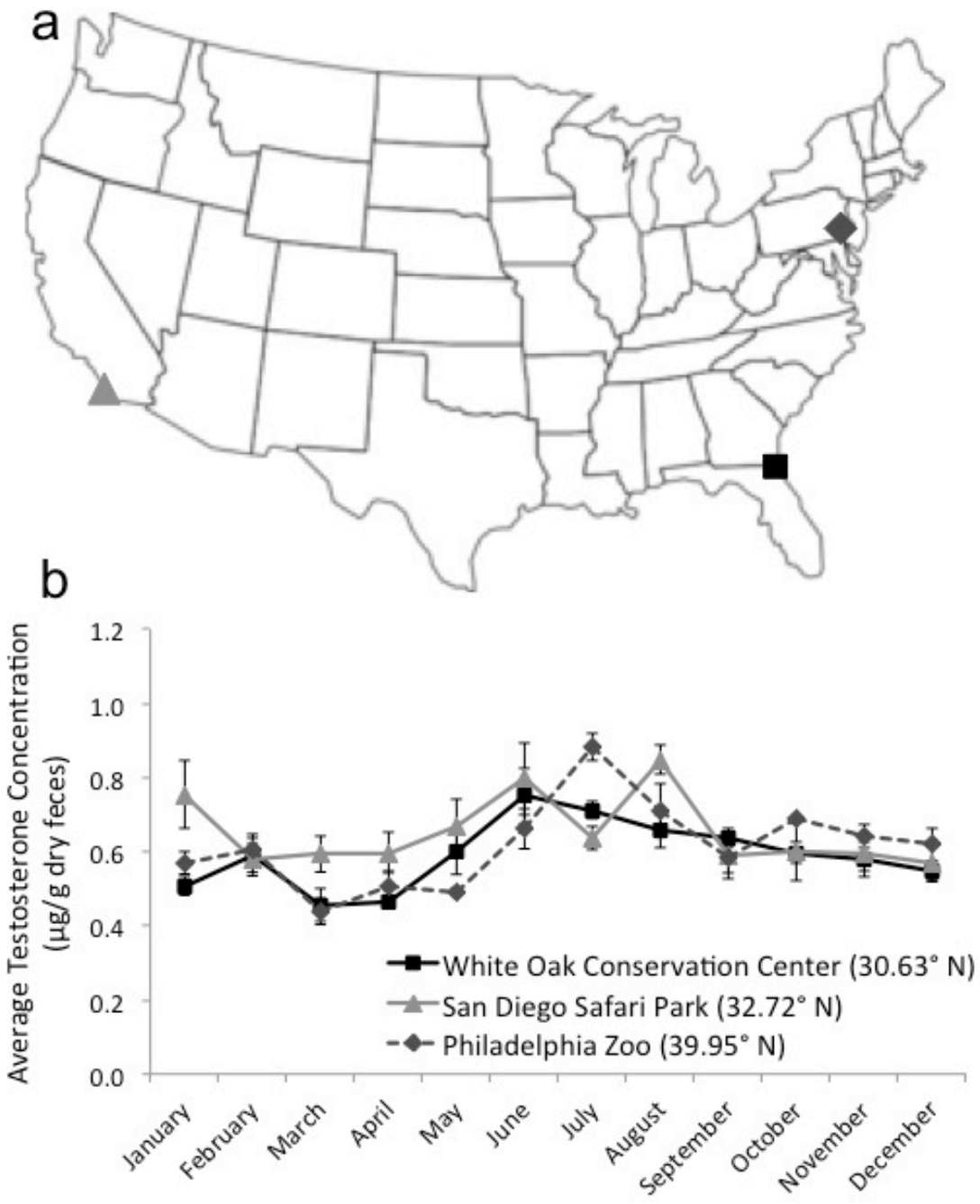


Figure 2.5 Location of geographically disparate zoological institutions (a) and average monthly testosterone concentrations of male cheetahs housed in each (b)

Effects of Group Housing on Male Reproductive Physiology

Males housed in coalitions had significantly ($F_{1,16} = 3.47$; $P < 0.01$) higher baseline testosterone concentrations (0.539 ± 0.053 μg/g dry feces) than those held alone (0.412 ± 0.088 μg/g dry feces) (Figure 2.6). Average testosterone concentrations were not different ($F_{1,16} = 1.42$; $P = 0.06$), however, between coalition (0.650 ± 0.034 μg/g dry feces) and singleton males (0.624 ± 0.101 μg/g dry feces). Glucocorticoid mean ($F_{1,16} = 1.93$; $P = 0.20$), baseline ($F_{1,16} = 0.14$; $P = 0.43$), and peak frequency ($F_{1,16} = 1.76$; $P = 0.41$) concentrations were also similar between coalition (mean: 0.500 ± 0.051 μg/g dry feces; baseline: 0.226 ± 0.021 μg/g dry feces; peak frequency: 0.158 ± 0.020 peak days/total sample days) and singleton males (mean: 0.536 ± 0.096 μg/g dry feces; baseline: 0.229 ± 0.016 μg/g dry feces; peak frequency: 0.165 ± 0.031 peak days/total sample days).

Ejaculate characteristics showed a pattern similar to that for hormone metabolites. Total sperm and total motile sperm per ejaculate were significantly higher ($F_{1,16} = 2.69$; $P = 0.02$ and $F_{1,16} = 2.79$; $P = 0.01$, respectively) in coalition (37.14 ± 1.31 x 10^6 and 26.09 ± 1.33 x 10^6, respectively) than in singleton males (10.54 ± 1.52 x 10^6 and 6.18 ± 1.60 x 10^6, respectively) (Figure 2.7a). Percent normal sperm, however, was higher ($F_{1,16} = -2.28$; $P = 0.04$) in singleton males (31.81 ± 4.00%) than coalition males (25.47 ± 3.40%). Total sperm and total motile sperm were both negatively correlated with percent normal sperm ($r_{21} = 0.39$; $P = 0.04$ and $r_{21} = 0.35$; $P = 0.04$ respectively) (Figure 2.7b). Coalition males maintained more ($F_{1,16} = 2.25$; $P = 0.04$) total normal sperm per ejaculate (10.92 ± 2.30 x 10^6) than singleton males (4.46 ± 1.58 x 10^6) (Figure 2.7c). All other analyzed

testicular/ejaculate characteristics including, total testicular volume ($F_{1,16} = 0.78$; $P = 0.43$), semen volume ($F_{1,16} = 1.81$; $P = 0.09$), sperm concentration ($F_{1,16} = 1.13$; $P = 0.28$), percent motile ($F_{1,16} = 1.00$; $P = 0.38$), and progressive status ($F_{1,16} = 0.67$; $P = 0.52$) were not different between males housed as coalitions and those held as singletons.

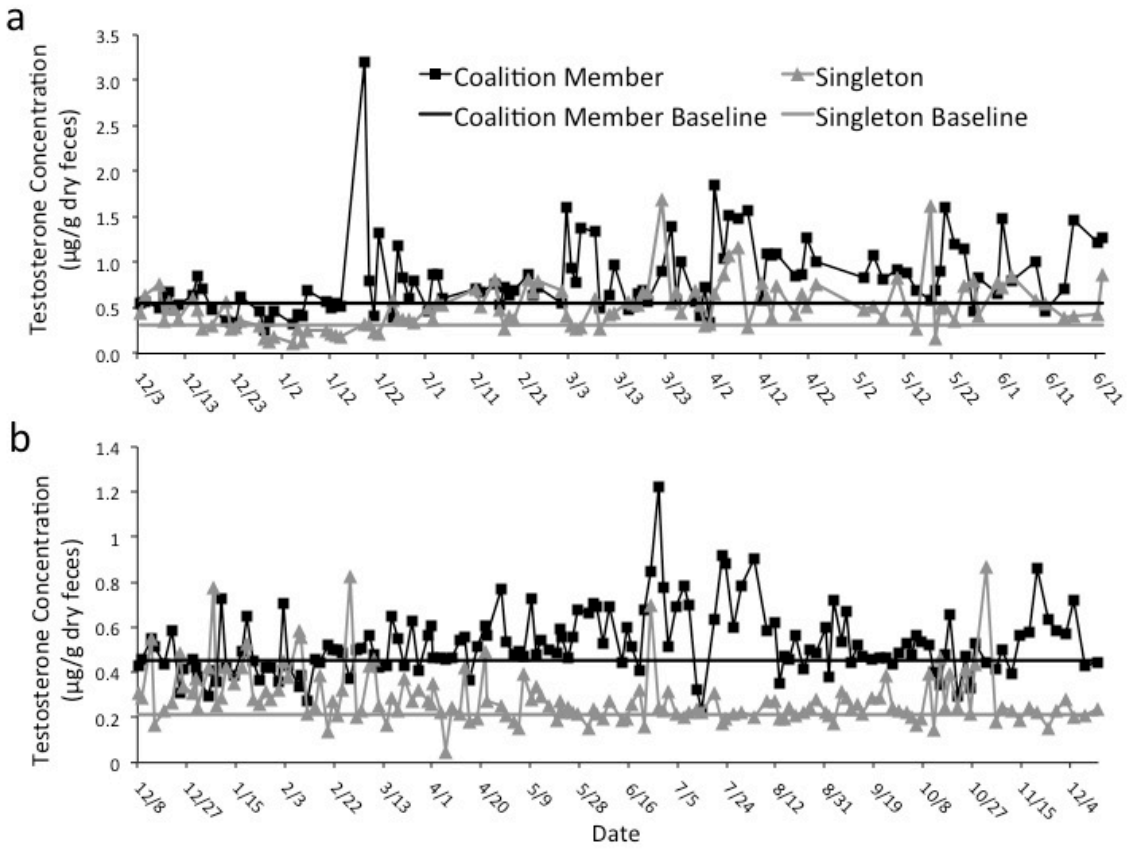


Figure 2.6 Representative testosterone metabolite profiles for coalition and singleton male cheetahs housed at the same institution illustrating significantly different baseline concentrations

Within Coalition Differences in Male Reproductive Physiology

Identification of males in each coalition that initiated the most and the fewest agonistic and affiliative behaviors with coalition members was completed using total frequency of initiated behaviors per hour for each male (Table 2.5). A significantly higher ($t_9 = 2.39$; $P = 0.04$) average fecal testosterone metabolite concentration was found in males that initiated the most agonistic and affiliative behaviors with coalition members (0.685 ± 0.048 μg/g dry feces) compared to males that initiated the fewest interactions with coalition members (0.629 ± 0.028 μg/g dry feces) (Figure 2.8). There was no difference between baseline testosterone ($t_9 = 0.72$; $P = 0.25$) in coalition males that initiated the most (0.510 ± 0.028 μg/g dry feces) or fewest behaviors with coalition members (0.499 ± 0.037 μg/g dry feces). Glucocorticoid average ($t_9 = 0.34$; $P = 0.37$), baseline ($t_9 = 0.10$; $P = 0.46$), or peak frequency ($t_9 = 1.07$; $P = 0.16$) concentrations were also not different between coalition males that initiated the most (average: 0.515 ± 0.067 μg/g dry feces; baseline: 0.217 ± 0.021 μg/g dry feces; peak frequency: 0.157 ± 0.028 peak days/total sample days) or fewest behaviors with coalition members (average: 0.534 ± 0.099 μg/g dry feces; baseline: 0.215 ± 0.033 μg/g dry feces; peak frequency: 0.189 ± 0.031 peak days/total sample days).

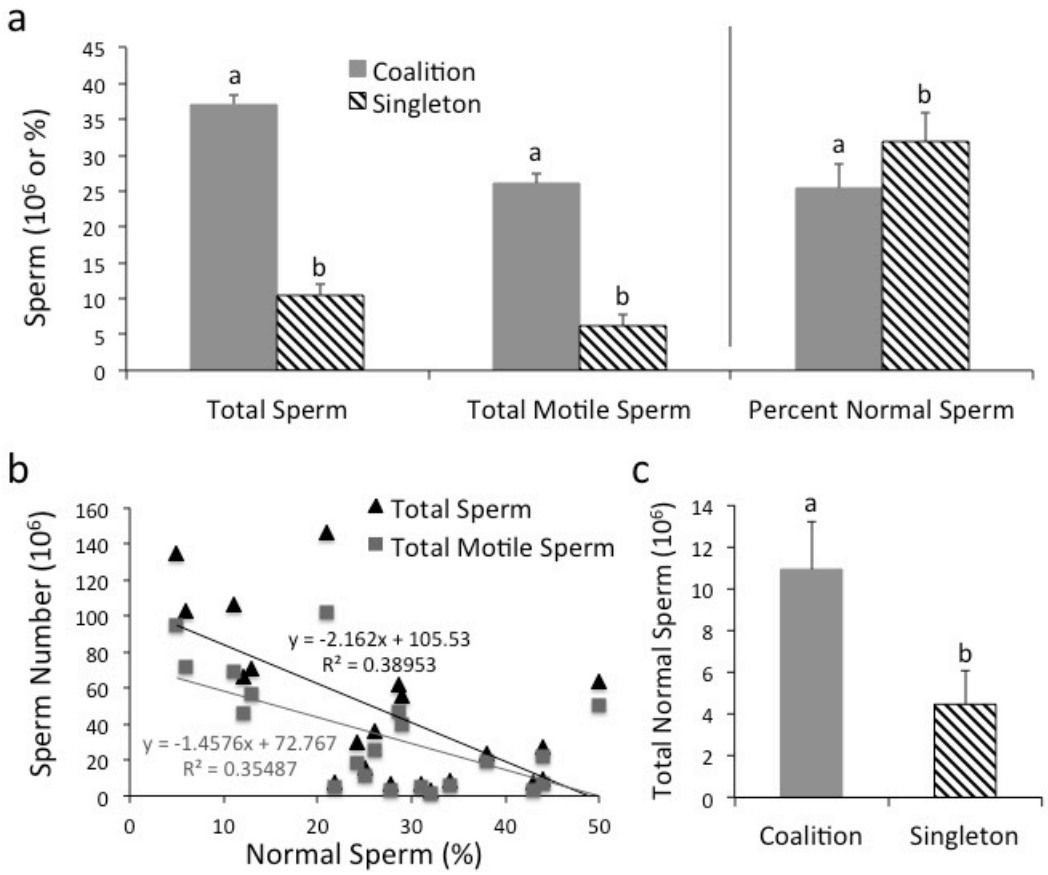


Figure 2.7 Ejaculate characteristics of male cheetahs (n = 6 coalitions of 2 – 3 males each, n = 16 males; n = 5 singletons) analyzed by grouping status (a), relationships between sperm metrics (b), and new combined sperm metric analyzed by male grouping status (c)

Table 2.5 Frequency of behaviors initiated by male cheetahs housed in coalitions (n = 23)

Male (coalition.individual)	Initiated Agonistic Behaviors (frequency/hour)	Initiated Affiliative Behaviors (frequency/hour)	Total Initiated Behaviors (frequency/hour)
Coalition A			
A.1	2.50	1.50	4.00*
A.2	0.63	3.25	3.88
A.3	1.63	0.75	2.38**
Coalition B			
B.1	0.88	0.38	1.25*
B.2	0.63	0.13	0.75**
B.3	0.63	0.25	0.88
Coalition C			
C.1	0.50	2.25	2.75*
C.2	0.00	1.63	1.63
C.3	0.00	0.13	0.13**
Coalition D			
D.1	1.25	1.63	2.88*
D.2	0.63	0.75	1.38**
Coalition E			
E.1	1.63	6.38	8.00*
E.2	1.00	5.25	6.25**
Coalition F			
F.1	2.00	0.25	2.25*
F.2	1.75	0.25	2.00**
Coalition G			
G.1	1.50	1.75	3.25
G.2	0.75	0.88	1.63**
G.3	1.13	3.00	4.13*
Coalition H			
H.1	0.63	0.00	0.63*
H.2	0.25	0.00	0.25**
Coalition I			
I.1	0.13	0.25	0.38**
I.2	0.50	1.13	1.63*
I.3	0.25	0.88	1.13

*most initiated behaviors within coalition
**fewest initiated behaviors within coalition

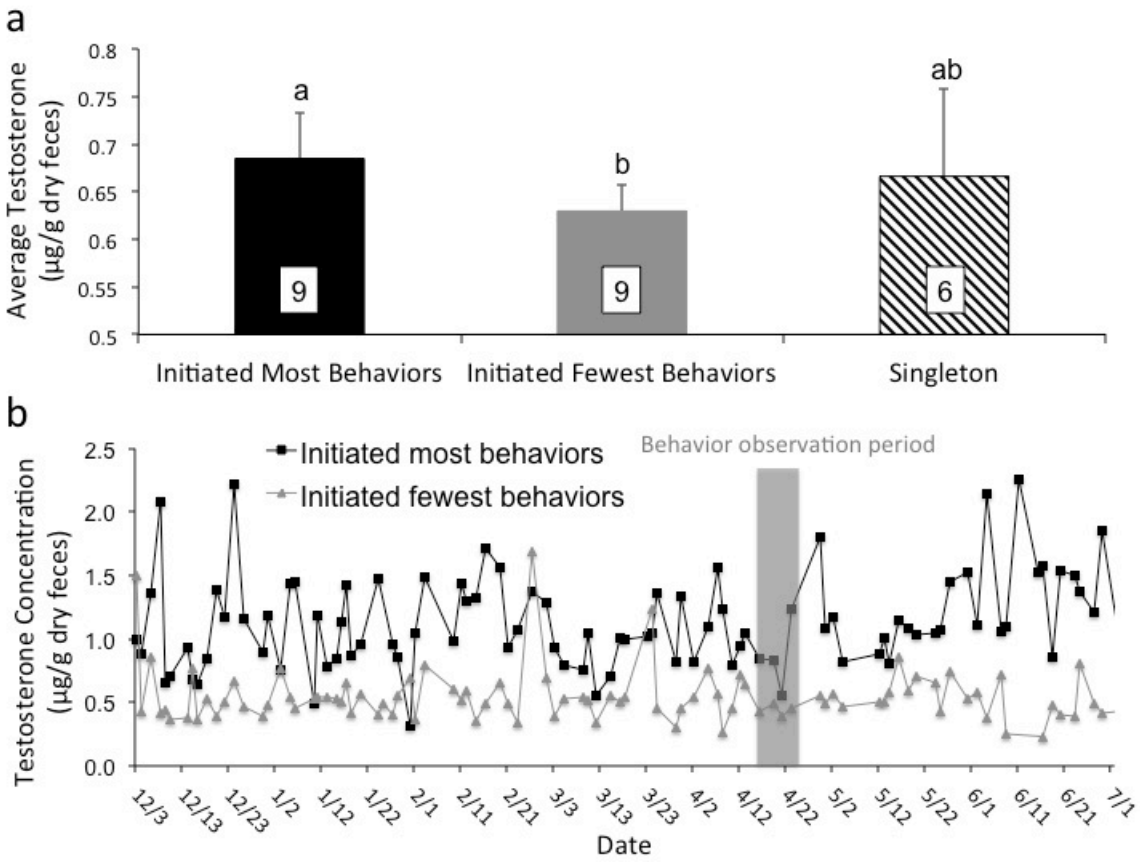


Figure 2.8 Average testosterone metabolite concentrations for male cheetahs in coalitions (n = 9) who initiated most and fewest behaviors and singleton males (n = 6) (a); representative testosterone profiles for males that initiated most and fewest behaviors within the same coalition (b)

Several ejaculate characteristics (sperm concentration, total and total motile sperm per ejaculate) were significantly higher ($P < 0.05$) for male cheetahs housed in coalitions and initiating the most behaviors with coalition members compared to males initiating the fewest behaviors and males housed as singletons (Figure 2.9). Sperm concentration was higher ($t_9 = 2.82$; $P = 0.04$) in coalition males that initiated the most behaviors (39.94 ± 1.47 x 10^6/ml) than males that initiated the fewest (16.20 ± 1.60 x 10^6/ml). However, coalition male sperm concentrations were not different from those of singletons (21.73 ±

1.57 x 10^6/ml) ($F_{1,16}$ = 1.13; P = 0.28). Total sperm per ejaculate was higher (t_{11} = 2.69; P = 0.01) in coalition males that initiated the most behaviors (41.07 ± 1.36 x 10^6) compared to singletons (11.68 ± 1.43 x 10^6), but males that initiated the fewest behaviors (21.50 ± 1.56 x 10^6; $P > 0.05$) were not different from either of the two other male groups. Finally, total motile sperm was higher in coalition males initiating the most behaviors (29.81 ± 1.34 x 10^6; $P < 0.05$) than any other males. Males that initiated the fewest (t_9 = 2.90; 13.09 ± 1.58 x 10^6) and singletons (t_9 = 2.89; 7.05 ± 1.50 x 10^6) did not have different motile sperm counts (t_{12} = 1.01; P = 0.17).

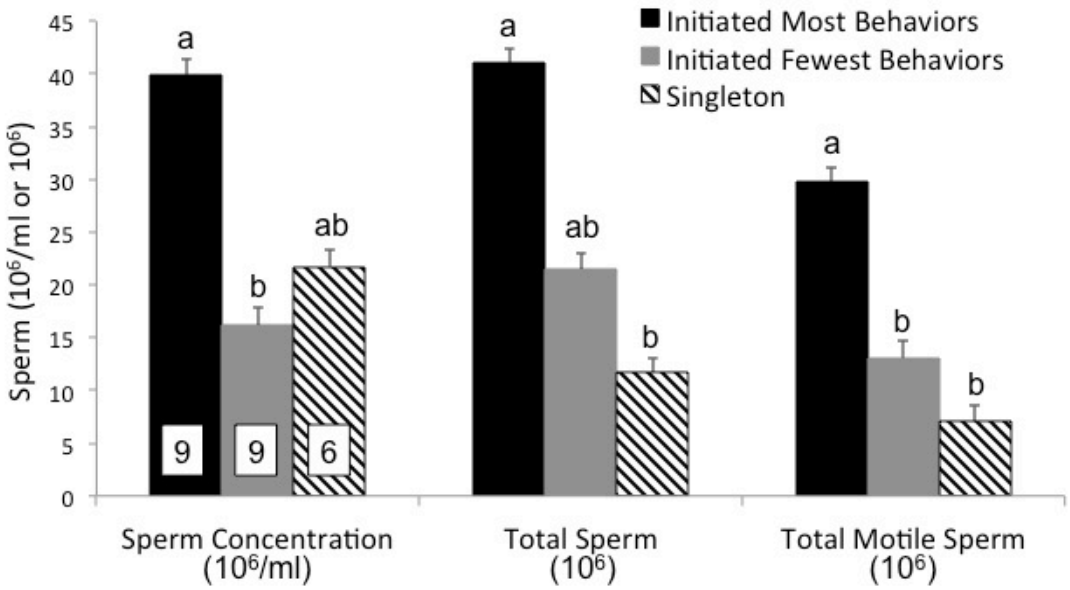


Figure 2.9 Ejaculate characteristics for male cheetahs held as singletons (n = 6) and males in coalitions (n = 18) separated by those that initiated the most and the fewest behaviors with coalition members

Discussion

As previous studies in females have shown (Caro 1994, Wielebnowski et al. 2002b, Kinoshita et al. 2011), this species has a remarkably complicated and unique social structure. Our lack of a full understanding about cheetah sociality has hurt attempts to successfully breed this species in captivity for decades. My study has helped to fill a knowledge gap, and indicated directions for future work required to increase reproductive success in managed collections of the cheetah. My main findings were: 1) fecal androgen metabolite concentrations remained unchanged throughout the year across males at all institutions, 2) longitudinal profiles of glucocorticoid metabolite excretion exhibited a large amount of variation both within and between individual males, 3) higher baseline testosterone concentrations and quantities of motile, normal sperm per ejaculate were found in males in coalitions compared to those housed as singletons, and 4) males that initiated more interactions with coalition members had higher average testosterone and motile sperm per ejaculate than other coalition members. The physiology of male cheetahs is markedly impacted by social structure and living in a group yields improved physiological metrics related to reproductive success.

