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Stephanie Marie Dloniak

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PATTERNS

SOCIOENDOCRINOLOGY OF SPOTTED HYENAS: PATTERNS OF ANDROGEN AND GLUCOCORTICOID EXCRETION WITHIN A UNIQUE SOCIAL SYSTEM

By

Stephanie Marie Dloniak

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Zoology
Program in Ecology, Evolutionary Biology and Behavior

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ABSTRACT

SOCIOENDOCRINOLOGY OF SPOTTED HYENAS:
PATTERNS OF ANDROGEN AND GLUCOCORTICOID EXCRETION WITHIN A UNIQUE SOCIAL SYSTEM

By

Stephanie Marie Dloniak

Socioendocrinology is specifically concerned with the effects of an animal's social environment on the interactions between hormones and behavior. Here, I examine how unique aspects of the social system of the spotted hyena, *Crocuta crocuta*, are mediated by hormone-behavior interactions in individuals of the species. Working with data collected over 10 years from a free-ranging clan of spotted hyenas in Kenya, I validate methods to quantify androgen and glucocorticoid concentrations in fecal samples collected from known individual hyenas. I then use these techniques to address three issues central to spotted hyena socioendocrinology. I first investigate the relationships among intra- and intersexual interactions on male androgen levels. In other polygynous species, aggressive competition causes elevated androgen levels in males. I find that the best predictor of increased androgens in male spotted hyenas is the degree of interaction with dominant females. This suggests that attempts to explain variation in androgen-behavior relationships in male vertebrates should consider intersexual interactions as well as male-male competition.

Female spotted hyenas are both morphologically and behaviorally masculinized. I test whether maternal effects of androgens are involved in

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behavioral masculinization in this species. I find that mothers of high social rank have greater fecal androgen concentrations during pregnancy than do low-ranking mothers. This variation in maternal androgens is related to variation in rates of aggression and sexual play in offspring. These results indicate a possible non-genetic, androgen-mediated effect of a mother's social environment on the organization of behavioral patterns in hyenas, similar to the effect of maternal deposition of androgens in bird eggs on chick development and behavior.

Finally, I examine predictors of glucocorticoid concentrations in hyenas. I show that time of day of sample is an important covariate that must be controlled for. Social status is not a predictor of glucocorticoid concentrations in adult hyenas of either sex. In female hyenas, reproductive status influences glucocorticoid concentrations, whereas in males, prey availability has an important effect. Therefore, different physiological and ecological variables predict stress hormone concentrations in males and females of this species, and social status per se does not appear to be an important variable.

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Perhaps the best part of writing this dissertation is being able to thank all of the wonderful individuals who have helped me complete it. This research was made possible by help and encouragement from many different people both in the US and in Kenya. First and foremost, I would like to thank my advisor, Kay Holekamp, for giving me the opportunity to work in her lab and to live and work in the Masai Mara for almost two years. Kay has been extremely patient with me and has always challenged me to live up to my potential. Being a part of her project has made me a better person, and I consider myself very lucky to have worked with her. I am also particularly grateful to Jeff French, one of my committee members. Jeff and his family welcomed me into their home numerous times when I traveled to Omaha to do lab work, which made those visits comfortable and fun. Jeff even made a trip to Kenya to do a bit of field work himself, in addition to a climb up Mt. Kenya. Not many graduate students get to literally climb a mountain with a committee member! Committee members Cheryl Sisk and Laura Smale were always available to discuss data and ideas, and I thank them as well.

My family and friends have given me unconditional support throughout my graduate career. My mom, Sue Matz, has sent me about a hundred care packages, as well as countless positive thoughts, in both Michigan and in Kenya. My dad, Frank Dloniak, has always sent cheesecake and has never let me down when I needed him. Over the years, my brother, Brian, provided many breaks

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from work and outlets from the academic world. My family may not quite get why working with hyena poop is fun or how I can be happy living without running water, but they have always encouraged me to live the life of my choice and to work hard. My very best friend, Howard Saunders, has done more than words can say. The last few years have been the best of all because of him, even though we have had to spend so much time apart. At least we have many years of tent-time, birding, and playing in the African bush ahead of us. My buddy Moose always welcomed me home, both after long days at school and after two years in Kenya.

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I was fortunate to collaborate with some excellent scientists while conducting my research. Jeff French taught me how to do fecal extractions and hormone assays in his laboratory, and assisted with most of my lab work. Ned Place, Steve Glickman, and Mary Weldele graciously performed hormone challenges on the hyenas in the captive colony at the University of California Berkeley Field Station. Mary collected many hyena fecal samples that were crucial for my research, and Ned and Steve also provided valuable feedback on my results. I thank Peggy Ostrom for allowing me to use the lyophilizer in her lab. Dave Kersey and Janine Brown, of the Conservation and Research Center of the Smithsonian Institution, completed the HPLC analyses in Chapter 2, and Janine provided valuable insight in the interpretation of HPLC results.

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Elly Kirkman always provided a hot shower and a break from fisi camp, and their visits to fisi camp on the pikipiki were always greeted with excitement when we heard them coming from over a kilometer away. Lars, Tina, Jonas, and Kristofer always gave me non-vegetarian breakfasts and great conversation at Dream Camp. Nasha Silantoi was a wonderful and inspirational break from my daily routine, and our conversations often reminded me of what's important in life. Matt Walpole was always fun to discuss conservation and life issues with. Ian McRae was an extremely helpful mechanic and friend. Anthony and Fiona Cheffings always entertained me and allowed me to vent frustrations in Nairobi. John Watkin was a good friend, a great listener, and a wonderfully bad influence. I also thank him for sending me random car parts on flights to the Mara. Lastly, Simo was my "nurse" when I had malaria, and was also the absolute best source of logistical advice and vehicle help a girl in the bush could ever want. Countless others helped me out at various times, and I thank them all.

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TABLE OF CONTENTS

Table 2.1.
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expressed

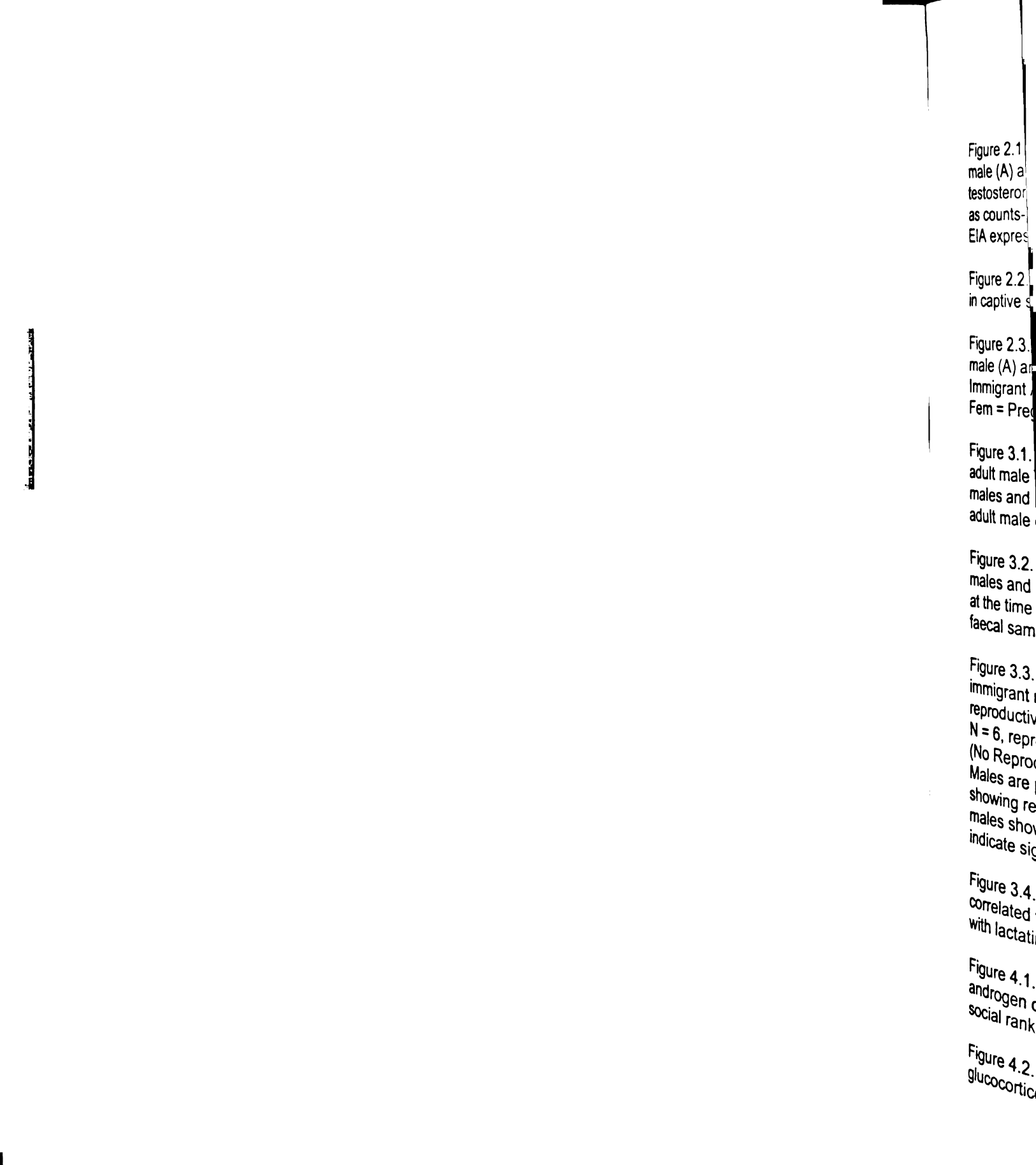
Table 4.1.
relation to
hyenas. Va

Table 5.1.
captive spo
are express
challenge.

Table 5.2.
male Talek

Table 5.3.
hyenas. W

LIST OF TABLES



LIST OF FIGURES

Figure 4.3
of gestatio
gestation.

Figure 4.4
day of ges
gestation.

Figure 4.5
male (A) a
were 2-6 m

Figure 4.6.
in male (A)
were 2-6 m

Figure 4.7.
directed by
period whe

Figure 5.1
challenge
indicated b

Figure 5.2
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Figure 5.3
glucocortic

Figure 5.4
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Figure 5.5
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Figure 5.6
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CHAPTER 1

GENERAL INTRODUCTION

Behavioral endocrinology is the study of how hormones influence the behavior of animals. It joins together the fields of endocrinology, neuroscience, and psychology to ask questions about how changes in the endocrine system influence the brain and behavior (Becker and Breedlove, 2002). Hormone action is influenced by both internal and external stimuli, and for individuals living in social groups, interactions with other group members can represent significant external stimuli. Socioendocrinology is the branch of behavioral endocrinology specifically concerned with the effects of an animal's social environment on the interactions between hormones and behavior (Bercovitch and Ziegler, 1990). My dissertation investigates various facets of the socioendocrinology of wild spotted hyenas (*Crocuta crocuta*), a large carnivore that lives in large, stable social groups.

Socioendocrinology

The goal of socioendocrinology is to understand the links among the social environment, hormones, and behavior in order to know how they modulate the reproductive success of individuals living in social groups (Bercovitch and Ziegler, 1990). Studies that monitor variation in both endocrine physiology and behavior are able to link the mechanisms mediating behavior with the functional significance of behavior. Such studies therefore have the potential to increase our understanding of the evolution of social organization, mating systems, and

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life history strategies (Ketterson and Nolan, 1992). Socioendocrinology provides a framework in which to connect evolutionary biology with reproductive physiology, and thus bridges the traditional gap between proximate and ultimate levels of analysis.

Where natural selection has favored individuals who live in groups and cooperate with group-mates, elaborate social systems have evolved along with specific mechanisms for maintaining order within social groups. In stable groups in which individuals interact repeatedly over time, a dominance hierarchy often determines priority of access to resources within each group (Pusey and Packer, 1997). Social dominance has a dramatic effect on reproduction in cooperatively breeding species in that dominant members of such societies almost completely monopolize reproduction (birds: Stacey and Koenig, 1990; mammals: Solomon and French, 1986). The influence of dominance on reproduction may be mediated by the hypothalamic-pituitary-gonadal (HPG) axis, the normal function of which is necessary for reproduction. Within the HPG axis, gonadotropin releasing hormone (GnRH) is secreted from neurosecretory cells in the hypothalamus, and acts on the pituitary to regulate release of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). The gonadotropins then act on the gonad, where FSH affects gametogenesis and LH causes the release of sex steroids. In males, the testes produce androgens including testosterone (T), whereas in females the ovaries produce estrogens and progestins (Norman and Litwack, 1997). Concentrations of the gonadotropins and sex steroids may be directly affected by dominance status,

though relationships between dominance and hormone concentrations vary across species, indicating that factors other than social dominance may interact to influence hormones and reproduction. (Bercovitch and Clarke, 1995; Zielinski and Vandenbergh, 1993).

In this dissertation, I investigate whether social status and other variables influence the functioning of the HPG axis in a social carnivore exhibiting female dominance over males. I inquire whether social status, male-male aggressive competition, or intersexual interactions influence androgen concentrations in male spotted hyenas. I also inquire whether social status influences androgen levels during pregnancy in female hyenas, and whether variation in prenatal androgen exposure corresponds to variation in sexual play and aggression in cubs. Lastly, I test the relative importance of social dominance in the regulation of stress hormone concentrations in this species.

One fundamental tenet of socioendocrinology is that individuals exhibit an adaptive flexibility enabling them to adjust both mating and parental effort on the basis of their current social surroundings. The occurrence of this flexibility can result in alternative mating strategies for maximizing individual reproductive success (Bercovitch and Ziegler, 2002). One excellent example of the effects of dominance on reproductive activity is found in the naked mole-rat (*Heterocephalus glaber*). Naked mole-rats are eusocial subterranean rodents with extreme division of labor in reproduction. Each group typically has one queen who mates with one to three breeding males (Jarvis, 1991). The rest of the colony members of both sexes are reproductively suppressed, but not sterile

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(Faulkes et al., 1990; 1991; Faulkes and Abbott, 1991; 1997). Non-breeding females are anovulatory (Faulkes et al., 1990), and non-breeding males have lower plasma LH and T levels than do breeding males (Faulkes et al., 1991). Both non-breeding females and non-breeding males in a colony rapidly become reproductively active when socially suppressive cues from the dominant individuals are removed (Faulkes and Abbott, 1991). Thus, socially regulated physiological suppression of the HPG axis has evolved for maintenance of order in this unusual social system.

Among primate males living in groups, variation in reproductive success is likely to arise from the adoption of alternative reproductive strategies by individual group members (Bercovitch and Goy, 1990), and each alternative strategy may be associated with a different endocrine profile. In rhesus macaques (*Macaca mulatta*), socially dominant males have greater reproductive success than subordinate males because of a suite of social, sexual, and physiological traits that foster mating with multiple females (Bercovitch and Nurnberg, 1996). Alpha male mandrills (*Mandrillus sphinx*) have greater reproductive success, more pronounced secondary sex characteristics, and higher T concentrations than subordinate males (Wickings and Dixson, 1992; Dixson et al., 1993). In these mandrills, abrupt changes in social status are also associated with dramatic changes in physical appearance, circulating T, and reproductive activity (Setchell and Dixson, 2001). Lastly, orangutan males (*Pongo pygmaeus*) show perhaps the most startling physical manifestation of a socioendocrine mechanism, with alternative paths of development leading to two

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different adult morphs, one with and one without secondary sexual traits such as larger body size, cheek flanges, and enlarged laryngeal sacs (Kingsley, 1982; Graham and Nadler, 1990). Social suppression of endocrine levels is associated with the lack of development of these characteristics, but not with reduced fertility in this species (Maggioncalda et al., 1999; 2000).

Recently, Sannen et al. (2003) showed that urinary androgen levels in male and female bonobos (*Pan paniscus*) are similar, whereas those in male and female chimpanzees (*Pan troglodytes*) differ substantially. These authors suggested that this species difference in male-female androgen ratio is related to their different social systems and differing levels of aggression among males. This pattern is predicted by the "Challenge Hypothesis." The challenge hypothesis is one of the best known, and most tested, hypotheses in the current socioendocrine literature. Wingfield and colleagues (1990; 2000) proposed this hypothesis to account for variation in the male androgen response to male-male aggression among avian species. In brief, this hypothesis suggests that the relationship between T and aggression during the breeding season in males of a given species can be predicted by two variables: the level of aggressive male-male competition and the extent of paternal care characteristic of that species' mating system. This hypothesis has mainly been tested in high-latitude, seasonally breeding birds, but it has more recently been applied with some success to mammalian species.

Although much current socioendocrine research focuses on relationships among hormones, reproduction, and intrasexual competition, another key area of

research involves study of effects on hormones exerted by members of the opposite sex. Intersexual social interactions can strongly influence hormone levels in many species of birds and mammals, causing androgen levels in males to increase following their pairing with females (e.g. birds: Moore, 1983; Pinxten et al., 2003; mammals: Katongole et al., 1971; Rose et al., 1972; Jainudeen et al., 1972; Bernstein et al., 1977). One of the clearest examples of intersexual effects on reproductive endocrinology in mammals is reflex ovulation, whereby copulation results in a surge of circulating LH in females, and this LH then acts on follicular tissue in the ovary to induce ovulation (Marie and Anouassi, 1986). A more subtle intersexual effect occurs in red deer (*Cervus elephas*), where ovulation may be accelerated as a result of roaring among males (McComb, 1987). Thus socioendocrine mechanisms can serve to coordinate the sexes for successful reproduction.

Another broad area of socioendocrine research has focused on the ways in which individual differences in the stress response can reflect variation in social dominance status among gregarious animals. In vertebrates, stressors (aversive stimuli) are known to activate the hypothalamic-pituitary-adrenal (HPA) axis, resulting in the release of glucocorticoids (GCs, stress hormones) from the adrenal cortex (Sapolsky, 2002). Individual differences in the physiological stress response often relate to an individual's dominance status within its social group (e.g. Bronson and Eleftheriou, 1964; Christian and Davis, 1964; Coe et al., 1979; Creel et al., 1997). However, the relationship between social rank and patterns of the stress response is not consistent among mammals, indicating that there is no

simple relationship that holds across species between social status and any aspect of stress physiology (Clarke and Boinski, 1995; Sapolsky, 1993; Abbott et al., 1997; Abbott et al., 2003).

Influential early work on this issue was conducted with winner-loser studies in captive rodents and monkeys (Louch and Higginbotham, 1967; Bronson and Eleftheriou, 1964; Manogue, 1975). In these studies, both winners and losers of staged encounters showed a strong stress response, but the response was larger among losers. In stable social groups, subordinate olive baboons (*Papio anubis*) and naked mole-rats have higher levels of GCs than dominant individuals (Virgin and Sapolsky, 1997; Faulkes and Abbott, 1997). However, some recent studies with female mammals have shown that dominant individuals have higher GC levels than subordinates (eg. Saltzman et al., 1994; Creel et al., 1996; 1997; Sands and Creel, 2004). In cooperatively breeding carnivores, the highest ranking individuals with the vast majority of reproductive success have higher stress hormone levels than do subordinate individuals in the group (reviewed in Creel, 2001; Creel and Sands, 2003). The diversity of stress hormone-dominance relationships in mammals appears to reflect, among other things, the extraordinary variety of social systems observed in this taxonomic group.

One of the most exciting new areas of socioendocrine research is that involving the study of maternal effects. Socioendocrine factors can affect ontogenetic trajectories, beginning in utero and lasting until death. For example, neuroendocrine feedback mechanisms play a prominent role in mediating

reproductive processes in post-pubescent animals, but variation in fetal and neonatal development influences adult neuroendocrine profiles. Prenatally stressed rhesus macaques develop different neuroendocrine feedback sensitivities than do control subjects not exposed to prenatal stress (Clarke et al., 1994). Blockage of elevated neonatal T in male common marmosets (*Callithrix jacchus*) results in attenuation of the pubertal rise in T (Lunn et al., 1994). Social interactions can influence a pregnant female's glucocorticoid or sex steroid levels, which may in turn have permanent effects on offspring development. These organizational effects can have profound influences on adult behavior and reproduction, and thus create natural variation on which selection may act.

Spotted hyenas

Spotted hyenas are the second largest, and most abundant, large carnivore in sub-Saharan Africa (Feldhammer et al., 1999). The social system of the spotted hyena is unique, and the hyena is therefore a particularly interesting species in which to investigate relationships between hormones and behavior. Hyenas live in dynamic fission-fusion groups of up to 80 individuals, called clans, that are structured by hierarchical rank relationships (Kruuk, 1972; Tilson and Hamilton, 1984; Frank, 1986; Holekamp and Smale, 1990). Each clan defends an exclusive group territory containing one or two active communal denning sites, where females rear their offspring (Kruuk, 1972; East et al., 1989). Female hyenas are philopatric, but almost all males disperse from their natal clans between the ages of 2 and 5 years (Frank, 1986; Henschel and Skinner, 1987;

Smale et al., 1997; Van Horn et al., 2003). All adult females are socially dominant to all adult males not born in the clan (Kruuk, 1972; Smale et al., 1993), and adult females are more aggressive than adult immigrant males (Kruuk, 1972; Frank, 1986; Mills, 1990; Szykman et al., 2003).

Social rank is important in this species because it determines an individual's priority of access to food (Kruuk, 1972; Tilson and Hamilton, 1984; Frank, 1986; Mills, 1990). A individual's social rank is not determined by fighting ability or size, but rather it is "inherited" from it's mother (Holekamp and Smale, 1991; 1993; Smale et al., 1993; Frank, 1986). There is a strong effect of social rank on female reproductive success, with higher ranking individuals doing much better than lower ranking individuals (Frank et al., 1995; Holekamp et al., 1996; Holekamp and Smale, 2000). Social rank of individuals within the adult immigrant male hierarchy is acquired by queuing, rather than by fighting (East and Hofer, 2001; Engh et al. 2002). Males are seldom observed to engage in aggressive interactions, and what little aggression does occur is typically very mild.

In addition to this unique social system, female hyenas are about 10% heavier than males (Hamilton et al., 1986), and their genitalia are virilized to resemble the genitals of males. The clitoris is enlarged and forms an erectile pseudopenis, the labia are fused to form a pseudo-scrotum, and the urogenital tract passes through the pseuduopenis (Matthews, 1939; Neaves et al., 1980; Frank et al., 1990; Cunha et al., 2003). Female hyenas urinate, copulate, and give birth through the elongated clitoris. Due to female dominance and morphological masculinization of the external genitalia, females appear to have

unusually tight control over reproduction (Kruuk, 1972; East et al., 2003). Molecular genetic data have revealed that high-ranking males do not monopolize reproduction, that both males and females mate with multiple partners, and that at least 20% of twin litters are of mixed paternity (Engh et al., 2002; East et al., 2003). Males show no paternal care (Kruuk, 1972).

The unique suite of social, behavioral, and morphological characteristics exhibited by spotted hyenas may be shaped and modulated by various endocrine mechanisms. The chapters of this dissertation examine some of these potential mechanisms. Exploring how endocrine mechanisms operate in this unusual, but extremely successful large mammal will shed light on important hormone-behavior relationships in this species in particular, and on some general principles that may be widely applicable in other mammals as well.

Overview of chapters

All of the data presented in this dissertation were collected from a single clan of wild spotted hyenas, the Talek clan, which defends a territory on the northeastern edge of the Masai Mara National Reserve in Kenya. The Talek clan has been monitored extensively since 1988, and the research presented here was only made possible due to the efforts of all of the people involved with the Mara Hyena Project over the years. I collected a relatively small proportion of these data, as I spent only 2 years in Kenya doing field work. Much of the data were collected by Kay Holekamp, Laura Smale, numerous field assistants, and

other graduate students. I therefore use "we" in each data chapter to indicate that this research was a collaborative effort.