This is the first study reporting longitudinal androgen profiles for male cheetahs and correlations with ejaculate quality and glucocorticoid concentrations. In agreement with previous work measuring cheetah ejaculate quality for evidence of seasonality (Crosier et al. 2007), excreted androgen metabolite concentrations remained unchanged throughout the year. My results confirm that male cheetahs are not seasonal breeders and are similar to other felids such as margay (*Leopardus wiedii*) (Brown et al. 2001), oncilla

(*Leopardus tigrinus*) (Brown et al. 2001), and Iberian lynx (*Lynx pardinus*) (Ganan et al. 2010). Longitudinal profiles of glucocorticoid metabolite excretion exhibited a remarkable amount of variation both within and between individual males. Highly variable glucocorticoid concentrations are similar to what has been observed in individual cheetahs in earlier reports (Jurke et al. 1997, Wells et al. 2004). High variability may be mitigating an effect on glucocorticoid concentrations due to grouping in this study.

Contrary to my hypothesis, higher baseline testosterone concentrations and quantities of motile, normal sperm per ejaculate were found in males in coalitions compared to those housed as singletons. Increased ejaculate quality in coalition males indicates a clear reproductive benefit to housing males in closely associated groups in captivity. All coalitions of males utilized in the present study were brothers kept together from birth or similarly aged individuals housed together from a very young age (i.e., two months old). It is known that male cheetahs exhibit behaviors indicative of distress (e.g., increased vocalization, pacing), similar to mother-young interactions of social mammals, when separated from other member(s) of their coalition, regardless of genetic relatedness, indicative of a strong psychological bond between members (Caro 1993, 1994, Ruiz-Miranda et al. 1998). The existence of a strong bond between coalitions members may be related to benefits males receive by forming coalitions in the wild, such as increased foraging success and ability to acquire and maintain territories, as well as decreased need for vigilance against predators and kleptoparasites (Caro 1994). Existence within a coalition may free males from some survival pressures and provide increased allocation of energy to reproductive function and success. Males living singly in the wild are known

to collaborate with other single males, or even with already established coalitions, and remain together for extended periods of time, perhaps because of these benefits (Caro 1994).

Interestingly, although several ejaculate characteristics were higher for coalition males, singleton males had a higher percentage of normal spermatozoa per ejaculate than coalition males. Investigation of this relationship showed a negative correlation between both total and total motile sperm and percent normal sperm, an indication that males producing higher numbers of sperm per ejaculate are doing so at the expense of sperm quality, a situation known to also occur in other teratospermic felids (Pukazhenthi et al. 2006). Coalition males were found to have more total normal sperm per ejaculate, showing the positive effect of grouping on ejaculate characteristics in cheetahs. This suggests that percent of abnormal spermatozoa, a well-known and analyzed ejaculate characteristic in cheetah, should not be considered as the only indicator of testicular functionality in the cheetah, a known teratospermic species. Changes can occur in quantity of sperm per ejaculate that are not directly proportional to morphology of spermatozoa.

Within male cheetah coalitions, there are extremely low levels of aggression, even over small or limited resources (Caro 1993). Therefore, subtle relationship nuances between coalition members are often difficult to discern. Conversations with keeping staff at each institution have revealed that they may not be able to determine coalition hierarchy despite their regular interaction with specific individuals. Reported difficulty may be exaggerated by differing levels of aggressive behavior displayed by individual

males toward human keepers that does not directly correlate with male status within a coalition. Within long-term social groups in multiple mammal species, aspects of social rank are not simply reflected by the outcome of agonistic interactions, but are rather a culmination of subtle interactions between group mates (Sapolsky 1990, Virgin and Sapolsky 1997, Burks et al. 2004, Creel 2005, Freeman et al. 2010).

Social interaction results among *ex situ* cheetahs in the present study indicated detectable behavioral differences between males in coalitions that were correlated with reproductive physiological differences. Therefore, at any given time, breeding success in wild individuals may not be shared equally among coalition members. Until these results, hypotheses about differences in reproductive success within coalitions were based primarily on speculation and the high degree of relatedness between most coalition members (Caro 1994). It is unknown if and how frequently shifts in coalition status occur in this species. Regular hierarchy shifts between members could result in relatively egalitarian increases in reproductive potential for all males within a coalition over time. A more egalitarian society would help to explain the observation of low levels of aggression between coalition males (Caro 1993), despite data supporting inequality in breeding success at a given point. Future studies will need to explore if changes in the reproductive hierarchy occur over time and whether these correlate with shifts in testicular functionality.

Based on results, in order to avoid known, aggression-related associations of the term ‘dominant’ I have chosen the descriptor Highest Reproductive Fitness (HRF) to indicate males initiating most behaviors within coalitions. Therefore, males initiating

fewest behaviors will be referred to as Lower Reproductive Fitness (LRF) males. Within coalitions, my data revealed higher average testosterone concentrations in HRF than LRF males, although neither was different from singleton concentrations. Higher testosterone, along with higher sperm quality in HRF males is indicative of some degree of social suppression of reproductive functionality occurring in males of this species. It is unlikely that suppression is occurring through a hypothalamic-pituitary-adrenal axis mechanism of suppression, however, as glucocorticoid concentrations were not different between coalition members. Within other socially living carnivores displaying reproductive suppression, such as African wild dogs (Creel et al. 1997) and dwarf mongooses (Creel 2005), dominant males have been found to have higher testosterone but similar glucocorticoid concentrations as subordinate males, also supporting a mechanism not involving 'social-stress'.

Instead, reproductive suppression may be occurring through specific behavioral interactions linked to suppression of the hypothalamic-pituitary-gonadal (HPG) axis within the brain, similar to shoving behavior of dominant female naked mole-rats (*Heterocephalus glaber*), which disrupts GnRH release in subordinate animals (Smith et al. 1997, Edrey et al. 2011). In one of the only other social felids, male lions (*Panthera leo*) maintain reproductive suppression of subordinates within coalitions by aggressive behaviors that physically prevent subordinates from breeding with females (Packer and Pusey 1982). Due to the low levels of aggressive and agonistic behavior observed within male cheetah coalitions (Caro 1993), disruption to HPG axis activity in LRF cheetahs

could be due to exposure to a pheromone produced by the HRF male within the coalition at the time.

In species such as the cheetah where there is reduced investment in parental care by males and a sexually receptive female is likely to have access to multiple males (Caro 1994), greater selectivity of choice of mating partner by females is likely to occur (Clutton-Brock 2007). The role of female mate choice in determining reproductive outcome when a female cheetah encounters a coalition of males is unknown. Successful breeding attempts in captivity often involve little aggression displayed by both partners and solicitation behavior by the female such as rolling and/or lordosis posturing (Cheetah Husbandry Manual 2009). This may be indicative of an element of female mate choice contributing to reproductive encounters in this species. In lions, in cases where estrous females outnumber males, 'excess' females are able to select preferred males for breeding (Packer and Pusey 1983). In these case, females preferred darker maned males, a trait positively correlated with higher serum testosterone, age, longer pride tenures, and better overall male fitness (West and Packer 2002). Although cheetahs lack a male ornament, there may be other physiological cues indicating individual male fitness to females. HRF males within coalitions in this study exhibited higher excreted testosterone concentrations than LRF males, one of the correlates of a preferred trait in lions. Higher testosterone may be related to fitness benefits in cheetahs other than just increased ejaculate quality measured in this study. It is therefore possible that female cheetahs will exhibit behaviors indicative of preference for HRF males within coalitions. Future studies assessing female preference in the captive environment will address this possibility.

My study supports a lack of seasonality displayed in testosterone concentrations of male cheetah held *ex situ* as well as a significant effect of grouping on testicular functionality. Although more work must be done to reveal the specific nature and related mechanisms of changes occurring in reproductive functionality within groups, this research indicates management implications on group housing of male cheetahs. Increased reproductive functionality of some members within male coalitions over singletons suggests that increased breeding success may be achieved through long-term group housing of male cheetahs.

CHAPTER THREE: REPRODUCTIVE METRICS IN MALE CHEETAHS (*ACINONYX JUBATUS*) MANAGED *EX SITU* ARE INFLUENCED BY ENVIRONMENTAL CONDITIONS

Abstract

Cheetahs in *ex situ* collections are charismatic 'ambassadors' that help to educate the public while providing unique research opportunities. Most of what is known about the physiology and veterinary/nutritional requirements of the species has been learned from studying *ex situ* collections. Such information is impossible to collect from free-living animals. Despite the value of captive cheetahs, these populations are not self-sustaining (i.e., more animals die every year than are born). Only approximately 20% of cheetahs in North American zoos have ever reproduced, and this poor fecundity is related in part to suboptimal management. There has been a tendency in zoological collections to construct breeding facilities in more spacious, naturalistic environments that are 'off-display' to the public. A retrospective analysis of data from North American zoos has revealed that more than 90% of cheetah litters produced in the last decade occurred in these off exhibit breeding centers. In the current study, I examined the effect of three environmental factors: 1) public exhibit status, 2) number of keepers on staff at each institution, and 3) number of conspecifics (of both sexes) in nearby enclosures on ejaculate quality, fecal adrenal and gonadal hormone metabolite concentrations, and behavior of male cheetahs. I hypothesized that reproductive fitness of male cheetahs is

enhanced by housing animals off exhibit, having fewer keepers on staff, and maintaining fewer conspecifics present in nearby enclosures.

My results revealed that cheetah ejaculate quality is negatively affected by exposure to high numbers of human individuals; both indirectly by housing on exhibit to the public and directly though interaction with numerous keepers. Exposure to high numbers of adult male and female conspecifics, however, does not appear to impact hormonal, seminal, or behavioral metrics in this dataset. I also found that behavioral activity level is negatively associated with adrenal output and may be indicative of individual 'stress'. These results have direct implications for management of this species because, increased breeding success may be achieved by housing male cheetahs with minimal human exposure. Future studies are needed to fully understand the complicated milieu of environmental effects on cheetah reproductive potential.

Introduction

The cheetah (*Acinonyx jubatus*) is recognized as threatened due to extirpation from most of its historic range, with continuing declines resulting in only about 10,000 individuals remaining in the wild today (Durant et al. 2008). Wild cheetahs are notoriously difficult to study due to inherent elusiveness. Those animals in *ex situ* collections are useful for educating the public, understanding basic species biology, and in developing methods applicable to wild counterparts (e.g., developing anesthetic protocols and monitoring/understanding disease sensitivity). Although scientists have been examining cheetahs for more than 30 years, this species has failed to consistently reproduce in captivity (Lindburg et al. 1993, Marker-Kraus and Grisham 1993). Findings of the Association of Zoos and Aquariums Species Survival Plan (SSP) show that only about 20% of animals in the North American population have ever reproduced (2013). This results in continued reliance upon imported cheetahs from Africa to sustain *ex situ* population numbers (Marker 2012).

Although the cheetah is known for low genetic variation (O'Brien et al. 1983, O'Brien et al. 1987) and the production of 75% malformed spermatozoa per ejaculate (Wildt et al. 1993), the relatively high fecundity of wild cheetahs indicates that these traits do not cause poor reproductive success (Caro 1994). Rather, it appears that *ex situ* cheetahs require specific husbandry and management techniques to encourage breeding success (Lindburg et al. 1993, Caro 1994, Brown et al. 1996b). It has been well documented that *ex situ* management techniques and related environmental factors (e.g., relocation, exposure to a high number of human keepers, housing in small/barren

enclosures, transfer to enclosures on exhibit to the public, and presence of conspecifics/predator species in nearby enclosures) affect adrenal glucocorticoid concentrations believed to be a measure of individual 'stress' in many felid species (Carlstead et al. 1992, Carlstead et al. 1993a, Carlstead et al. 1993b, Wielebnowski et al. 2002a, Wielebnowski et al. 2002b, Wells et al. 2004, Moreira et al. 2007). For example, adult cheetahs relocated to on exhibit areas are more likely to have higher fecal glucocorticoid metabolite concentrations after movement, particularly if the individual was held in an off exhibit enclosure previously (Wells et al. 2004).

There has been a trend in zoological collections to construct breeding facilities in more spacious, naturalistic environments that are 'off-display' to the public. A retrospective analysis of data from North American zoos has revealed that more than 90% of the cheetah litters produced in the last decade occurred in these off exhibit breeding centers (Marker 2012, 2013). Additionally, fecal glucocorticoid metabolite concentrations are higher in female cheetahs classified as non-reproductive, based on behavioral indicators of estrus and male interaction, than reproductive females (Jurke et al. 1997). Unfamiliar/unrelated female cheetahs housed in pairs, however, are known to experience prolonged periods of ovarian acyclicity, which is rapidly reversed upon separation, even by a single fence line (Wielebnowski et al. 2002b). A relationship between environmental 'stressors' and decreased reproductive activity has been documented in a variety of species including several small felids (Mellen 1991, Moreira et al. 2007), Syrian hamsters (*Mesocricetus auratus*) (Jasnow et al. 2001), and black (*Diceros bicornis*) and white rhinoceros (*Ceratotherium simum*) species, (Carlstead and

Brown 2005, Metrione and Harder 2011). Despite the negative relationship between stress and reproductive function suggested by these data, to date, there has been little exploration of what, or how, environmental factors exert an impact on reproduction in cheetahs.

In an effort to address the current paucity of information in this area, my study examined the effect of three environmental factors that are highly variable between *ex situ* institutions on reproductive functionality of male cheetahs: 1) public exhibit status, 2) number of keepers on staff at each institution, and 3) number of conspecifics (of both sexes) in nearby enclosures. These factors are known to increase glucocorticoid concentrations in multiple felid species (Carlstead et al. 1992, Carlstead et al. 1993b, Wielebnowski et al. 2002a). To investigate the effect of these factors, I conducted long-term data collection from a relatively large population of adult male cheetahs in North American managed collections, all recommended for breeding by the cheetah SSP (2013). The first phase of this study was to investigate the effect of these three environmental factors on seminal characteristics as an indicator of reproductive potential. The second part of the study was to determine the relationship between adrenal and testicular hormone concentrations, behavior/activity levels, and observed seminal effects from these environmental factors. My hypothesis was that reproductive fitness in the male cheetah is enhanced in individuals that are housed in institutions that are off exhibit to the public, have fewer keepers on staff, and contain fewer conspecifics of both sexes present in nearby enclosures. I hypothesized that this effect is reflected in better quality ejaculates, higher excreted androgens, lower glucocorticoids, and higher activity levels.

Materials and Methods

Animals and Environmental Conditions

This study utilized twenty-nine captive-born, adult male cheetahs housed in seven Association of Zoos and Aquariums (AZA) accredited institutions across the United States. Animals were housed and managed according to the Cheetah SSP (2013) and approved husbandry guidelines for the species (2009). The Institutional Animal Care and Use Committees (IACUC) of all involved facilities approved animal procedures. Males ranged in age from two to 12 years, considered prime reproductive years (Crosier et al. 2007), and were recommended for breeding by the Cheetah SSP at the time of data collection (2013). Cheetahs were assumed to be reproductively mature at two years and likely to exert an effect on surrounding conspecifics at this point (Crosier et al. 2007).

Males were considered housed on exhibit to the public if they were exposed (visual and assumed olfactory and auditory contact) to more than 50 different people each month for five or more months of the year. Off exhibit males do not fall into this category. Keeper number was the number of individual humans that had direct contact (e.g., through feeding, shifting between enclosures, training, offering enrichment, cleaning, etc.) with the cheetah on a regular basis. Conspecific number was the total number of male or female cheetahs two years of age or older that were housed at the same institution within visual and/or olfactory range of the male for the majority of the fecal collection period. These data were generated for each individual male both through contact with head cheetah managers and by administration of a simplistic survey near the end of the fecal collection period.

Fecal Collection and Preparation

Freshly voided feces were collected three to four days per week for a period of seven to thirteen months for each male into clean plastic bags, labeled with animal number and date. Samples were then stored or shipped frozen (-20°C) to the SCBI for processing. A non-digestible and harmless marker (e.g., glitter, birdseed, lentils, corn) was added to the diet of coalition males to identify individual fecal samples. All samples (n = 4,653) were then freeze-dried (Lyophilizer; Labconco) to remove water, pulverized using a rubber mallet, and stored in labeled plastic tubes at -20°C until processing.

Steroid hormone metabolites were extracted from fecal samples using previously described methods (Graham and Brown 1996). After 0.2 g of well-mixed fecal powder from each sample was boiled in 5 ml of 90% ethanol for 20 minutes, extracts were centrifuged to remove particulates and the supernatant was transferred into a glass tube. The pellet was resuspended in an additional 5 ml of 90% ethanol, vortexed for 30 seconds, and recentrifuged. Combined ethanol supernatants were dried under air and then resuspended in 1 ml of 100% methanol. Methanol extracts were dried under air and then resuspended in 1 ml of BSA-free phosphate buffer ($NaH_2PO_4 + Na_2HPO_4 + NaCl + H_2O$; pH = 7.0). Extracts were sonicated (15 minutes) and vortexed (30 seconds) prior to decanting into a plastic tube for freeze storage (-20°C) until evaluation for hormone metabolite concentration.

The efficiency of steroid extraction from feces was evaluated by adding radiolabeled hormone (^{3}H-cortisol or ^{3}H-testosterone; 4000-8000 dpm) to each fecal sample prior to boiling extraction. The minimum recovery of radiolabeled hormone from

fecal extracts acceptable for enzyme immunoassay (EIA) was 60% and the mean recovery across males was ~75%. Fecal extracts were diluted 1:20 and 1:200 concentration in BSA-free phosphate buffer for cortisol and testosterone EIA respectively. Samples considered too dilute (binding > 80% of maximum binding) were run at a higher concentration (1:10 – 1:100), and samples too concentrated (binding < 20%) were run at a lower concentration (1:200 – 1:2000).

Fecal Glucocorticoid Metabolite Analysis

Glucocorticoid metabolite concentrations in diluted fecal extracts were determined using a cortisol EIA validated for use in cheetahs (Young et al. 2004). This cortisol EIA employed a polyclonal antibody (1:8500; R4866; C. Munro, University of California, Davis, CA) raised in rabbits against cortisol-3-carboxymethyloxime linked to bovine serum albumin and cross-reacts with cortisol 100%, prednisolone 9.9%, prednisone 6.3%, cortisone 5%, and < 1% with corticosterone, desoxycorticosterone, 21-desoxycortisone, testosterone, androstenedione, androsterone, and 11-desoxycortisol (Young et al. 2004). The antibody was added to 96-well microtiter plates (Nunc-Immuno, Maxisorp; Fisher Scientific) and allowed to equilibrate for 12 – 48 hours (4°C). Unbound antibody was removed with wash solution and diluted samples, in duplicate, and standards, in triplicate, (0.05 ml; 78 – 20,000 pg/ml; Sigma Diagnostics) were added to the plate. A peroxidase enzyme-conjugated cortisol (1:20,000; 0.05 ml; C. Munro) was then added to each well containing sample or standard and the plate was allowed to incubate for one hour (23°C) before unbound components were removed. A chromagen solution was added (0.1 ml) to each well and incubated ~15 minutes before optical

densities were determined using a microplate reader (Dynex MRX, reading filter 405 nm, reference filter 540nm).

Sensitivity of the assay at maximum binding was 3.9 pg/well. The inter-assay coefficient of variation for two internal controls was 8.36% (mean binding – 27.88%) and 4.84% (mean binding – 68.12%) and intra-assay coefficient of variation was < 10% (n = 364 assays). Serially diluted pooled fecal extracts demonstrated displacement curves parallel to those of standard hormone preparations. Recovery of added standard to fecal extract ($y = 0.90x – 18.02$, $r = 0.99$) demonstrated significant recovery ($P < 0.05$). Several measures were calculated to summarize fecal glucocorticoid values during the longitudinal steroid evaluation of each male; 1) an overall mean of all samples for the collection period, 2) a mean baseline that excludes all values greater than the overall mean plus 1.5 standard deviations (SD) (Graham and Brown 1996), and 3) a peak frequency that divides the number of samples greater than three times baseline by total number of samples collected for that male (Young et al. 2004).

Fecal Androgen Metabolite Analysis

There is currently no published EIA to quantify fecal androgen metabolites in the cheetah. Physiological validation of the utilized EIA is described in Chapter 2. The number and relative proportions of testosterone metabolites in cheetah fecal extracts was determined by reverse-phase high-performance liquid chromatography (HPLC; (Brown et al. 1996a)). The selected EIA relied on a polyclonal anti-androgen (1:7500) antibody (R156/7; C. Munro, University of California, Davis, CA) used to measure testosterone metabolites from fecal extracts in other species such as the giant panda (Aitken-Palmer et

al. 2012). This antibody cross-reacts with testosterone 100%, 5α-dihydrotestosterone 57.37%, < 1% with androstenedione, androsterone, androsteneolone, cholesterol, and β-estradiol, and < 0.02% with progesterone, pregnenolone, and hydrocortisone. Microtiter plates were run in the same way as for glucocorticoid analysis; diluted samples in duplicate, standards in triplicate (0.05 ml; 46 – 12,000 pg/ml; 17β-hydroxy-4-androstein-3-one; Steraloids), and a peroxidase enzyme-conjugated testosterone (1:15,000; 0.05 ml; C. Munro) was added to the plate. The plate was then allowed to incubate for two hours (23°C) before unbound components were removed, a chromagen solution added, and optical densities read.