Acquiring blood samples for traditional endocrine analyses is somewhat difficult with large free-ranging carnivores. Spotted hyenas must be immobilized in order to collect blood, and this process disrupts normal behavior. However, recent advances in non-invasive endocrine methods have made possible the quantification of steroid hormones in feces. Luckily, researchers on the Mara Hyena Project had the foresight to begin collecting and freezing fecal samples from known individual hyenas in 1993. In order to investigate the socioendocrinology of spotted hyenas using this long-term data set, it was first necessary to validate methods for non-invasive quantification of steroid hormone concentrations in fecal samples collected and frozen over the last ten years. In Chapter 2, I collaborate with Dr. Jeffrey French of the University of Nebraska at Omaha, and with scientists studying captive hyenas at the University of California Berkeley, in order to validate an enzymeimmunoassay with which to measure fecal androgen concentrations in spotted hyenas. We employ HPLC analysis, as well as challenges with GnRH in captive hyenas. We test the effects of potential procedural covariates in fecal androgen concentrations to determine whether it is necessary to control for variables like time of day of sample deposition, bone in the diet, or time elapsed between deposition and freezing of the sample. Lastly, we confirm that fecal androgen patterns mirror plasma testosterone patterns, indicating that our assay measures biologically relevant variation in androgens in

wild hyenas. The work presented in Chapter 2 has recently appeared in *General and Comparative Endocrinology* (Dloniak et al., 2004).

Next, the noninvasive methods we developed in Chapter 2 are used to address relationships among fecal androgens, social status, reproductive state, and behavior in free-ranging spotted hyenas. In Chapter 3, my goals were to determine the best predictors of elevated androgen levels in adult males of this species, and to elucidate whether sexually motivated aggression against other males or interaction with females had a stronger relationship with fecal androgen concentrations. In many polygynous mammals and birds, elevated androgens are associated with increased male-male aggression during the breeding season (eg. Wingfield et al., 1990; 2000). However, male spotted hyenas show very low levels of aggressive male-male competition over females, whereas they expend considerable effort actively courting females. Here, I show that androgen concentrations in male spotted hyenas are related to courtship interactions and degree of association with attractive adult females. These results suggest that the biggest social challenge a male hyena may face is interacting with attractive, but socially dominant females. The work presented in Chapter 3 is currently in review at *Animal Behaviour*.

The prevailing hypothesis suggested to account for the unusual array of 'masculine' morphological and behavioral traits in the female spotted hyena is that elevated androgens mediate the development of large, aggressive females that are able to dominate males in competitive feeding situations, with a byproduct of genital masculinization (Gould, 1981; Gould and Vrba, 1982;

reviewed in Frank, 1997). In Chapter 2, I confirm that pregnant female hyenas have higher levels of androgens in both blood and feces than do lactating females. In Chapter 4, I investigate the effect of social rank on maternal androgens during pregnancy, and the effect of variation in prenatal maternal androgens on offspring behavior, thus testing whether maternal effects of androgens influence behavioral development in this species. I show that high-ranking females have higher levels of fecal androgens during gestation than do low-ranking females. To my knowledge, this is the first documentation of an effect of social status on androgen levels during gestation in a wild mammal. In addition, I show that prenatal androgen concentrations are positively correlated with rates of aggression and sexual play in cubs. These results may demonstrate a non-genetic form of inheritance related to female social status in mammals that may be comparable to the phenomenon observed in some birds in which variation in the amount of T deposited by mothers in eggs generates variation in offspring behavior and performance (e.g. Schwabl, 1996; Eising et al., 2001).

Finally, in Chapter 5, I attempt to determine the best predictors of stress hormone concentrations in spotted hyenas. In order to investigate what variables influence stress levels in spotted hyenas, it was first necessary for me to validate an assay for fecal glucocorticoids in hyenas, with methods similar to those described for the androgen assay in Chapter 2. Using this new methodology, I then develop models testing the power of variables such as social status, local prey availability, and female reproductive status, to predict fecal glucocorticoid concentrations in adult hyenas. I find that important procedural covariates,

namely time of day of fecal sample deposition and female age at time of sampling, must be controlled for when investigating other variables of potential interest. In addition, factors other than social status are important as predictors of fecal glucocorticoid excretion in hyenas. Reproductive state and prey availability have significant effects in female and male hyenas, respectively, indicating that physiological and ecological factors are more important influences on stress hormones than social status in this species.

CHAPTER 2

NON-INVASIVE MONITORING OF FECAL ANDROGENS IN SPOTTED HYENAS

INTRODUCTION

During fetal development, female spotted hyenas (*Crocuta crocuta*) are exposed to unusually high levels of androgens, and females are heavily masculinized in various aspects of their morphology and behavior (Lindeque and Skinner, 1982; Glickman et al., 1987; Glickman et al., 1992; Licht et al., 1992). The external genitalia of females closely resemble those of males in that the clitoris is elongated to form a fully erectile pseudopenis, and the vaginal labia are fused to form a pseudoscrotum (Matthews, 1939; Neaves et al., 1980; Frank et al., 1990). Although adult females may weigh more than adult males, the sexes are monomorphic with respect to other adult body size measurements (Matthews, 1939; Kruuk, 1972; Hamilton et al., 1986, Van Horn et al., 2003). Females are socially dominant to adult breeding males (Kruuk, 1972; Smale et al., 1993; Smale et al., 1997), and females are more aggressive than adult males (Frank, 1986; Hamilton et al., 1986; Monaghan and Glickman, 1992; Szykman et al., 2003).

Spotted hyenas live in social groups called clans. Adult male and female clan members have separate stable linear dominance hierarchies (Kruuk, 1972; Tilson and Hamilton, 1984; Frank, 1986; Holekamp and Smale, 1990), and both male and female offspring "inherit" their mother's social rank (Holekamp and Smale, 1991; Smale et al., 1993). Most males eventually disperse from their

natal clans between the ages of 24 and 76 months (Frank, 1986; Henschel and Skinner, 1987; Smale et al., 1997, East and Hofer, 2001, Van Horn et al., 2003) and attempt to immigrate into new clans in search of mating opportunities (Holekamp and Smale, 1998; Engh et al., 2002). In males of many animal species, androgens vary according to social rank, aggressive behavior or dispersal status (e.g., rhesus monkeys: Rose et al., 1971, 1972; red deer: Lincoln et al., 1972; elephants: Jainudeen et al., 1972; primates: Bernstein et al., 1974; ground squirrels: Holekamp et al., 1984; vervet monkeys: Steklis et al., 1985; rabbits: Farabollini, 1987; birds: Wingfield et al., 1987; olive baboons: Alberts et al., 1992; macaques: Zumpe and Michael, 1996; cattle: Lunstra et al., 1998; spotted hyenas: Holekamp and Smale, 1998). Availability of a method for assessing relationships among androgens, behavior, and social rank in free-living spotted hyenas would therefore allow us to address a wide array of intriguing questions.

Matthews (1939) first proposed that the behavioral and morphological masculinization of the female spotted hyena might be associated with androgens, but this question was not addressed systematically until 40 years later. Although considerable headway has been made in hormone studies utilizing plasma samples from captive hyenas (see Glickman et al., 1992; Licht et al., 1992; Yalcinkaya et al., 1993; Licht et al., 1998; Drea et al., 1998; Glickman et al., 1998; Place et al., 2002; Drea et al., 2002), the study of circulating androgen levels in free-ranging spotted hyenas has yielded conflicting results (Goymann et al., 2001a). For example, Racey and Skinner (1979) found that mean plasma

testosterone (T) levels did not differ between the sexes, whereas Frank et al. (1985) concluded that adult males had significantly higher levels of serum T than adult females, and suggested that variation in T within each sex is related to social status. The most recent study addressing plasma androgen levels in wild spotted hyenas indicated that adult females had lower plasma T and dihydrotestosterone (DHT) levels than adult males, but androstenedione (A4) levels did not differ between the sexes (Goymann et al., 2001a).

A review of the existing literature on sex differences in circulating androgens in spotted hyenas (Goymann et al., 2001a) identified some of the variables that may contribute to differences among results from previous studies, including different blood collection procedures, various extraction and assay methods, and lack of knowledge about the reproductive or social status of individuals. Furthermore, the problem of small sample size has plagued all studies involving measures of circulating androgens in free-ranging spotted hyenas. Drawing blood from large carnivores in the wild involves immobilizing each sampled animal, and this invasive procedure represents a large effort that yields limited sample sizes. Multiple samples from the same individual are difficult to achieve by darting, as it can be stressful to the hyena (van Jaarsveld and Skinner, 1992) and conditions are often unsuitable for obtaining repeated measures from specific individuals. Fecal steroid hormone analysis offers an appealing alternative to blood sampling in order to answer questions about hormone–behavior relationships in the spotted hyena. This technique has recently been used to measure fecal androgens in an array of wild mammals (for

example; African wild dogs: Creel et al., 1997; sifakas: Brockmann et al., 1998; muriquis: Strier et al., 1999; ring-tailed lemurs: Cavigelli and Pereira, 2000; mongoose lemurs: Curtis et al., 2000; hairy-nosed wombats: Hamilton et al., 2000; meerkats: Moss et al., 2001; Japanese macaques: Barrett et al., 2002). Multiple fecal samples can be collected from an individual non-invasively, without disrupting normal behavior. Within a well-studied population, each sample can be placed in the context of the individual's known age, reproductive status, social rank, and observed behavior. In contrast to plasma samples, large numbers of fecal samples can often be collected from animals assigned to particular reproductive and social rank categories, yielding sample sizes appropriate for analyses of the relationships between these variables and androgens. Although Goymann et al. (2001b, 2003) have recently examined the relationship between ecological and social variables and glucocorticoid hormones excreted in hyena feces, no methods have been described to measure excreted androgens in this species.

Our aim here was to validate a fecal androgen enzymeimmunoassay (EIA) for use in spotted hyenas. After demonstrating assay parallelism and confirmation of immunoreactive androgen metabolites by HPLC, we assessed the biological validity of our androgen EIA. We first injected captive hyenas with LHRH, expecting to see a post-injection increase in fecal androgens. We also determined whether variation in diet might affect fecal androgen levels by systematically manipulating the proportion of the diet derived from bone in captive hyenas, and then measuring excreted androgens in samples collected

after feeding. Using archived frozen fecal samples collected from wild hyenas over the last decade, we inquired whether time of day of sample deposition, time elapsed before freezing the sample, or time elapsed between freezing and assay had any systematic effects on fecal androgen concentrations. Finally, we compared patterns of excreted and circulating androgens collected from particular groups of wild hyenas (immigrant vs. natal adult males, pregnant vs. lactating females) among which we expected to observe differences in plasma androgens.

METHODS

Captive study site, subject animals, and sample collection

All captive hyenas were housed at the University of California Berkeley Field Station for Behavioral Research. These individuals were of known age and reproductive status. Some hyenas were housed individually and others were housed in small groups (for more details see Berger et al., 1992). To identify feces produced by group-housed hyenas, their food was treated with food coloring. All fecal samples were collected between 0800 and 1200. These were immediately mixed thoroughly and stored in individual containers at -80° C until extraction and assay.

LHRH challenges were conducted on 5 gonadally-intact adult hyenas (3 males and 2 females). Fecal samples were collected daily for 7 days prior to treatment to establish baseline levels of fecal androgen excretion. On the day of LHRH challenge, animals were immobilized with ketamine and xylazine

administered by blow dart, and anesthetized with isoflorane inhalant. Each hyena then received a single i.v. injection of gonadotropin releasing hormone (1 mg/kg LHRH, L-7134, Sigma Chemical Co., St. Louis, MO). Each hyena was allowed to recover from anesthesia and released back to its home enclosure. Fecal samples were collected on the day of challenge as well as for 8 days after the challenge.

Although wild hyenas ingest highly variable amounts of meat and bone as they feed on ungulate prey, captive hyenas in this colony are normally fed a standard zoo carnivore mix (Nebraska Brand Feline Food, Central Nebraska Packing, Inc., North Platte, NE) and small amounts of bone every day (Berger et al., 1992). In order to assess whether variation in the amount of bone in the diet influences measurement of fecal androgens, we varied the bone content in the diet of 5 adult females. Two of these females were ovariectomized for another experiment. At the start of this experiment some individuals were fed only feline diet while others were fed a small amount of feline diet plus 3 or 4 sheep neck bones. After 1 to 3 days on their respective diets, diets were reversed in all subjects, and reversed again another 1 to 3 days later. Fecal samples were collected each day from all subjects. For statistical analysis, a given fecal sample represented the previous day's diet.

Field study site, subject animals, and sample collection

Our field study site was the Talek area of the Masai Mara National Reserve in southwest Kenya. The subject population was one large, stable *Crocuta* clan inhabiting a home range of approximately 65 km^2 (Boydston et al.,

2001). The Talek hyenas have been monitored intensively since June 1988, and all hyenas in the clan were identifiable based on each individual's unique spot pattern and other distinguishing marks. Sex was determined by the dimorphic glans morphology of the erect phallus (Frank et al., 1990). Ages of individuals born in the Talek clan were estimated to within 7 days based on pelage, size, appearance, and behavior of cubs when first observed. Adult natal males were Talek-born males between 24 and 60 months of age that had not yet dispersed from the Talek home range. Immigrant males were adult males that had dispersed from natal clans elsewhere and had been present in the Talek clan for at least 6 months. Ages of immigrant males were estimated based on toothwear (Van Horn et al., 2003). Date of first appearance in the Talek clan was recorded for each immigrant male, and tenure was calculated as time elapsed since joining the clan (Holekamp and Smale, 1998). Female reproductive state was determined by behavioral observations or by assessment during immobilization. A female was pregnant if she gave birth to cubs within 110 days after sampling (Matthews, 1939), or if fetuses were observed in her uterine horns during immobilization, using a Hitachi portable ultrasound machine. A female was lactating if she was observed to nurse cubs around the time of sampling and/or milk could be expressed from teats when she was immobilized. Critical incident sampling (Altmann, 1974) of all observed aggressive and appeasement behaviors was used to determine social ranks of individuals. Social ranks were assigned based on a matrix of outcomes of dyadic agonistic interactions (Martin and Bateson, 1988), as described previously (Smale et al., 1993).

Fecal samples were collected either during early morning (0530 – 0900h) or evening (1700 – 2000h) observation periods. Samples were collected whenever a hyena defecated, upon direct and unambiguous observation. Samples were first collected into plastic bags at the site of defecation, and later approximately 6 ml of mixed sample were transferred to multiple 2 ml cryovials for freezing in liquid nitrogen. Ninety-four percent of the samples were frozen within 12 h of collection, and all samples were frozen within 48 h of collection. Samples were stored in liquid nitrogen until shipped on dry ice to the United States, where they were stored at -20° C or colder until extraction and assay.

Between 1990 and 2002, 33 adult immigrant males, 13 adult natal males, 16 pregnant females, and 33 lactating females in the Talek clan were anesthetized with Telazol (2.5 mg/kg body mass) administered in a lightweight plastic dart fired from a CO_2 rifle. All immobilizations took place between 0630 and 0900 hours, when hyenas were found resting. Within 10-17 minutes of anesthetic injection, we drew a blood sample from each hyena's jugular vein into a heparinized vacutainer tube, and then proceeded to take an array of body and dental measurements, as described elsewhere (Van Horn et al., 2003). Hyenas typically recovered from anesthesia within 60 min. All immobilizations were performed in accordance with Kenyan law and with NIH animal treatment guidelines. Blood was centrifuged at 1000*g* for 5 min, then plasma was drawn off and stored in liquid nitrogen until it was shipped on dry ice to the United States, where it was stored at –80° C until radioimmunoassay.

Radioimmunoassay of plasma testosterone in wild hyenas

Duplicate aliquots of plasma from each sample from each wild hyena were assayed for total testosterone (T) using coated tube I^{125} kits from Diagnostic Products Corporation (Los Angeles, CA), as described by Holekamp and Smale (1998). The T kit was previously validated for use with this species by demonstrating parallelism between serial dilutions of plain and T-spiked plasma and the standard curve generated using kit calibrators. The detection limit of the assay was 0.04 ng/ml. Cross-reactivity of the T anti-serum with 5-alpha-dihydrotestosterone was 3.3%, and was less than 0.1% with any other androgen. The mean coefficient of variation between T assays (N = 11) was 7.1%. The mean intra-assay coefficient of variation for high and low T control tubes run with each T assay was 6.8%.

High performance liquid chromatography (HPLC) of fecal samples

Fecal samples from 6 adult male and 6 pregnant adult female wild spotted hyenas were selected for two pools (male and female, respectively) to be subjected to HPLC for determination of androgen metabolites. Samples were collected between 1995 and 2001, and stored frozen until extraction. Each animal contributed approximately 0.5 grams of feces to its sex-specific pool, and steroids were extracted with ethanol (see below). Samples were spiked with ~7000 cpm of ^{3}H-testosterone, ^{3}H-androstenedione, and ^{3}H-dihydrotestosterone and air-dried. Samples were reconstituted in 500 µl of phosphate buffered saline (PBS; pH 5.0), sonicated, and vortexed to remove any particulates sticking to the

surface of the glass tube. Fecal metabolites were first recovered by: 1) priming a Spice ™ C18 sample preparation cartridge (Analtech, Inc., Newark, DE) with 3ml of methanol followed by 3 ml of dH_2O; 2) loading the 500 µl PBS sample onto the cartridge; 3) pushing 5 ml of dH_2O through the cartridge; and 4) pushing 5 ml of methanol through the cartridge, and collecting this portion into a 12 x 75 mm glass tube. The methanol portion was dried under air and the residue was resuspended in 300µl of methanol. HPLC was conducted by injecting 50 µl of the reconstituted sample onto the column (Reverse Phase Microsorb™ MV 100 C18, 5um diameter particle size, Varian Analytical Instruments, Woburn, MA). A mobile phase of 45% acetonitrile in water over 80 min at room temperature (RT) was used at a rate of 1 ml/min. For determination of retention times of ^3H reference standards, aliquots (100 µl) from each fraction were counted for radioactivity. The remainder of the fraction was dried down and resuspended in 250 µl PBS and analyzed by enzyme immunoassay (EIA) to evaluate androgen immunoreactivity (see below).

Extraction and Assay of Androgens from Fecal Samples

Approximately 0.5 g wet weight of each fecal sample were placed frozen in an open whirl-pak bag and lyophilized overnight (Labconco Freeze-Dry System 10-269). After lyophilization, each sample was ground to a fine powder with a mortar and pestle. In a 16 x 125 mm culture tube, 0.2 g of powdered feces were mixed with 5 ml absolute ethanol. Tubes were capped and vortexed, and then placed on an orbital shaker for 12-14 h. The samples were again vortexed

to resuspend particulate matter from the side of the tube, placed in a block heater, and gently boiled for 20 min. The remaining suspension was centrifuged for 15 min at 1000*g* to pellet the solid fecal material. The ethanol supernatant was poured into a clean 12 x 75 mm culture tube and evaporated to dryness under compressed air in a warm-water bath. Samples were reconstituted in 1.0 ml PBS and stored frozen at -20° C until assay.

Fecal androgens were assayed using a modified version of a previously described testosterone EIA (Nunes et al., 2000). The assay utilized a testosterone antibody (R156/7) and a testosterone conjugate (horseradish peroxidase; HRP) kindly provided by Dr. Bill Lasley and Coralie Munro of the University of California, Davis. When used with plasma, this antibody cross-reacts 57.4% with dihydrotestosterone, < 0.3% with androstenedione, and < 0.1% with androsterone, dihydroepiandrosterone, estradiol, and progesterone. Stock antibody was diluted 1:15,000 in bicarbonate coating buffer and T-HRP was diluted 1:15,000 in PBS. Testosterone standards (Sigma) ranged from 7.8 – 1,000 pg per well in halving dilutions. Wells of microtiter plates (Nunc Maxisorp) were coated with 50 µl antibody, and diluted fecal sample extract (1:50 in PBS) and 50 µl T-HRP were incubated in duplicate wells for 2h. Unbound hormone was removed by washing the plates, and 100 µl of substrate (ABTS-H_2O_2) were then added to each well. Plates were read on a Dynex plate reader when optical density in B_0 wells reached 1.0. Serial dilutions of pooled extracts from multiple males and females produced displacement curves that were parallel to the displacement curve produced by T standards. Quality control in the assay was

monitored by measuring T concentrations in two sets of pools on each plate. One pool was diluted to yield a high concentration of T in 50 µl (~ 30% binding) and the other to yield a low concentration of T (~ 70% binding). The inter-assay coefficients of variation were 7.8% (high pool) and 4.5% (low pool). The intra-assay coefficient of variation, based on these same pools, averaged 9.58 +/- 3.9 % (n = 35 assays).

Archived sample collection, processing and storage conditions

Conditions and time in storage can affect assessments of steroid hormone concentrations in fecal samples (Khan et al., 2002, Terio et al., 2002), and circadian variation has also been reported for some excreted steroids (Sousa and Ziegler, 1998). We therefore inquired whether time of collection or variation in processing and storage conditions systematically affected fecal androgen levels in archived samples from wild hyenas. We extracted and assayed 539 samples from 39 adult females and 302 samples from 32 adult immigrant males, collected between 1993 and 2001 in the Talek clan, for which time of day of sample and time elapsed between defecation and freezing had been recorded. We tested for effects of these variables, as well as time elapsed between freezing and assay, on fecal androgen levels measured in male and female hyenas separately.

Comparison of fecal and plasma androgens

A strong biological validation of a fecal hormone assay includes demonstration of differences in fecal hormone concentrations among groups that also vary in circulating hormone concentrations. In order to do this in spotted hyenas, we analyzed archived frozen fecal samples collected from the Talek clan between January 1993 and June 2001 that could be compared with plasma samples acquired during immobilizations. Fecal samples from 16 adult natal males, 25 immigrant males, 25 pregnant females, and 22 lactating females were accessed in order to compare patterns in fecal androgens with patterns in plasma testosterone among similar groups of immobilized hyenas. A single fecal sample for each hyena was selected for assay. Plasma T may vary with immigrant male tenure (Holekamp and Smale, 1998; Goymann, 2000), so we assigned each immigrant a tenure for the day on which his sample was collected. Tenure of males whose samples were used in the immigrant male group showed a random distribution between 6 and 36 months, so samples were not biased toward males with long or short tenure. Age estimates for immigrant males at time of fecal sampling ranged between 32 and 98 months.

Statistical analysis

All statistical treatment of data followed Zar (1996). Prior to statistical analysis, all data sets were tested for departures from normality and homoscedasticity. When such departures were detected, we employed the

nonparametric equivalents of the appropriate parametric statistical tests. Analyses were considered significant when $P < 0.05$.

RESULTS

HPLC analysis

HPLC fractions assayed with the testosterone EIA revealed the presence of 6 immunoreactive fecal metabolites in males and 7 metabolites in females (Figure 1). Three of the major peaks in both sexes were clearly associated with the elution of T, A4, and dihydrotestosterone (DHT), respectively. Highly polar fecal metabolites were noted in fractions 10 – 25, with 2 distinct peaks within these fractions for both males and females. One apolar peak was detected in males (fractions 72-83) and 2 apolar peaks in females (fractions 65-71 and 75-79), but these accounted for only a small proportion of the total immunoreactivity. Overall, patterns of immunoreactive androgen metabolites were strikingly similar in the fecal pools from males and females, although A4 and DHT were present at higher levels in females, relative to the T peak, than they were in males. Thus, our EIA has a broad ability to detect multiple androgen metabolites excreted in the feces of both male and female hyenas.

LHRH challenges

All five captive hyenas treated with LHRH responded physiologically to the challenge with an increase in fecal androgens from average baseline levels (Figure 2, Table 1). The latency to peak androgen varied from one to three days

post-LHRH, and averaged 2.4 days. Peak fecal androgen levels after challenge were significantly higher than average levels prior to challenge, but levels six to eight days after challenge were no longer different from initial baseline levels (Repeated measures ANOVA followed by Tukey test for multiple comparisons, $F = 7.32$, $p = 0.016$).

Effect of bone in diet

The presence or absence of bone in the diet did not affect mean androgen concentrations in feces collected the next day from adult female hyenas in captivity. No differences in fecal androgens were found when hyenas switched from a meat plus bone diet to one consisting of only meat (meat plus bone = 625.74 +/- 381.02 ng/g, meat = 614.41 +/- 191.10; Wilcoxon paired sample test: $Z = 0.944$, $p = 0.35$) or the reverse (meat = 839.96 +/- 186.12 ng/g, meat plus bone = 1218.10 +/- 529.58 ng/g; Wilcoxon paired sample test: $Z = 0.404$, $p = 0.68$).