Sensitivity of the testosterone EIA at maximum binding was 2.3 pg/well. The inter-assay coefficient of variation for two internal controls was 10.64% (mean binding – 24.14%) and 5.01% (mean binding – 66.18%) and intra-assay coefficient of variation was < 10% (n = 326 assays). Serially diluted pooled fecal extracts demonstrated displacement curves parallel to those of standard hormone preparations. Recovery of added standard to fecal extract ($y = 1.08x - 3.80$, $r = 0.99$) demonstrated significant recovery ($P < 0.05$). Several measures were calculated to summarize fecal testosterone values during the longitudinal steroid evaluation of each male, 1) an overall mean of all samples for the collection period and 2) a mean baseline that excludes all values greater than the overall mean plus 1.5 standard deviations (SD).

Semen Collection, Evaluation, and Processing

A minimum of one semen collection at least two months after commencement of sampling was done on each male. Ejaculates from three groups of three males each were

also collected opportunistically after they were moved between institutions following completion of fecal collection for this study. Semen collection was completed for these males at least five months after translocation. Methods utilized for anesthesia and semen collection and evaluation were similar to previous studies on cheetah (Wildt et al. 1983, Wildt et al. 1988, Wildt et al. 1993, Crosier et al. 2006). To induce a surgical plane of anesthesia, medetomidine hydrochloride, midazolam, and butorphanol (Domitor®, Pfizer Inc., La Jolla, CA; 22 – 25 μg/kg, 0.2 mg/kg, and 0.3 mg/kg respectively) were injected intramuscularly by hand using a restraint-cage technique or using a pole-syringe or air-pressured darting system if necessary according to protocols described in the Cheetah Husbandry Manual (2009). If needed, propofol (0.5 – 4 mg/kg, intravenously) was administered during the electroejaculation to maintain an appropriate level of anesthesia. Laboratory calipers were used to measure testicular length and width, which was then used to estimate testicular volume for each male (Howard et al. 1990). A rectal probe of either 1.6 of 1.9 cm in diameter with three longitudinal electrodes and an electrostimulator (P.T. Electonics, Boring, OR) were used to administer 80 stimuli (2 – 5 V) over a 30-minute interval (Wildt et al. 1983, Wildt et al. 1993).

Semen was collected in pre-warmed, sterile collection vials, and an aliquot (3 μl) was immediately assessed for sperm percent motility and forward progressive status (scale: 0 – 5, five rating equivalent to rapid, straightforward progress (Howard et al. 1990)). A 15 – 20 μl aliquot of raw semen was fixed in 100 μl of 0.3% glutaraldehyde in phosphate buffered saline (pH, 7.4) for assessing sperm morphology (1000x). Spermatozoa were classified as normal or as having one of the following abnormalities:

1) head abnormalities including micro-, macro-, and bi-cephalic; 2) acrosomal abnormalities including missing or damaged acrosomal membranes; 3) midpiece abnormalities including abnormal or missing midpiece, and a bent midpiece with or without retained cytoplasmic droplet; 4) flagellar abnormalities including tightly coiled flagellum, bent flagellum with or without retained cytoplasmic droplet, bi-flagellate, retained proximal or distal droplet; and 5) other abnormalities including spermatid and bent neck (Crosier et al. 2007). Total volume of the ejaculate was measured using a pipette and the remainder, after motility and morphology aliquots were removed, was immediately diluted with an equal volume of sterile Ham's F10 culture medium (HF10; Irvine Scientific, Santa Ana, CA) supplemented with 20 mM Hepes, 5% (v:v) fetal calf serum (Irvine Scientific), pyruvate (1 mM), glutamine (2 mM), 10,000 IU/ml penicillin, 10 mg/ml streptomycin, and 20 mg/ml neomycin (Sigma Chemical, St. Louis, MO). Sperm concentration was determined using a hemocytometer (Wildt et al. 1983). Diluted samples were centrifuged (Eppendorf Mini-Spin, Hamburg, Germany) for 8 minutes at 100*g* and the supernatant was aspirated from the sperm pellet. The pellet was then resuspended in cryodiluents and slow-cooled before being loaded into 0.25 ml straws and cryopreserved over liquid nitrogen using a previously determined protocol (Crosier et al. 2006) for genome banking and utilization by additional studies.

Behavioral Data Collection

Behavioral evaluations were conducted on eight successive days during the period of fecal collection. Coalitions of males were observed for one-hour and singletons for a 30-minute period each day in their home enclosure to establish baseline behavioral

frequencies and activity level. Instantaneous sampling (two minute time intervals for coalitions males and one minute intervals for singletons) was used to estimate time spent in any behavior, and continuous scan sampling was used to measure behavioral event frequencies (Martin and Bateson 2007). One-hour sessions were completed in the morning between 0600 and 1000 and before feeding whenever possible, to avoid discrepancies between males in displayed behaviors and activity level due to time of day or state of hunger. Observations were recorded using methods and definitions of observable behaviors described in the Cheetah Husbandry Manual (2009), and consistent with previously published ethograms for the cheetah (Wielebnowski and Brown 1998, Wielebnowski et al. 2002b) (Table 3.1). To maintain consistency, a single researcher conducted all behavior observations.

In order to test for repeatability of baseline behavior data gathered across multiple environmental conditions (e.g., temperature or season), a subset of males (six individuals in two coalitions) was observed approximately every ten days for the duration of the fecal collection period by the same researcher collecting all behavioral data. This multi-seasonal data was compared to data collected for the same six males during eight successive days, as was protocol for all males in this study, to determine effects of seasonal variations on behavioral frequencies and activity levels. There were no differences ($P > 0.05$) between these data sets indicating behavioral data collection as described for this study was representative of baseline behaviors exhibited by individual males, regardless of season or temperature.

Table 3.1 Behavioral state and event definitions for *ex situ* male cheetahs

Behavioral States	
Laying	Side of body in contact with ground, legs to side, eyes open
Standing	As implied
Sleeping	Laying with eyes closed
Crouching	Legs under body like standing, but legs bent, body close to ground
Sitting	As implied
Walking	Forward locomotion at relxed speed, includes trot
Pacing	Walking back and forth across same area repeatedly
Running	Rapid forward locomotion with full extension of limbs and tail
Out of Sight	Cheetah is unable to be seen by observer to assess state
Behavioral Events	
Rub	Rubs face, head, or neck on object or conspecific
Roll	Rolls from one side to another, back on ground, paws in the air
Sniff	Olfactory examination of ground, object, or conspecific
Groom	Lick, chew, or otherwise clean self or conspecific
Stutter	Repetitive short throat calls
Meow	Soft call, similar to that of domestic cat
Chirp	High pitched, short call, corners of mouth drawn back
Growl	Low, drawn out "snarling" sound, mouth open showing teeth
Hiss	Silent, drawn out expulsion of air with mouth and eyes wide open
Tread	Scraping ground with rear legs without forward locomotion
Urinate	Excretion of urine from a sitting or squat position
Urine Spray	Sprays urine directly behind from standing position with tail raised
Defecate	As implied

Statistical Analysis

Prior to analysis, statistical outliers were determined (Hoaglin and Iglewicz 1987) and replaced with the mean for all sperm, hormone, and behavior variables (Sokal and Rohlf 2012). Normality and homoscedasticity were verified using an Anderson-Darling and a Levene's test, respectively (Grafen and Hails 2002). Three sperm metrics, total sperm, total motile sperm, and sperm concentration were log transformed to achieve normality. To maintain statistical integrity, these three sperm metrics were back

transformed for visual representation. Relationships between hormone, sperm, and behavioral metrics were evaluated using a Pearson's correlation coefficient (Grafen and Hails 2002). Hormonal metrics analyzed included mean and mean baseline concentration for both glucocorticoid and testosterone metabolite excretion, and peak frequency of glucocorticoid metabolite excretion. Sperm metrics analyzed included total testicular volume, ejaculate volume, sperm concentration, total sperm per ejaculate, percent motile sperm per ejaculate, progressive status, total motile sperm per ejaculate, and percent normal sperm per ejaculate (Crosier et al. 2007). Behavioral metrics analyzed were principle component scores resulting from principle component analysis (PCA).

A retrospective analysis was completed on sperm metrics from the Smithsonian National Zoological Park's (NZP) historical sperm collection database, including ejaculate characteristics from semen collections completed during the last 33 years at more than 25 institutions worldwide. Historical sperm metrics were analyzed for effect of exhibit status using a student's *t*-test (Grafen and Hails 2002). Sperm metrics from opportunistic semen collections completed on nine males moved between institutions were analyzed using a Wilcoxon signed-rank test (Grafen and Hails 2002). All statistical procedures were completed using SAS software (version 9.2, Cary, NC). Effects were considered significant at $P < 0.05$ and data are reported as mean ± standard error of the mean (SEM).

The effect of environmental factors on sperm metrics and subsequently hormonal and behavioral metrics were analyzed using a nested, multivariate mixed model that held exhibit status (on or off exhibit to the public), number of keepers (institutions with ≤3 or

>3 keepers on staff), and number of conspecifics (≤11 conspecifics or >11 conspecifics of either sex held in nearby enclosures) as fixed factors, while controlling for the effects of institution, age, grouping (coalition or singleton), and interaction between grouping, exhibit status, and institution, as well as between exhibit status and institution. Specific numerical cutoffs for number of keepers (≤3 or >3 keepers) and conspecifics (≤11 conspecifics or >11 conspecifics) were selected based on what would give the model the most statistical power utilizing the population of males grouped at institutions participating this study. However, the effects of two factors, exhibit status and number of conspecifics, could not be differentiated by the model. This was because most off exhibit facilities managed high numbers of cheetahs while on exhibit institutions held few individuals. Therefore, the model was only able to indicate whether or not there was a significant impact of these two factors together on specific variables, but it was unable to distinguish which factor was having the greater effect. For post-hoc analysis, the model had already determined that age had no effect on identified significant variables and we chose to analyze only coalition males to control for the effect of grouping on the results, leaving 23 males to be analyzed. Mann-Whitney U tests (Grafen and Hails 2002) were then conducted on each variable deemed significant by the original model for these males across groups based on exhibit status and ≤11 or >11 conspecifics, separately.

All behavioral state data were converted to proportion of time each male spent per hour in each state. Behavioral event frequencies were reported as average frequency per hour for each male for each behavior (Martin and Bateson 2007). ‘Laying’ and ‘sleeping’ behavioral states were combined into a single ‘resting’ score, and ‘meow’ and ‘chirp’

behavioral events were combined into a 'meowchirp' category to achieve normality for these metrics. The behavioral event 'hiss' was removed from analysis because only a single incidence of this behavior was observed in a single individual. Principle component analysis (PCA) was used to identify correlated groups of behavioral state and event data (Martin and Bateson 2007). Components with eigenvalues of more than one were retained, labeled according to the variables that showed the highest loadings, and analyzed as behavioral metrics in the statistical model (Wielebnowski 1999). Principle component scores required no removal of outliers or transformations to achieve normality before analysis in multivariate mixed model.

Results

Ejaculate Characteristics

The statistical model revealed a significant effect of exhibit status on the ejaculate characteristics total sperm ($F_{1,16} = 2.42$; $P = 0.03$), total motile sperm ($F_{1,16} = 2.25$; $P = 0.04$), and sperm concentration ($F_{1,16} = 2.26$; $P = 0.04$). The model also reported a significant effect of number of conspecifics on the same three ejaculate characteristics, total sperm ($F_{1,16} = 2.47$; $P = 0.03$), total motile sperm ($F_{1,16} = 2.29$; $P = 0.04$), and sperm concentration ($F_{1,16} = 2.27$; $P = 0.04$). These two variables were determined to be confounded in the model, therefore, separate post-hoc analysis of significant variables for both factors, exhibit status and number of conspecifics, was performed. Post-hoc analysis revealed off exhibit males to have higher total sperm ($U = 215$; $60.97 \pm 11.42 \times 10^6$; $P = 0.02$), total motile sperm ($U = 211$; $42.28 \pm 8.15 \times 10^6$; $P = 0.04$), and sperm concentration ($U = 213$; $63.83 \pm 16.22 \times 10^6$/ml; $P = 0.03$) than those housed on exhibit

to the public (total sperm: 20.46 ± 7.39 x 10^6, total motile sperm: 15.42 ± 5.91 x 10^6, sperm concentration: 19.31 ± 7.62 x 10^6/ml) (Figure 3.1).

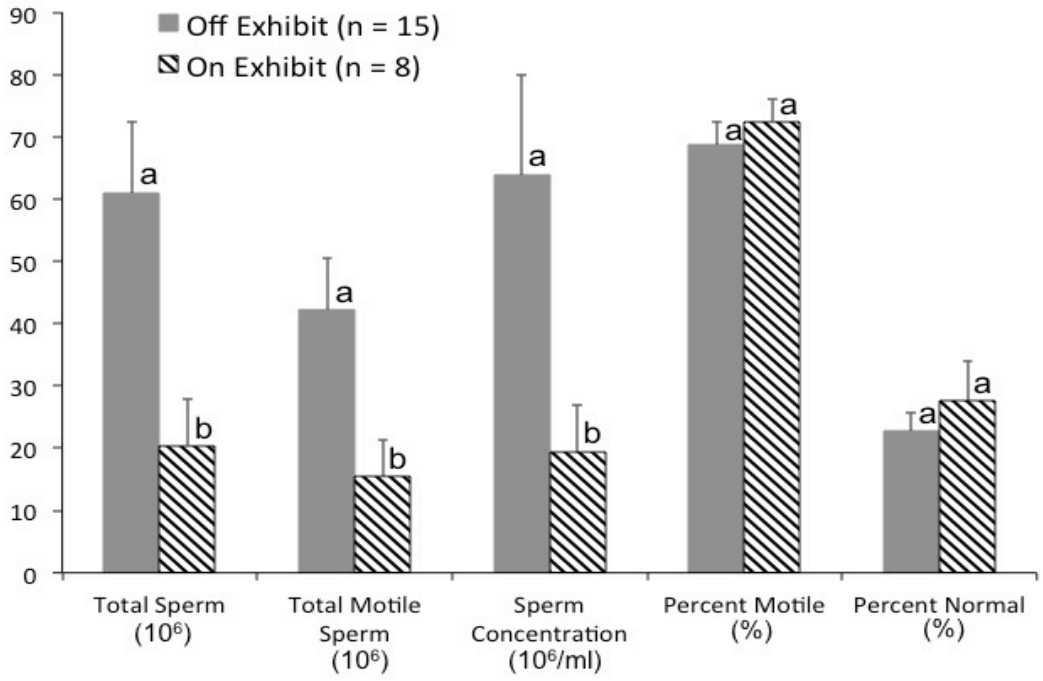


Figure 3.1 Ejaculate characteristics for *ex situ* male cheetahs (n = 23) housed on or off exhibit to the public

These results led to a retrospective analysis of the Smithsonian National Zoological Park's (NZP) historical sperm collection database completed using our definition of exhibit status. Results were the same as those obtained in this study (Figure 3.2). Off exhibit males had higher total sperm (t_{48} = 3.49; 83.92 ± 14.06 x 10^6; $P < 0.01$), total motile sperm (t_{47} = 3.34; 62.00 ± 11.31 x 10^6; $P < 0.01$), and sperm concentration (t_{50} = 3.95; 64.49 ± 9.35 x 10^6/ml; $P < 0.01$) than males housed on exhibit to the public

(total sperm: 32.98 ± 3.86 x 10^6, total motile sperm: 23.13 ± 2.77 x 10^6, sperm concentration: 25.68 ± 3.00 x 10^6/ml). Within the historical collection database, off exhibit males had similar percent motile (t_{112} = 1.41; 69.65 ± 1.68%; P = 0.08) and percent normal values (t_{47} = 1.20; 24.41 ± 2.87%; P = 0.11) to on exhibit males (percent motile: 66.47 ± 1.53%; percent normal: 20.40 ± 1.73%).

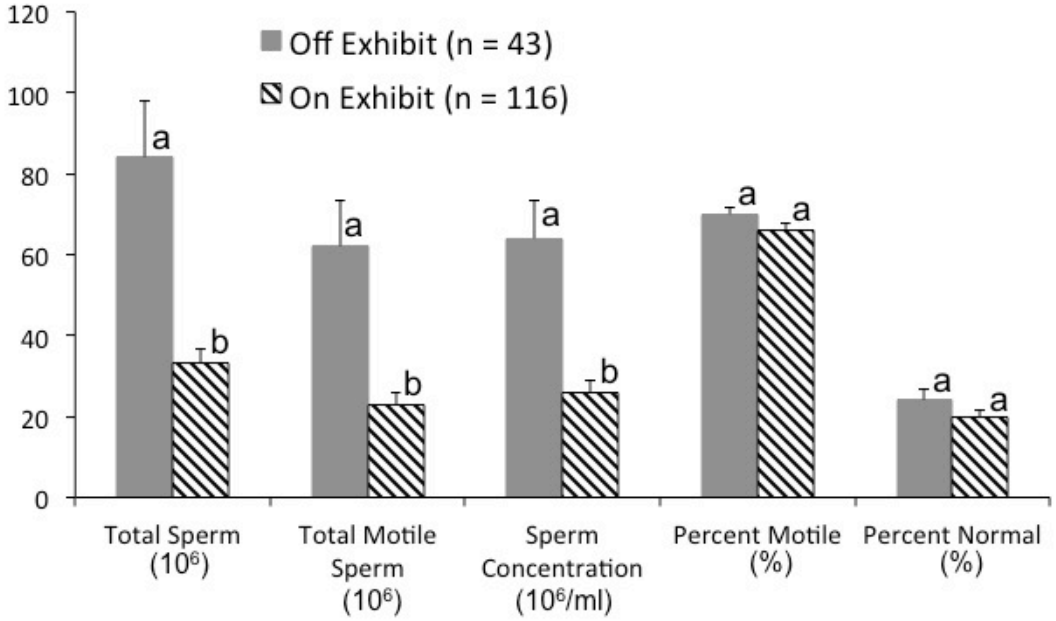


Figure 3.2 Ejaculate characteristics from male cheetah electroejaculations recorded in the NZP historical sperm collection database while housed both on and off exhibit to the public

Ejaculates analyzed from males collected after relocation to new institutions yielded no differences (P > 0.05) (Figure 3.3), but a trend of increased seminal quality can be observed in one group of three males moved from an on exhibit to an off exhibit institution (Figure 3.3a).

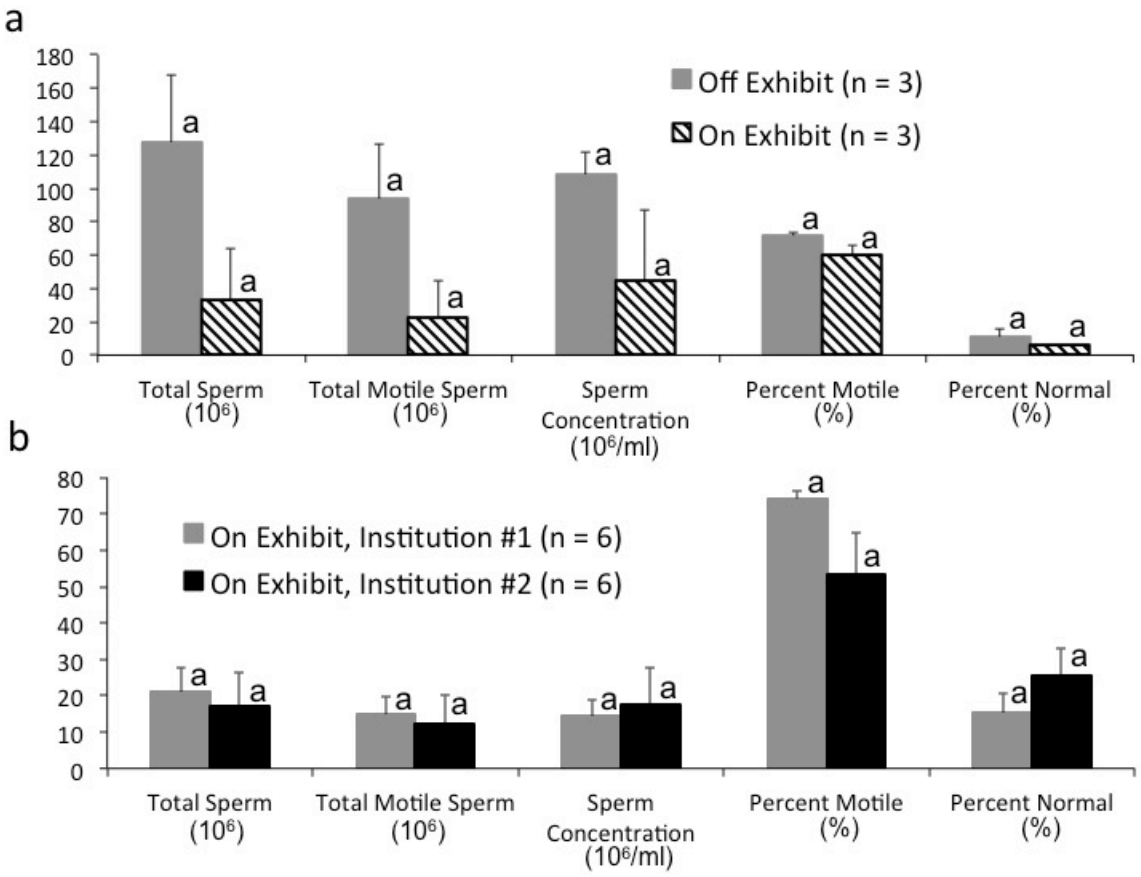


Figure 3.3 Seminal characteristics opportunistically collected from male cheetahs moved between on and off exhibit institutions (a), and between two on exhibit institutions (b)

Post-hoc analysis revealed no differences ($P > 0.05$) in ejaculate characteristics for males housed at institutions with ≤11 conspecifics or >11 conspecifics in nearby enclosures (Figure 3.4). However, my model did reveal that males at institutions with three or fewer keepers on staff had more total ($F_{1,16} = 2.69$; $26.92 \pm 1.31 \times 10^6$; $P = 0.02$) and total motile ($F_{1,16} = 2.45$; $17.16 \pm 1.34 \times 10^6$; $P = 0.03$) sperm per ejaculate compared to males housed in facilities with more than three keepers on staff (total sperm: 19.95 ±

1.43 x 10^6, total motile sperm: 13.72 ± 1.45 x 10^6) (Figure 3.5). All other sperm metrics were not different ($P > 0.05$) between institutions with different numbers of keepers.

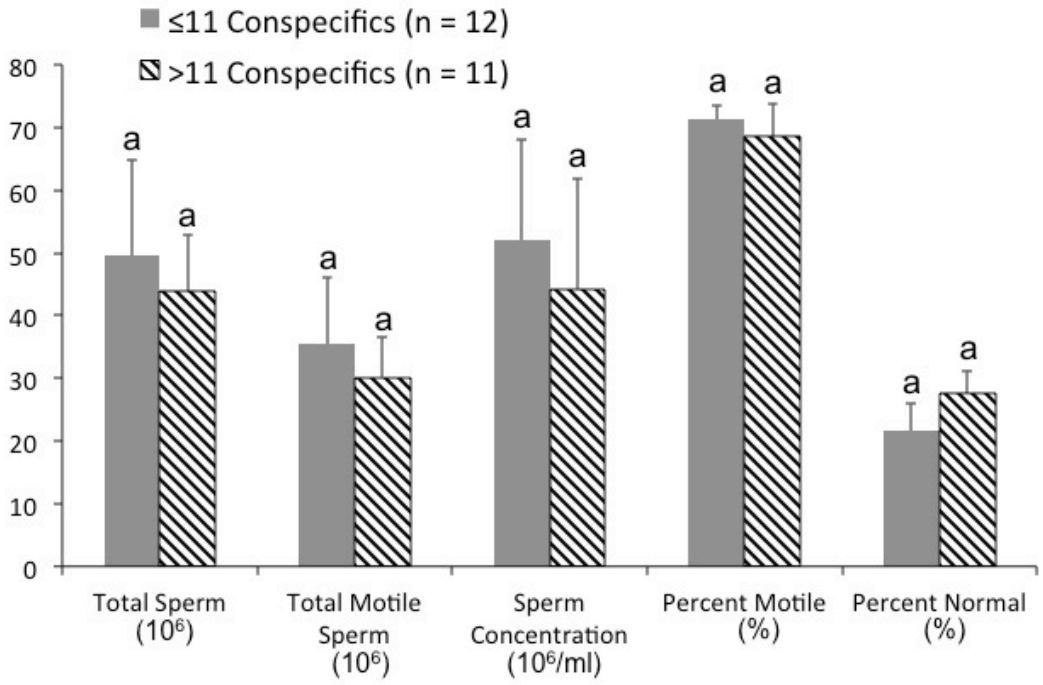


Figure 3.4 Ejaculate characteristics for *ex situ* male cheetahs (n = 23) housed at institutions with ≤11 or >11 conspecifics in nearby enclosures

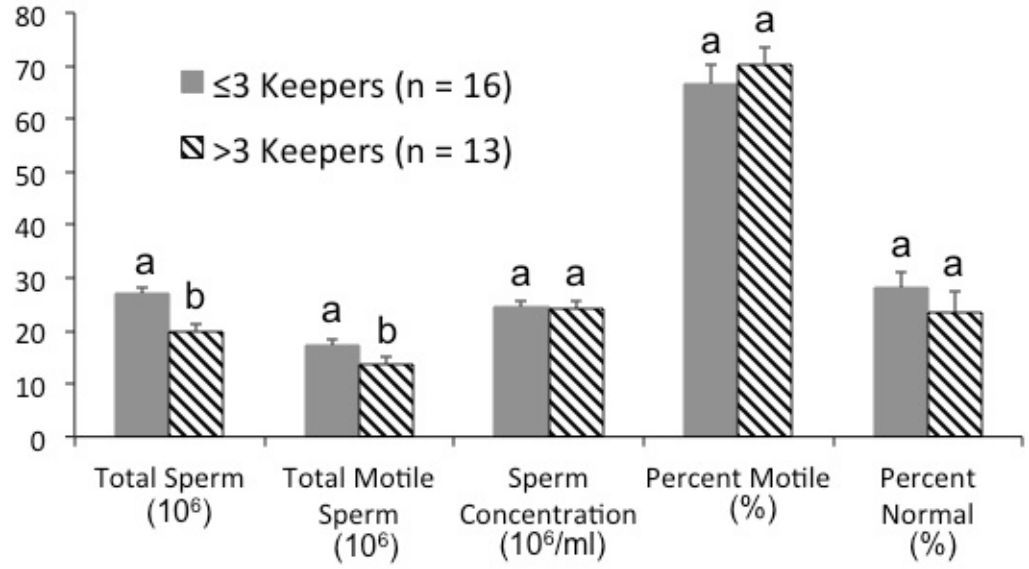


Figure 3.5 Ejaculate characteristics for *ex situ* male cheetahs (n = 29) housed at institutions with ≤3 or >3 keepers on staff

Hormone Concentrations and Behaviors

There were no differences ($P > 0.05$) in adrenal or gonadal hormone concentrations (i.e., average or baseline) between males for any of the environmental factors investigated (Table 3.2). Individual male profiles were highly variable, and these variations did not correlate with seminal characteristic differences between males of different exhibit status or keeper number (Figure 3.6). Additionally, there was no correlation ($P > 0.05$) between testosterone concentrations (i.e., average and baseline), glucocorticoid concentrations (i.e., average, baseline, and peak frequency), and testicular/ejaculate metrics (i.e., total testicular volume, ejaculate volume, sperm concentration, total sperm per ejaculate, percent motile sperm per ejaculate, progressive status, total motile sperm per ejaculate, and percent normal sperm per ejaculate).

Table 3.2 Gonadal and adrenal hormone metabolite concentrations for *ex situ* male cheetahs (n = 29) housed in different environmental conditions

Environmental Condition	Testosterone Concentration (µg/g)		Glucocorticoid Concentration (µg/g)	
	Average (±SEM)	Baseline (±SEM)	Average (±SEM)	Baseline (±SEM)
Off Exhibit (n = 17)	0.62 ± 0.02	0.47 ± 0.02	0.47 ± 0.05	0.19 ± 0.01
On Exhibit (n = 12)	0.70 ± 0.04	0.52 ± 0.03	0.41 ± 0.05	0.20 ± 0.02
≤3 Keepers (n = 16)	0.62 ± 0.03	0.47 ± 0.02	0.43 ± 0.04	0.18 ± 0.01
>3 Keepers (n = 13)	0.69 ± 0.04	0.52 ± 0.03	0.46 ± 0.06	0.23 ± 0.03
≤11 Conspecifics (n = 15)	0.62 ± 0.03	0.47 ± 0.02	0.47 ± 0.04	0.21 ± 0.02
>11 Conspecifics (n = 14)	0.68 ± 0.04	0.52 ± 0.03	0.52 ± 0.06	0.23 ± 0.02

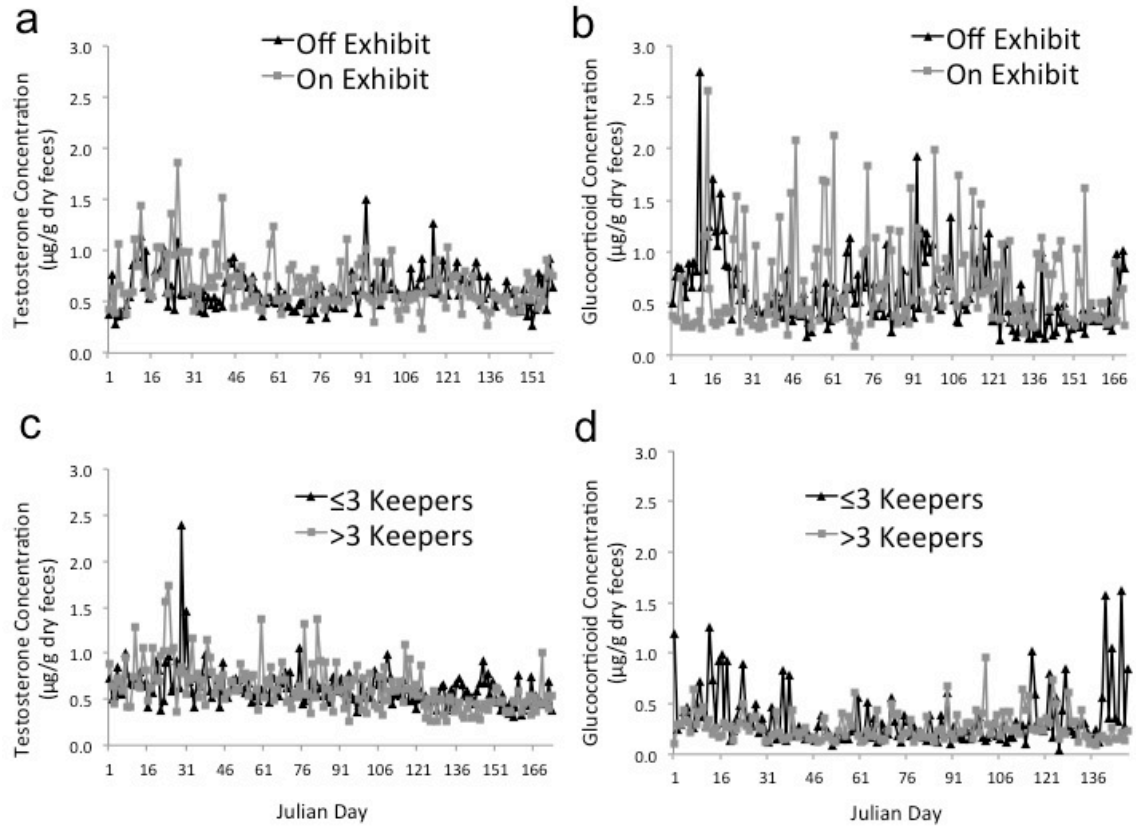


Figure 3.6 Gonadal (a and c) and adrenal (b and d) hormone profiles of two representative male cheetahs in each environmental condition – exhibit status (a and b) and keeper number (c and d)

All behavioral states were grouped into two principle components with eigenvalues of more than one, together accounting for 64% of the total variation (Table 3.3). The states standing, walking, pacing, running, and negative resting were grouped as the first principle component and these were collectively termed 'active states'. Sitting and crouching states were grouped as the second principle component and these were together termed 'inactive states'.

Table 3.3 Component loadings for principle components analysis of 7 behavioral state variables for 29 adult male cheetahs

	Component 1	Component 2
Variance explained	0.39	0.25
Label	Active States	Inactive States
Resting	**-48**	-40
Standing	**40**	-33
Crouching	-2	**68**
Sitting	18	**36**
Walking	**45**	-36
Pacing	**47**	6
Running	**39**	6

Values are multiplied by 100 and rounded to the nearest integer. Bold = highest loading/variable

The mixed effects model revealed that males housed in institutions with three or fewer keepers on staff spent more time per hour ($F_{1,16} = 3.91$; $P < 0.01$) in an active state (56.04 ± 5.35%) than males in institutions with more than three keepers on staff (34.97 ± 5.41%) (Figure 3.7a). There was no difference in time spent in an active state between males housed on or off exhibit to the public ($F_{1,16} = 1.26$; $P = 0.23$), or between males housed at institutions with ≤11 conspecifics or >11 conspecifics in nearby enclosures ($F_{1,16} = 1.53$; $P = 0.14$). Average time spent in an active state per hour was also negatively correlated ($r = -0.64$; $P < 0.01$) with baseline glucocorticoid (Figure 3.7b) and average glucocorticoid metabolite concentration ($r = -0.43$; $P = 0.04$). There were no additional relationships between behavioral state scores and hormonal or sperm metrics determined by correlation analysis ($P > 0.05$).

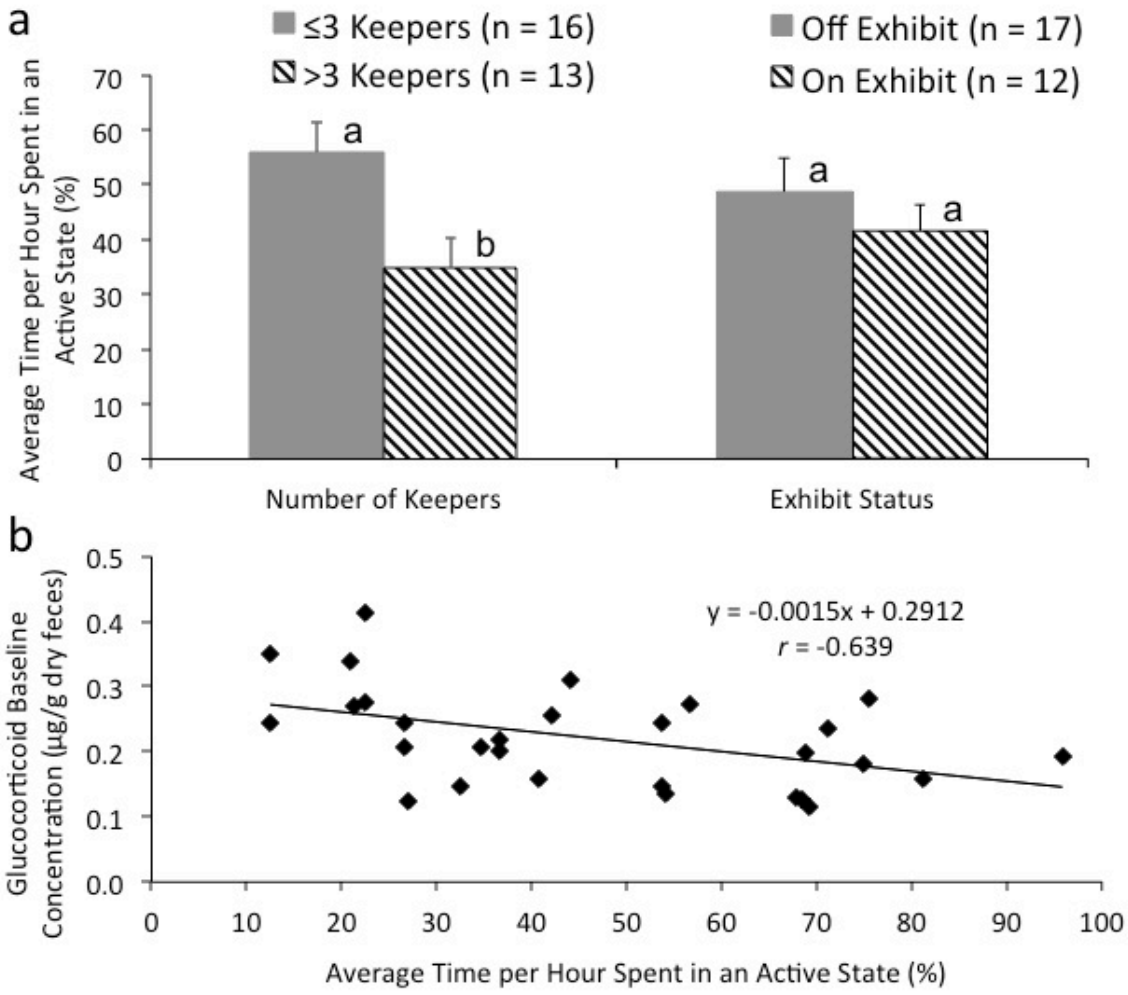


Figure 3.7 Average time per hour male cheetahs spent in an active behavioral state across the environmental factors number of keepers and housing status (a), relationship between average time males spent in an active state and baseline glucocorticoid concentration (b)

Behavior frequencies analyzed by PCA yielded four principle components with eigenvalues of more than one, together accounting for 66% of the total variation (Table 3.4). The behavioral events rub, roll, and negative meowchirp were grouped as the first principle component and these were collectively termed 'body marking' behaviors as they reflected the individual making bodily contact with objects or group mates. Sniff and

stutter behaviors were grouped as the second principle component. These were together termed 'interest' behaviors representing investigatory behavior and vocalizations made by males when displaying interest in nearby females. Urine spray, urinate, and tread were grouped as the third component and collectively referred to as 'urine marking' behaviors. The fourth and final principle component grouped the behaviors groom, growl, and negative defecate into a category labeled 'wash/threaten' behaviors. Behavior component scores were analyzed with the statistical model and returned no differences ($P > 0.05$) between males of different environmental conditions (i.e., exhibit status, number of keepers, or number of conspecifics). There were no correlations between behavior scores and hormonal or sperm metrics ($P > 0.05$).

Table 3.4 Component loadings for principle components analysis of 11 behavioral event variables for 29 adult male cheetahs

	Component 1	Component 2	Component 3	Component 4
Variance explained	0.21	0.19	0.14	0.12
Label	Body marking	Interest	Urine marking	Wash/threaten
Rub	**43**	27	-23	12
Roll	**47**	-3	18	22
Sniff	29	**44**	6	-18
Groom	38	1	-38	**43**
Stutter	-15	**47**	29	28
Meowchirp	**-41**	10	20	17
Growl	-15	34	22	**53**
Tread	26	-32	**45**	-4
Urinate	18	-39	**47**	29
Urine Spray	18	29	**34**	-34
Defecate	16	22	21	**-37**

Values are multiplied by 100 and rounded to the nearest integer. Bold = highest loading/variable

Discussion

This research has addressed a significant knowledge gap on the interaction of environment and reproductive potential in male cheetahs in managed collections. Results of this work are vital to improving current management/husbandry protocols to increase reproductive success *ex situ*. My research has generated three main findings: 1) cheetah ejaculate quality was negatively affected by exposure to high numbers of human individuals, both indirectly by housing on exhibit to the public and directly though interaction with numerous keepers, 2) cheetah activity levels were negatively associated with adrenal output and may be indicative of individual 'stress', and 3) exposure to high numbers of adult male and female conspecifics did not impact reproductive potential in male cheetahs. I conclude that the reproductive physiology of male cheetahs is markedly impacted by exposure to high numbers of people and housing *ex situ* males off exhibit to the public with few keepers yields improved reproductive potential.

Exposure to high numbers of people is connected to elevated adrenal activity and/or low reproduction in a number of species in managed collections, such as black rhinos (Carlstead and Brown 2005), clouded leopards (Wielebnowski et al. 2002a), as well as in free ranging populations of carnivores such as the pine marten (Barja et al. 2007). Previous work has shown an increase in adrenal gland activity when cheetahs experience public exposure (Wells et al. 2004). However, this is the first study to report distinct decreases in reproductive functioning in cheetahs in response to exposure to high numbers of individual humans. My study suggests that the sensitivity to human exposure in cheetahs goes beyond just exposure of enclosures to the public, but also includes

interactions with a keeper staff made up of more than just a few rotating individuals. Numerous keepers interacting directly with males may prevent the establishment of a regular routine, shown to be important in the reduction of adrenal responses and stress-related behaviors in laboratory cats (Carlstead et al. 1993b).

In addition to obvious natural breeding implications, impact on ejaculate quality in males exposed to high numbers of humans has significance for semen cryopreservation for artificial insemination as well as cheetah genome resource banking (Wildt and Roth 1997). Overall low seminal quality combined with the sensitivity of cheetah spermatozoa to the freeze-thaw process makes cryopreservation of male ejaculate a practical option only when motile sperm number per milliliter of ejaculate is above 30×10^6 total motile sperm/ml (Wildt et al. 1993, Wildt and Roth 1997, Pukazhenthi et al. 2000, Crosier et al. 2006, Comizzoli et al. 2009, Terrell et al. 2012). The established sperm quality cutoff ensures survival of adequate numbers of viable spermatozoa for potential success of post-thaw fertilization procedures. Electroejaculation and cryopreservation of spermatozoa from individual males requires significant allocation of manpower and equipment, as well as the acceptance of the dangers of inducing anesthesia in wild species. These are risks and resources that are wasted when collected material is of inadequate quality to preserve and/or bank for future use. My results suggest that males housed on exhibit to the public may not produce ejaculates of sufficient quality to warrant electroejaculation attempts on these animals.

My study did not reveal increased glucocorticoid concentrations associated with decreased testicular functionality, indicating that a ‘stress’ related mechanism is not

responsible for depressed hypothalamic-pituitary-gonadal (HPG) axis functioning. However, previous work indicates stress related reproductive suppression might be the case. Cheetahs moved from off exhibit to on exhibit enclosures were likely to exhibit increased glucocorticoid concentrations (Wells et al. 2004), while some female cheetahs behaviorally classified as non-reproductive had higher adrenal activity than reproductive females (Jurke et al. 1997). It is likely that high intra- and inter-individual variability of glucocorticoid concentrations made effects of environmental factors difficult to discern in our study. Studies collecting samples for endocrine analysis over long-term periods from individual cheetahs moved between types of facilities are needed to resolve this issue.

My analysis of behavior may support stress-related reproductive suppression, as higher activity levels were associated with lower glucocorticoid baseline concentrations and institutions with fewer keepers on staff had more active males with higher ejaculate quality. Several exotic felid species display lower activity levels associated with high glucocorticoid concentrations, such as leopard cats and clouded leopards, upon exposure to environmental stressors (Carlstead et al. 1993a, Wielebnowski et al. 2002a). In the clouded leopard, environmental stressors eliciting this response include exposure to a high number of human keepers and housing of animals on exhibit to the public (Wielebnowski et al. 2002a). Based on my findings, relative activity level of a male cheetah may be indicative of glucocorticoid concentrations and therefore give insight into the reproductive potential of that individual. As pacing behavior was included in our study as an 'active' behavior, this suggests that, cheetah pacing is not a behavioral stereotypy associated with a stressful condition, but possibly part of a behavioral

repertoire indicating a healthy activity level. In addition, cheetahs hiding and/or being predominantly stationary may indicate elevated adrenal functioning in response to perceived stressors (Carlstead et al. 1993a, Wielebnowski et al. 2002a).

The number of conspecifics in nearby enclosures did not affect ejaculate quality or adrenal and gonadal hormone metabolite concentrations in male cheetahs. It is possible that this effect was diminished by the observed impact of human exposure. An investigation of reproductive physiology in cheetahs exposed to different numbers of conspecifics, but at similar human exposure levels are needed to resolve this issue. My previous work (Chapter 2) demonstrated a marked impact of male grouping on reproductive potential, possibly demonstrating the importance of conspecific presence on this species. However, effects of grouping on physiology of female cheetahs are rapidly reversed when individuals are separated, even with a simple fenceline (Wielebnowski et al. 2002b). This may reflect a lack of sensitivity in cheetah to this particular aspect of *ex situ* management. It is clear that more work is necessary with respect to exposure to conspecifics affecting the reproductive potential of cheetahs in managed collections.

The present study shows that management of environmental factors has significant impacts on the reproductive potential of captive cheetahs. Human exposure appears to be one factor that needs to be controlled when making plans to breed or collect genetic material from males in managed collections. My results suggest management protocols should be adjusted to place males of high genetic value away from public exposure under the care of a small number of keeping staff. Minimal human exposure

will likely increase reproductive success in managed collections and move us closer to achieving a self-sustaining population of captive cheetah.