Archived sample collection, processing and storage conditions

Variation in sample collection time, processing methods, and storage conditions had no systematic influence on measured concentrations of fecal androgens. Although there was considerable variation in hormone concentrations, there were no pronounced effects of time of day of deposition on androgen levels. Mean levels in morning samples did not differ from evening

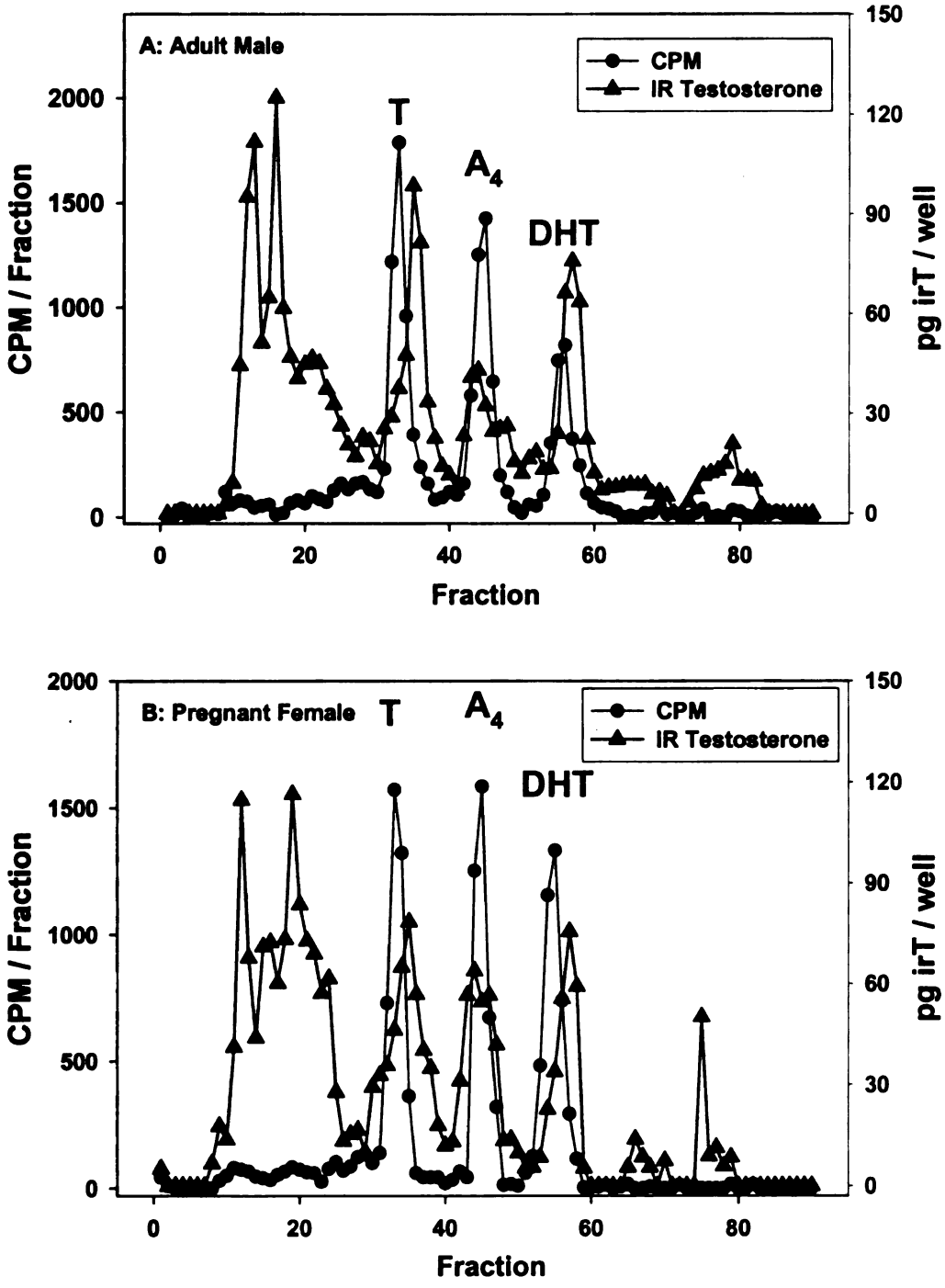

Figure 2.1. HPLC profiles of immunoreactive androgen metabolites in separate male (A) and pregnant female (B) fecal extract pools. Elution of ^{3}H-labeled testosterone[T], androstenedione[A_4], and dihydrotestosterone[DHT] expressed as counts-per-minute per fraction, and immunoreactive androgens measured by EIA expressed as pg irT per well.

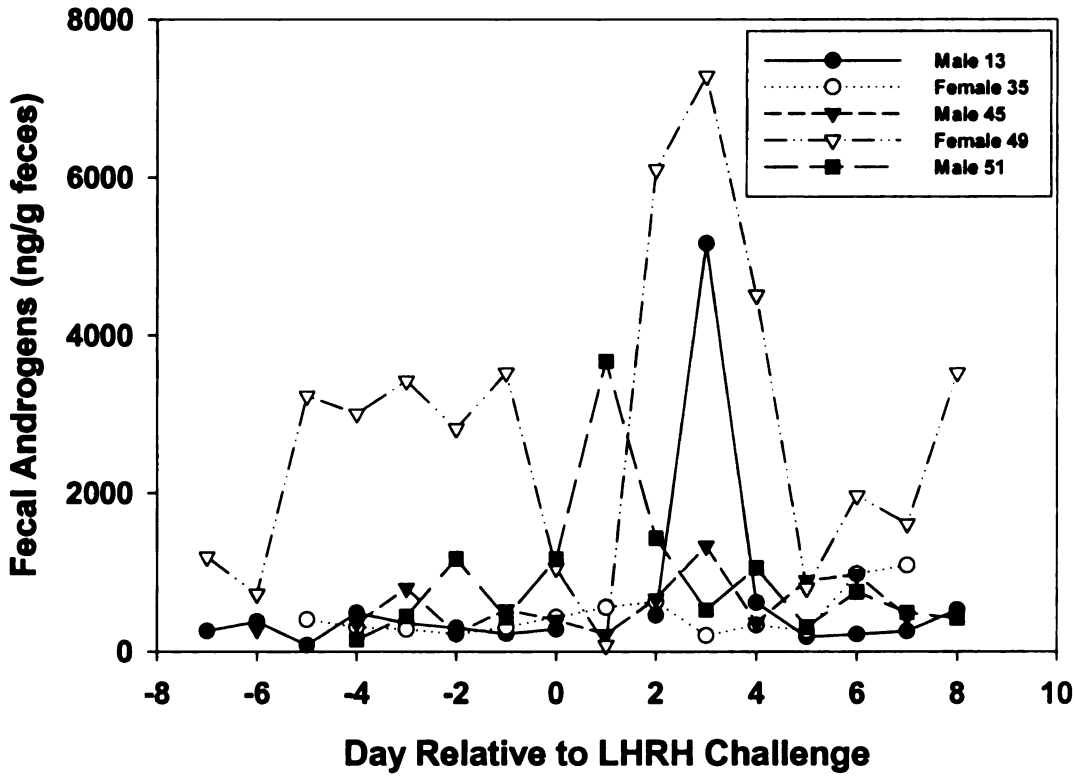

Figure 2.2. Changes in fecal androgen excretion in response to LHRH challenge in captive spotted hyenas. LHRH was administered on day 0.

Table 2.1. Individual and mean response patterns to LHRH challenge in five captive spotted hyenas. Androgen values indicate mean ± s.e.m. and are expressed as ng androgen per g lyophilized feces.

Hyena ID	Baseline Androgen	Maximum Androgen	% Change	Days to Maximum
Male 13	298.46 ± 48.84	5167.0	1733.89	3
Female 35	325.71 ± 37.45	629.35	193.32	2
Male 45	431.61 ± 84.45	1331.67	308.82	3
Female 49	2371.88 ± 414.03	7281.50	307.09	3
Male 51	673.01 ± 209.24	3671.11	545.47	1
All	820.14 ± 393.52	3616.13 ± 1224.39	440.92	2.4 ± 0.89

samples in adult immigrant male hyenas, whether levels were analyzed within (t tests; t's < 2.30, p's > 0.06), or across individuals (am = 652.64 +/- 193.88 ng/g, pm = 509.62 +/- 165.38 ng/g; paired sample t-test; t = 0.552, p = 0.592). Although two adult females had significantly different morning and evening mean values, they differed in opposing directions, and there was no overall difference in morning and evening levels within individual females (all other t tests; t's < 3.64, p's > 0.06) or across all females (am = 1325.336 +/- 136.311 ng/g, pm = 1125.739 +/- 125.392 ng/g; paired sample t test; t = 1.456, p = 0.161). Likewise, the latency to process and freeze samples was not associated with significant variation in excreted androgen. The number of minutes elapsed between sample collection and freezing was not correlated with fecal androgen levels in either females (R_p = - 0.057; F = 1.742; p = 0.187) or males (R_p = -0.082; F = 1.998; p = 0.158). Finally, long-term storage of samples while frozen was not associated with systematic changes in fecal androgen. The number of days frozen until extraction and assay (ranging from 46 to 2673 days) was not correlated with fecal androgen levels in either males (R_p = 0.024; F = 0.169; p = 0.681) or females (R_p = 0.092; F = 0.824; p = 0.115).

Comparisons of patterns in fecal androgens with patterns in plasma T

Differences in fecal androgens mirrored those in plasma T in wild males and females. Mean plasma T and fecal androgens both varied with dispersal status in adult males (Figure 3A). Adult immigrant males had higher plasma T levels than adult natal males (Mann Whitney U test: U = 97.5, p = 0.0043), as

well as higher fecal androgen concentrations (Mann Whitney U test: U = 125, p = 0.045). Mean fecal androgens and plasma T also varied with reproductive status in adult females (Figure 3B). Pregnant females had higher fecal androgen concentrations (Mann Whitney U test: U = 104, p = 0), and higher plasma T values than did lactating females (Mann Whitney U test: U = 58.5, p = 0). In addition, we tested whether either category of adult males differed from pregnant females in either plasma T or fecal androgen concentrations. Immigrant males had significantly higher concentrations of both plasma T and fecal androgens than both adult natal males and pregnant females, which did not differ from each other (ANOVAs followed by Tukey tests: plasma T, F = 12.54, p = 0; fecal androgens, F = 10.99, p = 0).

DISCUSSION

The goal of this study was to determine the extent to which we can reliably measure androgens in the feces of spotted hyenas and make available a non-invasive measurement tool to investigate relationships between androgens and behavior in this species. Our results show that multiple fecal androgen metabolites can be reliably measured by use of our EIA in both male and female spotted hyenas. The assay measures variation in fecal androgen concentrations that is biologically significant, and we have also shown similar relationships in plasma and feces for both males and females. In addition, frozen archived samples can be analyzed without concern regarding variation in the amount of

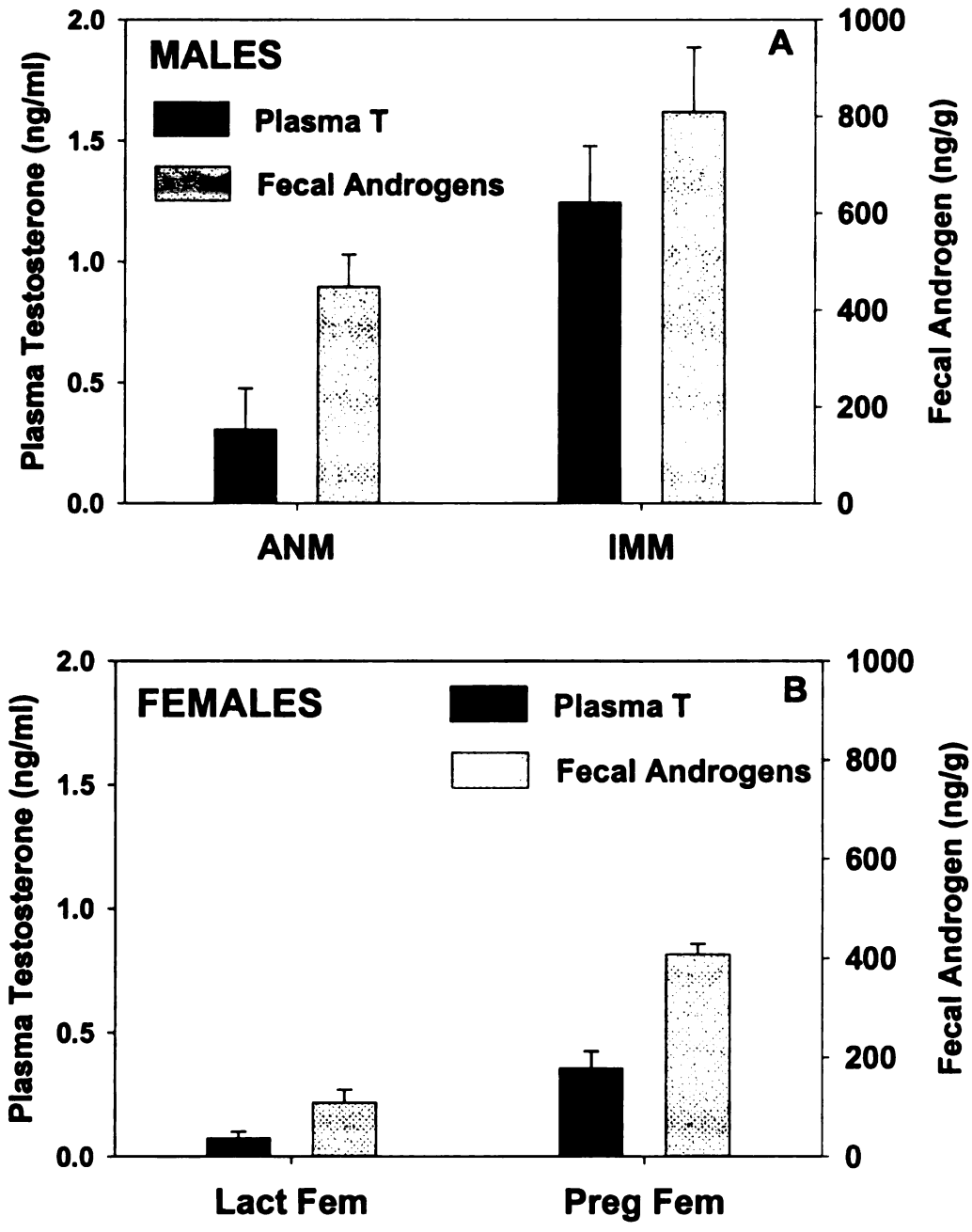

Figure 2.3. Relationship between plasma and fecal hormone concentrations in male (A) and female (B) wild hyenas. ANM = Adult Natal Males; IMM = Immigrant Adult Males; Lact Fem = Lactating, Nonpregnant Adult Females; Preg Fem = Pregnant Adult Females.

bone in the diet, the time of defecation, or the length of time the sample has been frozen. We subjected male and female fecal sample pools to HPLC analysis to determine which androgen metabolites show immunoreactivity in our T assay system. The HPLC analysis revealed that both male and female spotted hyenas excrete multiple immunoreactive androgen metabolites, with markedly similar patterns for the two sexes. Three of the large peaks of immunoreactivity corresponded to the elution times for testosterone, androstenedione, and dihydrotestosterone. A broad peak of immunoreactivity was detected in early fractions, where more polar steroids are eluted. Similar broad, early peaks in metabolite excretion have been noted in studies of androgen excretion in marmosets (Möhle et al., 2002) and maned wolves (Velloso et al., 1998), and in estrogen and progestin excretion in felids (Brown et al., 1994). These peaks likely represent conjugated androgens (glucuronide and/or sulfate conjugates) that nonetheless cross-react with the T antibody (Möhle et al., 2002; Brown et al., 1996; J.L. Brown, pers. comm.). Our assay thus permits estimation of multiple androgen metabolites in spotted hyena feces.

Steroid hormone conversion and metabolism, excretion dynamics in the gut, variation in uptake of hormones by target tissues, binding proteins, and even defecation rate all undoubtedly influence the makeup of the pool of androgen metabolites in fecal samples. Although the exact makeup of the metabolite pool is highly variable, the other carnivore species sampled to date excrete multiple androgen metabolites, some of which remain unidentified (domestic cats: Brown et al., 1996; wild dogs: Monfort et al., 1997; cheetahs: Brown et al., 1998; maned

wolves; Velloso et al., 1998; red wolves: Walker et al., 2002). In these species, the number of androgen metabolites that show immunoreactivity in a particular T or androgen assay is also quite variable. In the case of the spotted hyena, we have shown that all of the detected fecal androgen metabolites show immunoreactivity in our T assay, in both males and females.

LHRH is a potent stimulator of the hypothalamic-pituitary-gonadal (HPG) axis, therefore the first step of the biological validation of our assay was to administer LHRH challenges to 5 captive adult spotted hyenas. As predicted, LHRH treatment led to substantial increases in excreted fecal androgen in the three males and two females tested in this study. There was an average 5-fold increase in excreted androgens across individuals, ranging from 2 to 17-fold. The timing of peak androgen excretion was somewhat variable in that three hyenas responded maximally after three days, one responded after two days, and one responded the day after injection with LHRH. Goymann et al. (1999) found similar variation in peak glucocorticoid excretion in feces of captive spotted hyenas after ACTH injection. While the average latency to peak excretion of fecal glucocorticoids after injection was 24 h, some hyenas exhibited peak excretion rates more than 48 h after injection. Variation in the efficiency of i.v. infusion, variable thresholds in sensitivity to LHRH, variation in dietary intake or fecal output during the course of the experiment, and individual differences in steroid hormone metabolism and excretion might all factor into the variation in the peak response to LHRH challenge. Regardless, the predicted response to LHRH challenge was seen in all five hyenas, showing that our EIA is capable of

measuring physiologically-induced elevations in androgen activity within individuals. In addition, we now know that changes in circulating levels of androgen are typically expressed in fecal samples 1 to 3 days later, which is important for future studies investigating relationships between behavior and fecal androgen levels in this species.

Dietary variation may influence concentrations of excreted steroid hormones. For example, an increase in dietary fiber has a negative effect on progestogen excretion in adult female baboons (Wasser et al., 1993). While fiber is not necessarily of concern in carnivores, bone constitutes a variable proportion of the spotted hyena diet. Social rank affects nutritional status in female hyenas by means of priority of access to food (Holekamp et al., 1996), and higher ranking hyenas may be ingesting less bone than lower ranking hyenas. Finding variation in fecal androgens with the presence or absence of bone could force us to treat high and low ranking hyenas differently. Therefore, we tested whether the presence of bone had an effect on fecal androgen concentrations in captive females. Our results indicate no obvious effect of bone, therefore allowing us to treat high and low ranking hyenas similarly in future studies.

Slight variations in collection and storage conditions could also affect the ability to extract and detect androgen in hyena fecal samples. Hot weather and humidity could potentiate bacterial degradation of the hormone product. In the field, reluctance to disturb our study animals, behavioral sampling protocols, the presence of other large mammals such as lions, and the distance back to basecamp can make it difficult to quickly collect, process, and freeze samples. In

addition, for this project, samples were collected opportunistically in both mornings and evenings. Other species exhibit diurnal variation in excreted hormone levels (e.g., marmosets: Sousa and Ziegler, 1998). However, we found no effect of variation in collection and storage conditions, and our data revealed no significant differences in fecal androgen concentrations in morning and evening samples, suggesting little circadian variation in the excretion of this class of steroids.

Steroid concentrations in fecal samples may change with long-term storage. For example, concentrations of fecal glucocorticoids in baboon feces stored in ethanol at either room temperature or -20° C for 90-120 days differed from concentrations in the same samples measured after 180 days of storage (Khan et al., 2002). However, our data suggest that long-term frozen storage without the use of ethanol does not systematically affect androgen concentrations in spotted hyena feces. These findings are significant, because they suggest that minor variations in sample collection and processing, even delays of up to 48 hours between deposition and freezing, do not affect fecal androgen concentrations systematically in this species. Furthermore, the ability to measure meaningful levels of androgenic steroids in samples that have been frozen for long periods of time allows us to retrospectively examine hormone-behavior-life-history relationships by accessing samples collected during the long-term study of the Talek hyenas that has endured for more than a decade.

In most mammals, males have significantly higher circulating T than females. Thus, a good test of the validity of a particular androgen assay is often

the ability to differentiate males and females on the basis of excreted androgens. However, as described earlier, the demonstration of consistent sex differences in plasma androgens in free-ranging spotted hyenas has been problematic. In the LHRH challenges presented here, one female (#49) had higher baseline androgen than the three males (and had the highest post-LHRH levels), but LHRH-induced elevations in excreted androgens reduced the differences between this female and the three males. Inspection of the plasma T and A4 levels of this female, which were measured as part of another study (Place et al., 2002), showed that she had circulating T levels higher than the other female, but well below the levels of the males. However, this female had very high circulating A4 levels at the beginning of the experiment, and her plasma A4 levels 120 minutes after the LHRH challenge were five times higher than levels in the males. Thus it appears that the relative concentrations of each of the androgen metabolites shown in figure 1 may vary among the endocrine responses of individual hyenas challenged with LHRH.

The results from our comparisons of plasma T and fecal androgens in wild hyenas provide valuable insight towards answering questions concerning sex differences in androgens in this species. Previous studies have shown that plasma T increases in captive females during pregnancy (Licht et al., 1992; Glickman et al., 1992). In one study of wild hyenas, no differences in plasma T were noted between pregnant and lactating females (Goymann et al. 2001a), but that sample included only four pregnant females. Here, using large numbers of both fecal and plasma samples from adult females in various reproductive states,

we found that wild pregnant females do indeed show higher levels of both circulating T and excreted androgens than do lactating females. Also of significance is the fact that pregnant females had similar levels of androgens to those of adult natal males, but not to those of immigrant males. Lactating females had the lowest androgen levels among all groups of adults. This convincingly shows that it is important to account for female reproductive state and male dispersal status when comparing androgenic hormones in male and female hyenas.

In conclusion, we are confident that fecal androgen analysis will be a useful tool in future research with wild spotted hyenas. Our results indicate that we can measure biologically meaningful variation in fecal androgen concentrations among groups of hyenas known to vary with respect to in concentrations of plasma androgens. Future studies can now focus on investigating the unusual relationships between sex, social status, reproductive state, aggression, sexual behavior and levels of excreted androgens in this species.

CHAPTER 3

FECAL ANDROGEN LEVELS IN ADULT MALE SPOTTED HYENAS REFLECT INTERACTIONS WITH SOCIALLY DOMINANT FEMALES

INTRODUCTION

Linking physiology with reproductive behavior is critical to our understanding of the proximate mechanisms involved in the regulation of reproductive success, and socioendocrine studies can shed light on the sources of individual variation in reproductive success (Bercovitch, 1999; Bercovitch and Ziegler, 2002). In male vertebrates, androgens, particularly testosterone (T), influence a variety of morphological and behavioral processes related to reproduction and social behavior. Testosterone is essential for the formation of secondary sexual characteristics (Wickings and Dixson, 1992; Dixson, 1998; Maggioncalda et al., 1999; Gonzalez et al., 2001), plays a crucial role in the initiation and maintenance of sperm production and sexual behavior (Wickings et al., 1986; Baum, 2002), and has been linked to the expression of aggressive behavior and social status (Harding, 1981; Bouissou, 1983; Albert et al., 1990; Whitten, 2000). Androgen levels can be quite variable among individuals in a population, and this variation may be associated with individual differences in behavior, physiology, and reproductive success. The relationship between androgens and behavior is often modulated by social factors. For example, levels of T in primates often do not correlate with dominance status or aggressive behaviour under stable social conditions (Sapolsky, 1983; Cavigelli and Pereira, 2000), whereas a positive correlation is found in situations characterized by

unstable hierarchies or other social challenges (Sapolsky, 1983; Steklis et al., 1986; Bernstein et al., 1992; Cavigelli and Periera, 2000; Brockman et al., 2001).

Although originally developed for use in seasonally-breeding avian species, the 'Challenge Hypothesis' (Wingfield et al., 1990; Wingfield et al., 2000) has recently been employed as a theoretical framework to help predict patterns in the relationship between T and aggression associated with mating in various species of mammals (dwarf mongoose, *Helogale parvula*: Creel et al., 1993; wild dog, *Lycaon pictus*: Creel et al., 1997; ring-tailed lemur, *Lemur catta*: Cavigelli and Periera, 2000; Verreaux's sifaka, *Propithecus verreauxi*: Brockman et al., 2001; tufted capuchin monkey, *Cebus apella nigritus*: Lynch et al., 2002; redfronted lemur, *Eulemur fulvus rufus*: Ostner et al., 2002; spotted hyena, *Crocuta crocuta*: Goymann et al., 2003; arctic ground squirrel, *Spermophilus parryii*: Buck and Barnes, 2003; chimpanzee, *Pan troglodytes*: Muller and Wrangham, 2004). As originally formulated, the main premise of the challenge hypothesis is that the relationship between T and aggression during the breeding season in males of a given species can be predicted by the level of aggressive male-male competition and extent of paternal care characteristic of that species. In species where males are intensively involved in parental care and engage in relatively low rates of male-male aggressive competition (often monogamous species), the hypothesis predicts that males should maintain a breeding baseline level of T and respond to occasional aggressive challenge with a relatively large increase in T to facilitate aggressive competition. By contrast, in species showing no paternal care and higher rates of male-male aggression (often polygynous

species), the hypothesis predicts that males should maintain relatively high levels of T during the entire breeding season to facilitate a heightened state of aggression. Therefore, the physiological response of a male to challenge in this situation should be smaller than in the previous category of males, because T is already at or near the effective maximum (Wingfield et al., 1990; Creel et al., 1993; Wingfield et al., 2000).