CHAPTER FOUR: THE INFLUENCE OF MANAGEMENT ON GONADAL AND ADRENAL HORMONES IN FEMALE CHEETAHS (*ACINONYX JUBATUS*)

Abstract

Cheetahs in *ex situ* collections are charismatic 'ambassadors' that help to educate the public while providing unique research opportunities. Most of what is known about the physiology and veterinary/nutritional requirements of the species has been learned from studying *ex situ* collections. Such information is impossible to collect from elusive, free-living animals. Despite the value of captive cheetahs, these populations are not self-sustaining (i.e., more animals die every year than are born). Only about 20% of cheetahs in North American zoos have ever reproduced, and this poor fecundity appears mostly related to suboptimal management. There has been a tendency in zoological collections to construct breeding facilities in more spacious, naturalistic environments that are 'off-display' to the public. A retrospective analysis of data from North American zoos has revealed that more than 90% of cheetah litters produced in the last decade occurred in these off exhibit breeding centers compared to traditional urban zoos. In the current study, I examined the effect of three environmental factors on gonadal and adrenal hormone metabolite concentrations of female cheetahs: 1) public exhibit status, 2) number of conspecifics (of both sexes) in nearby enclosures, and 3) male presence. Secondarily, I assessed a subset of several groups of sisters that were housed together since birth for effects of long-term cohabitation of highly related females on hormone

metabolite concentrations. I hypothesized that females housed at institutions that are on exhibit to the public, contain a high number of conspecifics in nearby enclosures, and have no males present have lower estrogen and higher glucocorticoid metabolite concentrations, and spend more time in an inactive ovarian state than females housed at institutions off exhibit, with few conspecifics in nearby enclosures, and males present. My results revealed that gonadal and adrenal concentrations were similar between females. Also, although group housing of unfamiliar females has been shown to suppress ovarian hormonal activity, long-term group housing of highly related females does not decrease ovarian or increase adrenal hormone concentrations. There was also a positive relationship between short-term glucocorticoid and estrogen production in females. This research has contributed to closing a significant knowledge gap regarding the effect of environmental factors on the hormonal output of female cheetahs in managed collections. Conclusions from this work will allow for management protocols to be adjusted allowing long-term group housing of female cheetahs from a young age (e.g., sister groups can remain together) to maximize use of enclosure space without concern for future reproductive potential of those individuals. These results also add to our understanding of the significance of fecal glucocorticoid concentrations in predicting reproductive potential of females held *ex situ*.

Introduction

The cheetah (*Acinonyx jubatus*) is recognized as threatened due to extirpation from most of its historic range, with continuing declines resulting in only about 10,000 individuals remaining in nature today (Durant et al. 2008). Wild cheetahs are notoriously difficult to study due to inherent elusiveness. Those in *ex situ* collections are useful for educating the public, understanding basic species biology (e.g., behavior, reproduction, genetics, veterinary, and nutritional requirements), and in developing methods applicable to wild counterparts (e.g., developing anesthetic protocols and monitoring/understanding disease sensitivity). Although scientists have been examining cheetahs for more than 30 years, the species resists consistent reproduction in captivity (Lindburg et al. 1993, Marker-Kraus and Grisham 1993). Under the Association of Zoos and Aquariums Species Survival Plan (SSP), findings reveal that only about 20% of animals in the North American population have ever reproduced, causing reliance upon imported cheetahs from Africa to sustain *ex situ* population numbers (Marker 2012, 2013).

Although the species is known for low genetic variation (O'Brien et al. 1983, O'Brien et al. 1987) and the production of 75% malformed spermatozoa per ejaculate (Wildt et al. 1993), the relatively high fecundity of free-ranging cheetahs indicates that these traits do not ultimately cause poor reproductive success (Caro 1994). Rather, it appears that *ex situ* cheetahs require specific husbandry and management techniques to encourage breeding success (Lindburg et al. 1993, Caro 1994, Brown et al. 1996b). It has been well documented that *ex situ* management techniques and related environmental factors (e.g., relocation, exposure to a high number of human keepers, housing in

small/barren enclosures, transfer to enclosures on exhibit to the public, and presence of conspecifics/predator species in nearby enclosures) affect adrenal glucocorticoid concentrations believed to be a measure of individual 'stress' in many felid species (Carlstead et al. 1992, Carlstead et al. 1993a, Carlstead et al. 1993b, Wielebnowski et al. 2002a, Wielebnowski et al. 2002b, Wells et al. 2004, Moreira et al. 2007). For example, adult cheetahs relocated to on exhibit areas are more likely to have higher fecal glucocorticoid metabolite concentrations after movement, particularly if the individual was held in an off exhibit enclosure previously (Wells et al. 2004).

There has been a tendency in zoological collections to construct breeding facilities in more spacious, naturalistic environments that are 'off display' to the public. A retrospective analysis of data from North American zoos has revealed that more than 90% of cheetah litters produced in the last decade occurred in these off exhibit breeding centers compared to traditional urban zoos (Marker 2012, Cheetah SSP 2013). Additionally, fecal glucocorticoid concentrations have been found to be higher in female cheetahs exhibiting no behavioral indicators of estrus and eliciting no male interest during scent exposure than females that have produced cubs (Jurke et al. 1997). Unfamiliar/unrelated female cheetahs housed in pairs experience prolonged instances of ovarian inactivity (Wielebnowski et al. 2002b), or periods of time with an absence of episodic increases in estradiol concentration. Episodic estradiol increases are believed to be indicative of developing follicular waves (Brown et al. 1996b). These acyclic periods, however, are rapidly reversed upon separation of the females, even by a single fence line. A relationship between environmental 'stressors' and decreased reproductive activity has

been documented in a variety of species including several small felids (Mellen 1991, Moreira et al. 2007), Syrian hamsters (Jasnow et al. 2001), and black and white rhinoceros species, (Carlstead and Brown 2005, Metrione and Harder 2011). In Chapter Three of this dissertation, it was reported that male cheetahs held on exhibit to the public have reduced seminal quality compared to males housed off exhibit, although testosterone concentrations were similar. Despite the negative relationship between 'stress-related' environmental factors and reproductive functionality suggested by these data, to date, there has been little exploration beyond anecdotal into what, or how, environmental factors exert an impact on reproductive metrics in cheetahs and the role of glucocorticoids in these effects.

In an effort to address the current paucity of information in this area, my study examined the influence of the environmental factors: 1) exhibit status, 2) number of conspecifics (of both sexes) in nearby enclosures, and 3) male presence/absence on captive female cheetah gonadal and adrenal fecal hormone metabolite concentrations. In addition, I assessed a subset of several groups of adult sisters that were housed together since birth for effects of long-term cohabitation of highly related females on gonadal and adrenal hormone metabolite concentrations. I hypothesized that females housed at institutions that are on exhibit to the public, contain a high number of conspecifics in nearby enclosures, and/or have no males present have lower estrogen and higher glucocorticoid metabolite concentrations, and spend more time in an inactive ovarian state than females housed at institutions off exhibit, with few conspecifics in nearby enclosures, and/or males present. I also hypothesized that long-term group housing of

highly related females does not affect hormone concentrations compared to singly housed females. This work addresses a gap in our knowledge of the female hormonal response to environmental factors. Results from this will also be directly applicable to improve management protocols to limit the effect of *ex situ* housing/husbandry on female reproductive potential.

Materials and Methods

Animals

This study utilized twenty-two captive-born, adult female cheetahs housed in ten Association of Zoos and Aquariums (AZA) accredited institutions across the United States (Table 4.1). Animals were housed and managed according to the Cheetah SSP and approved husbandry guidelines for the species (2009, 2013). The Institutional Animal Care and Use Committees (IACUC) of all involved facilities approved fecal collection procedures. Females ranged in age from two to ten years (Table 4.1), considered prime reproductive age (Crosier et al. 2011).

Females were considered housed on exhibit to the public if they were exposed (visual and assumed olfactory contact) to more than 50 different people each month for five or more months of the year. Off exhibit females do not fall into this category. Conspecific number was established as the total number of male or female cheetahs two years of age or older that were housed at the same institution within visual and/or olfactory range of the female for the majority of the fecal collection period. Cheetahs were assumed to be reproductively mature at two years of age, based on work in males (Crosier et al. 2007). Females analyzed with no male presence had been held in an

institution with no male cheetahs for a minimum of six months before commencement of fecal collection. Females with male presence had been housed in an institution with males over two years of age for at least six months before the start of fecal collection. These data were generated for each individual female both through contact with head cheetah managers and by examination of current studbook data (Marker 2012).

Table 4.1 Demographic and housing information about female cheetahs (n = 22) used in this study

Facility	Studbook Number	Age (years)	Group Housed?	Male(s) Present?
Birmingham Zoo	5757*	3.2	Yes	No
Birmingham Zoo	5759*	3.2	Yes	No
Chehaw Wild Animal Park	6532*	4.9	Yes	No
Chehaw Wild Animal Park	6533*	4.9	Yes	No
Chehaw Wild Animal Park	6534*	4.9	Yes	No
Dickerson Park Zoo	5756*	8.4	No	Yes
Dickerson Park Zoo	5758*	8.4	No	Yes
White Oak Conservation Center	5992	3.8	No	Yes
White Oak Conservation Center	4177	7.1	No	Yes
White Oak Conservation Center	4494	6.2	No	Yes
Philadelphia Zoo	5674*	5.1	No	Yes
Fort Worth Zoo	3961*	7.6	No	Yes
Mesker Park Zoo	6480*	7.0	No	No
SCBI	5816	6.6	No	Yes
SCBI	6163	9.0	No	Yes
SCBI	4453	7.4	No	No
SCBI	4568	8.0	No	No
SCBI	6592	2.1	No	No
Smithsonian National Zoological Park	6339*	3.7	No	Yes
Fossil Rim Wildlife Center	3527	10.2	No	Yes
Fossil Rim Wildlife Center	3979	7.3	No	Yes
Fossil Rim Wildlife Center	4261	5.3	No	Yes

* indicates inclusion in cohabitation analysis (some singles for comparison)

Fecal Collection and Preparation

Freshly voided feces were collected four to five days per week for a period of 12 to 24 weeks for each female. Samples were placed into clean plastic bags, labeled with animal number and date and then stored or shipped frozen (-20°C) to the Smithsonian Conservation Biology Institute (SCBI) for processing. A non-digestible and harmless marker (e.g., glitter, birdseed, lentils, corn) was added to the diet of grouped females to identify individual fecal samples. All samples (n = 2,093) were then freeze-dried (Lyophilizer; Labconco) to remove water, pulverized using a rubber mallet, and stored in labeled plastic tubes at -20°C until processing.

Steroid hormone metabolites were extracted from fecal samples using previously described methods (Graham and Brown 1996). Briefly, after 0.2 g of well-mixed fecal powder from each sample was boiled in 5 ml of 90% ethanol for 20 minutes, extracts were centrifuged to remove particulates and the supernatant was transferred into a glass tube. The pellet was resuspended in an additional 5 ml of 90% ethanol, vortexed for 30 seconds, and recentrifuged. Combined ethanol supernatants were dried under air and then resuspended in 1 ml of 100% methanol. Methanol extracts were dried under air and then resuspended in 1 ml of BSA-free phosphate buffer ($NaH_2PO_4 + Na_2HPO_4 + NaCl + H_2O$; pH = 7.0). Extracts were sonicated (15 minutes) and vortexed (30 seconds) prior to decanting into a plastic tube for freeze storage (-20°C) until measurement of hormone metabolite concentration.

The efficiency of steroid extraction from feces was evaluated by adding radiolabeled hormone (^{3}H-cortisol or ^{3}H-progesterone; 4000-8000 dpm) to each fecal

sample prior to boiling extraction. The minimum recovery of radiolabeled hormone from fecal extracts acceptable for enzyme immunoassay (EIA) was 60% and the mean recovery across females was ~75%. Fecal extracts were diluted to a 1:20 concentration in BSA-free phosphate buffer for cortisol and estradiol EIA. Samples considered too dilute (binding > 80% of maximum binding) were run at a higher concentration (1:10), and samples too concentrated (binding < 20%) were run at a lower concentration (1:200).

Fecal Glucocorticoid Metabolite Analysis

Glucocorticoid metabolite concentrations in diluted fecal extracts were determined using a cortisol EIA validated for use in cheetahs (Young et al. 2004). This cortisol EIA employed a polyclonal antibody (1:8500; R4866; C. Munro, University of California, Davis, CA) raised in rabbits against cortisol-3-carboxymethyloxime linked to bovine serum albumin and cross-reacts with cortisol 100%, prednisolone 9.9%, prednisone 6.3%, cortisone 5%, and < 1% with corticosterone, desoxycorticosterone, 21-desoxycortisone, testosterone, androstenedione, androsterone, and 11-desoxycortisol (Young et al. 2004). The antibody was added to 96-well microtiter plates (Nunc-Immuno, Maxisorp; Fisher Scientific) and allowed to equilibrate for 12 – 48 hours (4°C). Unbound antibody was removed with wash solution and diluted samples, in duplicate, and standards, in triplicate, (0.05 ml; 78 – 20,000 pg/ml; Sigma Diagnostics) were added to the plate. A peroxidase enzyme-conjugated cortisol (1:20,000; 0.05 ml; C. Munro) was then added to each well containing sample or standard and the plate was allowed to incubate for one hour (23°C) before unbound components were removed. A chromagen solution was added (0.1 ml) to each well and incubated ~15 minutes before optical

densities were determined using a microplate reader (Dynex MRX, reading filter 405 nm, reference filter 540 nm).

Sensitivity of the assay at maximum binding was 3.9 pg/well. The inter-assay coefficient of variation for two internal controls was 10.56% (mean binding – 27.31%) and 6.02% (mean binding – 66.45%) and intra-assay coefficient of variation was < 10% (n = 113 assays). Serially diluted pooled fecal extracts demonstrated displacement curves parallel to those of standard hormone preparations. Recovery of added standard to fecal extract ($y = 0.90x – 18.02$, $r = 0.99$) was significant ($P < 0.05$). Several measures were calculated to summarize fecal glucocorticoid values during the longitudinal steroid evaluation of each female; 1) an overall mean of all samples for the collection period, 2) a mean baseline that excludes all values greater than the overall mean plus 1.5 standard deviations (SD) (Graham and Brown 1996), and 3) a peak frequency that divides the number of samples greater than three times baseline by total number of samples collected for that male (Young et al. 2004).

Fecal Estrogen Metabolite Analysis

Estrogen metabolite concentrations in diluted fecal extracts were determined using an estradiol EIA validated for use in cheetahs (Brown et al. 1994). This estradiol EIA employed a polyclonal antibody (1:10,000; R4972; C. Munro, University of California, Davis, CA) raised in rabbits against 17β-estradiol and cross-reacts with 17β-estradiol 100%, estrone 3.33%, and < 0.01% with estrone sulfate, progesterone, testosterone, cortisol, and corticosterone (Brown et al. 1994, Crosier et al. 2011). Microtiter plates were run in the same way as for glucocorticoid analysis after addition of

phosphate buffer (0.05 ml) to each well of the plate; diluted samples in duplicate, standards in triplicate (0.02 ml; 97.5 – 25,000 pg/ml; 17β-estradiol; Sigma Diagnostics), and a peroxidase enzyme-conjugated 17β-estradiol (1:50,000; 0.05 ml; C. Munro) was added to the plate. The plate was then allowed to incubate for two hours (23°C) before unbound components were removed, a chromagen solution added, and optical densities determined.

Sensitivity of the assay at maximum binding was 1.95 pg/well. The inter-assay coefficient of variation for two internal controls was 7.49% (mean binding – 27.48%) and 8.55% (mean binding – 70.72%) and intra-assay coefficient of variation was < 10% (n = 98 assays). Serially diluted pooled fecal extracts demonstrated displacement curves parallel to those of standard hormone preparations. Recovery of added standard to fecal extract ($y = 1.28x – 4.03$, $r = 0.99$) demonstrated significant recovery ($P < 0.05$). During analysis of fecal estrogen concentrations, a 'peak' value was considered to be the highest in a group of elevated concentrations (greater than 1.5 times baseline), and anestrous periods were more than 28 days between estrogen peak events (that is, greater than twice the estimated estrous cycle duration) (Brown et al. 1996b). Measures calculated to summarize fecal estrogen values during the steroid evaluation for each female were as follows: 1) an overall mean of all samples for the collection period, 2) a mean baseline that excludes all values greater than the overall mean plus 1.5 standard deviations (SD), 3) a peak frequency that divides the number of peak samples by the total number of samples collected for that female, 4) number of anestrous periods occurring during the time of fecal collection, and 5) length of anestrous periods determined by dividing

number of days spent anestrous by total number of days collected (Brown et al. 1994, Brown et al. 1996b, Crosier et al. 2011).

Statistical Analysis

Prior to analysis, statistical outliers were determined (Hoaglin and Iglewicz 1987) and replaced with the mean for all hormone variables (Sokal and Rohlf 2012). Normality and homoscedasticity were verified using an Anderson-Darling and a Levene's test, respectively (Grafen and Hails 2002). Relationships between all hormone metrics were evaluated using a Pearson's correlation coefficient (Grafen and Hails 2002). Post-hoc comparison of hormone metrics between anestrous periods and cyclic periods of ovarian activity within females (n = 11) was completed using a paired *t*-test after appropriate normality and variance checks described above (Grafen and Hails 2002). All statistical procedures were completed using SAS software (version 9.2, Cary, NC). Effects were considered significant at $P < 0.05$ and data are reported as mean ± standard error of the mean (SEM).

Differences in hormone metrics between grouped females and those housed singly was tested using a Mann-Whitney U test (Grafen and Hails 2002). A total of 11 females (n = 5 in two groups of 2 – 3 females each; n = 6 singly housed; Table 4.1) were included in this analysis. Singly housed females for comparison to those in groups were chosen so all included females were on exhibit with ≤10 conspecifics in nearby enclosures to eliminate the effect of these environmental factors on variables. One group of three female cheetahs (6532, 6533, 6534) and two singly housed females (5756, 5758) were excluded from the final analysis because of a limited fecal collection period (6 – 8

weeks), compared to the rest of the females in the study (Table 4.1). Thus a total of 17 female cheetahs were analyzed within the statistical model for the three main environmental factors.

The effect of environmental factors on hormonal metrics was analyzed using a mixed model that held exhibit status (on or off exhibit to the public), number of conspecifics (≤10 conspecifics or >10 conspecifics of either sex held in nearby enclosures), and male presence as fixed factors, while controlling for the effects of age and interaction between the three fixed factors, exhibit status, number of conspecifics, and male presence. A specific numerical cutoff for number of conspecifics (≤10 or >10 conspecifics) was selected based on what would give the model the most statistical power based on the population of females utilized for this study.

Results

In our dataset, the three environmental factors, exhibit status, number of conspecifics, and male presence, had no effect ($P > 0.05$) on measured gonadal and adrenal hormone metrics (Figure 4.1). Similarly, measured hormonal metrics for females housed in groups (2 – 3 individuals) were not different ($P > 0.05$) from those of singly housed females (Table 4.2).

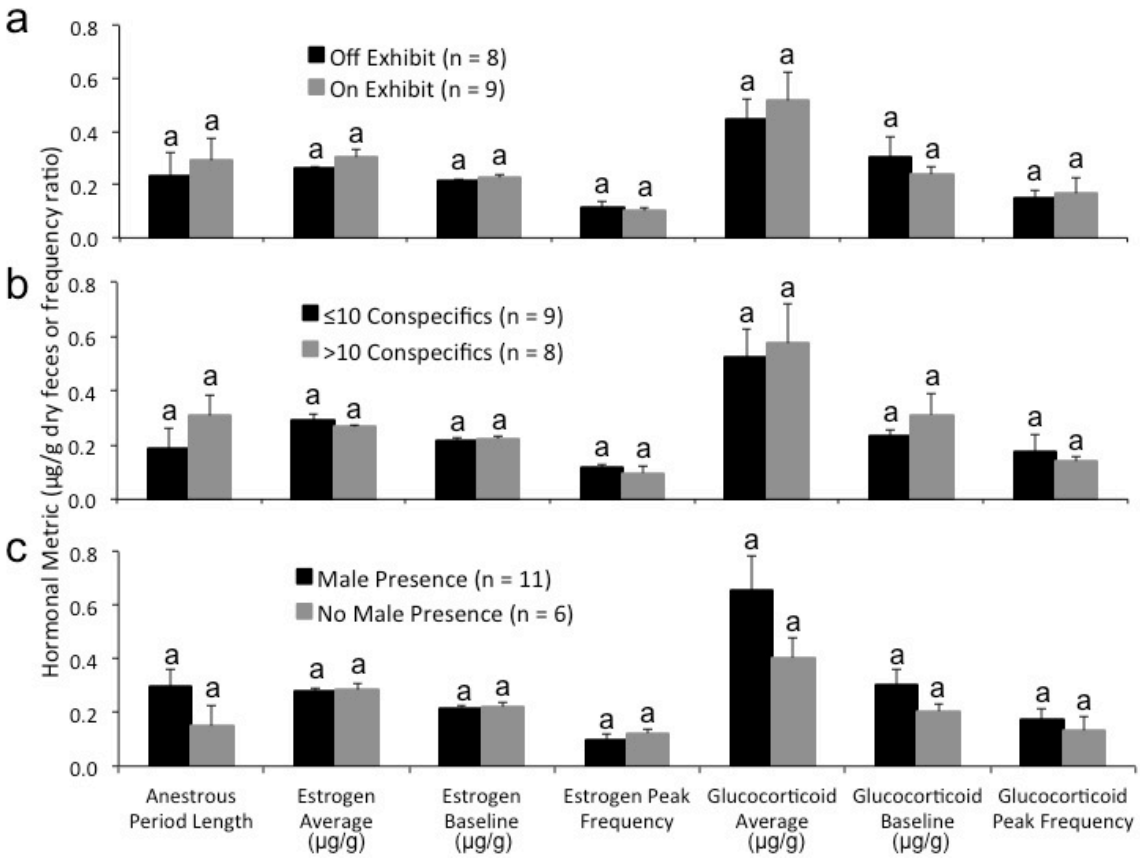


Figure 4.1 Gonadal and adrenal hormone metrics compared between female cheetahs (n = 17) of different environmental states: (a) on or off exhibit to the public, (b) ≤10 or >10 conspecifics present in nearby enclosures, and (c) male presence or no male presence

Table 4.2 Gonadal and adrenal fecal hormone metabolite concentrations (± SEM) for female cheetahs that are group-housed compared to those that are singly-housed

Metric	Group-housed (n = 5)	Singly-housed (n = 6)
Anestrous period length	0.05 ± 0.046	0.13 ± 0.092
Estrogen metabolite concentration (μg/g)		
Average	0.27 ± 0.010	0.35 ± 0.038
Baseline	0.22 ± 0.016	0.23 ± 0.016
Peak Frequency	0.13 ± 0.016	0.12 ± 0.014
Glucocorticoid metabolite concentration (μg/g)		
Average	0.61 ± 0.167	0.73 ± 0.142
Baseline	0.38 ± 0.091	0.33 ± 0.046
Peak Frequency	0.07 ± 0.021	0.19 ± 0.081

Longitudinal hormonal profiles for females housed in groups were similar to those for singly housed females, as number ($U = 27.5$; $P = 0.64$) or lengths ($U = 27$; $P = 0.56$) of anestrous periods were not different (Figure 4.2). Both groups exhibited periodic increases in estrogen metabolite concentration. Additionally, there were no visually discernable, synchronous patterns detected between females housed within the same group.

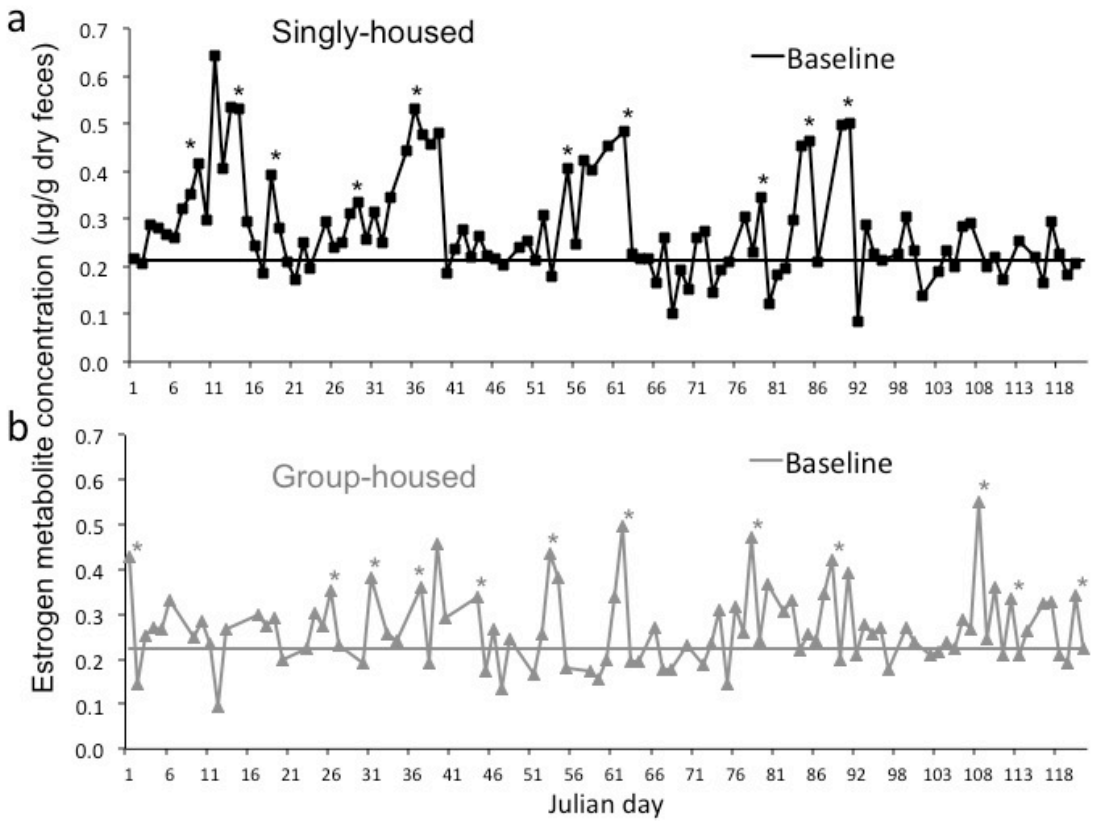


Figure 4.2 Longitudinal fecal estrogen metabolite profiles for a singly-housed female cheetah (a) and a group-housed female cheetah (b), asterisks indicate estrogen peaks

Post-hoc analysis of individual females that experienced both anestrous periods and cyclic periods of ovarian activity yielded on average, a higher percentage ($t_{11} = 3.58$; $P < 0.01$) of samples containing glucocorticoid peak concentrations occurring during periods of ovarian activity (73.31 ± 6.52%) than anestrous periods (26.69 ± 6.52%). Further analysis revealed increased glucocorticoid peak frequency ($t_{11} = 3.64$; $P < 0.01$) and average glucocorticoid concentration ($t_{11} = 3.90$; $P < 0.01$) during ovarian cyclic periods (0.19 ± 0.03 and 0.53 ± 0.06 µg/g dry feces, respectively) than during anestrous

periods (0.10 ± 0.02 and 0.42 ± 0.04 µg/g dry feces, respectively) (Figure 4.3). This effect can also be observed in representative female profiles (Figure 4.4).

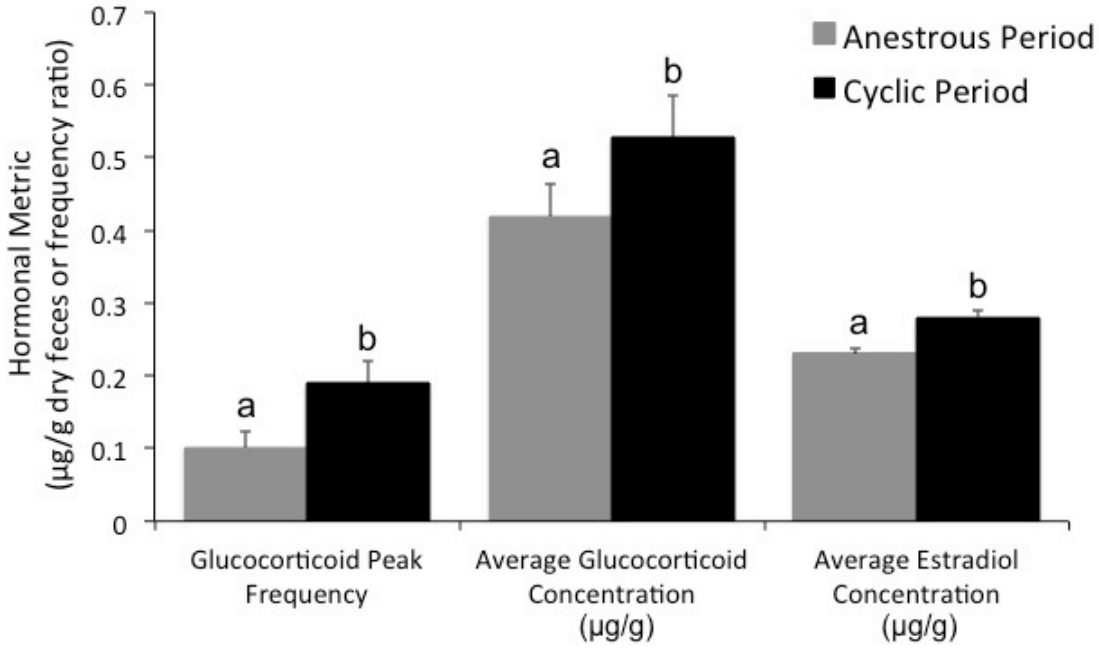


Figure 4.3 Adrenal and gonadal hormone metrics measured within female cheetahs (n = 11) during anestrous periods and cyclic ovarian periods

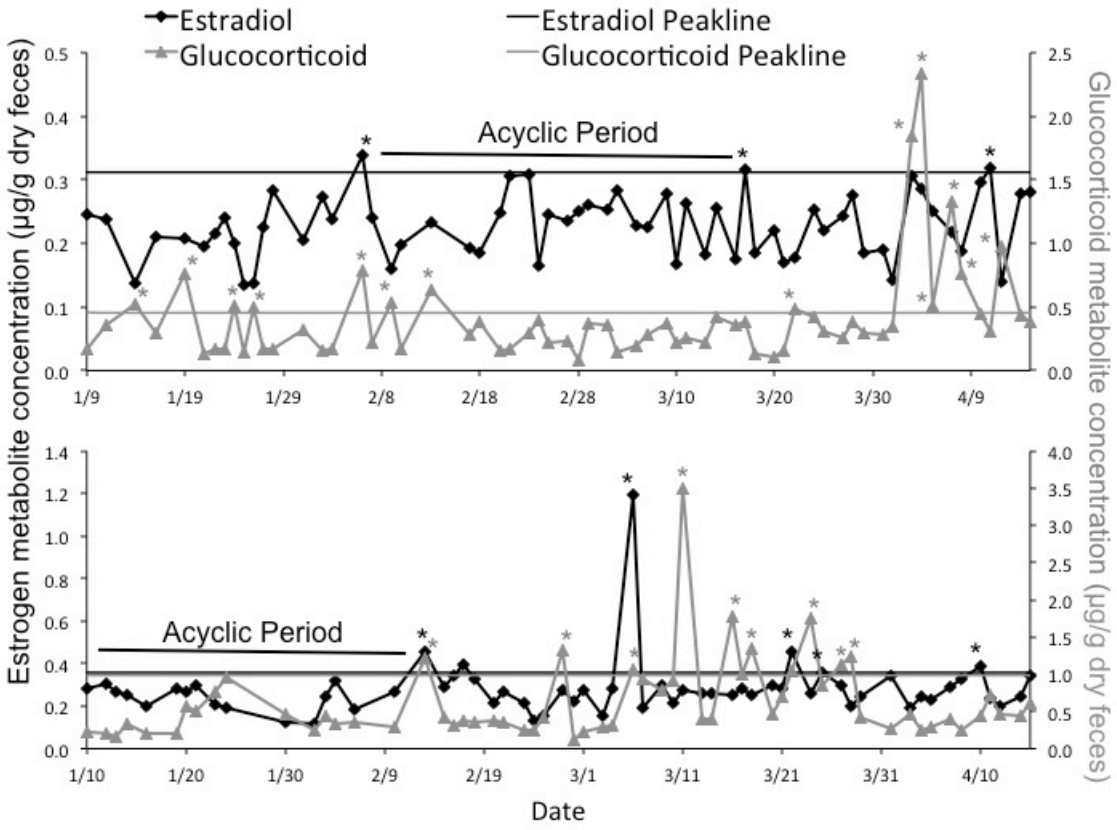


Figure 4.4 Representative female cheetah estrogen and glucocorticoid metabolite concentration profiles, asterisks indicate peaks

Discussion

The present study has addressed a significant knowledge gap regarding the effect of environmental factors on the hormonal output of female cheetahs in managed collections. Results of this work will contribute to modifications of current management/husbandry protocols to encourage captive reproduction. Application of management techniques that improve breeding success is a crucial step toward establishment of a self-sustaining *ex situ* population in this species. My research has generated three main findings: 1) gonadal and adrenal hormone metabolite concentrations

were not different in female cheetahs at institutions exposed to the public, housing a high numbers of conspecifics, or lacking male cheetahs compared to those housed off exhibit, near few conspecifics, or in the presence of male cheetahs, 2) long-term group housing of highly related females does not affect hormone concentrations compared to singly housed females, and 3) there is a positive relationship between short-term glucocorticoid and estrogen production. I conclude that there is no hormonal indication of decreased ovarian function or increased physiological stress in response to environmental management conditions in female cheetahs, but the relationship between the hypothalamic-pituitary-adrenal (HPA) and the hypothalamic-pituitary-gonadal (HPG) axes was positive in regard to short-term increases.

It was found that male cheetahs experienced a reduction in seminal quality in response to exposure to large numbers of humans, but a direct correlation with gonadal or adrenal hormones was not observed (Chapter 3). Also, suppression of ovarian hormone excretion has been documented in unfamiliar female cheetahs in response to group housing, but this was not correlated with glucocorticoid concentration (Wielebnowski et al. 2002b). Although there was no hormonal evidence in the present study of decreased reproductive potential in female cheetahs in response to several common environmental stressors among captive felids, these results may not be entirely indicative of ovarian functionality. It is possible for gonadal function to be affected in this species without observing differences in hormonal metrics. In order to more directly evaluate ovarian functionality in this species, a highly invasive and impractical collection and assessment of oocyte quality would be necessary. More realistically, an investigation of ovarian

response to administration of exogenous gonadotropins of females in different environmental conditions might be conducted. This, however, requires a laparoscopic surgical procedure to view the ovaries that is much more invasive than electroejaculation procedures on males.

Although the methods utilized by this study were the most practical option, I believe this does not necessarily allow a conclusion of no effect of environmental conditions on female reproductive potential to be made. Individual variation in glucocorticoid concentrations has been reported to be high in this species, even when measuring within individuals moved between enclosures (Wells et al. 2004). The outcome of the present study would dictate a concurrent examination of ovarian and adrenal hormonal output during movement of individual female cheetahs between institutions of different types, such as on or off exhibit to the public. A relationship between environmental 'stressors' and decreased reproductive activity has been documented in several felid species (Mellen 1991, Moreira et al. 2007), indicating that this hypothesis should be examined more fully in cheetahs.

Although the similarity I observed between hormone profiles of long-term group housed and singly housed females seems to be in contrast to previous reports of ovarian shutdown in group housed female cheetahs (Wielebnowski et al. 2002b), the high degree of relatedness between the females in my study contributes to the understanding of social structure nuances in this species. Both groups of female cheetahs analyzed in my study were littermates, kept together since birth. This level of familiarity is similar to a group housed, mother-daughter pair that exhibited no aggressive behaviors and continued

ovarian hormone cyclicity, in contrast to other 'unfamiliar' grouped females displaying open aggression and experiencing ovarian shutdown (Wielebnowski et al. 2002b). More recently, Kinoshita and colleagues (2011) reported an instance of continued ovarian hormonal activity in two females that had been housed together long-term and were not known to exhibit aggressive behaviors. Although female behavior was not analyzed in this study, long-term cohabitation and a high degree of relatedness between females would suggest that levels of aggression were low between grouped females. Together, these reports indicate that maintenance of highly related females in groups, or those kept together from a young age showing a low degree of aggression, does not reduce female reproductive potential. This has management implications for those institutions where space is an issue and long-term group housed females have been separated to increase their reproductive potential. These females are likely not experiencing ovarian suppression if levels of aggression are low and therefore, it is possible for these females to be bred successfully while being group housed. Separation near the time of parturition may be necessary, however, as behavior toward the paired female's offspring would be unknown.

My observation of a higher incidence of variable glucocorticoid excretion occurring unexpectedly with periods of enhanced estrogen production may be a by-product of increased physical and adrenal activity occurring near times of maximal ovarian activity. Glucocorticoids are known to mediate physiological responses to perceived stressors, but they can also function as metabolic regulators during predictable events such as reproduction. In many species, such as various birds (Crossin et al. 2013),

giant pandas (Kersey et al. 2010), gray wolves (Eggermann et al. 2013), and Canada lynx (Fanson et al. 2012), it has been found that excreted glucocorticoid concentration is higher during times of increased breeding frequency for one or both sexes. These increases in glucocorticoids may be due to the increased energetic demands of reproduction or released in response to stressors associated with increased interaction with conspecifics (Crossin et al. 2013, Eggermann et al. 2013).

In female cheetah, increased glucocorticoids during times of cyclic ovarian hormonal output may be required to support appropriate activity levels and physiological needs associated with soliciting and supporting successful reproduction and/or pregnancy. This hypothesis is contrary to previous dogma maintaining that increased glucocorticoids are negatively associated with reproductive functionality in this and other closely related felids (Mellen 1991, Jurke et al. 1997, Wells et al. 2004, Moreira et al. 2007). My results suggest that the relationship between the HPA and the HPG axes is more complicated than previously thought in this species. Although increases in long-term baseline concentrations of glucocorticoids may be associated with depressed reproductive functioning (Jurke et al. 1997, Wielebnowski et al. 2002b, Wells et al. 2004), a high frequency of short glucocorticoid increases has a role in supporting an individual female's reproduction. Future studies analyzing glucocorticoid metabolite excretion patterns as indicators of overall individual well being of cheetahs in managed collections should consider all physiological roles of glucocorticoids. My study, with others, has concluded that females may exhibit both positive and negative relationships between the HPA and the HPG axes.

This research has contributed to closing a significant knowledge gap regarding the effect of environmental factors on the hormonal output of female cheetahs in managed collections. Conclusions from this work will allow for the addition of important details to management protocols concerning group housing of female cheetahs such as, if females are kept or placed together from a young age, they can remain together without concern for negative effects of grouping on future breeding ability. Through describing hormonal output of female cheetahs, my results also allow for the organization of future studies to be focused in such a way as to yield solid conclusions concerning the physiology of this species. If fecal glucocorticoid concentrations are to be used to predict reproductive potential, long-term profiles need to be generated both before and after any manipulations to establish individual baselines and fluctuation patterns to be analyzed for changes. In this way, my study has taken the next step in the quest to ultimately improve reproductive success of this species in managed collections in order to achieve a self-sustaining captive population and a reliable preventative mechanism against extinction of the cheetah in the wild.

CHAPTER FIVE: OVERALL SIGNIFICANCE AND FUTURE DIRECTIONS

The research in this dissertation has filled gaps in our knowledge of the social structure, reproductive biology, and adrenal functioning of the cheetah in managed collections. My results highlight the uniqueness of this charismatic carnivore and have helped to focus future research efforts attempting to address the difficulty surrounding successful breeding of this species *ex situ*. In working through the Smithsonian Conservation Biology Institute, I was able to acquire participation by numerous institutions therefore ensuring extended data collection from a large proportion of valuable animals in the current SSP population. My study marks the first time longitudinal endocrine analysis has been completed on males of this species, providing irreplaceable information from a substantial subset of animals vital to the genetic diversity of the SSP population. This work will provide the foundation for future studies attempting to maximize reproductive potential of male cheetahs housed in managed collections. I also utilized my unique access to numerous individuals to expand on previously published reports on fecal hormone metabolite concentrations in female cheetahs held *ex situ*. My results provide critical support for earlier suppositions, while also offering new information on the relationship between adrenal and gonadal axis hormones in this species.

In my first study, we were able to validate a testosterone enzyme immunoassay (EIA) to measure fecal androgen metabolite concentrations in cheetahs. This was then used to generate the first ever long-term endocrine profile of males of this species. Results provided concrete evidence to support previous notions based on seminal quality that male cheetahs do not experience a reproductive seasonality. This is information that adds to the understanding of the overall reproductive biology of the cheetah, in both *ex situ* and *in situ* populations. Additionally, my first study concluded that testosterone concentrations and ejaculate characteristics were increased for coalition males over those held as singletons. This effect was mostly due to testosterone and sperm quality increases in coalitions males initiating the most behaviors with group members. These results have direct implications for management of this species because, as these data show, increased breeding success may be achieved through long-term group housing of male cheetahs. Future studies will: 1) monitor coalition males opportunistically for changes in seminal quality and/or hormonal output upon loss of group members and in singletons after potential establishment of familiarity with new group members to verify my result, 2) behaviorally monitor coalitions long-term for shifts in dominance hierarchy and investigate if these shifts correspond to concurrent changes in hormonal and/or seminal output.

My second study also utilized the newly validated testosterone EIA for this species to investigate differences in gonadal and adrenal hormone metabolite concentrations, as well as seminal and behavioral differences as predictors of reproductive potential between males under different environmental conditions. Based on

my results, I concluded that the reproductive physiology of male cheetahs is markedly impacted by exposure to high numbers of people and housing *ex situ* males off exhibit to the public with few keeper caretakers yields improved reproductive potential. I also found that relative behavioral activity level of a male cheetah may be indicative of glucocorticoid concentrations and therefore give insight into the reproductive potential of that individual. From these results, direct management suggestions have been made to directly improve reproductive potential of males in managed collections. My results have also introduced new potential monitoring techniques of individual well being, capable of being carried out by institutions without access to endocrine or seminal examination capabilities. Future research building on my results will add data from males collected in multiple environmental conditions to assess if changes occur in endocrine, seminal, or behavioral metrics. This has the potential to support my conclusions to provide irrefutable evidence to institutions to which significant changes to management protocols have been suggested. This will likely increase reproductive success in managed collections and take one step closer to achieving a self-sustaining population of captive cheetah.

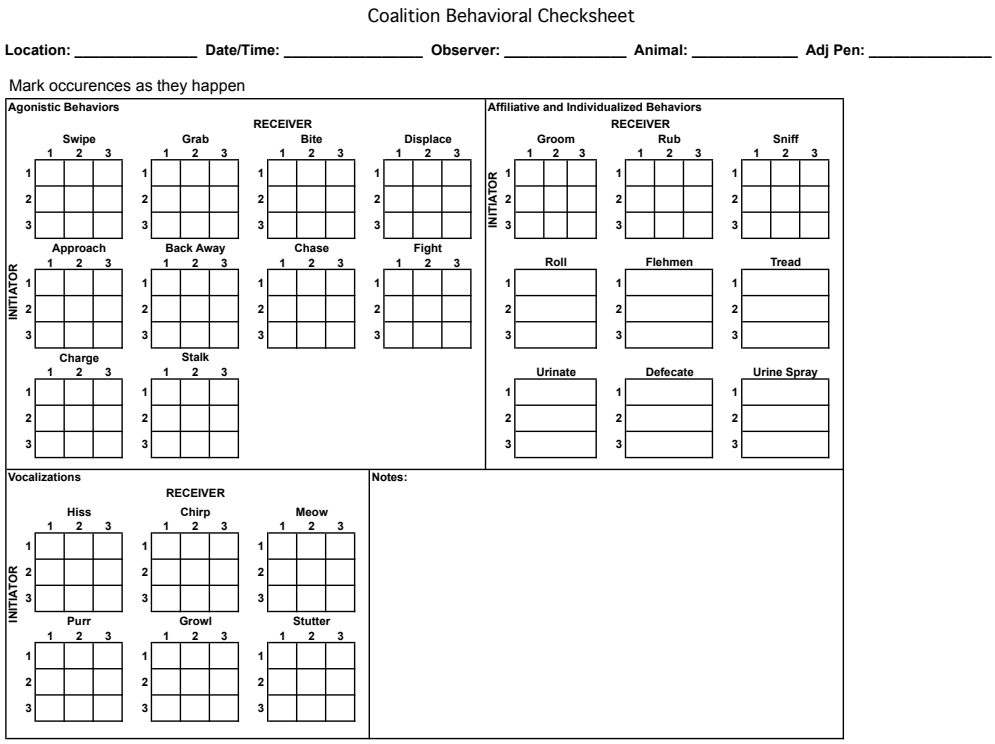
The third study of this dissertation, based on results obtained from males of this species, aimed to examine the influence of similar environmental factors on captive female cheetah gonadal and adrenal fecal hormone metabolite concentrations. This work was done to address significant knowledge gaps and support suppositions made by previous studies completed by other groups, but lacking sufficient data for distinct conclusions. I concluded that hormonally there is no indication of decreased ovarian

functioning or increased physiological stress in response to several differing environmental conditions in female cheetahs in managed collections. Also, group housing of highly related/familiar females is not detrimental to ovarian functionality. Additionally, my results contend that the relationship between gonadal and adrenal hormone axes is more complicated than previously thought in this species. Although increases in long-term baseline concentrations of glucocorticoids may be associated with depressed reproductive functioning, a high frequency of short glucocorticoid increases has a role in supporting an individual female's reproductive potential. Conclusions from this work will allow for the addition of important details to management protocols concerning group housing of female cheetahs. These results also add to our understanding of the significance of fecal glucocorticoid concentrations in predicting reproductive potential of females held *ex situ*. Future studies further describing the hormonal excretion patterns of female cheetahs will take into account the high degree of inter and intra individual variation in concentration to organize methodology capable of discerning effect of central factors despite significant variation.