Although the vast majority of studies testing the challenge hypothesis have focused on the relationship between androgens and reproductive aggression among same-sexed individuals, the original hypothesis also suggests that physiologically influential social challenges may occur during interactions with potential mates as well as those occurring during interactions with rival males (Wingfield et al., 1990, Wingfield et al., 2000). Male white-crowned sparrows (*Zonotrichia leucophyrs*) that are caged with estradiol-implanted females have higher levels of T than do males housed with non-receptive females (Moore, 1983). Pinxten et al. (2003) demonstrated that testosterone levels in European starlings (*Sturnus vulgaris*) were positively influenced by female presentation. Most recently, Moore et al. (2004) found that the challenge hypothesis was not supported in an equatorial population of rufous-collared sparrows (*Zonotrichia capensis*), and these authors suggested that male-female interactions may be responsible for patterns of T observed in this species. Plasma T levels also rise in many male mammals after mating or exposure to a novel receptive female (Katongole et al., 1971; Rose et al. 1972; Jainudeen et al., 1972; Purvis and Haynes, 1974; Sanford et al., 1974; Macrides et al., 1975).

These studies suggest that, when investigating the sources of variation in androgen concentrations in males, it may be important to consider the influence of intersexual interactions in addition to the influence of aggressive competition with rival males.

Here, we set out to evaluate the influence of inter- and intrasexual interactions on male androgen excretion in a gregarious carnivore, the spotted hyena. These hyenas live in dynamic fission-fusion groups of up to 80 individuals, called clans. Each clan contains one to several matrilines of adult females and their offspring, as well a number of immigrant males. Clans are structured by hierarchical rank relationships (Kruuk ,1972; Tilson and Hamilton, 1984; Frank,1986; Holekamp and Smale, 1990), and all adult females are socially dominant to all adult males not born in the clan (Kruuk ,1972; Smale et al., 1993). Adult males are less aggressive than adult females (Kruuk, 1972; Frank, 1986; Mills, 1990; Szykman et al., 2003), and the social rank of individuals within the male hierarchy is acquired by queuing, rather than by aggression (East and Hofer, 2001; Engh et al., 2002). Males are seldom observed to engage in aggressive interactions, and what little aggression does occur is typically very mild. Due to female dominance and morphological masculinization of the external genitalia, females appear to have unusually tight control over reproduction (Kruuk, 1972; East et al., 2003), and the awkward courtship behaviour of the male hyena reflects his extreme motivational conflict with regard to approaching females (Holekamp and Smale, 1998; Szykman, 2001). Molecular genetic data have revealed that high-ranking males do not monopolize reproduction, that both

males and females mate with multiple partners, and that at least 20% of twin litters are of mixed paternity (Engh et al., 2002; East et al., 2003). Males show no paternal care (Kruuk, 1972), although they can recognize their own offspring (Van Horn et al., in press).

Female hyenas are philopatric, but males disperse sometime after reaching reproductive maturity at approximately 2 years of age. Males may remain in their natal clans for up to 52 months after puberty (Smale et al.1997; East and Hofer, 2001; Van Horn et al., 2003), so there are two classes of adult males in every clan: natal males born in the clan and immigrant males born elsewhere. Although natal males are socially dominant to all immigrant males, immigrants engage in higher rates of sexual and courtship behavior, have higher average levels of plasma testosterone (Holekamp and Smale, 1998; Holekamp and Sisk, 2003; Dloniak et al., 2004), and sire 30 times as many offspring as do natal males (Engh et al., 2002).

Goymann et al. (2003) recently described a relationship in spotted hyenas between elevated plasma T and the occurrence of male defense of one or more females. However, low intensity male-male reproductive aggression in the form of female defense is not very common among male hyenas, and we therefore suggest that the relationship between mate defense and androgens may be confounded by the presence of a female and/or by courtship interactions between the sexes. We therefore set out to test the hypothesis that intersexual interactions constitute a significant social challenge for male hyenas, and that these interactions correlate with androgen concentrations. We recently

developed an assay to quantify fecal androgen (fA) concentrations in this species non-invasively (Dloniak et al. 2004), and we used that assay here with samples collected from adult male spotted hyenas to inquire about the influence of the following variables on fA concentrations: social status, tenure in the clan, reproductive aggression among males, male-male aggression not associated with the defense of a female, courtship, and degree of association with females in particular reproductive states. If male-male competition represents a significant challenge for male hyenas, fA concentrations should vary with the occurrence of male-male aggression. On the other hand, if interactions with behaviorally dominant yet attractive females constitute significant challenges for males, we expected fA concentrations in males to vary with social measures related to interactions with females. We also predicted that relationships between androgens and behavior would differ between adult natal and immigrant males, based on differences in behavior and physiology between these two groups of males that were documented in earlier studies.

METHODS

Study Site and Animals

The study was conducted in the Talek area of the Masai Mara National Reserve, an area of open rolling grassland in southwest Kenya. The subject population was one large, stable spotted hyena clan, inhabiting a home range of about 65km^2 (Boydston et al., 2001). The Talek clan has been intensively studied since 1988, and the boundaries of the clan's home range have been stable at

least since 1979 (Frank, 1986). All hyenas in the clan were known individually by their unique spot patterns, and sex was determined by the dimorphic glans morphology of the erect phallus (Frank et al., 1990). Data used in this study were gathered between July 1995 and July 2002, during which time observers monitored the Talek hyenas 20-31 days per month for approximately 6 hours per day. During this study period, the number of adult immigrant males, adult natal males, and adult females in the clan ranged from 11-21, 1-9, and 21-31, respectively.

Adult natal males were those that had been born in the Talek clan, and were older than 24 months (the approximate age of puberty) but had not yet dispersed. Ages of adult natal males were known +/- 7 days, using methods described by Holekamp and Smale (1998). Ages of the adult natal males monitored in this study ranged from 26 to 68 months. Adult immigrant males were those that had been born outside Talek and then immigrated into the Talek clan at dispersal. Because immigrant males originated in clans other than our study population, exact ages of immigrants were unknown. However, we could reliably estimate (to +/- 6 months) the ages of immigrant males based on tooth-wear measures obtained during routine immobilizations (Van Horn et al., 2003). Estimated ages of immigrant males in this study ranged from 30 to 150 months. In addition, we recorded the date of first appearance in the Talek home range of each immigrant male, thus allowing us to calculate his tenure in the clan at the time of each sampling, based on time elapsed since his initial appearance in the clan.

We monitored the extent to which adult males associated with adult females that were either attractive (close to conception) or lactating (relatively unattractive). Spotted hyenas in East Africa breed year-round (Holekamp et al. 1999), thus at any given time there may be pregnant, lactating, and cycling females present in a clan. Lactating females were within the third or fourth month of lactation, based on direct observation of nursing bouts and known birth dates of cubs. Attractive females were within one month prior to a known conception. We calculated the conception date for each litter born to each female by subtracting 110 days, the length of the gestation period in *Crocuta*, from the birth date of that litter (Schneider, 1926). Birthdates could be reliably estimated to +/- 7 days (Smale et al., 1993).

Behavioral Observations

Behavioral data presented here were collected throughout the 7-year study period, except for courtship data, which were collected only from May 1997 through August 1999, and May 2000 through May 2002. We conducted daily behavioral observations from vehicles between 0530 and 0930 hours and between 1630 and 2000 hours. We located hyenas while driving daily circuits around the Talek home range, visiting high points in the area and scanning with binoculars, and by radiotracking collared individuals. We initiated an observation session when we first drove up to one or more hyenas separated from others by at least 200m, and the session ended when all hyenas moved out of sight, or when we drove to a new location. Data documenting agonistic behavior during

observation sessions were collected using critical incident sampling (Altmann 1974) of all aggression and appeasement behaviors observed in all social contexts. All courtship behaviors were also recorded as critical incidents. Social ranks were assigned based on a matrix of outcomes of dyadic agonistic interactions (Martin and Bateson, 1988), as described previously (Smale et al., 1993). Adult females (together with their offspring) and adult immigrant males were ranked in separate dominance hierarchies. Since the number of immigrant males present during the study varied over time, we calculated standardized social rank for each immigrant at each time of sampling by assigning the highest-ranking male a rank of +1 and the lowest-ranking male the rank –1, with all other males spread evenly between these two individuals (East and Hofer, 2001; Goymann et al., 2003).

Agonistic Behavior

Appeasement behaviors used to determine social ranks included head-bob, carpal crawl, giggle, squeal, back-off, and submissive-posture (tail between the legs, head down, body lowered and bent) (Kruuk 1972; Holekamp and Smale, 1998). Aggression included lunge, snap, bite, chase, displace, push, stand over, and intention movement to bite (Kruuk, 1972; Holekamp and Smale, 1998). We used social context to distinguish between reproductive and non-reproductive aggression among males. In order for a male to show reproductive aggression, he had to be within 10m of an adult female and show aggression (including at least a low-level threat, such as displace) toward another adult male

who approached to within 10m of that female. If the context of the aggression was not related to a female (such as during feeding with other males), we considered it to be non-reproductive aggression. We discarded any aggressive interactions for which context was ambiguous.

Courtship Behavior

Courtship behavior included extended following, approach/avoid display, present, bowing display, paw the ground, and mount (see Holekamp and Smale, 1998; Szykman, 2001 for full definitions of behaviors). These behaviors were exhibited by adult male hyenas only toward females and indicated male sexual interest (Holekamp and Smale, 1998; Szykman, 2001). Although these behaviors were often accompanied by appeasement gestures, courtship behaviors alone could not be used to determine social rank because males never directed them at other males. We did not include either male aggression towards females (baiting behavior: Kruuk, 1972; Szykman et al., 2003) or copulations in our analyses due to small sample sizes for these rarely observed events.

Association Index

We wanted to assess whether the degree of association between a male and a particular female influenced androgen concentrations in the male's feces. Due to the highly dynamic, fission-fusion nature of spotted hyena society, the best measure of association between any two individuals is an association index (AI). We therefore calculated AIs around a male's fecal sample date, between

that male and all members of each of the 2 classes of adult female, attractive and lactating. We used the twice-weight index of association (Cairns & Schwager 1987):

$$(A+B)_{together} / [(A_{without\ B}) + (B_{without\ A}) + (A + B)_{together}]$$

where $(A_{without\ B})$ represents the number of observation sessions in which male A was observed but female B was not present, $(B_{without\ A})$ represents the number of observation sessions in which female B was present but male A was not, and $(A + B)_{together}$ represents the number of sessions in which both male A and female B were present (as in Syzkman et al., 2001). Specifically, for 69 fecal samples from 22 immigrant males we separately calculated male AIs with both lactating and attractive females present in the clan for the time period encompassing three weeks before, and one week after, the date of fecal sample collection.

Fecal Sample Collection, Processing, and Androgen Assay

During the study period, fecal samples were collected from 16 adult natal males and 26 adult immigrant males, yielding totals of 25 and 159 samples, respectively. A fecal sample was collected whenever a hyena was directly observed to defecate during an observation session. Samples were first collected into plastic bags at the site of defecation, then mixed and transferred to 3ml cryovials. Samples were stored in liquid nitrogen until transported on dry ice to the United States, where they were stored at –20°C or colder until processing. Samples were then extracted and assayed for androgens as described by Dloniak et al. (2004). In brief, steroids in 0.2g lyophilized feces were extracted

with 100% ethanol. Fecal extracts were reconstituted and diluted 1:50 in PBS and measured for immunoreactive androgens by enzymeimmunoassay (EIA). The assay utilized a testosterone antibody (R156/7) and a testosterone conjugate (horseradish peroxidase) kindly provided by Dr. Bill Lasley and Coralie Monroe of the University of California at Davis. The mean coefficient of variation between assays (N = 10) was 8.5%, based on high and low pools in each assay. The intra-assay coefficient of variation for high and low pools run within each assay was 7.3%. Dloniak et al. (2004) demonstrated that neither time of day at which the sample was collected, nor the length of time the sample was kept in frozen storage before extraction and assay systematically influence fA concentrations in spotted hyenas, so we did not include these variables in our analyses.

Statistical Analyses

Adult natal and immigrant male hyenas behave very differently, have different mean plasma T and fA concentrations, respond differently to challenge with gonadotropin releasing hormone (Holekamp and Smale, 1998; Dloniak et al., 2004; Holekamp and Sisk, 2003), and we collected many more faecal samples from immigrants than from natal males. Therefore, we analyzed the relationships between fA and behavior in each of these two groups of males separately. We first inquired whether rates of courtship were higher than rates of reproductive aggression in 6 adult natal males and 12 adult immigrant males present in the Talek clan for the entire period between May 2000 and May 2002,

using Wilcoxon matched pairs analyses. In adult natal males we then investigated relationships among fA, age, and social rank at the time of sample using multiple regression. Due to non-independence of some data points, we included individual hyena identity (ID) as a random effect variable in a generalized linear mixed model with backward selection. We next inquired whether natal males showing non-reproductive aggression, reproductive aggression, or courtship behavior during the three days prior to, and on the day of, a fecal sample had greater fA concentrations than natal males not showing these behaviors at the time of sampling. We chose this time-frame based on the fact that circulating androgens are represented in hyena feces one to four days later (Dloniak et al., 2004). Three separate analyses were done using Mann-Whitney U tests, and since the same samples were used in multiple analyses we corrected for the experimental error rate by applying a Bonferroni adjustment in significance testing (Rice, 1989). Due to the small number of samples obtained from natal males, we did no further analysis of this dataset.

In immigrant males, we first used multiple regression to investigate relationships among fA, estimated age, standardized social rank, and tenure in the clan. The majority of adult immigrant males were sampled repeatedly throughout the study, and the number of samples per individual varied greatly (1 – 17). Due to this unbalanced data set, we included hyena ID as a random effect variable in the initial regression. ID had no significant effect, so we repeated the analysis without ID included. We next used subsets of the available samples to analyze the relationships between fA and behavior in immigrants. Here we used

Wilcoxon matched pairs to determine whether fA concentrations in males showing non-reproductive aggression, reproductive aggression, or courtship behavior were different than concentrations in the same males when they were not showing these behaviors. Here, no sample was used in more than one analysis, and a male was represented by only one paired sample within an analysis. Lastly, we again used multiple regression to investigate the relationship between immigrant male fA and AIs with lactating and attractive females. All statistical analyses followed Zar (1996) and were done using Statistica 8.0. Statistical significance was set at $P \leq 0.05$ and nonparametric tests were employed when necessary. Results are presented as means +/- standard errors.

RESULTS

Rates of Reproductive Aggression and Courtship

A matched-pairs comparison of rates of courtship and reproductive aggression in 6 adult natal males, all of which were present in the Talek clan throughout a two-year period, showed that these males directed courtship behaviour toward females at significantly higher rates than those at which they directed reproductive aggression toward other males (Wilcoxon matched pairs, $Z = 2.201$, $P = 0.041$; Figure 1). The corresponding analysis in 12 immigrant males present in the Talek clan during the same time interval as the 6 natal males showed that immigrants also courted females at much higher rates than they showed reproductive aggression (Wilcoxon matched pairs, $Z = 3.059$, $P = 0.002$;

Figure 1). Reproductive aggression in adult males generally consisted of low-level threats, and these were always directed toward subordinate males.

Adult Natal Males

We found no relationship among social rank, age, and fA concentrations in adult natal males, controlling for hyena ID as a random effect in a mixed model multiple regression (whole model R = 0.413, $F_{3,21}$ = 1.4389, P = 0.26). Because we found no effect of social rank or age in natal males, we did not control for these variables in our subsequent analyses of behavior. Concentrations of fA were similar between males showing non-reproductive aggression and males failing to show any non-reproductive aggression (Mann-Whitney U test: Z = -0.28, P = 0.77, aggression N = 9, $\bar{x} \pm$ SE = 386.32 ±115.48 ng/g, no aggression N = 16, $\bar{x} \pm$ SE = 315.27 ± 88.70 ng/g). We found no significant difference between fA concentrations in natal males showing courtship behavior prior to a faecal sample and those in males showing no courtship behavior (Mann Whitney U test: Z = 0.11, P = 0.93, courtship N = 10, $\bar{x} \pm$ SE = 308.12 ± 87.62 ng/g, no courtship N = 15, $\bar{x} \pm$ SE = 362.66 ±101.63 ng/g). Lastly, although there was a slight tendency for natal males showing reproductive aggression to have greater fA concentrations than natal males failing to show reproductive aggression, this difference was not statistically significant when the Bonferroni adjustment was applied, possibly because so few natal males were observed showing reproductive aggression (Mann-Whitney U test: Z = -2.00, P = 0.045, reproductive aggression N = 2, $\bar{x} \pm$ SE = 851.66 ± 52.71 ng/g, no reproductive

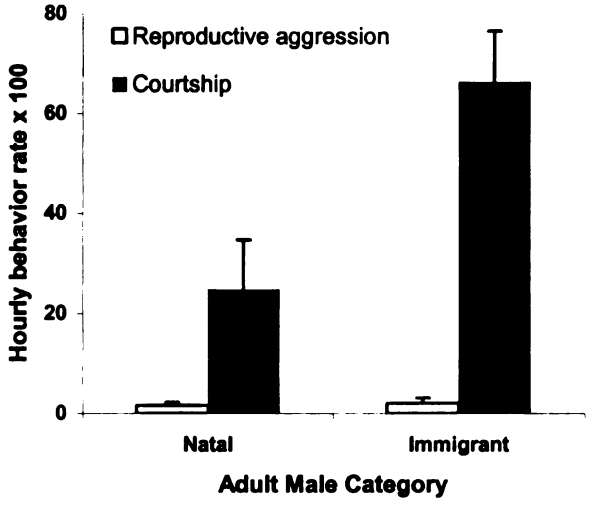

Figure 3.1. Paired rates of reproductive aggression and courtship observed in adult male Talek hyenas from May 2000 through May 2002. N = 6 adult natal males and 12 adult immigrant males. Rates of behavior were compared within adult male category.

aggression N = 23, $\bar{x} \pm$ SE = 296.43 $\pm$ 67.49 ng/g). Interestingly, the fecal samples of the two males who showed reproductive aggression had the highest concentrations of fA among all samples collected from adult natal males, and both of these males were also involved in courting females prior to the sample collection.

Immigrant Males

We first used multiple regression to investigate whether there was an influence of male social status, age, or tenure on fA concentrations among immigrants. The overall model here was significant (whole model R = 0.25, $F_{4,134}$ = 4.78, P = 0.01), with tenure (P = 0.03, Figure 3.2A) and standardized social rank (P = 0.03, Figure 3.2B) retained, but hyena ID and estimated age were removed from the model by backwards selection. However, social rank and tenure were closely and positively correlated (R = 0.90), neither variable explained significant variation in fA (social rank partial R = 0.03, tenure partial R = -0.13), and the partial correlations with fA concentration were in opposing directions. These results show that there is no simple, systematic relationship between social status or tenure and fA concentrations in immigrant male hyenas.

Samples from immigrant males were sufficiently abundant to permit use of paired analyses of the relationships between androgens and specific behaviors. Although a male's fA concentrations did not vary significantly based on whether or not he exhibited any non-reproductive aggression (Wilcoxon matched-pairs: Z = 1.29, N = 13, P = 0.20, Figure 3.3A), fA concentrations were significantly

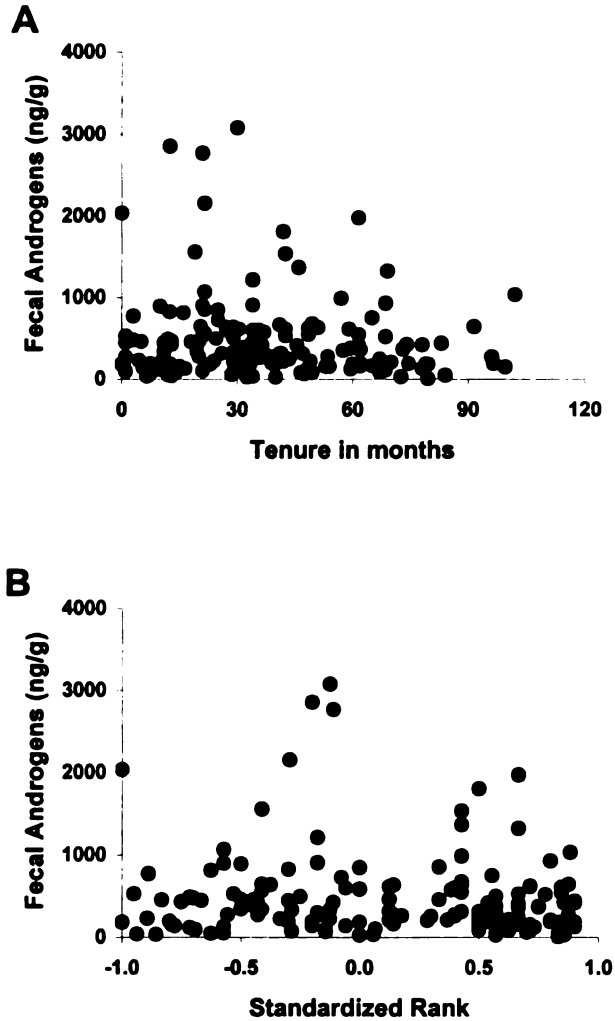

Figure 3.2. Relationships between fecal androgen concentrations in immigrant males and their tenure in the Talek clan (A) and their standardized social rank (B) at the time of sampling. The highest social rank is indicated as 1.0. N = 159 faecal samples from 26 immigrant male hyenas.

elevated in samples collected from immigrant males showing reproductive aggression compared to samples collected from the same males when they were not showing reproductive aggression (Wilcoxon matched-pairs: $Z = 1.99$, $N = 6$, $P = 0.046$; Figure 3.3B). In addition, fA concentrations were greater in samples from immigrant males showing courtship behaviour than in samples from the same males when they did not show courtship (Wilcoxon matched-pairs: $Z = 3.31$, $N = 16$, $P = 0.0009$, Figure 3.3C). Very few males ever showed reproductive aggression that was associated with a fecal sample (6 out of 26 males). In addition, five of the six males engaged in reproductive aggression (3.3B) that was associated with a fecal sample were also seen courting a female during the days prior to the sample. In the courtship analysis (3.3C) we only included samples collected from males showing courtship behavior without reproductive or non-reproductive aggression, therefore our results indicate that there is an effect of courtship that is independent of reproductive aggression, and that there is not a significant additional effect of reproductive aggression on fA concentrations after courtship is considered.

Thus far, there is a potential confound in our behavioral analyses in that female presence is required for courtship and reproductive aggression, but not for non-reproductive aggression. Therefore we next tested whether or not there was an influence on fA concentrations of the extent to which immigrant males associated with females. The multiple regression model of AI was significant (whole model $R = 0.565$, $F_{2,66} = 15.506$, $P < 0.00001$). The AI between males and lactating females was not correlated with the AI between these same males

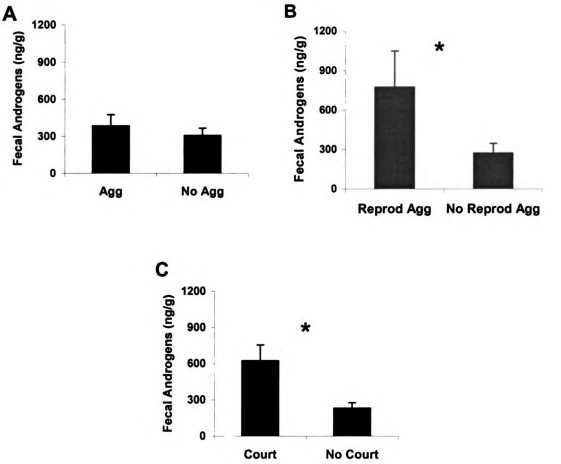

Figure 3.3. Differences in faecal androgen concentrations between samples when immigrant males did and did not emit particular behaviours. (A) N = 13, non-reproductive aggression (Agg) and no non-reproductive aggression (No Agg), (B) N = 6, reproductive aggression (Reprod Agg) and no reproductive aggression (No Reprod Agg), (C) N = 16, courtship (Court) and no courtship (No Court). Males are paired between bars within each histogram. Note that 5 of 6 males showing reproductive aggression (B) were also observed courting a female. No males showing courtship (C) also showed reproductive aggression. Asterisks indicate significant differences.

and attractive females (R = 0.035). fA concentrations were positively correlated with AI between males and attractive females (partial R = 0.558, P < 0.00001; Figure 3.4A), but not with AI between males and lactating females (partial R = 0.127, P = 0.213; Figure 3.4B). This suggests that the degree of association between a male and females near their times of conception influences his androgen levels.

DISCUSSION

Whereas behavior was not a useful predictor of fA concentrations in adult natal males, reproductive aggression and courtship were good predictors of elevated fA concentrations in adult immigrant males. Furthermore, degree of association with females near their times of conception was positively related to fA levels in immigrant males, whereas association with lactating females showed no such relationship. These data suggest that associating closely with cycling females influences reproductive physiology in the male spotted hyaena.

Our results indicate that age does not systematically influence fA concentrations in adult male spotted hyaenas, regardless of dispersal status. Furthermore, neither adult natal nor immigrant males showed systematic status-dependent variation in fA concentrations within their social hierarchies. Although a lack of a simple relationship between plasma T and social status has been shown previously in immigrant male hyenas (Goymann et al., 2003; Holekamp and Sisk, 2003), we demonstrate here for the first time that fA concentrations show the same pattern as plasma T, and that the lack of relationship between

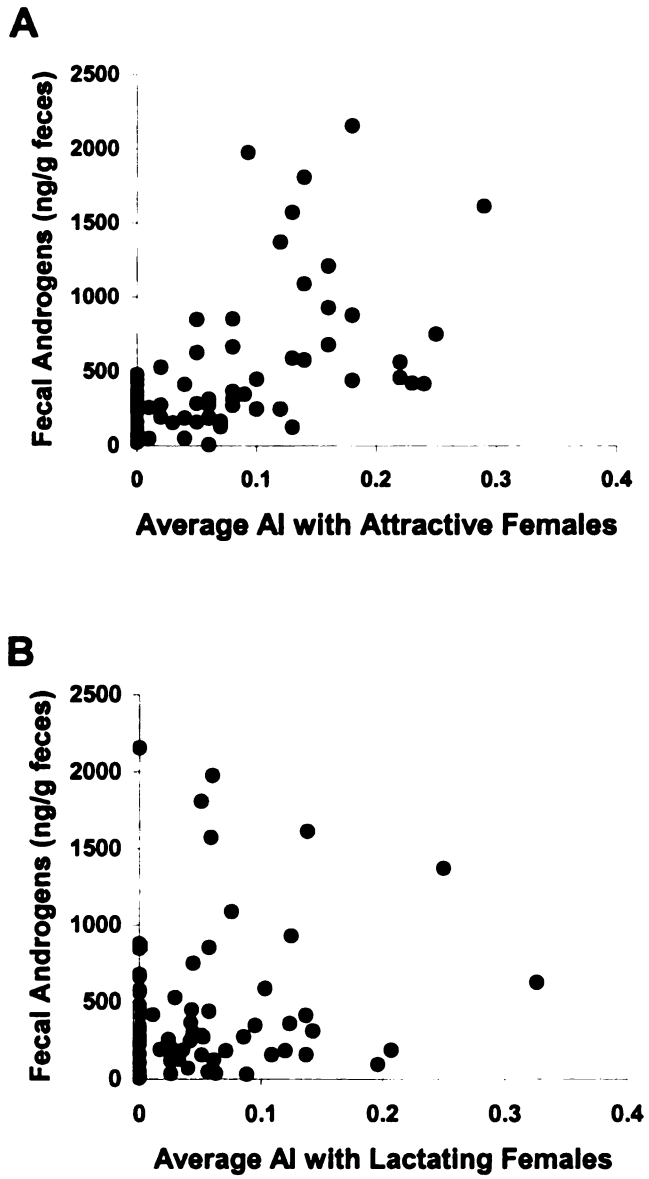

Figure 3.4. Fecal androgen concentrations in immigrant males are positively correlated with their association index (AI) with attractive females (A), but not with lactating females (B). N = 69 fecal samples from 22 immigrant male hyenas.

androgens and social rank also holds true for adult natal males. Social ranks in natal males are based on the social ranks of their mothers (Holekamp and Smale, 1993; Smale et al., 1993; Engh et al., 2000). Furthermore, immigrant males only gain status in their new clan by advancing in a queue as higher-ranking males die or emigrate (East and Hofer, 2001; Engh et al., 2002). Thus, neither natal nor immigrant males gain social status through fighting ability or size. These data therefore resemble those from previous studies in other species showing that androgen levels are not closely related to male social status within a stable hierarchy (Eaton and Resko, 1974; Gordon et al., 1976; Bernstein et al., 1983; Steklis et al., 1986; Alberts et al., 1992; Sapolsky ,1993; Goymann et al,. 2003).

The challenge hypothesis is often interpreted to predict high testosterone levels in males during bouts of reproductive aggression, regardless of other important social factors that might also affect relationships between androgens and behavior in social mammals. Goymann and colleagues (2003) recently showed that male spotted hyenas defending a female had higher levels of plasma T than did non-defending males in the Serengeti. We report here a similar result in Talek hyenas, in that immigrant males had higher concentrations of fA when showing reproductive aggression and courting than when they were not showing reproductive aggression. However, our data are not consistent with Goymann et al.'s (2003) conclusion that defending males have higher levels of T due to a higher interaction rate with other males. If a higher interaction rate with other males per se were enough to raise androgen levels, we should have

detected significantly higher concentrations of fecal androgens in males showing non-reproductive aggression than in males showing no aggressive behavior. Instead, our results indicate that the context in which aggression occurs is quite important. Also, our results suggest that male-male interactions over a female do not constitute a particularly challenging situation for male hyenas. Males rarely fight over females, aggression intensities are low and consist mostly of low-level threats among males, and the outcome of any male-male interaction is nearly perfectly predictable due to the stable social hierarchy and queuing system among immigrants.

In their efforts to apply the challenge hypothesis to spotted hyaenas, Goymann et al. (2003) acknowledged that there was a possibility that T levels were elevated in male hyenas defending prospective mates due to being close to females. However, male-female interactions were not investigated in that study. The present work directly addressed this possibility by investigating whether the main social challenge for a male spotted hyena is male-male competition or interacting with dominant females. Although we did find a significant relationship between reproductive aggression and androgens, most males showing reproductive aggression were also concurrently engaged in active courtship. Males showing courtship alone also had elevated androgen levels. These results demonstrate that interacting with females is associated with elevated androgen levels independent of whether or not a male is also engaging in defensive aggressive interactions with other males over access to the females. This supports our hypothesis that interacting with females represents a substantial

challenge to adult immigrant males. It is quite obvious upon observation that spotted hyena males are conflicted between tendencies to approach and flee during interactions with dominant females. Males sometimes even engage in brief running mounts and awkward dismounts when interested in a female, as well as masturbation next to a female. Males may need to reach some threshold of sexual stimulation in order to overcome this approach-avoidance conflict, to actively court and eventually mate with a female.

The challenge hypothesis has proven to be a useful starting point for formulating predictions concerning androgen-behavior relationships in male vertebrates. However, the results presented here, as well as a small number of recent studies (Creel et al., 1993; Peters et al., 2001; Lynch et al., 2002; Moore et al., 2004), suggest that the hypothesis may be limited in its direct application to social mammals and some birds. The challenge hypothesis appears to lack predictive power for patterns in androgen responsiveness to challenge in species with low ratios of paternal care to male-male aggression (Fig. 4.7 in Wingfield et al., 2000). This suggests that factors influencing male fitness other than direct male-male aggressive competition with other males modulate hormone-behavior relationships in these species. Instead of attempting to modify the challenge hypothesis for application to complex social systems, we suggest that future research should focus on testing the influence of additional variables that may affect hormone-behavior relationships in free-living animals, including alternative reproductive tactics and male-female interactions.

We found that degree of association with attractive females positively influenced fA concentrations in immigrant male hyenas. The reproductive state of the females with which a male associated was clearly important, as males associating closely with females in the month prior to a conception had greater fA concentrations than males associating with lactating females. An endocrine response of a male to a female may play a role in synchronizing reproductive behavior and physiology between the sexes when mating opportunities are somewhat unpredictable. Our findings suggest that, in spotted hyenas, androgen levels are increased by interaction with stimulating females, and that this increased androgen might then function to facilitate courtship and sexual behavior, as well as occasional aggressive defense of females (Moore, 1984; Wingfield et al., 1987, 1990; Beletsky et al., 1995; Balthazart et al., 1996).

We were unable to investigate the relationship between fA concentrations in adult natal males and the intersexual association patterns involving these males because we had too few fecal samples from adult natal males in the months before known conceptions. However, we found no relationship between courtship or reproductive aggression and fA concentrations in adult natal males whereas we did find such relationships in immigrant males. The results presented here, as well as those by Holekamp and Smale (1998) and Holekamp and Sisk (2003), strongly support the idea that males who are adults but have not yet dispersed differ both behaviorally and physiologically from males who have dispersed and immigrated into a new clan, and should be considered as such. East and Hofer (2002) and Goymann et al. (2003) maintain that some natal male

hyenas in the Serengeti never disperse. Instead, these natal males are reported to drop in social rank to the top of the immigrant male hierarchy in their natal clans, and to begin competing for females at that time. These non-dispersing natal males submit to all adult females, including those of lower maternal rank, in all contexts (M.L. East pers. comm.). Although some adult natal males in the Talek clan do show reproductive aggression and court females, they all do so at lower rates than immigrant males (Holekamp and Smale, 1998; this study). The 6 adult natal males for which we calculated rates of reproductive aggression and courtship were aggressive towards, but were never seen to submit to, lower-ranking females in contexts other than courting, such as feeding. Thus adult natal males in the Talek clan do not ever fall in social rank, as they are sometimes reported to do in the Serengeti. This discrepancy appears to represent a very interesting difference between the Talek and Serengeti populations, because it suggests an alternative male strategy occasionally adopted by males in one population but not the other.

Szykman et al. (2001) recently showed that immigrant male spotted hyaenas associate most closely with females that are most likely to be fertile, and now we have shown that male androgen levels increase with this association. However, Szykman (2001) also examined the strength of associations between females and the males known to sire litters born to these females, and found that known sires varied greatly in how tightly they associated with their future mates during the month preceding conception. This suggests that immigrant male hyenas may adopt alternative reproductive tactics. If elevated androgen levels

are costly to male hyenas as they are reported to be in other vertebrate species (Folstad and Karter, 1992; Wedekind and Folstad, 1994), then only males who can afford the cost of associating closely with females are likely to do so. Another potential cost to male hyenas of associating closely with breeding females is the energetic cost of having these dominant females monopolize food resources. In response to these potential costs, alternative reproductive tactics in the male spotted hyena may be mediated by variation in androgenic and stress hormones.

CHAPTER 4

ANDROGENS DURING PREGNANCY IN WILD SPOTTED HYENAS: THE INFLUENCE OF SOCIAL RANK AND MATERNAL EFFECTS ON CUB BEHAVIOR

INTRODUCTION

Absolute female dominance is uncommon among mammals, but occurs in some lemurs (Jolly, 1984; Kappeler, 1990; Young et al., 1990) and in spotted hyenas (*Crocuta crocuta*). Hyenas live in dynamic fission-fusion groups of up to 80 individuals, called clans, that are structured by hierarchical rank relationships (Kruuk, 1972; Tilson and Hamilton, 1984; Frank, 1986; Holekamp and Smale, 1990). All adult females are socially dominant to all adult males not born in the clan (Kruuk, 1972; Smale et al., 1993), and adult females are more aggressive than adult males (Kruuk, 1972; Frank, 1986; Mills, 1990; Szykman et al., 2003). In addition, females are about 10% heavier than males (Hamilton et al., 1986) and their genitalia are virilized and resemble the genitals of the males. The clitoris is enlarged and forms an erectile pseudopenis, the labia are fused to form a pseudo-scrotum, and the urogenital tract passes through the pseduopenis (Matthews, 1939; Neaves et al., 1980; Frank et al., 1990; Cunha et al., 2003). Female hyenas thus urinate, copulate, and give birth through the elongated clitoris.

The prevailing hypothesis suggested to account for this unusual array of masculine morphological and behavioral traits in the female spotted hyena has been that elevated androgens produce large, aggressive females that are able to dominate males in competitive feeding situations, with a byproduct of genital

masculinization (Gould, 1981; Gould and Vrba, 1982; reviewed in Frank, 1997). In mammals, including humans, masculinization and defeminization of the reproductive system and behavior are induced by testosterone (T) or its metabolites (androgens) during certain critical periods of perinatal development, and hyperandrogenism during fetal life results in the physical virilization of female fetuses (reviewed in Breedlove and Hampson, 2002). The current evidence suggests that, in general, adult female hyenas do not have unusually elevated levels of T when compared to male hyenas (reviewed in Goymann et al., 2002, Dloniak et al., 2004), indicating that females are not simply more aggressive than males due to higher levels of circulating T. However, in captivity, pregnant female hyenas experience a great increase in various androgens during the second half of gestation, and these concentrations cross the placenta and reach the developing fetus (Yalcinkaya et al., 1993; Licht et al., 1998). In wild hyenas, it has recently been shown that females have higher plasma T and fecal androgen metabolite concentrations when pregnant than when lactating (Dloniak et al., 2004). These data indicate that spotted hyenas are exposed to high concentrations of androgens during fetal life, which could account for both the external genital and behavioral masculinization of female offspring (Glickman et al., 1987; Yalcinkaya et al., 1993; Glickman et al., 1998; Licht et al., 1998).

Recent research on captive hyenas has cast some doubt on this hypothesis, however. Drea et al. (1998) showed that pregnant female hyenas given anti-androgens during pregnancy still produce male and female cubs with masculinized external genitalia. Although there were definite de-masculinizing

effects of prenatal anti-androgens on both male and female external genitalia, this experiment indicated that the formation of the external genitalia of both male and female spotted hyenas involves more than early androgen exposure, and therefore presently can not be a mere side-effect of selection for a hormonally mediated trait (Drea et al., 2002). Based on this experiment and the fact that adult female hyenas do not always have unusually elevated circulating T levels, East and Hofer (2002) concluded that the assumed link between androgens and aggression does not exist in this species. However, although research has begun to investigate the links between prenatal androgens and external genital masculinization, the relationship between prenatal exposure to androgens and the organization of behavior patterns has not yet been examined in spotted hyenas. The organizational effects of androgens have been able to account for the development of virtually all behavioral sex differences among juvenile mammals studied to date (Goy, 1997; Stockman et al, 1986; Pellis et al., 1992; Olioff and Stewart, 1978; Orgeur, 1995). In particular, sexual play and aggression are known to be affected by organizational androgen effects of in a wide array of mammals (reviewed in Becker et al., 2002). In addition, the influence of androgens on behavioral and genital masculinization of a fetus can be uncoupled (Goy et al., 1988; Goy, 1996). Therefore, the current evidence is certainly not sufficient to rule out a maternal effect of prenatal androgens on behavior in this species.

Maternal effects can occur whenever variation among maternal phenotypes provides an additional, usually non-genetic, source of variation

among offspring phenotypes (Mousseau and Dingle, 1991). These maternal effects represent a mechanism by which current environmental conditions experienced by the mother can influence offspring phenotype, potentially enhancing offspring fitness (Bernardo, 1996; Mousseau and Fox, 1998). In birds and other oviparous species, the egg contents are an important route through which maternal effects can be transmitted. Mothers in good condition often produce relatively large eggs, and egg size is positively correlated with hatchling body size, growth rate, and survival prospects (reviewed in Williams, 1994; Christians, 2002). Variation in maternal investment also occurs by differential transfer of hormones to eggs during their production. Recent studies on birds have shown that nestlings hatching from eggs with relatively high maternal androgen concentrations exhibit increased growth rates (Schwabl, 1996; Eising et al., 2001), accelerated embryonic development (Eising et al., 2001), enhanced development of the hatching muscle (Lipar and Ketterson, 2000), and enhanced social rank (Schwabl, 1993). Importantly, the social environment of a laying bird can influence the amount of androgen transferred to the eggs. Breeding density (Schwabl, 1997; Reed and Vleck, 2001), female social rank (Müller et al., 2002), and aggressive interactions (Whittingham and Schwabl, 2002) have all been shown to influence the amount of T allocated to eggs.

Among wild female spotted hyenas living in a clan, social rank determines priority of access to food (Kruuk, 1972; Tilson and Hamilton, 1984; Frank, 1986; Mills, 1990), and thus profoundly influences energy intake. A female's social rank is not determined by fighting ability or size, but rather is "inherited" from her

mother (Holekamp and Smale, 1991; 1993; Smale et al., 1993; Frank, 1986). There is a strong effect of social rank on female reproductive success, with higher ranking individuals displaying earlier age at first parturition, shorter interbirth intervals, earlier weaning, and enhanced growth of cubs (Frank et al., 1995; Holekamp et al., 1996; Holekamp and Smale, 2000). Within a clan, female spotted hyenas therefore vary considerably with respect to condition depending on their social rank. Interestingly, the highest level of circulating T thus far reported in a free-ranging female spotted hyena was in an alpha female that happened to be in the last trimester of gestation, suggesting a possible effect of rank on circulating T levels in pregnant hyenas (Frank et al., 1985). Thus, maternal transfer of androgens to hyena cubs may represent a mechanism for adaptive maternal control of offspring phenotype in hyenas, as is also suggested to occur in birds. In this study, we tested the hypothesis that maternal effects of androgens during pregnancy influence offspring behavior in wild hyenas. This hypothesis predicts that variation in fecal androgen concentrations in pregnant female spotted hyenas should be related to female social status, and this variation in turn should be related to rates of sexual play and aggression observed in offspring.

In rodents, stress experienced by a mother during pregnancy has also been shown to influence offspring development and behavior via increased glucocorticoids. Prenatally stressed males show reduced male-typical sexual behavior (reviewed in Ward and Reed, 1985) and increased aggression (Marchlewska-Koj et al., 2003). Prenatally stressed females are partially

masculinized (Kinsley and Bridges, 1988) and show decreased fecundity and fertility (Herrenkohl, 1979). Because social rank may influence stress hormones levels in female hyenas (Goymann et al., 2001), we also investigated the role of female glucocorticoid concentrations as a covariate in our analyses of maternal androgen levels and offspring behavior.

METHODS

Study Population

The study was conducted in the Talek area of the Masai Mara National Reserve, an area of open rolling grassland in southwest Kenya. The study animals were members of one large, stable spotted hyena clan, inhabiting a home range of about 65km^2 (Boydston et al., 2001). Life history data are available for all hyenas in the Talek clan from 1988 to the present. Adult hyenas were known individually by their unique spot patterns, and cubs were identified on the basis of distinctive scars, relative sizes, and patterns of molt from the black pelage of young cubs to the spotted patterns of older ones. Sex was determined by the dimorphic glans morphology of the erect phallus (Frank et al., 1990). Data presented here were collected between July 1993 and July 2002, during which time observers monitored the Talek hyenas 20-31 days per month for approximately 6 hours per day. Data documenting agonistic behavior among adult females during observation sessions were collected using critical incident sampling (Altmann, 1974) of all aggressive and appeasement behaviors observed in all social contexts. Social ranks were assigned based on a matrix of

outcomes of dyadic agonistic interactions (Martin and Bateson, 1988), as described previously (Smale et al., 1993).

Fecal Sample Processing and Assays

We collected 53 fecal samples from a total of 43 pregnancies of 27 adult female hyenas over 10 years. We could calculate a female's day of gestation for a given fecal sample by back-calculating from the birthdate of cubs resulting from each pregnancy. When cubs were first seen, day of birth +/- 7 days was estimated (Smale et al., 1993). The number of days between the sample and the birthdate was subtracted from 110 days, the length of the gestation period in spotted hyenas (Schneider, 1926), to give the day of gestation corresponding to each sample. Age and current social rank of each female at each time of sampling were also known from long-term records.

A fecal sample was collected when a hyena was directly observed to defecate during an observation session. Each sample was first collected into a labeled plastic bag at the site of defecation, then mixed and transferred to 3ml cryovials. Samples were stored in liquid nitrogen until transported on dry ice to the United States, where they were stored at –20°C or colder until processing.

Samples were extracted and assayed for fecal androgen metabolites (fA) as described by Dloniak et al. (2004). In brief, steroids in 0.2g lyophilized feces were extracted with 100% ethanol. Fecal extracts were reconstituted and diluted 1:50 in phosphate buffered saline (PBS) and measured in duplicate for immunoreactive androgens by enzymeimmunoassay (EIA). The androgen assay

utilized a testosterone antibody (R156/7) and a testosterone conjugate (horseradish peroxidase) kindly provided by Dr. Bill Lasley and Coralie Monroe of the University of California Davis. In hyena feces, this assay measures both conjugated and unconjugated androgens, including testosterone, androstenedione, and dyhydrotestosterone. Samples were assayed as part of a much larger study (Dloniak et al., 2004). The mean duplicate coefficient of variation for only the samples used in this study was 9.4%.

Fecal extracts were also assayed for fecal glucocorticoid metabolites (fGC) with a modified version of an assay previously validated for use with spotted hyena feces (Goymann et al., 1999). Reconstituted fecal extracts were diluted 1:20 in steroid diluent and measured in duplicate for immunoreactive glucocorticoids by radioimmunoassay kit (RIA). This assay kit utilizes a corticosterone antibody (ICN Biomedicals, Costa Mesa, CA) that crossreacts with the major glucocorticoid metabolites present in the feces of spotted hyenas, as well as many other mammals (Goymann et al., 1999; Wasser et al., 2000). The samples used in this study were assayed as part of a larger study. The interassay coefficient of variation for the entire study was 11.11% (N = 9 assays, a total of 788 samples). The intra-assay coefficient of variation was 5.3 +/- 0.4%. The mean duplicate coefficient of variation for only the samples used in this study was 4.5%.

Offspring Behavior

Subject animals for cub behavioral analyses included all cubs born to females for which we had fecal samples from the second half of gestation, that survived to at least 6 months of age. We chose to use only samples from the second half of gestation in order to limit the effects of day of gestation of sample, and to have comparable measures of fA in females at the time concentrations are the most variable. This included 35 cubs in 8 mixed-sex twin litters, 2 male-male litters and 4 female-female litters, as well as 4 singleton males and 3 singleton females. Spotted hyenas are born in an isolated natal den where they live either alone or with a littermate sibling (East et al., 1989). At 2 to 4 weeks of age they are brought to the clan's communal den where multiple litters of various ages live together in a shared burrow system. Cubs reside at the communal den until 8-12 months of age. The communal den serves as the social center of the clan and is periodically visited by all clan members, including adult females, adult males, and older juveniles that no longer reside at the den. Den-dwelling cubs spend much of the day underground, typically entering the den 2-3 hours after sunrise and emerging in the late afternoon, approximately 2-3 hours before sunset. Behavioral data from cubs were therefore collected between 0600 and 0900h, and between 1600 and 2000h.

Behavioral data were collected using critical incident sampling (Altmann, 1974) of all cubs between the ages of 2-6 months, and cubs were observed for an average of 36.1 +/- 3.8 hours during this period. We recorded all mounts and aggressions of cubs. Aggression included the following behaviors: lunge, snap,

bite, chase, displace, push, stand over, and intention movement to bite (Kruuk, 1972; Smale et al., 1993). Aggressive behavior could easily be distinguished from rough and tumble play because it was always accompanied by distinctive elements of a threatening posture with ears forward and the head and tail raised (Kruuk, 1972). We considered aggression against a littermate separately from all other aggressions, since a different set of variables may affect intra-litter aggression than those affecting aggression among non-littermates. Play-mounting involved one cub approaching another from behind, raising up on the hind legs, and placing the forepaws on the other cub's back with a posture like that exhibited by adult males during copulation. The phallus was often, but not always, erect during play mounting. For each cub, hourly rates of mounting and aggression were calculated by dividing the numbers of observed behaviors of each type by the number of hours each animal was observed during the four-month interval when cubs were 2-6 months of age. At this age, cubs have not yet attained their maternal social ranks (Holekamp and Smale, 1993; Smale et al., 1993), so patterns of aggressive behavior among cubs are not yet shaped by the rank relationships existing among their mothers. We focused on this age interval because play-mounting generally disappears from the behavioral repetoire of cubs by 6 months of age (Holekamp and Smale, 1998).