Further investigation and application of the methods discussed in this dissertation have the potential to improve the overall reproductive success of cheetahs in managed collections by adjustment of current management and husbandry protocols. To maximize reproductive potential of individuals in the *ex situ* cheetah population, it is essential that identified sensitivities of this unique species to environmental conditions be addressed and appropriate measures taken to avoid reproductive suppression by these factors. Thus,

further development of the research discussed herein is essential for maintaining a healthy and viable cheetah population indefinitely.

APPENDIX



Minute:	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46	48	50	52	54	56	58	60
Laying																														
Standing																														
Sleeping																														
Crouching																														
Sitting																														
Walking																														
Pacing																														
Running																														
Out of Sight																														
PROXIMITY																														
Male 1-Male 2																														
Male 1-Male 3																														
Male 2-Male 3																														
	Individual Code: **1=** **2=** **3=**														Proximity Code: **C=contact** **B=body length** **>=greater than body length**															

Figure 1. Cheetah behavioral checksheet used to record behaviors of males housed in coalitions of 2 - 3 individuals

Cheetah Health and Temperament Survey

Facility_____ **Cheetah**_____ **SB#**_____

Your Name_____ **Title**_____ **Date**_____

(All personal information will be held strictly confidential. It will only be used if follow-up questions are necessary.)

Health Information:

1. When was this cheetah born? _____

   Was he born in captivity? _____ If no, at what age was he removed from the wild? _____

2. How long has this cheetah been at your facility? _____

3. Please list the average weight of this male _____

   Is he considered normal, overweight or underweight? (circle one)

   How often do you weigh this cheetah? _____

4. Has this male naturally produced any pregnancies with females at your facility? _____ If yes, please list the month and year, surviving litter size, and female involved for each.

5. Does he have any chronic physical conditions or health problems? (Use the back if necessary)

6. On average, how many times a year is non-routine (examples: for symptoms of an illness, significant injury) veterinary care required for this male? _____

7. Does he display any unusual or stereotypic behaviors (examples: cage biting, hair plucking)? _____ If yes, please explain.

   How often are these behaviors displayed?

   a. > once a day c. > once a week e. > once a month

   b. Daily d. Weekly f. Monthly

7. Is he currently on any medication? _____ If yes, what? _____

   Dosage: _____ For how long? _____

   What is the reason for the medication?

Behavior and Temperament Information:

1. Is this male held in a coalition? _____ If no, skip to question #10.

2. How does the overall size of this male compare to the other males in the coalition?

   a. Largest b. Average c. Smallest

3. Does this male act more aggressively or submissively towards the other male(s) in the coalition?

   a. Always aggressive towards other males
   b. Usually aggressive towards some or all males
   c. Equally aggressive and submissive
   d. Usually submissive in response to some or all males
   e. Always submissive to other males
   f. Neither aggressive nor submissive
   g. Depends on other member involved
   h. Unknown

4. In relation to others, how solicitous is this male of interactions (both positive and negative) with coalition members?

   a. Solicits the most interactions
   b. Solicits more interactions than receives
   c. Solicits and receives interactions equally
   d. Usually receives more interactions than solicits
   e. Only receives interactions
   f. Neither solicits nor receives
   g. Depends on other member involved
   h. Unknown

5. When presented a **new or preferred enrichment activity** while in the presence of other coalition members but when behavior is not being controlled by keepers, this male typically:

   a. Blocks other from it
   b. Resists sharing it
   c. Freely shares
   d. Waits until others are done with it
   e. Actively avoids the object out of deference
   f. Shows no interest
   g. Unknown

6. When presented a **new or preferred food item** while in the presence of other coalition members but when behavior is not being controlled by keepers, this male typically:

   a. Blocks other from the food
   b. Resists letting others have some
   c. Freely shares
   d. Waits until others are done with it
   e. Actively avoids the food out of deference
   f. Shows no interest
   g. Unknown

7. The majority of the time, what would you say is the status of this male within the hierarchy of the coalition?
   a. Dominant b. Middle c. Subordinate

8. Upon what do you base your hierarchy assessment?

9. Has the status of this male within the coalition changed within the last year? _____
   If yes, please explain.

10. In general, how does this male respond when presented with novel items?
    a. Extremely inquisitive
    b. Inquisitive and curious
    c. Typical/Interested response
    d. Interested but timid
    e. Extremely timid/fearful
    f. Shows no interest in novel items

11. How does this male behave towards you?
    a. Tries to exert dominance over you
    b. Generally defiant/standoffish
    c. Sometimes defiant and/or misbehaves
    d. Well-behaved
    e. Always acts submissive and subordinate
    f. I do not work directly with this male
    g. Other _____
    _____

12. How does this male behave towards people other than his regular/routine keepers?
    a. Acts overtly aggressive
    b. Interested without showing aggressive behavior
    c. Uninterested
    d. Timid and/or fearful
    e. Other _____
    _____

13. Has the personality/temperament of this cheetah changed within the last year? _____
    If yes, please explain.

14. Please add any additional comments that you think would provide more insight to the temperament and/or coalition status of this male. (Use the back if necessary.)

Cheetah Socio-environmental Survey

Facility_____ **Your Name**_____

Title_____ **Date**_____

(All personal information will be held strictly confidential. It will only be used if follow-up questions are necessary.)

Facility Information:

1. How many cheetah **enclosures** do you have at your facility? _____

2. What are the approximate dimensions of the enclosures? (Or if you prefer, please send a copy of the cheetah enclosure schematic.)

3. For each of the following, please circle the appropriate response:

a. Are your cheetah enclosures typically open to the public?	Yes	No	Not applicable
b. Is your entire facility typically open to the public?	Yes	No	Not applicable
c. Are cheetahs held mostly outdoors during the day at your facility?	Yes	No	Not applicable
d. Can cheetahs move freely between indoor and outdoor enclosures?	Yes	No	Not applicable
e. Can cheetahs move freely between outdoor enclosures?	Yes	No	Not applicable
f. Can cheetahs move freely between indoor enclosures?	Yes	No	Not applicable
g. Do cheetahs have access to heated areas during cold weather?	Yes	No	Not applicable
h. Do cheetahs have access to shaded areas within the enclosure?	Yes	No	Not applicable
i. Do enclosures contain natural vegetation (trees, bushes, tall grass)?	Yes	No	Not applicable
j. Does the enclosure have areas where cheetahs can hide from the view of people?	Yes	No	Not applicable
k. Does the enclosure have areas where cheetahs can hide from the view of other cheetahs?	Yes	No	Not applicable

4. How many cheetahs are currently held at your facility? _____ males _____females

If any cheetahs are held together, please briefly describe the group (size and sex).

5. Are your cheetahs housed adjacent to (or in the same building with) any other species? _____
   If yes, please list them.

6. Are your male cheetahs regularly able to establish:

Visual (sight) contact with	male cheetahs _____	female cheetahs _____
Olfactory (smell) contact with	male cheetahs _____	female cheetahs _____
Auditory (hearing) contact with	male cheetahs _____	female cheetahs _____

7. Are your female cheetahs regularly able to establish:

Visual (sight) contact with	male cheetahs _____	female cheetahs _____
Olfactory (smell) contact with	male cheetahs _____	female cheetahs _____
Auditory (hearing) contact with	male cheetahs _____	female cheetahs _____

8. Please enter the number of the following changes to the cheetahs at your facility within the last 2 years (Enter 0 if there have not been any):

	Young successfully reared	**Deaths**	**Transfers out**	**Transfers in**
Male cheetahs	_____	_____	_____	_____
Female cheetahs	_____	_____	_____	_____

Keeper Information:

9. How many cheetah keepers are on staff? _____

10. How many cheetah keepers are on duty at one time? _____

11. On average, how many minutes a day do keepers interact with the cheetahs? _____

Cheetah Diet:

12. Please use the space below (a-h) to list the items that normally make up the cheetah's diet, including any rewards or supplements. (Use the back if you need more space.)

Circle frequency of the items use during a typical week:	**Daily**	**5-6 d/wk**	**3-4 d/wk**	**1-2 d/wk**	**<1 d/wk**
a. _____	1	2	3	4	5
b. _____	1	2	3	4	5
c. _____	1	2	3	4	5
d. _____	1	2	3	4	5
e. _____	1	2	3	4	5
f. _____	1	2	3	4	5
g. _____	1	2	3	4	5
h. _____	1	2	3	4	5

Enrichment and Interaction:

13. Generally, how often are each of the following presented to the cheetahs at your facility? (Circle one)

a. Target training	**Daily**	**Weekly**	**Monthly**	**Several times a year**	**≤ once a year**
b. Scent enrichment	**Daily**	**Weekly**	**Monthly**	**Several times a year**	**≤ once a year**
c. Food enrichment	**Daily**	**Weekly**	**Monthly**	**Several times a year**	**≤ once a year**
d. Sound enrichment	**Daily**	**Weekly**	**Monthly**	**Several times a year**	**≤ once a year**

14. For cheetahs held in groups, are the activities in question #12 presented:
    a. To each cheetah alone
    b. To the entire group at one time
    c. Some activities are presented to the entire group and others to each cheetah alone
    d. Not applicable

15. Are there cheetahs at your facility that you are trying breed naturally? _____ If yes, please list their names, stud book numbers, and frequency and duration of introductions.

Figure 2. Survey given to cheetah keeping staff at each institution during the fecal collection period

REFERENCES

2009. Husbandry Manual for the Cheetah (*Acinonyx jubatus*). White Oak Conservation Center, Yulee, FL.

2013. Cheetah Species Survival Plan (SSP). Silver Spring, MD.

Adachi, I., S. Kusuda, H. Kawai, M. Ohazama, A. Taniguchi, N. Kondo, M. Yoshihara, R. Okuda, T. Ishikawa, I. Kanda, and O. Doi. 2011. Fecal progestagens to detect and monitor pregnancy in captive female cheetahs (*Acinonyx jubatus*). Journal of Reproduction and Development **57**:262-266.

Aitken-Palmer, C., R. Hou, C. Burrell, Z. H. Zhang, C. D. Wang, R. Spindler, D. E. Wildt, M. A. Ottinger, and J. Howard. 2012. Protracted reproductive seasonality in the male Giant Panda (*Ailuropoda melanoleuca*) reflected by patterns in androgen profiles, ejaculate characteristics, and selected behaviors. Biology of Reproduction **86**:10.

Asa, C. S., R. E. Junge, J. S. Bircher, G. A. Noble, K. J. Sarri, and E. D. Plotka. 1992. Assessing reproductive cycles and pregnancy in cheetahs (*Acinonyx jubatus*) by vaginal cytology. Zoo Biology **11**:139-151.

Barja, I., G. Silvan, S. Rosellini, A. Pineiro, A. Gonzalez-Gil, L. Camacho, and J. C. Illera. 2007. Stress physiological responses to tourist pressure in a wild population of European pine marten. Journal of Steroid Biochemistry and Molecular Biology **104**:136-142.

Berardelli, R., I. Karamouzis, V. D'Angelo, C. Zichi, B. Fussotto, R. Giordano, E. Ghigo, and E. Arvat. 2013. Role of mineralocorticoid receptors on the hypothalamus-pituitary-adrenal axis in humans. Endocrine **43**:51-58.

Brown, E. W., R. A. Olmsted, J. S. Martenson, and S. J. Obrien. 1993. Exposure to FIV and FIPV in wild and captive cheetahs. Zoo Biology **12**:135-142.

Brown, J. L. 2006. Comparative endocrinology of domestic and nondomestic felids. Theriogenology **66**:25-36.

Brown, J. L. 2011. Female reproductive cycles of wild female felids. Animal Reproduction Science **124**:155-162.

Brown, J. L., L. H. Graham, N. Wielebnowski, W. F. Swanson, D. E. Wildt, and J. G. Howard. 2001. Understanding the basic reproductive biology of wild felids by monitoring of faecal steroids. Journal of Reproduction and Fertility Supplement:71-82.

Brown, J. L., K. A. Terio, and L. H. Graham. 1996a. Fecal androgen metabolite analysis for non invasive monitoring of testicular steroidogenic activity in felids. Zoo Biology **15**:425-434.

Brown, J. L., S. K. Wasser, D. E. Wildt, and L. H. Graham. 1994. Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured noninvasively in feces. Biology of Reproduction **51**:776-786.

Brown, J. L., and D. E. Wildt. 1997. Assessing reproductive status in wild felids by non-invasive faecal steroid monitoring. International Zoo Yearbook **35**:173-191.

Brown, J. L., D. E. Wildt, L. H. Graham, A. P. Byers, L. Collins, S. Barrett, and J. G. Howard. 1995. Natural versus chorionic gonadotropin-induced ovarian responses in the clouded leopard (*Neofelis nebulosa*) assessed by fecal steroid analysis. Biology of Reproduction **53**:93-102.

Brown, J. L., D. E. Wildt, N. Wielebnowski, K. L. Goodrowe, L. H. Graham, S. Wells, and J. G. Howard. 1996b. Reproductive activity in captive female cheetahs (*Acinonyx jubatus*) assessed by faecal steroids. Journal of Reproduction and Fertility **106**:337-346.

Burks, K. D., J. D. Mellen, G. W. Miller, J. Lehnhardt, A. Weiss, A. J. Figueredo, and T. L. Maple. 2004. Comparison of two introduction methods for African elephants (*Loxodonta africana*). Zoo Biology **23**:109-126.

Carlstead, K., and J. L. Brown. 2005. Relationships between patterns of fecal corticoid excretion and behavior, reproduction, and environmental factors in captive black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros. Zoo Biology **24**:215-232.

Carlstead, K., J. L. Brown, S. L. Monfort, R. Killens, and D. E. Wildt. 1992. Urinary monitoring of adrenal responses to psychological stressors in domestic and nondomestic felids. Zoo Biology **11**:165-176.

Carlstead, K., J. L. Brown, and J. Seidensticker. 1993a. Behavioral and adrenocortical responses to environmental changes in leopard cats (*Felis bengalensis*). Zoo Biology **12**:321-331.

Carlstead, K., J. L. Brown, and W. Strawn. 1993b. Behavioral and physiological correlates of stress in laboratory cats. Applied Animal Behaviour Science **38**:143-158.

Caro, T. M. 1993. Behavioral solutions to breeding cheetahs in captivity: Insights from the wild. Zoo Biology **12**:19-30.

Caro, T. M. 1994. Cheetahs of the Serengeti Plains: Group living in an asocial species. The University of Chicago Press, Chicago.

Caro, T. M., and D. A. Collins. 1987. Male cheetah social organization and territoriality. Ethology **74**:52-64.

Clutton-Brock, T. 2007. Sexual selection in males and females. Science **318**:1882-1885.

Comizzoli, P., A. E. Crosier, N. Songsasen, M. S. Gunther, J. G. Howard, and D. E. Wildt. 2009. Advances in reproductive science for wild carnivore conservation. Reproduction in Domestic Animals **44 Suppl 2**:47-52.

Cowlishaw, G., and R. I. M. Dunbar. 1991. Dominance rank and mating success in male primates. Animal Behaviour **41**:1045-1056.

Creel, S., N. M. Creel, M. G. L. Mills, and S. L. Monfort. 1997. Rank and reproduction in cooperatively breeding African wild dogs: Behavioral and endocrine correlates. Behavioral Ecology **8**:298-306.

Creel, S. F. 2005. Dominance, aggression, and glucocorticoid levels in social carnivores. Journal of Mammalogy **86**:255-264.

Creel, S. R., and P. M. Waser. 1991. Failures of reproductive suppression in dwarf mongooses (*Helogale parvula*): Accident or adaptation? Behavioral Ecology **2**:7-15.

Crosier, A. E., P. Comizzoli, T. Baker, A. Davidson, L. Munson, J. Howard, L. L. Marker, and D. E. Wildt. 2011. Increasing age influences uterine integrity, but not ovarian function or oocyte quality, in the cheetah (*Acinonyx jubatus*). Biology of Reproduction **85**:243-253.

Crosier, A. E., J. N. Henghali, J. Howard, B. S. Pukazhenthi, K. A. Terrell, L. L. Marker, and D. E. Wildt. 2009. Improved quality of cryopreserved cheetah (*Acinonyx jubatus*) spermatozoa after centrifugation through Accudenz. Journal of Andrology **30**:298-308.

Crosier, A. E., L. Marker, J. Howard, B. S. Pukazhenthi, J. N. Henghali, and D. E. Wildt. 2007. Ejaculate traits in the Namibian cheetah (*Acinonyx jubatus*): influence of age, season and captivity. Reproduction, Fertility and Development **19**:370-382.

Crosier, A. E., B. S. Pukazhenthi, J. N. Henghali, J. Howard, A. J. Dickman, L. Marker, and D. E. Wildt. 2006. Cryopreservation of spermatozoa from wild-born Namibian cheetahs (*Acinonyx jubatus*) and influence of glycerol on cryosurvival. Cryobiology **52**:169-181.

Crossin, G. T., R. A. Phillips, C. R. Lattin, L. M. Romero, and T. D. Williams. 2013. Corticosterone mediated costs of reproduction link current to future breeding. General and Comparative Endocrinology **193**:112-120.

Dallman, M. F., N. Levin, C. S. Cascio, S. F. Akana, L. Jacobson, and R. W. Kuhn. 1989. Pharmacological evidence that the inhibition of diurnal adrenocorticotropin secretion by corticosteroids is mediated via type I corticosterone-preferring receptors. Endocrinology **124**:2844-2850.

De Kloet, E. R., E. Vreugdenhil, M. S. Oitzl, and M. Joels. 1998. Brain corticosteroid receptor balance in health and disease. Endocrine Reviews **19**:269-301.

Dierenfeld, E. S. 1993. Nutrition of captive cheetahs: Food composition and blood parameters. Zoo Biology **12**:143-150.

Donoghue, A. M., J. G. Howard, A. P. Byers, K. L. Goodrowe, M. Bush, E. Blumer, J. Lukas, J. Stover, K. Snodgrass, and D. E. Wildt. 1992. Correlation of sperm viability with gamete interaction and fertilization *in vitro* in the cheetah (*Acinonyx jubatus*). Biology of Reproduction **46**:1047-1056.

Durant, S., L. Marker, N. Purchase, F. Belbachir, L. Hunter, C. Packer, C. Breitenmoser-Wursten, E. Sogbohossou, and H. Bauer. 2008. *Acinonyx jubatus*. IUCN 2013 Red List of Threatened Species, http://www.iucnredlist.org.

Eaton, R. L. 1970. Hunting behavior of the cheetah. Journal of Wildlife Management **34**:56-67.

Edrey, Y. H., T. J. Park, H. S. Kang, A. Biney, and R. Buffenstein. 2011. Endocrine function and neurobiology of the longest-living rodent, the naked mole-rat. Experimental Gerontology **46**:116-123.

Eggermann, J., J. Theuerkauf, B. Pirga, A. Milanowski, and R. Gula. 2013. Stress-hormone levels of wolves in relation to breeding season, pack size, human activity, and prey density. Annales Zoologici Fennici **50**:170-175.

Evermann, J. F., M. K. Laurenson, A. J. McKeirnan, and T. M. Caro. 1993. Infectious disease surveillance in captive and free-living cheetahs: An integral part of the species survival plan. Zoo Biology **12**:125-133.

Fanson, K. V., N. C. Wielebnowski, T. M. Shenk, and J. R. Lucas. 2012. Comparative patterns of adrenal activity in captive and wild Canada lynx (*Lynx canadensis*). Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology **182**:157-165.

Freeman, E. W., B. A. Schulte, and J. L. Brown. 2010. Investigating the impact of rank and ovarian activity on the social behavior of captive female African Elephants. Zoo Biology **29**:154-167.

French, S. S., R. McLemore, B. Vernon, G. I. H. Johnston, and M. C. Moore. 2007. Corticosterone modulation of reproductive and immune systems trade-offs in female tree lizards: Long-term corticosterone manipulations via injectable gelling material. Journal of Experimental Biology **210**:2859-2865.

Funder, J. W. 1997. Glucocorticoid and mineralocorticoid receptors: Biology and clinical relevance. Annual Review of Medicine **48**:231-240.

Ganan, N., A. Sestelo, J. J. Garde, F. Martinez, A. Vargas, I. Sanchez, M. J. Perez-Aspa, J. V. Lopez-Bao, F. Palomares, M. Gomendio, and E. R. S. Roldan. 2010. Reproductive traits in captive and free-ranging males of the critically endangered Iberian lynx (*Lynx pardinus*). Reproduction **139**:275-285.

Gottelli, D., J. L. Wang, S. Bashir, and S. M. Durant. 2007. Genetic analysis reveals promiscuity among female cheetahs. Proceedings of the Royal Society B-Biological Sciences **274**:1993-2001.

Grafen, A., and R. Hails. 2002. Modern statistics for the life sciences. Oxford University Press Inc., New York.

Graham, L. H., and J. L. Brown. 1996. Cortisol metabolism in the domestic cat and implications for non-invasive monitoring of adrenocortical function in endangered felids. Zoo Biology **15**:71-82.

Graham, L. H., K. L. Goodrowe, J. I. Raeside, and R. M. Liptrap. 1995. Non-invasive monitoring of ovarian function in several felid species by measurement of fecal estrasiol-17-beta and progestins. Zoo Biology **14**:223-237.

Hayward, M. W., M. Hofmeyr, J. O'Brien, and G. I. H. Kerley. 2006. Prey preferences of the cheetah (*Acinonyx jubatus*) (Felidae : Carnivora): morphological limitations or the need to capture rapidly consumable prey before kleptoparasites arrive? Journal of Zoology **270**:615-627.

Herrick, J. R., J. B. Bond, M. Campbell, G. Levens, T. Moore, K. Benson, J. D'Agostino, G. West, D. M. Okeson, R. Coke, S. C. Portacio, K. Leiske, C. Kreider, P. J. Polumbo, and W. F. Swanson. 2010. Fecal endocrine profiles and ejaculate traits in black-footed cats (*Felis nigripes*) and sand cats (*Felis margarita*). General and Comparative Endocrinology **165**:204-214.

Hoaglin, D. C., and B. Iglewicz. 1987. Fine-tuning some resistant rules for outlier labeling. Journal of the American Statistical Association **82**:1147-1149.

Howard, J. 1999. Assisted reproductive techniques in nondomestic carnivores. Pages 449-457 *in* M. E. Fowler and R. E. Miller, editors. Zoo and Wild Animal Medicine: Current Therapy IV. W.B. Saunders Co., Philadelphia.