Statistical Analyses

We first investigated the relationship between social rank and fecal steroid concentrations among pregnant females. We employed mixed model multiple

regressions (one for fA and one for fGC) that included the continuous predictor variables of social rank, day of gestation, and age. Due to an unbalanced design with some repeated measures, we also included individual hyena (ID) as a random effect variable. This method effectively doubled our sample size, but may still be considered pseudoreplication due to the fact that we had more than one sample from some females. Therefore we also re-ran these two analyses on a data set consisting of only a single sample from each female (N=27, one sample from each female chosen at random) to determine the robustness of the significant effects found in the larger data set.

Based on the multiple regression results, we next ran additional analyses on the subset of fecal samples that would be used to investigate the relationship between maternal hormones and cub behavior. We knew the sex composition of each of these litters, therefore we could also include litter composition (all male, all female, or mixed sex) as a variable. We investigated the relationship between maternal hormones and cub behavior with separate ANCOVAs for aggression and play mounting. We included sex of cub, maternal fA, residuals of maternal fGC from the previous analysis, and maternal rank as predictor variables. The residuals of the fGC values were used in these analyses in order to control for the significant effect of day of gestation on fGC that was present in this data subset (see below). Lastly, we investigated whether maternal fA, residuals of fGC, or maternal social rank were related to dominant cub aggression towards it's subordinate littermate with a multiple regression. We only considered dominant cub aggression because aggression in the other direction happens too

rarely to consider here. Significance was set at $p = 0.05$. All statistical analyses were performed using Statistica 8.0 and followed Zar (1995).

RESULTS

Maternal hormones during gestation

We first investigated patterns in fA and fGC concentrations throughout gestation in pregnant female hyenas. The multiple regression for fA concentrations was significant (whole model $R = 0.61$, $F_{5,47} = 9.62$, $p = 0.004$; Figure 4.1). Female ID did not have a significant effect and was therefore removed from the model by backwards selection. fA concentrations increased as gestation progressed (day of gestation partial $R = 0.45$, $p < 0.001$), and higher-ranking females had higher fA concentrations than did lower-ranking females during pregnancy (rank partial $R = -0.46$, $p < 0.001$). Age of the mother did not have a significant effect on fA concentrations among pregnant females (age $F = 0.71$, $p = 0.41$). In addition, when we re-ran the multiple regression on just one sample from each female (N=27), we still had a significant model (whole model $R = 0.62$, $F_{2,24} = 7.39$, $p = 0.003$) with female social rank (partial $R = -0.35$, $p = 0.049$) and day of gestation (partial $R = 0.41$, $p = 0.02$) significantly influencing fA concentrations.

The multiple regression model for fGC concentrations was also significant (whole model $R = 0.62$, $F_{5,47} = 10.43$, $p < 0.001$; Figure 4.2). Female ID was not significant and was removed from the model by backwards selection. Day of gestation of sample explained a significant proportion of the variance in fGC

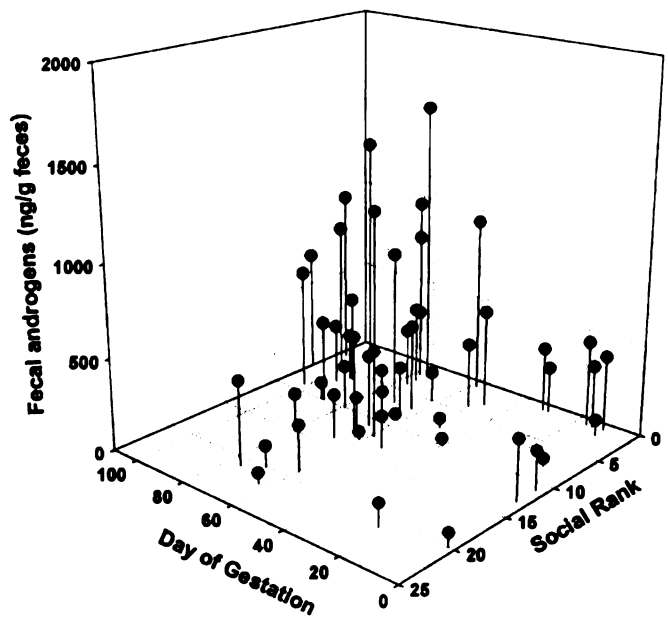

Figure 4.1. Relationships among maternal social rank, day of gestation, and fecal androgen concentrations in pregnant female hyenas. By convention, the highest social rank possible is 1.

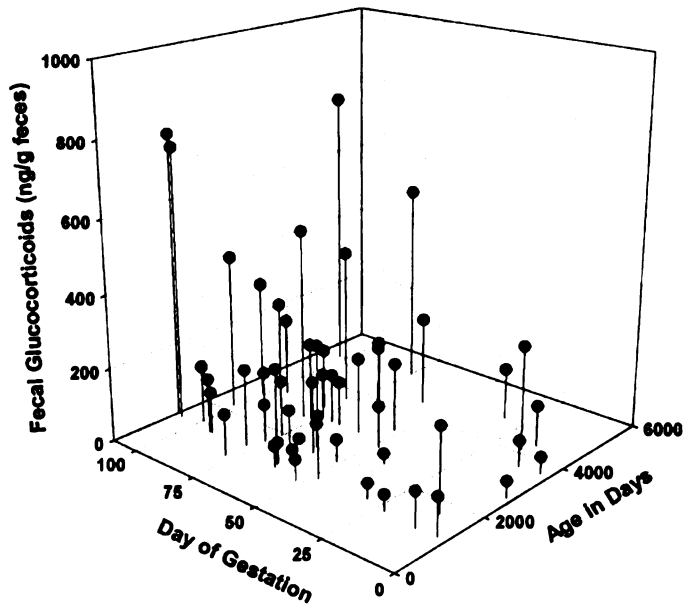

Figure 4.2 Relationships among age of mother, day of gestation, and fecal glucocorticoid concentrations in pregnant female spotted hyenas.

concentrations, with fGCs increasing in the second half of gestation (day of gestation partial R = 0.57, $p < 0.001$). Social rank had no effect on fGC concentrations in pregnant females (rank F = 0.63, p = 0.43), but older females had greater fGC concentrations than younger females during gestation (age partial R = 0.38, p = 0.006). When we re-ran the multiple regression on just one sample from each female (N = 27), we still had a significant model (whole model R = 0.70, $F_{2,24} = 11.29$, $p < 0.001$), including significant effects of maternal age (partial R = 0.47, p = 0.016) and day of gestation (partial R = 0.62, $p < 0.001$).

We next determined whether these relationships between maternal rank and day of gestation and fecal hormone concentrations still held when we only looked at the samples collected during the second half of gestation that would be used for our analyses of cub behavior. We used a mixed model multiple regression and tested for an effect of litter composition on maternal hormones in these samples by including it as a covariate. Within the subset of data there was still a significant multiple regression model of fA concentrations (whole model R = 0.67, $F_{4,16} = 4.54$, p = 0.016). A significant proportion of the variance was explained by maternal rank, with females of high rank having greater fA concentrations than females of low rank (rank partial R = -0.46, p = 0.02, Figure 4.3A). However, among the samples collected exclusively during the second half of gestation, day of gestation was not significant (day of gestation F = 3.16, p = 0.10, Figure 4.3B). Furthermore, there was no effect of litter composition on maternal fA concentrations (litter composition F = 0.12, p = 0.73; Table 4.1). The

multiple regression model for fGC concentrations was significant (whole model R = 0.83, $F_{4,16} = 8.82$, $p < 0.001$), with day of gestation influencing fGC concentrations (partial R = 0.75, $p < 0.001$, Figure 4.4A), but with age of mother not significant (F = 0.512, p = 0.48). There was no effect of social rank (F = 0.20, p = 0.66, Figure 4.4B) or litter composition (F = 0.24, p = 0.63, Table 4.1) on maternal fGC concentrations during the second half of gestation.

Maternal Hormones and Cub Behavior

We found that rates of aggression and mounting in cubs were positively related to maternal fA concentrations during the second half of gestation. Play mounting was almost always directed toward other den-dwelling cubs, rather than towards older juveniles or adults. The ANCOVA for cub mounting rate was significant (Whole model R = 0.681, $F_{4,30} = 6.48$, $p < 0.001$), with cub sex (F = 8.13, p= 0.008) and maternal androgens (F = 7.91, p = 0.009) significant, as well as a significant interaction between the two variables (sex x fA interaction F = 17.97, $p < 0.001$). In general, male cubs showed higher rates of play mounting than did female cubs (Figure 4.5A and B). Maternal fA concentrations were significantly and positively related to mounting rates in both sexes, however there was a more robust relationship in males than in females, as indicated by different slopes in the two sexes. Maternal fGC and maternal rank did not significantly influence mounting rates of cubs (fGC residuals F = 0.86, p = 0.36; rank F = 0.16, p = 0.69).

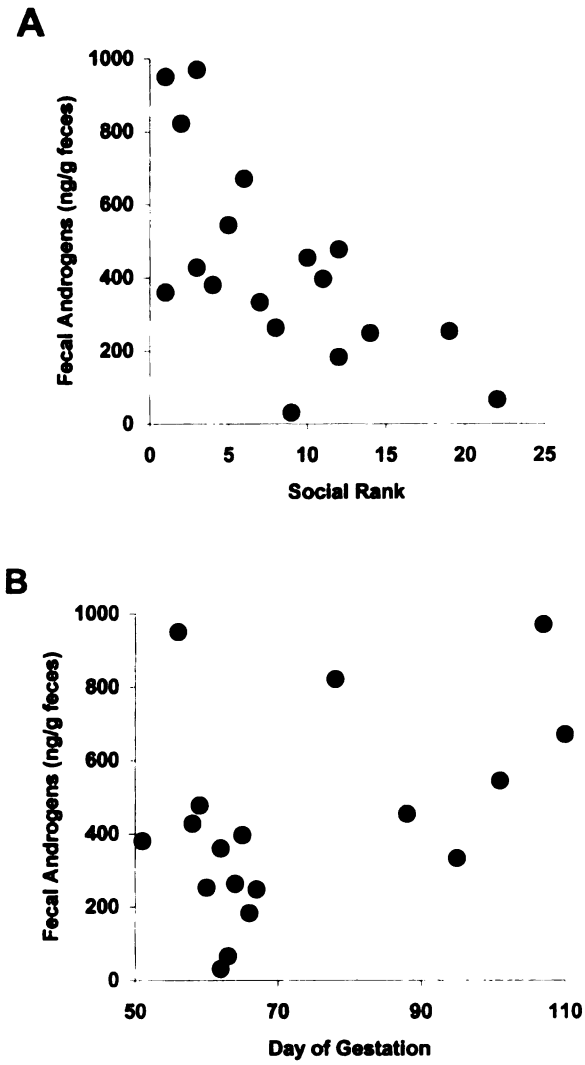

Figure 4.3. Relationships between fecal androgens and social rank (A) and day of gestation (B) in pregnant female spotted hyenas during the second half of gestation.

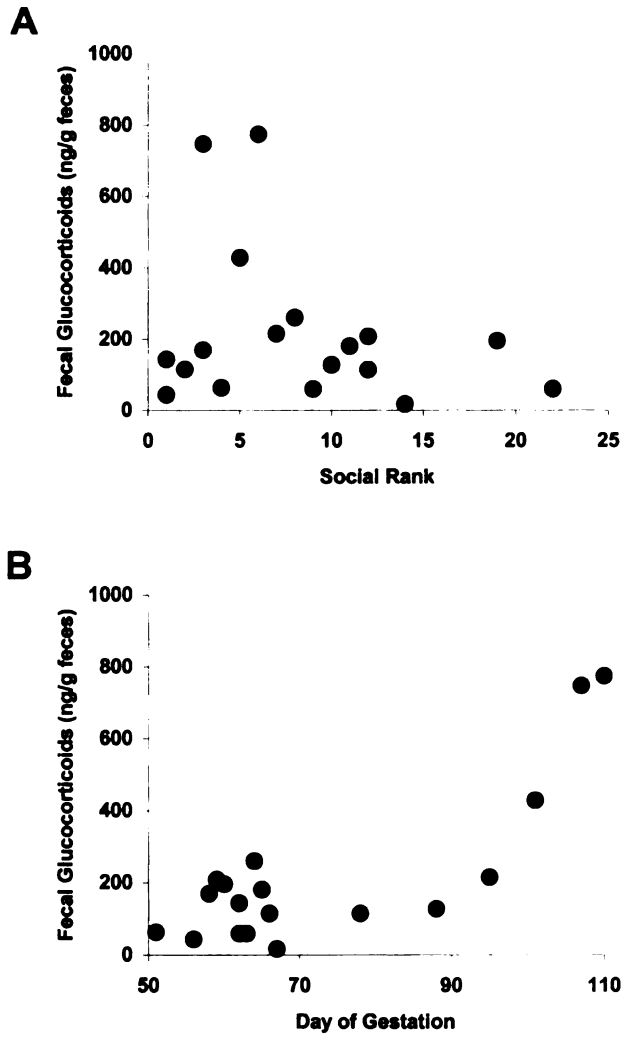

Figure 4.4. Relationships between fecal glucocorticoids and social rank (A) and day of gestation (B) in pregnant female spotted hyenas during the second half of gestation.

Table 4.1. Mean ±SE fecal androgen and glucocorticoid concentrations in relation to litter composition during the second half of gestation in spotted hyenas. Values are given as ng/g feces.

Litter Composition	fA	fGC	N
Female	447.28 ± 80.97	263.66 ± 95.98	7 (4 twin, 3 single)
Mixed	429.38 ±117.99	113.09 ± 25.60	8
Male	436.53 ± 34.42	369.28 ±126.53	6 (2 twin, 4 single)

Cubs directed aggressive behavior towards other den-dwelling cubs and older juveniles, and occasionally towards adult immigrant males and adult females. Aggressive behavior occurred in the context of competition over access to solid food as well as in other contexts. The ANCOVA applied to the aggression data was significant (whole model R = 0.53, $F_{4,30} = 2.92$, p = 0.03; Figure 4.6 A, B). Rates of aggression did not vary with cub sex (F = 0.0001, p = 0.99), and maternal rank did not directly influence aggression in cubs (F = 0.10, p = 0.75). However, there was a significant positive influence of maternal androgens on cub aggression rates (F = 7.37, p = 0.011), and a significant cub sex by maternal androgen interaction (F = 8.0813, p = 0.001). In male cubs, the relationship between maternal androgens and aggression was weak, whereas in female cubs the relationship was dramatic. Interestingly, the multiple regression model investigating the relationships between maternal fA, fGC residuals, maternal social rank, and rates of aggression directed only towards the subordinate sibling by the dominant cub within a twin litter was not significant (Whole model R = 0.52, F = 1.30, p = 0.33). This suggests that a different relationship exists between prenatal androgens and intralitter aggression than that existing between prenatal androgens and aggression towards members of the clan other than littermates (Figure 4.7).

DISCUSSION

We have shown here that fecal androgens increase during pregnancy in wild spotted hyenas, corroborating prior results obtained from plasma of captive

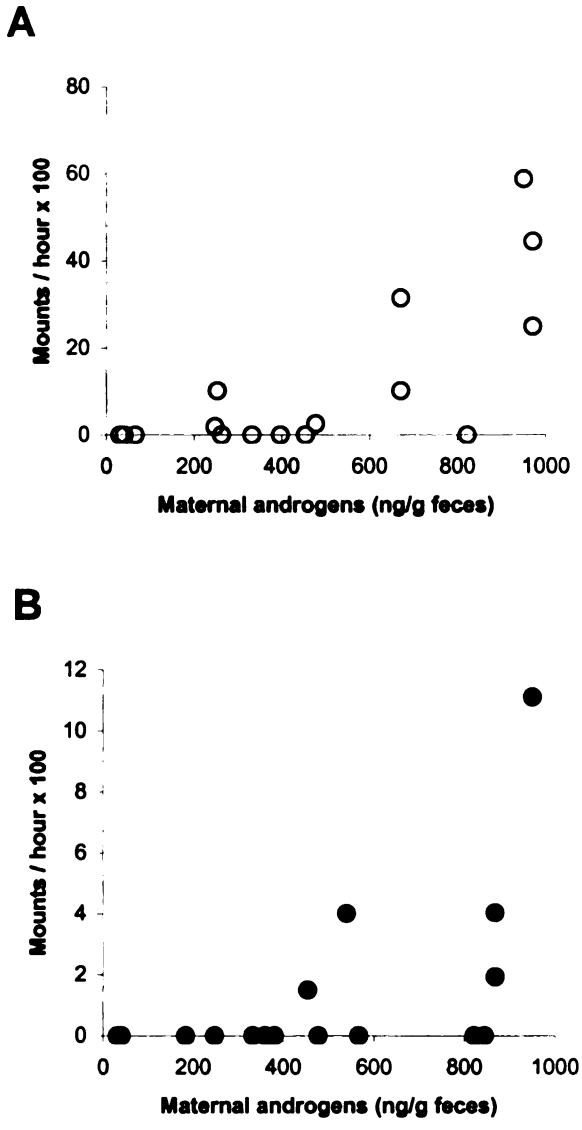

Figure 4.5. Relationships between maternal androgens and mounting rates of male (A) and female (B) cubs. Rates were calculated for the period when cubs were 2-6 months old. Note different scales of y axes.

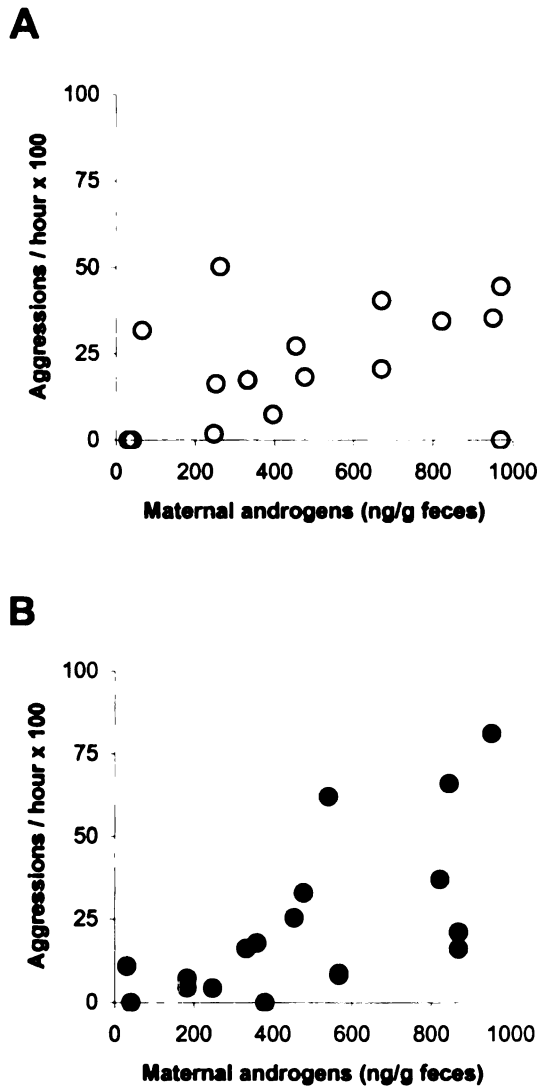

Figure 4.6. Relationships between maternal androgens and cub aggression rates in male (A) and female (B) cubs. Rates were calculated for the period when cubs were 2-6 months old.

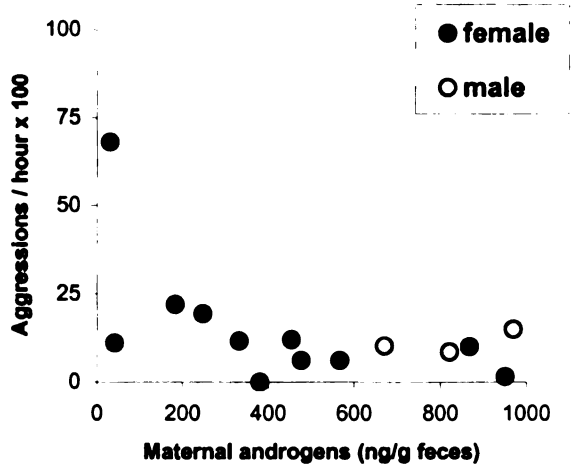

Figure 4.7. Relationship between maternal androgens and rates of aggression directed by dominant cubs towards their subordinate littermates, during the period when cubs were 2-6 months of age.

hyenas (Licht et al., 1992; Yalcinkaya et al; 1993; Licht et al., 1998). We have also shown that wild female hyenas vary in the degree to which fA concentrations increase during the second half of gestation. Females of higher social rank excrete greater concentrations of fA than females of lower social rank. In turn, this variation in maternal fA concentrations appears to influence cub behavioral development. Cubs born to females that have greater fA concentrations during the second half of gestation show higher rates of both aggression and sexual play than do cubs born to females with lower fA concentrations. Fecal glucocorticoid concentrations, on the other hand, were not influenced by maternal social rank, and natural variation in fGC concentrations during the second half of gestation did not appear to influence offspring aggression or mounting in this species.

Glucocorticoids in females

Although we did not detect an effect of maternal social rank on fGC concentrations in hyenas during pregnancy, we did see a significant effect of maternal age. Older females had greater fGC concentrations than younger females throughout pregnancy. Free-ranging yellow baboons (*Papio cynocephalus*) show a general pattern of increased basal plasma cortisol concentrations and glucocorticoid feedback resistance with increased age (Sapolsky and Altmann, 1991), and there is a considerable increase in basal glucocorticoid concentrations in aged rats (Sapolsky, 1992). Our finding that older female spotted hyenas have elevated fGC profiles during pregnancy

therefore probably does not indicate that older females are more "stressed" during pregnancy, but more likely indicates that fGC concentrations increase with female age in general. In the only published study investigating correlates of fGC concentrations in female spotted hyenas, age of female was not addressed (Goymann et al., 2001). Based on these results and the pattern of glucocorticoid increase with age in mammals, any future work in this species investigating correlates of fGC concentrations should certainly consider the influence of age as a covariate.

Goymann et al. (2001) showed that, in general, lactating female spotted hyenas had higher fGCs than non-lactating females, but these authors did not have a sufficient sample size to investigate whether fGCs were elevated in pregnant females. In addition, Goymann et al. reported a significant influence of social rank on fGCs in non-lactating females, with lower ranking females having higher fGC concentrations than higher ranking females. However, the amount of variance in fGC concentrations that this relationship explained was not reported, and the correlation was weak at best (Figure 3 in Goymann et al., 2001). In the same study, no relationship was observed between social rank and fGC concentrations in lactating females. Here, we did not detect an influence of social rank on fGC concentrations during pregnancy, but we did find a significant effect of day of gestation on fGC concentrations. A rise in glucocorticoids, beginning at mid-gestation and continuing through parturition, also occurs in female primates. This rise is attributed to the development of the transitional zone of the fetal adrenal glands, which begin to synthesize cortisol at about mid-gestation (Coulter

and Jaffe, 1998; Smith et al., 1999; Umezaki et al., 2001). The pattern of fGC excretion in female hyenas during pregnancy fits this explanation. Based on these results, it appears that low ranking female spotted hyenas are not abnormally stressed during pregnancy, and that gestational stress probably does not contribute to the lower reproductive success of lower ranking females in this species. Additional research is now needed to determine the possible effects of relatively old maternal age and extremely elevated fGCs (due to certain social situations) on cub development in this species.

Maternal effects of androgens

Our work contributes to an extensive body of research indicating that the prenatal sex hormone environment is extremely important with respect to both the morphological and behavioral development of mammalian offspring. Many studies have now investigated the effects of androgen administration or removal on mammalian development (reviewed in Ward and Ward, 1985; Baum, 1987; Hutchinson and Hutchinson, 1990; Goy, 1996). Fewer studies have investigated natural variation in prenatal exposure to androgens, and virtually all of these studies have been in rodents. In rats and mice, variation in intra-uterine position results in variation in the exposure of fetuses to prenatal hormones of opposite-sexed siblings, and thus to the organizing effects of those steroids on the developing fetus (Clemens et al., 1978; Drickamer, 1996). Female mice that have their first litter when they are middle-aged or adolescent have different T and estradiol profiles than do young adult females during pregnancy. In turn, the male

offspring of middle-aged and adolescent mice have lower body, epididymis, and testes weights than do the male offspring of young adult females (Wang and vom Saal, 2000). To our knowledge, ours is the first study to demonstrate a relationship between a free-ranging female mammal's social environment and her androgen profile during pregnancy. We do not yet know the mechanism by which higher ranking females have higher androgen levels during gestation. An increase in aggressive interactions or a nutritional mechanism seem plausible, and future research will investigate these possibilities.