Howard, J. G., J. L. Brown, M. Bush, and D. E. Wildt. 1990. Teratospermic and normospermic domestic cats: Ejaculate traits, pituitary-gonadal hormones, and improvement of spermatozoal motility and morphology after swim-up processing. Journal of Andrology **11**:204-215.

Howard, J. G., A. M. Donoghue, M. A. Barone, K. L. Goodrowe, E. S. Blumer, K. Snodgrass, D. Starnes, M. Tucker, M. Bush, and D. E. Wildt. 1992. Successful induction of ovarian activity and laparoscopic intrauterine artificial insemination in the cheetah (*Acinonyx jubatus*). Journal of Zoo and Wildlife Medicine **23**:288-300.

Howard, J. G., T. L. Roth, A. P. Byers, W. F. Swanson, and D. E. Wildt. 1997. Sensitivity to exogenous gonadotropins for ovulation induction and laparoscopic artificial insemination in the cheetah and clouded leopard. Biology of Reproduction **56**:1059-1068.

Howard, J. G., and D. E. Wildt. 2009. Approaches and efficacy of artificial insemination in felids and mustelids. Theriogenology **71**:130-148.

Jasnow, A. M., D. L. Drazen, K. L. Huhman, R. J. Nelson, and G. E. Demas. 2001. Acute and chronic social defeat suppresses humoral immunity of male Syrian hamsters (*Mesocricetus auratus*). Hormones and Behavior **40**:428-433.

Jewgenow, K., S. V. Naidenko, F. Goeritz, A. Vargas, and A. Dehnhard. 2006. Monitoring testicular activity of male Eurasian (*Lynx lynx*) and Iberian (*Lynx pardinus*) lynx by fecal testosterone metabolite measurement. General and Comparative Endocrinology **149**:151-158.

Johnston, L. A., D. L. Armstrong, and J. L. Brown. 1994. Seasonal effects on seminal and endocrine traits in the captive snow leopard (*Panthera uncia*). Journal of Reproduction and Fertility **102**:229-236.

Johnston, L. A., A. M. Donoghue, S. J. Obrien, and D. E. Wildt. 1991. Rescue and maturation *in vitro* of follicular oocytes collected from nondomestic felid species. Biology of Reproduction **45**:898-906.

Jurke, M. H., N. M. Czekala, D. G. Lindburg, and S. E. Millard. 1997. Fecal corticoid metabolite measurement in the cheetah (*Acinonyx jubatus*). Zoo Biology **16**:133-147.

Kersey, D. C., D. E. Wildt, J. L. Brown, Y. Huang, R. J. Snyder, and S. L. Monfort. 2010. Parallel and seasonal changes in gonadal and adrenal hormones in male giant pandas (*Ailuropoda melanoleuca*). Journal of Mammalogy **91**:1496-1507.

Kinoshita, K., M. Ohazama, R. Ishida, and H. Kusunoki. 2011. Daily fecal sex steroid hormonal changes and mating success in captive female cheetahs (*Acinonyx jubatus*) in Japan. Animal Reproduction Science **125**:204-210.

Kirby, E. D., A. C. Geraghty, T. Ubuka, G. E. Bentley, and D. Kaufer. 2009. Stress increases putative gonadotropin inhibitory hormone and decreases luteinizing hormone in male rats. Proceedings of the National Academy of Sciences of the United States of America **106**:11324-11329.

Laurenson, M. K., T. Caro, and M. Borner. 1992. Female cheetah reproduction. Research & Exploration **8**:64-75.

Leyhausen, P. 1990. Cats. English Language edition. McGraw-Hill, Inc., New York.

Leyhausen, P., and B. A. Tonkin. 1979. Cat behaviour. The predatory and social behaviour of domestic and wild cats. Garland STPM Press, New York.

Lindburg, D. G., B. S. Durrant, S. E. Millard, and J. E. Oosterhuis. 1993. Fertility assessment of cheetah males with poor quality semen. Zoo Biology **12**:97-103.

Ludwig, C., B. Wachter, S. Silinski-Mehr, A. Ganswindt, H. Bertschinger, H. Hofer, and M. Dehnhard. 2013. Characterisation and validation of an enzyme-immunoassay for the non-invasive assessment of faecal glucocorticoid metabolites in cheetahs (*Acinonyx jubatus*). General and Comparative Endocrinology **180**:15-23.

Maclusky, N. J., F. Naftolin, and C. Leranth. 1988. Immunocytochemical evidence for direct synaptic connections between corticotrophin-realeasing factor (CRF) and gonadotropin-releasing hormone (GnRH)-containing neurons in the pre optic area of the rat. Brain Research **439**:391-395.

Mann, D. R., D. C. Evans, V. L. Jacobs, and D. C. Collins. 1986. Influence of acute intracerebroventricular (ICV) administration of adrenocorticotropin (ACTH) on LH secretion in male rats: Effect of pretreatment (ICV) with ACTH antiserum on

the serum LH response to an acute ether stress. Journal of Endocrinology **108**:275-280.

Marker, L. L. 2012. International studbook cheetah (*Acinonyx jubatus)*. Cheetah Conservation Fund, Otjiwarongo, Namibia.

Marker, L. L., and A. J. Dickman. 2004. Dental anomalies and incidence of palatal erosion in namibian cheetahs (*Acinonyx jubatus jubatus*). Journal of Mammalogy **85**:19-24.

Marker, L. L., A. J. Dickman, R. M. Jeo, M. G. L. Mills, and D. W. Macdonald. 2003a. Demography of the Namibian cheetah, *Acinonyx jubatus jubatus*. Biological Conservation **114**:413-425.

Marker, L. L., A. J. Dickman, and D. W. Macdonald. 2005. Perceived effectiveness of livestock-guarding dogs placed on Namibian farms. Rangeland Ecology & Management **58**:329-336.

Marker, L. L., A. J. Dickman, M. G. L. Mills, R. M. Jeo, and D. W. Macdonald. 2008a. Spatial ecology of cheetahs on north-central Namibian farmlands. Journal of Zoology **274**:226-238.

Marker, L. L., M. G. L. Mills, and D. W. Macdonald. 2003b. Factors influencing perceptions of conflict and tolerance toward cheetahs on Namibian farmlands. Conservation Biology **17**:1290-1298.

Marker, L. L., J. R. Muntifering, A. J. Dickman, M. G. L. Mills, and D. W. Macdonald. 2003c. Quantifying prey preferences of free-ranging Namibian cheetahs. South African Journal of Wildlife Research **33**:43-53.

Marker, L. L., A. J. P. Wilkerson, R. J. Sarno, J. Martenson, C. Breitenmoser-Wuersten, S. J. O'Brien, and W. E. Johnson. 2008b. Molecular genetic insights on cheetah (*Acinonyx jubatus*) ecology and conservation in Namibia. Journal of Heredity **99**:2-13.

Marker-Kraus, L., and J. Grisham. 1993. Captive breeding of cheetahs in North American zoos: 1987-1991. Zoo Biology **12**:5-18.

Martin, P., and P. Bateson. 2007. Measuring behavior: An introductory guide. 3rd edition. Cambridge University Press, Cambridge.

Matteri, R. L., J. G. Watson, and G. P. Moberg. 1984. Stress of acute adrenocorticotropin treatment suppresses LHRH-induced LH release in the ram. Journal of Reproduction and Fertility **72**:385-393.

Mellen, J. D. 1991. Factors influencing reproductive success in small captive exotic felids (*Felis* spp.): A multiple regression analysis. Zoo Biology **10**:95-110.

Metrione, L. C., and J. D. Harder. 2011. Fecal corticosterone concentrations and reproductive success in captive female southern white rhinoceros. General and Comparative Endocrinology **171**:283-292.

Michael, R. P. 1961. Observations upon the sexual behaviour of the domestic cat (*Felis catus* L.) under laboratory conditions. Behaviour **18**:1-24.

Mor, I., D. Grisaru, L. Titelbaum, T. Evron, C. Richler, J. Wahrman, M. Sternfeld, L. Yogev, N. Meiri, S. Seidman, and H. Soreq. 2001. Modified testicular expression of stress-associated "readthrough" acetylcholinesterase predicts male infertility. Faseb Journal **15**:2039-2041.

Moreira, N., J. L. Brown, W. Moraes, W. F. Swanson, and E. L. A. Monteiro. 2007. Effect of housing and environmental enrichment on adrenocortical activity, behavior and reproductive cyclicity in the female tigrina (*Leopardus tigrinus*) and margay (*Leopardus wiedii*). Zoo Biology **26**:441-460.

Muniz, L., S. Perry, J. H. Manson, H. Gilkenson, J. Gros-Louis, and L. Vigilant. 2010. Male dominance and reproductive success in wild white-faced capuchins (*Cebus capucinus*) at Lomas Barbudal, Costa Rica. American Journal of Primatology **72**:1118-1130.

Munson, L., I. A. Gardner, R. J. Mason, L. M. Chassy, and U. S. Seal. 2002. Endometrial hyperplasia and mineralization in zoo felids treated with melengestrol acetate contraceptives. Veterinary Pathology **39**:419-427.

Munson, L., J. W. Nesbit, D. G. A. Meltzer, L. P. Colly, L. Bolton, and N. P. J. Kriek. 1999. Diseases of captive cheetahs (*Acinonyx jubatus jubatus*) in South Africa: A 20-year retrospective survey. Journal of Zoo and Wildlife Medicine **30**:342-347.

Munson, L., K. A. Terio, M. Worley, M. Jago, A. Bagot-Smith, and L. Marker. 2005. Extrinsic factors significantly affect patterns of disease in free-ranging and captive cheetah (*Acinonyx jubatus*) populations. Journal of Wildlife Diseases **41**:542-548.

Nelson, R. J. 2005. An introduction to behavioral endocrinology. Third edition. Sinauer Associates, Inc., Sunderland, Massachusettes.

Newton-Fisher, N. E., M. E. Thompson, V. Reynolds, C. Boesch, and L. Vigilant. 2010. Paternity and social rank in wild chimpanzees (*Pan troglodytes*) from the Budongo Forest, Uganda. American Journal of Physical Anthropology **142**:417-428.

Norris, D. O. 2006. Vertebrate endocrinology. Forth edition. Academic Press.

O'Brien, S. J., M. E. Roelke, L. Marker, A. Newman, C. A. Winkler, D. Meltzer, L. Colly, J. F. Evermann, M. Bush, and D. E. Wildt. 1985. Genetic Basis for Species Vulnerability in the Cheetah. Science **227**:1428-1434.

O'Brien, S. J., D. E. Wildt, M. Bush, T. M. Caro, C. Fitzgibbon, I. Aggundey, and R. E. Leakey. 1987. East African cheetahs: Evidence for two population bottlenecks? Proceedings of the National Academy of Sciences of the United States of America **84**:508-511.

O'Brien, S. J., D. E. Wildt, D. Goldman, C. R. Merril, and M. Bush. 1983. The cheetah is depauperate in genetic variation. Science **221**:459-462.

Packer, C., D. A. Gilbert, A. E. Pusey, and S. J. Obrien. 1991. A molecular genetic analysis of kinship and cooperation in African lions. Nature **351**:562-565.

Packer, C., and A. E. Pusey. 1982. Cooperation and competition within coalitions of male lions: Kin selection or game theory. Nature **296**:740-742.

Packer, C., and A. E. Pusey. 1983. Adaptations of female lions to infanticide by incoming males. American Naturalist:716-728.

Pelican, K. M., D. E. Wildt, B. Pukazhenthi, and J. Howard. 2006. Ovarian control for assisted reproduction in the domestic cat and wild felids. Theriogenology **66**:37-48.

Pukazhenthi, B., E. Noiles, K. Pelican, A. Donoghue, D. Wildt, and J. Howard. 2000. Osmotic effects on feline spermatozoa from normospermic versus teratospermic donors. Cryobiology **40**:139-150.

Pukazhenthi, B. S., K. Neubauer, K. Jewgenow, J. Howard, and D. E. Wildt. 2006. The impact and potential etiology of teratospermia in the domestic cat and its wild relatives. Theriogenology **66**:112-121.

Pukazhenthi, B. S., and D. E. Wildt. 2004. Which reproductive technologies are most relevant to studying, managing and conserving wildlife? Reproduction Fertility and Development **16**:33-46.

Reul, J., and E. R. Dekloet. 1985. Two receptor systems for corticosterone in rat brain: Microdistribution and differential occupation. Endocrinology **117**:2505-2511.

Rivier, C., and S. Rivest. 1991. Effect of stress on the activity of the hypothalamic-pituitary-gonadal axis: Peripheral and central mechanisms. Biology of Reproduction **45**:523-532.

Rodriguez-Llanes, J. M., G. Verbeke, and C. Finlayson. 2009. Reproductive benefits of high social status in male macaques (*Macaca*). Animal Behaviour **78**:643-649.

Roelke, M. E., J. S. Martenson, and S. J. Obrien. 1993. The consequences of demographic reduction and genetic depletion in the endangered Florida panther. Current Biology **3**:340-350.

Roth, T. L., W. F. Swanson, E. Blumer, and D. E. Wildt. 1995. Enhancing zona penetration by spermatozoa from a teratospermic species, the cheetah (*Acinonyx jubatus*). Journal of Experimental Zoology **271**:323-330.

Ruiz-Miranda, C. R., S. A. Wells, R. Golden, and J. Seidensticker. 1998. Vocalizations and other behavioral responses of male cheetahs (*Acinonyx jubatus*) during experimental separation and reunion trials. Zoo Biology **17**:1-16.

Sands, J., and S. Creel. 2004. Social dominance, aggression and faecal glucocorticoid levels in a wild population of wolves, *Canis lupus*. Animal Behaviour **67**:387-396.

Sapolsky, R. M. 1990. Adrenocortical function, social rank, and personality among wild baboons. Biological Psychiatry **28**:862-&.

Schramm, R. D., M. B. Briggs, and J. Reeves. 1994. Spontaneous and induced ovulation in the lion (*Panthera leo*). Zoo Biology **13**:301-307.

Senger, P. L. 2005. Pathways to pregnancy and parturition. 2nd revised edition. Current Conceptions, Inc., Pullman, Washington.

Sharp, N. C. C. 1997. Timed running speed of a cheetah (*Acinonyx jubatus*). Journal of Zoology **241**:493-494.

Smith, T. E., C. G. Faulkes, and D. H. Abbott. 1997. Combined olfactory contact with the parent colony and direct contact with nonbreeding animals does not maintain suppression of ovulation in female naked mole-rats (*Heterocephalus glaber*). Hormones and Behavior **31**:277-288.

Sokal, R. R., and F. J. Rohlf. 2012. Biometry. Fourth edition. W.H. Freeman and Company, New York, NY.

Spiering, P. A., M. J. Somers, J. E. Maldonado, D. E. Wildt, and M. S. Gunther. 2010. Reproductive sharing and proximate factors mediating cooperative breeding in the African wild dog (*Lycaon pictus*). Behavioral Ecology and Sociobiology **64**:583-592.

Stoebel, D. P., and G. P. Moberg. 1982. Repeated acute stress during the follicular phase and luteinizing hormone surge of dairy heifers. Journal of Dairy Science **65**:92-96.

Surbeck, M., R. Mundry, and G. Hohmann. 2011. Mothers matter! Maternal support, dominance status and mating success in male bonobos (*Pan paniscus*). Proceedings of the Royal Society B-Biological Sciences **278**:590-598.

Swanson, W. F., J. L. Brown, and D. E. Wildt. 1996. Influence of seasonality on reproductive traits of the male Pallas' cat (Felis manul) and implications for captive management. Journal of Zoo and Wildlife Medicine **27**:234-240.

Terio, K. A., S. B. Citino, and J. L. Brown. 1999. Fecal cortisol metabolite analysis for noninvasive monitoring of adrenocortical function in the cheetah (*Acinonyx jubatus*). Journal of Zoo and Wildlife Medicine **30**:484-491.

Terio, K. A., L. Marker, and L. Munson. 2004. Evidence for chronic stress in captive but not free-ranging cheetahs (*Acinonyx jubatus*) based on adrenal morphology and function. Journal of Wildlife Diseases **40**:259-266.

Terio, K. A., L. Marker, E. W. Overstrom, and J. L. Brown. 2003. Analysis of ovarian and adrenal activity in Namibian cheetahs. South African Journal of Wildlife Research **33**:71-78.

Terrell, K. A., D. E. Wildt, N. M. Anthony, B. D. Bavister, S. P. Leibo, L. M. Penfold, L. L. Marker, and A. E. Crosier. 2010. Evidence for compromised metabolic function and limited glucose uptake in spermatozoa from the teratospermic domestic cat (*Felis catus*) and cheetah (*Acinonyx jubatus*). Biology of Reproduction **83**:833-841.

Terrell, K. A., D. E. Wildt, N. M. Anthony, B. D. Bavister, S. P. Leibo, L. M. Penfold, L. L. Marker, and A. E. Crosier. 2011a. Glycolytic enzyme activity is essential for domestic cat (*Felis catus*) and cheetah (*Acinonyx jubatus*) sperm motility and viability in a sugar-free medium. Biology of Reproduction **84**:1198-1206.

Terrell, K. A., D. E. Wildt, N. M. Anthony, B. D. Bavister, S. P. Leibo, L. M. Penfold, L. L. Marker, and A. E. Crosier. 2011b. Oxidative phosphorylation is essential for felid sperm function, but is substantially lower in cheetah (*Acinonyx jubatus*) compared to domestic cat (*Felis catus*) ejaculate. Biology of Reproduction **85**:473-481.

Terrell, K. A., D. E. Wildt, N. M. Anthony, B. D. Bavister, S. P. Leibo, L. M. Penfold, L. L. Marker, and A. E. Crosier. 2012. Different patterns of metabolic cryo-damage in domestic cat (*Felis catus*) and cheetah (*Acinonyx jubatus*) spermatozoa. Cryobiology **64**:110-117.

Thibier, M., and O. Rolland. 1976. The effect of dexamethasone (DXM) on circulating testosterone (T) and luteinizing hormone (LH) in young postpubertal bulls. Theriogenology **5**:53-60.

Tilbrook, A. J., A. I. Turner, and I. J. Clarke. 2000. Effects of stress on reproduction in non-rodent mammals: The role of glucocorticoids and sex differences. Reviews of Reproduction **5**:105-113.

Virgin, C. E., and R. M. Sapolsky. 1997. Styles of male social behavior and their endocrine correlates among low-ranking baboons. American Journal of Primatology **42**:25-39.

Wells, A., K. A. Terio, M. H. Ziccardi, and L. Munson. 2004. The stress response to environmental change in captive cheetahs (*Acinonyx jubatus*). Journal of Zoo and Wildlife Medicine **35**:8-14.

West, P. M., and C. Packer. 2002. Sexual selection, temperature, and the lion's mane. Science **297**:1339-1343.

Wielebnowski, N., and J. L. Brown. 1998. Behavioral correlates of physiological estrus in cheetahs. Zoo Biology **17**:193-209.

Wielebnowski, N. C. 1999. Behavioral differences as predictors of breeding status in captive cheetahs. Zoo Biology **18**:335-349.

Wielebnowski, N. C., N. Fletchall, K. Carlstead, J. M. Busso, and J. L. Brown. 2002a. Noninvasive assessment of adrenal activity associated with husbandry and behavioral factors in the North American clouded leopard population. Zoo Biology **21**:77-98.

Wielebnowski, N. C., K. Ziegler, D. E. Wildt, J. Lukas, and J. L. Brown. 2002b. Impact of social management on reproductive, adrenal and behavioural activity in the cheetah (*Acinonyx jubatus*). Animal Conservation **5**:291-301.

Wildt, D. E., J. L. Brown, M. Bush, M. A. Barone, K. A. Cooper, J. Grisham, and J. G. Howard. 1993. Reproductive status of cheetahs (*Acinonyx jubatus*) in North American zoos: The benefits of physiological surveys for strategic planning. Zoo Biology **12**:45-80.

Wildt, D. E., M. Bush, K. L. Goodrowe, C. Packer, A. E. Pusey, J. L. Brown, P. Joslin, and S. J. Obrien. 1987a. Reproductive and genetic consequences of founding isolated lion populations. Nature **329**:328-331.

Wildt, D. E., M. Bush, J. G. Howard, S. J. Obrien, D. Meltzer, A. Vandyk, H. Ebedes, and D. J. Brand. 1983. Unique seminal quality in the South African cheetah and a

comparative evaluation in the domestic cat. Biology of Reproduction **29**:1019-1025.

Wildt, D. E., S. Y. W. Chan, S. W. J. Seager, and P. K. Chakraborty. 1981a. Ovarian activity, circulating hormones, and sexual behavior in the cat. I. Relationships during the coitus-induced luteal phase and the estrous period without mating. Biology of Reproduction **25**:15-28.

Wildt, D. E., and J. Grisham. 1993. Basic research and the cheetah SSP program. Zoo Biology **12**:3-4.

Wildt, D. E., S. J. Obrien, J. G. Howard, T. M. Caro, M. E. Roelke, J. L. Brown, and M. Bush. 1987b. Similarity in ejaculate-endocrine characteristics in captive versus free-ranging cheetahs of 2 subspecies. Biology of Reproduction **36**:351-360.

Wildt, D. E., L. G. Phillips, L. G. Simmons, P. K. Chakraborty, J. L. Brown, J. G. Howard, A. Teare, and M. Bush. 1988. A comparative analysis of ejaculate and hormonal characteristics of the captive male cheetah, tiger, leopard, and puma. Biology of Reproduction **38**:245-255.

Wildt, D. E., C. C. Platz, S. W. J. Seager, and M. Bush. 1981b. Induction of ovarian activity in the cheetah (*Acinonyx jubatus*). Biology of Reproduction **24**:217-222.

Wildt, D. E., W. F. Rall, J. K. Critser, S. L. Monfort, and U. S. Seal. 1997. Genome resource banks. Bioscience **47**:689-698.

Wildt, D. E., and T. L. Roth. 1997. Assisted reproduction for managing and conserving threatened felids. International Zoo Yearbook **35**:164-172.

Xiao, E., J. Luckhaus, W. Niemann, and M. Ferin. 1989. Acute inhibition of gonadotropin secretion by corticotropin-releasing hormone in the primate: Are the adrenal glands involved? Endocrinology **124**:1632-1637.

Young, K. M., S. L. Walker, C. Lanthier, W. T. Waddell, S. L. Monfort, and J. L. Brown. 2004. Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticold analyses. General and Comparative Endocrinology **137**:148-165.

BIOGRAPHY

Diana C. Koester graduated from Ottoville High School, Ottoville, Ohio, in 2004. She received her Bachelor of Science in Zoology from the Ohio State University in 2008.