In addition to showing that higher ranking female spotted hyenas have higher fA concentrations than lower ranking females during the second half of gestation, we also showed that this variance in fA was related to offspring phenotype. Cubs born to females with higher fA concentrations showed increased rates of both aggression and play mounting than did cubs born to females with lower fA concentrations. Thus the naturally-occurring variation in maternal fA concentrations in pregnant female hyenas appears to influence offspring behavior in ways similar to the effects of experimental administration or removal of androgens in lab mammals. Male hyena cubs exposed to low prenatal androgen concentrations are similar to male rats given flutamide (anti-androgen) prenatally, which show reduced rates of mounting and intromissions (Casto et al., 2003). Female spotted hyena cubs exposed to high prenatal androgen concentrations are similar to female infant rhesus monkeys given prenatal exogenous androgens, which results in masculinization of rough-and-tumble play and mounting behavior (Goy et al., 1971; 1988). Females treated prenatally with

T propionate showed increased amounts of rough-and-tumble play and mounting behavior, to levels intermediate between those of normal males and females (Goy et al., 1971; 1988). Interestingly, prenatal exposure to T propionate had no effect on the physical or behavioral development of male infant rhesus monkeys, suggesting that additional administration of maternal androgens does not always have similar effects in male and female offspring.

We found a sex difference in rates of mounting behavior in spotted hyena cubs that appeared to be independent of maternal androgen exposure. The same basic sex difference is seen in most juvenile mammals, and in other species this sex difference during the juvenile pre-pubertal period is promoted by differential exposure to testosterone earlier in development (Goy, 1997). Although our results indicate no effect of litter composition on fA concentrations in pregnant hyenas, it remains possible that males are exposed to slightly different hormone concentrations in utero. Secretory activity of the prenatal testes may elevate androgens locally in male fetuses at a stage of perinatal development during which androgen levels have not yet been measured. Within the hyena medial preoptic area and adjacent anterior hypothalamus there is a sexually dimorphic nucleus that is twofold larger in males (Fenstermaker et al., 1999). Although this is a modest difference compared to that observed in other mammals, it does support the idea male and female offspring may be exposed to slightly different prenatal hormonal environments in this species.

In red fronted lemurs (*Eulemur fulvus rufus*), another species exhibiting female dominance and masculinized external genitalia, it was also found that fA

concentrations were similar in 5 pregnant females regardless of litter composition. However, fecal estrogen (E) levels were much higher in pregnant females carrying a male fetus than in females carrying a female fetus (Ostner et al., 2003). These authors suggested that the relative ratio of E to T to which fetuses are exposed may be the variable that is important as a masculinization mechanism. This possibility now needs to be explored in hyenas. Another possible explanation of sex differences in hyenas is that testosterone during the early postnatal period, which appears to be elevated in males (Frank et al., 1991), might promote the development of play mounting. In addition, female hyena cubs that were exposed to prenantal antiandrogens had lower levels of circulating androstenedione during the first six months of life than did control cubs (Drea et al., 1998). Taken together with the results presented here, these data support the epigenetic hypothesis proposed by Yalcinkaya et al. (1993), which suggests that this antiandrogen-induced decrease in androgen levels in females results from modifications in ovarian morphology and function. In order to test this hypothesis directly, we would need to determine whether cubs resulting from pregnancies associated with higher androgens have higher androgens levels themselves than cubs resulting from pregnancies associated with lower androgens. Hyena cubs rarely defecate during observation sessions at the communal den and we do not attempt darting young cubs until they have left the den, so we currently have very little information concerning concentrations of excreted or circulating androgens in very young cubs and can't directly test this hypothesis yet.

Adaptive maternal effects?

The idea that naturally occurring variation in fetal exposure to androgens may be adaptive in mammals has basically been ignored. However, it seems reasonable that the plasticity of the prenatal organization of this physiological system would allow environmental factors acting on the mother to "hard-wire" the differentiated functions of an organ or tissue system to prepare the unborn animal optimally for the environmental conditions it will soon encounter after birth (Welberg and Seckl, 2001), analogous to the scenario proposed in some birds. vom Saal and Bronson (1980) proposed that, under certain ecological conditions, female mice with a particular set of characteristics might be more likely to reproduce than other females. Female house mice (*Mus musculus*) exposed to higher levels of androgens in utero, due to intrauterine position between 2 males, may have a reproductive advantage over other females when population density is high, because they are highly aggressive toward other females, they fiercely defend their young when lactating, and they enter puberty sooner when housed in groups (vom Saal and Bronson, 1980; Zielinski et al., 1992).

Maternal effects can have adaptive value if they improve the performance of offspring. The maternal transfer of androgens to cubs may be a mechanism for adaptive maternal control of offspring phenotype in hyenas, as it has often been suggested to be the case in birds. Female spotted hyena cubs showed a tight relationship between maternal androgen concentrations and rates of aggression. Increased aggressiveness in female offspring as they develop can easily be considered adaptive in this species due to the high level of competition for

access to food resources (Kruuk, 1972). Male hyena cubs showed play mounting behavior at much higher rates than females, and in both sexes the rate of mounting was positively correlated with maternal fA concentration. An increase in mounting rate may be considered adaptive for male hyenas, for, if any male mammal needs to practice mounting as much as possible, it is the male spotted hyena. Complete female masculinization makes copulation awkward for males, and in fact it has been shown that males exposed to less T in utero do not successfully copulate with females in captivity (Drea et al., 2002). It was suggested that this was an effect of feminized penile morphology. Based on our results, we suggest that prenatal androgen exposure may be just as important for behavioral as for morphological masculinization in spotted hyenas. It would be very interesting to test whether higher ranking sons exposed to higher prenatal androgen concentrations were more successful at immigrating into neighboring clans and siring cubs than sons exposed to lower prenatal androgen concentrations.

The results presented here represent the first definitive link between prenatal androgens and aggression in female spotted hyenas, and suggest an adaptive maternal effect of prenatal hormone exposure in a mammal. Our results do not support the alternative hypothesis put forth by East and colleagues that higher androgen levels in neonates were selected for increased sibling rivalry and facultative siblicide (East et al., 1993; East and Hofer, 2002). The lowest ranking cubs in this study showed relatively higher rates of sibling aggression than higher-ranking cubs, with no relationship to maternal androgen

concentrations. These results do, however, strongly indicate that the epigenetic hypothesis of selection for increased androgens influencing aggressive behavior in females in this species should not be thrown out just yet.

CHAPTER 5

CORRELATES OF FECAL GLUCOCORTICOID EXCRETION IN WILD ADULT SPOTTED HYENAS

INTRODUCTION

Animals respond to stress with a series of endocrine responses that increase the immediate availability of energy, in part by inhibiting physiological processes that are not required for immediate survival (Munck et al., 1984; Wingfield, 1994; Sapolsky, 2002). One of the primary responses to stress is an increase in the activity of the hypothalamic-pituitary-adrenal (HPA) axis, resulting in an increase in quantities of "stress hormones," or glucocorticoids (GCs), released into the bloodstream. The GCs most commonly released from the mammalian adrenal cortex are cortisol and corticosterone (Sapolsky, 2002). These steroids stimulate cardiovascular and musculoskeletal activity, and inhibit a variety of costly anabolic processes such as digestion, energy storage, growth, and reproduction (reviewed in Sapolsky et al., 2000; Sapolsky, 2002). Short-term elevations of GCs lead to adaptive behavioral and physiological processes (Wingfield et al., 1998), but chronic elevation may cause reproductive failure, disease, and loss of muscle mass (Pottinger, 1999; Sapolsky, 2002).

A general theoretical framework has been proposed to help explain how animals keep stress and GCs in balance. Allostasis is the process by which the stability of central physiological "life support" parameters is maintained in the face of environmental variation or variation among life history stages (McEwen, 1998; McEwen and Wingfield, 2003). Through their release during times of stress, GCs

and other hormones, such as cytokines and catecholamines, are primary mediators of allostasis (McEwen and Windfield, 2003). The "allostatic load" of an individual refers to it's cumulative physiological burden (Seeman et al., 2001) as it adjusts to variation over time in conditions associated with seasonal changes, parasite load, reproductive state, social status, injury, infection, and ageing. An increase in allostatic load is typically accompanied by a rise in GC levels, a response which may help the individual alter certain behavioral or physiological processes in order to avoid or resist the potential for chronic stress (Wingfield et al., 1998; Sapolsky et al., 2000).

A stressor is any stimulus (behavioral, environmental, demographic) that provokes a physiological stress response, as measured by an increase in GC concentrations (Creel et al., 2001; Sapolsky, 2002). For animals living in groups, psychosocial factors such as agonistic interactions may represent stressors influencing allostatic load (Goymann and Wingfield, 2004). In fact, in social species with dominance hierarchies, GC levels may be linked to dominance status within the group (reviewed in Creel et al., 2001; Abbott et al., 2003; Creel and Sands, 2003). A number of studies have found elevated cortisol levels in subordinate individuals based on winner-loser situations in captivity (e.g. Bronson and Eleftheriou, 1964; Louch and Higginbotham, 1967; Manogue, 1975). However, results are less clear in surveys of stable groups in social species, where escalated antagonistic encounters are relatively rare. For example, there is no consistent relationship between social status and GC measures in primates. Only a few primate studies have demonstrated that

subordinates have higher GC levels than dominants (squirrel monkeys: Manogue 1975; olive baboons: Virgin and Sapolsky, 1997). Opposite results, in which higher GC levels occurred among dominant individuals than among subordinates, were obtained in marmosets (Saltzman et al., 1998), ring-tailed lemurs (Cavigelli, 1999; Cavigelli et al., 2003), and Japanese macaques (Barrett et al., 2002). No reliable rank-related variation in GC measures was found in rhesus macaques (Bercovitch and Clarke, 1995), long-tailed macaques (van Schaik et al., 1991), cynomolgus monkeys (Stavisky et al., 2001), black tufted-ear marmosets (Smith and French, 1997), or capuchin monkeys (Lynch et al., 2002). Rather than social status per se, these primate studies suggest that better predictors of relationships between GC levels and dominance status may be whether or not subordinates are actually stressed more frequently than dominants, and whether or not individuals enjoy opportunities for social support by conspecifics (Abbott et al., 2003).

As in primates, among the few gregarious carnivore species in which GCs and social dominance have been studied there is no clear simple relationship between these variables. Among wolves, dwarf mongooses, and African wild dogs, dominant individuals typically have higher basal GC levels than subordinates, although in dwarf mongooses this only holds for females (Creel et al., 1992; 1996; 1997; Sands and Creel, 2004; reviewed in Creel et al., 2001; Creel and Sands, 2003). All of these species are cooperative seasonal breeders, in which reproductive success within any particular social group is generally monopolized by the alpha pair. In the one well-studied carnivore species that is a

plural breeder, the spotted hyena, no relationship was found between dominance status and GCs in males (Goymann et al., 2003), and a weak negative relationship was observed between social status and GC concentrations in non-lactating females (Goymann et al., 2001).

In addition to social status or its correlates, allostatic load and GC concentrations in gregarious mammals may also be strongly influenced by a number of other variables, including age and reproductive condition of sampled animals. In female mammals, reproduction often entails large energetic costs. The energy-intensive processes associated with reproduction activate catabolic processes that are part of HPA axis activation. Ovulation, pregnancy, and lactation can all be associated with elevated GC levels (rabbits: Kriesten and Murawski, 1988; humans: Allolio et al., 1990; Lockwood et al., 1996; marmosets: Smith and French, 1997; Saltzman et al., 1998; ringtailed lemurs: Cavigelli, 1999; Cavigelli et al.,2003; spotted hyenas: Goymann et al., 2001). A number of studies also indicate that ovarian hormones stimulate the HPA axis. For example, exogenous estrogen treatment increases cortisol levels in many primate species (Coe et al., 1986; Pepe et al., 1982; Smith and Norman, 1987; Stavisky et al., 2003).

In addition to social status and reproductive state, another potential influence on GC levels in gregarious species may be local food availability. Food deprivation increases circulating GC levels, such that mammals in the wild show clear negative correlations between measures of GC concentrations and food availability (mule deer: Saltz and White, 1991; elephants: Foley et al., 2001;

chimpanzees; Muller and Wrangham, 2003). Severe drought, during which food is inevitably scarce, causes a decrease in social interaction rates and a decrease in male plasma testosterone (T) levels in olive baboons (Sapolsky, 1986). Although GC concentrations were not measured in that particular study, elevated GC levels are associated with decreases in T in male baboons from this study population (Sapolsky, 1982; 1985; 1986), and it was concluded that the ecological stress of drought caused an increase in the amount of time animals spent foraging, a decrease in social interaction rates, and a decrease in T in baboons.

Here we investigated what factors predict GC levels in wild spotted hyenas, in order to determine the relative influence of social status and other factors on the HPA axis in this species. In addition to social status per se, we also examined effects on GCs of reproductive state and local prey availability. Spotted hyenas live in large, dynamic, fission-fusion social groups called clans. Hyenas are unique among social carnivores in that they are plural breeders with moderate reproductive skew (Holekamp and Smale, 2000). Each clan contains one to several matrilines of adult females and their offspring, as well a number of immigrant males. Clans are structured by hierarchical rank relationships (Kruuk,1972; Tilson and Hamilton, 1984; Frank, 1986; Holekamp and Smale, 1990), and all adult females are socially dominant to all adult males not born in the clan (Kruuk, 1972; Smale et al., 1993). Adult males are less aggressive than adult females (Kruuk, 1972; Frank, 1986; Mills, 1990; Szykman et al., 2003), and the social rank of individuals within the immigrant male hierarchy is acquired by

queuing, rather than by fighting (East and Hofer, 2001; Engh et al., 2002). Males are seldom observed to engage in aggressive interactions, and what little aggression does occur among them is typically very mild.

Spotted hyenas kill 65-95% of their own prey, mainly medium- and large-bodied ungulates (Kruuk, 1972; Mills, 1990; Holekamp et al., 1997, Cooper et al., 1999), and variation in local food availability may affect their ability to satisfy their energetic and nutritional needs. Although *Crocuta* usually breed throughout the year, in various populations distributed across the range of this species reproductive seasonality is known to be associated with seasonal variations in local prey abundance (Kruuk, 1972; Lindeque and Skinner, 1982; Mills, 1990; Cooper, 1993; Holekamp et al., 1999). Fluctuations in prey availability may be very important in affecting allostatic load and therefore GCs in this species.

We had two specific goals in this study. Our first objective was to extend the biological validation of a fecal glucocorticoid (fGC) assay for use in spotted hyenas. Goymann et al. (1999) developed a fGC enzymeimmunoassay for use in spotted hyenas, but certain potentially important procedural covariates have not been addressed until now. These covariates include time of day at which samples were deposited, length of time samples were in storage, and age of the hyena depositing the sample. Here, we employed a radioimmunoassay (RIA) kit utilizing the same antibody (ICN corticosterone) as in Goymann et al. (1999). We confirmed the biological validity of the hyena fGC RIA by injecting captive hyenas with adrenocorticotrophic hormone (ACTH), expecting to see a post-injection increase in fGC comparable to the results obtained in a similar experiment by

Goymann et al. (1999). We determined whether variation in diet affected fGC concentrations by systematically manipulating the proportion of the diet derived from bone in captive hyenas, and then measuring excreted glucocorticoids in samples collected after feeding. We also inquired whether time of day of sample deposition, time elapsed before freezing the sample, time elapsed between freezing and assay, or age of the sampled individual had any systematic effects on fGC concentrations in wild hyenas. After determining whether any of these procedural covariates significantly influenced fGC concentrations in hyenas, our second goal was to identify the best predictors of fGC concentrations in adult male and female hyenas, using samples collected over nine years from one large clan in the wild. Here we developed models testing the power of each of the following variables to predict fGC concentrations: social status, female reproductive state, local prey availability, male tenure in the clan, and interactions among these variables.

METHODS

Captive study site, subject animals, and sample collection

All captive hyenas were housed at the University of California Berkeley Field Station for Behavioral Research. These individuals were of known age and reproductive status. Some hyenas were housed individually and others were housed in small groups (for more details see Berger et al., 1992). To identify feces produced by group-housed hyenas, their food was treated with food coloring. All fecal samples were collected between 0800 and 1200h, were

immediately mixed thoroughly, and were then stored in individual containers at -80° C until extraction and assay.

Adrenocorticotrophic hormone (ACTH) challenges were conducted on 5 captive adult hyenas (4 males and 1 female). Fecal samples were collected for 7 days prior to treatment to establish baseline levels of fGC excretion. On the day of ACTH challenge, animals were immobilized with ketamine and xylazine administered by blow dart, and anesthetized with isoflorane inhalant. Each hyena then received a single injection of ACTH (100 IU ACTH, Sigma Chemical Co., St. Louis, MO). Each hyena was allowed to recover from anesthesia and released back to its home enclosure. Fecal samples were collected on the day of challenge as well as for 10 days after the challenge.

Although wild hyenas ingest highly variable amounts of meat and bone, captive hyenas in the Berkeley colony are fed a standard zoo carnivore mix (Nebraska Brand Feline Food, Central Nebraska Packing, Inc., North Platte, NE) and small amounts of bone every day (Berger et al., 1992). In order to assess whether variation in the amount of bone in the diet influences measurement of fGC concentrations, we varied the bone content in the diet of 5 adult females (as in Dloniak et al., 2004). At the start of this experiment some individuals were fed only feline diet while others were fed feline diet plus 3 or 4 sheep neck bones each day. After 1 to 3 days on their respective diets, diets were reversed in all subjects, and reversed again another 1 to 3 days later. Fecal samples were collected each day from all subjects. For statistical analysis, a given fecal sample represented the previous day's diet.

Field study site, subject animals, and sample collection

Our field study site was the Talek area of the Masai Mara National Reserve in southwest Kenya. The subject population was one large, stable *Crocuta* clan inhabiting a home range of approximately 65 km^2 (Boydston et al., 2001). The Talek hyenas have been monitored intensively since June 1988, and data used in this study were collected between January 1993 and July 2002. All hyenas in the clan were identifiable based on each individual's unique spot pattern and other distinguishing marks. Sex was determined by the dimorphic glans morphology of the erect phallus (Frank et al., 1990). Adult females were those born in the Talek clan that were at least 36 months of age. Ages of females at any given time were calculated from known birthdates, which were estimated +/- 7 days when cubs were first seen (Smale et al., 1993). Ages of females in this study ranged from 36 to 199 months. Immigrant males were adult males that had dispersed from natal clans elsewhere before joining the Talek clan. Ages of immigrant males were estimated based on measures of toothwear obtained during immobilizations (Van Horn et al., 2003). Estimated ages of immigrant males in this study ranged from 30 to 153 months. Date of first appearance in the Talek clan was recorded for each immigrant male, and his tenure on a given date was calculated as time elapsed since joining the clan (Holekamp and Smale, 1998). Critical incident sampling (Altmann, 1974) of all observed aggressive and appeasement behaviors was used to determine social ranks of individuals. Social ranks within each sex were assigned based on a matrix of outcomes of dyadic

agonistic interactions (Martin and Bateson, 1988), as described previously (Smale et al., 1993).

Female reproductive state was determined by behavioral observations or by assessment during immobilization. A female was considered to be pregnant if she gave birth to cubs within 110 days after sampling (Schneider, 1926), or if fetuses were observed in her uterine horns during immobilization, visualized with a Hitachi portable ultrasound machine. A female was lactating if she was observed to nurse cubs around the time of sampling and/or milk could be expressed from teats when she was immobilized. Reproductive state was considered to be "unknown" for females that were neither pregnant nor lactating at the time of sampling. These females may have been cycling, but they may also include individuals for which it was impossible to determine whether or not their cubs had been weaned at the time of sampling.

The Talek clan defends a territory at the northernmost edge of the Serengeti ecosystem, an area of open, rolling grasslands grazed year round by concentrations of several different ungulate species. The majority of the resident population consists of Thomson's gazelle (*Gazella thomsonii*), topi (*Damiscilus korrigum*), and impala (*Aepyceros melampus*). Each year, the resident antelope are joined for 3-4 months (usually June-September) by large migratory herds of wildebeest (*Connochaetes taurinus*) and zebra (*Equus burchelli*). Therefore although there are resident prey year-round, there is also a period of high abundance, and there are intermittent periods of relatively low prey availability (Holekamp et al., 1993; Holekamp et al., 1999). Local availability of food to Talek

hyenas was estimated by counting all prey animals found within 100m of 4 km-long transect lines in two different areas of the Talek clan home range (Holekamp et al., 1999). Both transects were run 2 times each month, between 0800 and 1000h. A fecal sample could thus be related to a prey count that occurred within 14 days of the sample. We divided prey counts into three categories (low, average, and high) for male and female samples separately. Within each sex, we calculated the median prey count associated with all fecal samples, and considered average prey abundance to be represented by the counts falling within one interquartile interval above and below the median prey count. High prey abundance then corresponded to counts in the top interquartile interval, and low prey abundance corresponded to counts in the bottom interquartile interval. In males, prey count ranges were as follows: low = 20-190; average = 199-452; high = 457-4094. In females, prey counts ranged as follows: low = 0-172, average = 173-515, high = 587- 4094.

Fecal samples were collected either during early morning (0530 – 0900h, AM samples) or evening (1600 – 2000h, PM samples) observation periods. Samples were collected whenever a hyena defecated, upon direct and unambiguous observation. Samples were first collected into plastic bags at the site of defecation, and later approximately 6 ml of mixed sample were transferred to multiple 2 ml cryovials for freezing in liquid nitrogen. Ninety-four percent of the samples were frozen within 12h of collection, and all samples were frozen within 48h of collection. Samples were stored in liquid nitrogen until shipped on dry ice

to the United States, where they were stored at -20° C or colder until extraction and assay.

Extraction and Assay of Glucocorticoids from Fecal Samples

Fecal samples were extracted in ethanol as described by Dloniak et al. (2004). Samples were then diluted 1:20 in ICN steroid diluent and fGCs were assayed with a double-antibody corticosterone radioimmunoassay kit (ICN corticosterone RIA, Cat. No. 07-120102). This kit utilizes a corticosterone antibody that demonstrates high cross-reactivities to the major glucocorticoid metabolites present in the feces of many mammalian and avian species (Goymann et al., 1999; Wasser et al., 2000). Serial dilutions of pooled extracts from multiple male and female hyenas, respectively, produced displacement curves that were parallel to the displacement curve produced by corticosterone standards. The interassay coefficient of variation for the entire study was 11.11% (N = 9 assays). The intra-assay coefficient of variation was 5.3 +/- 0.4%.

Procedural covariates

Collection conditions and amount of time spent in frozen storage before assay varied among fecal samples. Conditions and time in storage can affect assessments of steroid hormone concentrations in fecal samples in other species (Khan et al., 2002, Terio et al., 2002), and circadian variation has also been reported for some excreted steroids (Sousa and Ziegler, 1998; Muller and Lipson, 2003). We therefore inquired whether time of defecation or variation in

processing and storage conditions systematically affected fGC concentrations in samples from wild male and female hyenas separately. Individuals in this study were sampled repeatedly over almost 10 years. Since glucocorticoid levels are known to increase with age in some other mammals (Sapolsky and Altmann, 1991; Sapolsky, 1992), we also investigated whether age of individual hyenas at the time of sampling influenced their fGC concentrations.

Statistical analysis

All statistical treatment of data followed Zar (1995) and Quinn and Keough (2002). Prior to statistical analysis, all variables were tested for departures from normality and homoscedasticity. For the analyses involving wild hyenas, we used a total of 110 fecal samples from 10 adult males and 341 fecal samples from 21 adult females. Each male and female contributed at least 5 samples to the data sets, and samples from each individual were separated by at least 1 month. We first used general linear models to determine whether any of the following procedural covariates might systematically influence fGC concentrations in male or female hyenas: time of day of sample (AM or PM), time elapsed before freezing of sample (minutes), time elapsed before assay (days), and age in months of individual at the time of sampling. The distributions of two variables, number of minutes between collection and freezing of sample and fGC concentrations for both males and females, were obviously skewed when examined by use of normal probability plots. When natural log transformations were applied to these variables their distributions were normal, therefore all

analyses were done on the transformed variables. Although analyses used log-transformed fGC concentrations to meet the normality assumption, we present the results as raw fGC values (and mean +/- sem) for ease of interpretation of figures.

After we determined whether any of the procedural covariates were important predictors of variation in fGC concentrations, we sought the models that explained the greatest amount of variance in fGC excretion in adult hyenas of each sex. Here, we used general linear models with mixed categorical and continuous predictor variables. We included any significant covariates from the procedural covariate analyses as variables within these models. In building the male model, we investigated effects on fGC concentrations in each fecal sample of each of the following variables: prey abundance (high, average, or low), social rank (high, middle, low), tenure in the clan, and time of day of sample. Because tenure and social rank are closely correlated in immigrant male hyenas (Smale et al., 1997), these variables were never included concurrently in a model. In building the female model, we investigated the predictive power of prey abundance (high, average, or low), social rank (high, middle, low), reproductive state (pregnant, lactating, or unknown), and time of day of sample and age. Because repeated measures were obtained from all individuals, we also tested hyena identity (ID) as a possible predictor variable within each model to determine whether substantial differences existed among individuals over time, and whether ID alone might be important in any models of hyena fGC. We added and removed variables and interaction terms from the models until we had

a significant model that explained the greatest amount of variance in fGC concentrations within each sex (following Quinn and Keough, 2002).

RESULTS

ACTH challenges

Four of five captive hyenas treated with ACTH responded physiologically to the challenge with an increase in fecal glucocorticoids from average baseline levels (Figure 5.1, Table 5.1). The latency to peak fGC concentration varied from one to four days after ACTH injection, and averaged 2.25 days. Among the 4 hyenas that responded to ACTH, peak fGC concentrations after challenge were significantly higher than average baseline concentrations prior to challenge, but levels six to eight days after challenge were no longer different from initial baseline levels (Friedman ANOVA and Kendall coefficient of concordance, N = 4, df = 2, χ^2= 8.0, p = 0.018).

Effect of bone in diet

The presence or absence of bone in the diet did not affect mean fGC concentrations in feces collected the next day from adult female hyenas in captivity. No differences in fGC were found when hyenas switched from a meat plus bone diet to one consisting of only meat (meat plus bone = 131.03 +/- 32.24 ng/g, meat = 196.85 +/- 83.83; Wilcoxon paired sample test: Z = 0.67, p = 0.50) or the reverse (meat = 198.52 +/- 83.29 ng/g, meat plus bone = 183.12 +/- 51.12 ng/g; Wilcoxon paired sample test: Z = 0.13, p = 0.89).

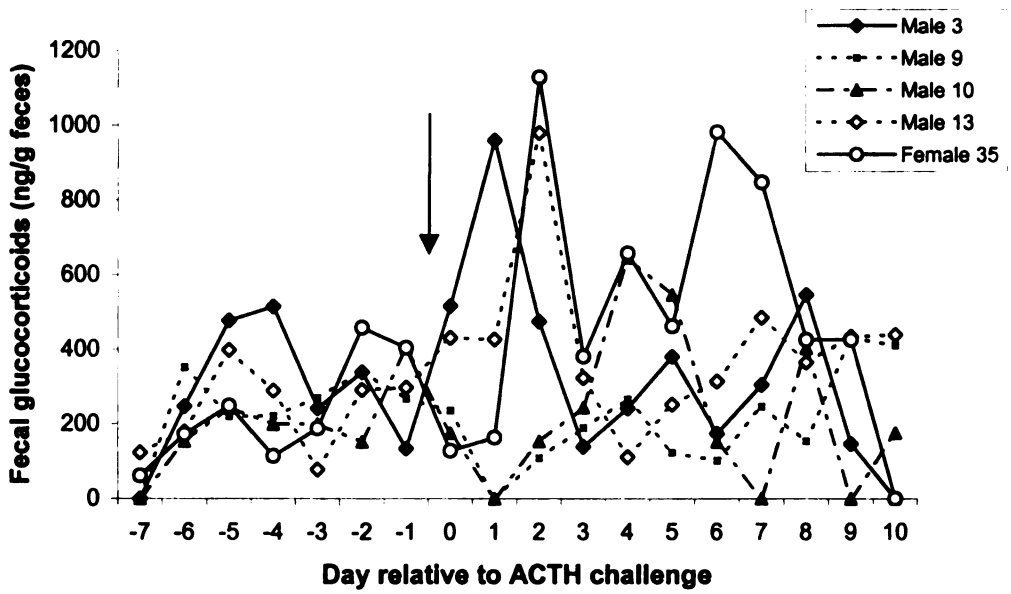

Figure 5.1. Changes in fecal glucocorticoid excretion in response to ACTH challenge in five captive spotted hyenas. ACTH was administered on day 0, as indicated by the arrow.

Table 5.1. Individual and mean response patterns to ACTH challenge in five captive spotted hyenas. Fecal glucocorticoid values indicate mean ± SEM and are expressed as ng glucocorticoid per g lyophilized feces. NR = no response to challenge.

Hyena ID	Baseline glucocorticoid	Maximum glucocorticoid	% Change	Days to Maximum
Male 3	325.52 ± 60.16	959.67	295	1
Male 9	278.78 ± 23.21	266.35	NR	NR
Male 10	225.15 ± 38.25	646.50	287	4
Male 13	236.52 ± 42.72	979.88	414	2
Female 35	235.11 ± 55.45	1128.94	480	2
All responders	255.58 ± 23.45	928.75 ± 101.37	369	2.25

Procedural covariates

Males

The general linear model investigating effects on fGC concentrations of the various procedural covariates in males was significant ($R^2 = 0.27$, $F_{4,105} = 8.85$, $p < 0.001$). Within this model, neither the number of minutes elapsed between sample collection and freezing ($F = 0.585$; $p = 0.446$) nor the number of days frozen until extraction and assay ($F = 0.044$; $p = 0.834$) influenced fGC concentrations in males. In addition, estimated age of male at the time of sampling was not related to fGC concentrations ($F = 0.099$, $p = 0.754$). However, the model did show a pronounced effect of time of day of sample deposition on fGC concentrations in males ($F = 30.98$, $p < 0.001$). We investigated the robustness of this effect by comparing paired mean AM and PM fGC concentrations in males. Each male had at least two AM and two PM samples. In all ten immigrant males, mean AM fGC values were higher than mean PM values (paired t-test; $t = 4.623$, $p = 0.001$, Figure 5.2).

Females

In females, the general linear model of procedural covariates was also significant, although it explained little variance in fGC concentrations ($R^2 = 0.08$, $F_{4,336} = 6.710$, $p < 0.001$). As observed in the male model, neither the number of minutes elapsed between sample collection and freezing ($F = 2.94$; $p = 0.12$), nor the number of days frozen until extraction and assay ($F = 1.47$; $p = 0.23$),

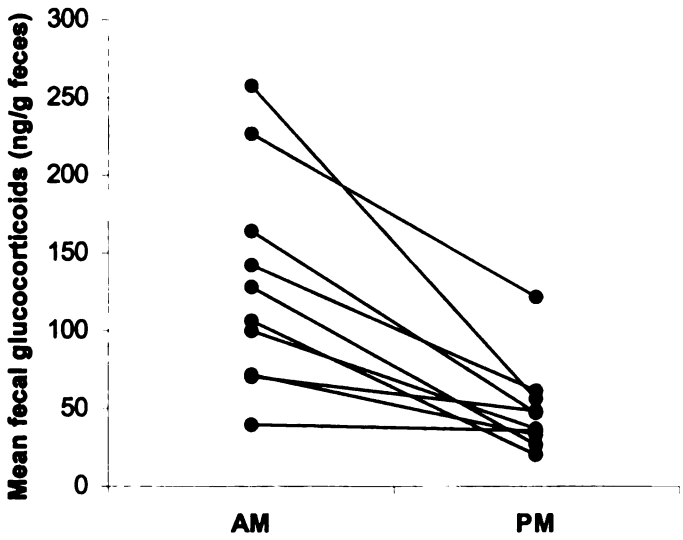

Figure 5.2. Paired comparison of mean morning (AM) and afternoon (PM) fecal glucocorticoid concentrations in 10 adult immigrant male hyenas.

significantly influenced fGC concentrations in females. However, we found significant effects of both age and time of day of sample in females. As in males, fGC concentrations in females were greater in samples collected in the morning than in those collected in the afternoon ($F = 13.087$, $p < 0.001$). We investigated the robustness of the time of day effect by paired comparison of mean AM and PM fGC concentrations in the 21 females. Each female had at least 2 AM and 2 PM samples. Of these females, 16 had greater mean fGC values for AM samples than for PM samples, two had greater PM than AM means, and three had similar AM and PM means (Paired t-test, $t = 2.493$, $p = 0.021$, Figure 5.3). Lastly, as females got older, fGC concentrations generally increased ($F = 7.26$, $p = 0.008$).

Models of glucocorticoid excretion in wild hyenas

Males

Due to the fact that time of day of sample had a significant effect on concentrations of fGCs in male hyenas, we included this variable within our model of ecological and social factors influencing male fGC levels. The best model of male fGC concentrations was one that included male tenure, time of day of sample, prey availability, and an interaction term for time of sample by prey availability. Of these variables, only time of sample and prey availability were significant (Table 5.2). Although included in the model, the interaction term of time of day and prey abundance was not significant, indicating that the influence of prey availability on fGC concentrations in males was similar in both AM and PM samples. Immigrant males had higher fGC concentrations when prey

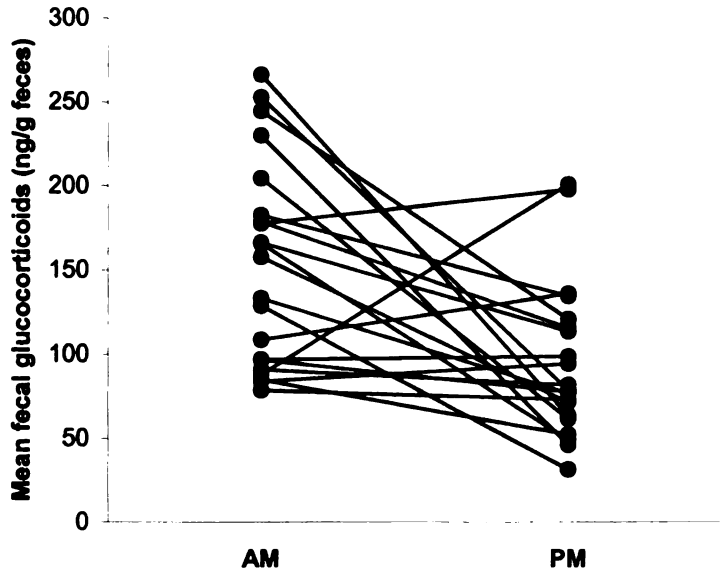

Figure 5.3. Paired comparison of mean morning (AM) and afternoon (PM) fecal glucocorticoid concentrations in 21 adult female hyenas.

Table 5.2. Best model of fecal glucocorticoid concentrations in adult male Talek hyenas. Whole model $R^2 = 0.30$, $F_{6,103} = 7.37$, $p < 0.001$.

Effect	SS	df	MS	F	P
Intercept	369.55	1	369.55	438.94	< 0.0001
Tenure	1.52	1	1.52	1.80	0.183
Time[a]	23.57	1	23.57	28.00	<0.0001
Prey[b]	5.79	2	2.90	3.44	0.036
Time*Prey	0.25	2	0.15	0.15	0.863
Error	86.72	103	0.84		

[a] Time represents time of day of sample (AM or PM)
[b] Prey represents prey abundance at time of sample (high, average, or low)

availability was low than when prey availability was average or high (Figure 5.4). Social status did not have a significant effect on fGC concentrations in any model, and it's inclusion as a predictor variable actually caused the amount of variation explained by the final model to decrease. Therefore, social status was not included in the best model of fGC concentrations in males.

Females

Because we found significant effects of age and time of day of sample on fGC concentrations in female hyenas in our previous analysis of procedural covariates, we included these variables when building our model of social and ecological effects on fGC concentrations in females. The best significant model only explained a small amount of the variance in fGC concentrations in female spotted hyenas (Table 5.3). This model included the variables age, time of sample, reproductive state, and an interaction term for time of sample by reproductive state. Although included in the model, the interaction term was not significant, indicating that the influence of reproductive state was similar in both AM and PM samples among females. Adult females had greater concentrations of fGCs when pregnant than when lactating or when reproductive status was unknown (Figure 5.5). Older females generally had greater fGC concentrations than younger females, and this effect of age was apparent in both pregnant and lactating females (Figure 5.6). Social status and prey availability did not have significant effects on fGC concentrations in any model, nor did their inclusion as predictor variables increase the amount of variance explained by the model.

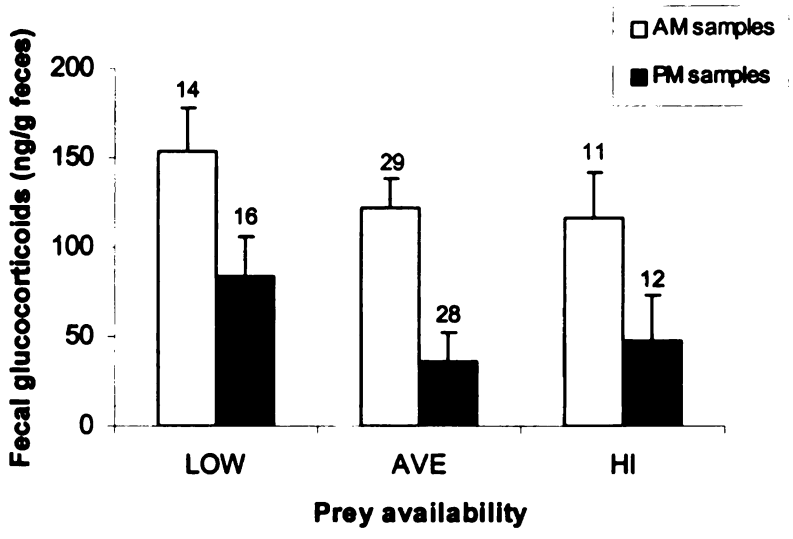

Figure 5.4. The influence of prey availability on fecal glucocorticoid concentrations in adult male spotted hyenas. LOW = lowest quartile of prey availability. AVE = middle 2 quartiles of prey availability. HI = upper quartile of prey availability. AM samples were collected between 0530 and 0900h, whereas PM samples were collected between 1600 and 2000h. Numbers above error bars indicate numbers of fecal samples in categories.

Table 5.3. Best model of fecal glucocorticoid concentrations in adult female Talek hyenas. Whole model $R^2 = 0.11$, $F_{6,334} = 6.66$, $p < 0.001$.

Effect	SS	df	MS	F	P
Intercept	872.62	1	872.62	1059.39	< 0.0001
Age	3.41	1	3.41	4.14	0.042
Time[a]	5.41	1	5.41	6.57	0.011
Repro[b]	19.60	2	9.80	11.90	<0.0001
Time*Repro	0.63	2	0.32	0.38	0.682
Error	275.12	334	0.82		

[a] Time represents time of day of sample (AM or PM)
[b] Repro represents reproductive status at time of sample (pregnant, lactating, or unknown)

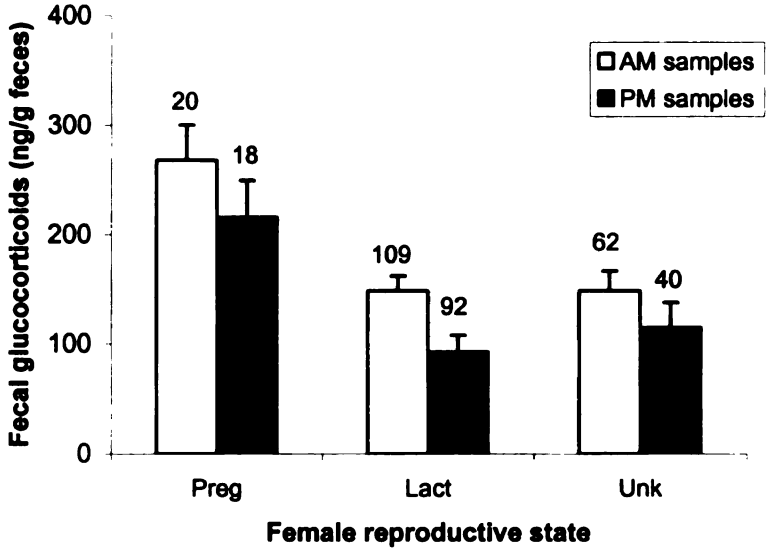

Figure 5.5. The influence of reproductive state on fecal glucocorticoid concentrations in adult female spotted hyenas. Preg = pregnant, Lact = lactating, and Unk = unknown. AM samples were collected between 0530 and 0900h, whereas PM samples were collected between 1600 and 2000h. Numbers above bars indicate numbers of fecal samples in categories.

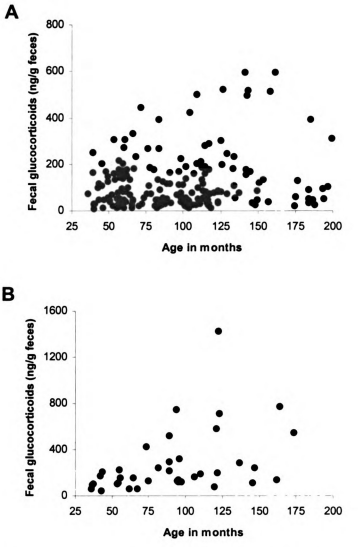

Figure 5.6. Relationships between age and fecal glucocorticoid concentrations in lactating (A) and pregnant (B) female spotted hyenas.

Therefore social status and prey availability were not included in the best model of fGC concentrations in female hyenas. Lastly, in order to compare our results to those of another study, we also ran one separate analysis using just the samples from females in an unknown reproductive state to determine whether social status was related to GC concentrations within this category. The correlation between fGC concentrations and social status was not significant in these females ($R^2 = 0.001$, $F_{1,100} = 0.12$, $p = 0.73$)

DISCUSSION

ACTH is a potent stimulator of the hypothalamic-pituitary-adrenal (HPA) axis. The first step in the biological validation of our assay was to administer ACTH challenges to 5 captive adult spotted hyenas. ACTH challenge led to substantial increases in excreted fGC in 4 of the 5 hyenas challenged in this study, with an average increase of 369% in excreted glucocorticoids across the responding individuals, ranging from 287 to 480%. The timing of peak glucocorticoid excretion was somewhat variable in that one hyena responded maximally after one day, two hyenas responded after two days, and one responded four days after injection with ACTH. A previous ACTH challenge experiment found similar variation in peak glucocorticoid excretion in feces of captive spotted hyenas after ACTH injection (Goymann et al., 1999). In that study, while the average latency to peak excretion of fGC after injection was about one day, some hyenas exhibited peak excretion rates more than two days after injection. Regardless of the slight variation in response patterns, the

predicted response to ACTH challenge was seen in all but one subject, showing that the ICN corticosterone RIA kit is capable of measuring physiologically-induced elevations in fGC concentrations within individual spotted hyenas.

Although a response in fGCs to ACTH challenge in spotted hyenas had been shown prior to this study (Goymann et al., 1999), possible procedural covariates that might be influencing the measurement of fGC in samples from wild hyenas had never been addressed. In spotted hyenas, bone constitutes a variable proportion of the diet, and finding variation in fGC concentrations with the presence or absence of bone might require correction for bone concentration in samples. Therefore, we tested whether the presence or absence of bone in the diet had an effect on fGC concentrations in captive female hyenas. Our results indicate no obvious effect of bone on fGC concentrations, so we do not need to adjust for bone content within samples. Slight variations in collection and storage conditions might also potentially affect the ability to extract and detect fGCs in hyena fecal samples, for reasons outlined previously (Dloniak et al., 2004). A study in baboons showed that fGC concentrations in fecal samples stored in ethanol at either room temperature or -20° C for 90-120 days differed from concentrations in the same samples measured after 180 days of storage (Khan et al., 2002). Therefore we explored this possibility, as well as the influence of the length of time between sample deposition and freezing, with our spotted hyena samples. Fortunately, our data suggest that variation in length of time elapsed before freezing and long-term frozen storage do not systematically affect fGC concentrations in spotted hyena feces.

We also investigated potential effects of two other procedural covariates on fGC concentrations in hyenas: time of day of sample and age of individual at the time of sampling. Numerous studies have described a circadian rhythm in the release of glucocorticoids in various mammals (e.g. Bottoms et al., 1972; Fulkerson et al., 1980; Hoffsis et al., 1970; Hudson et al., 1975; Ingram et al., 1999; Irvine and Alexander, 1994; Knutsson et al., 1997). Serum cortisol concentrations in diurnal mammals increase in the early morning and decrease in the evening, and concentrations in nocturnal animals show the opposite pattern. It is commonly assumed that using fecal samples integrates such variation by "pooling" an animal's daily fluctuations in circulating GC concentrations. However, the effect of time of day of sample on fecal steroid hormones is rarely examined. The results presented here show a clear effect of time of day of sample on fGC concentrations in spotted hyena feces. Both male and female hyenas had greater concentrations of fGCs in samples deposited in the morning than in samples deposited in the evening. This effect was less pronounced in females, but was nonetheless significant. In female marmosets, significantly higher levels of fecal cortisol were found in afternoon samples than in early morning samples (Sousa and Ziegler, 1998). Clearly it is important to consider time of day of sample when evaluating predictors of fGC concentrations. Certain variables, such as prey availability, may affect the general physiological state of an individual, and thus influence morning and evening samples in parallel. However, investigations of predictor variables such as acute social stress may need to control for circadian variation. For example, if the average time lag

between circulating and excreted GCs is between 12 and 24 hours, care must be taken to only collect fecal samples at one time of day, and corresponding behavioral observations should be done 12 to 24 hours prior to the sample. In spotted hyenas, our best estimate of the average time lag between circulating and excreted GCs is between 24 and 72 hours. A more precise estimate of time lag in wild hyenas will be necessary prior to any investigation of the influence of acute stressors on fecal GC concentrations in this species.

In addition to time of day, a female's age had a systematic effect on her fGC concentrations. fGC concentrations increased with age in female hyenas, although this relationship did not appear to be very strong and the model including this variable did not explain much of the variance in female fGC concentrations. This relationship seems to be driven by a lot of low fGC values from relatively young females under 5 years of age (Figure 5.6). GC concentrations also rise with age in rats (Sapolsky, 1992) and baboons (Sapolsky and Altmann, 1991). The mechanism proposed to account for this rise is increased GC feedback resistance with age. Although we do not yet know whether this mechanism is responsible for the age-related increase in GCs in female hyenas, our results clearly show that covariates such as age and time of day of sample simply should be checked before investigating potential behavioral and ecological predictor variables of steroid hormone excretion in any species.

In our models of fGC excretion in male and female hyenas, social status did not explain any of the variation in fGC concentrations in either sex, and therefore was not included in either model. In another study that investigated the

relationship between social status and fGC concentrations in male hyenas in the Serengeti, there was also no effect of social status on fGCs. We agree with the interpretation of these results offered by Goymann et al. (2003), that the lack of a relationship between social status and fGCs is probably a result of the low frequency and intensity of agonistic interactions among male spotted hyenas (Frank et al., 1986; Szykman et al., 2003; Dloniak et al., 2004). Low or high social status within the male social hierarchy may not affect variation in the allostatic load borne by this species. Our results in male hyenas thus support a growing body of evidence in the literature that GC levels are not primarily influenced by social status in species with low frequencies and intensities of agonistic interactions among males (reviewed in Sapolsky, 2002).

Social status also explained none of the variance in female hyena fGC concentrations in this study, and this variable was not included in the best female model. Similarly, no relationship between social status and GCs was found in studies of female macaques (van Schaik et al., 1991) or female chacma baboons (Weingrill et al., 2004). However, Goymann et al. (2001) reported a significant influence of social rank on fGCs in non-lactating female hyenas in the Serengeti, with lower ranking females having higher fGC concentrations than higher ranking females. However, the amount of variance in fGC concentrations that this relationship explained was not reported, and the relationship was weak (Figure 3 in Goymann et al., 2001). In the same study, no relationship was found between social rank and fGC concentrations in lactating females. Although we cannot directly compare our results with those of Goymann et al. due to methodological

differences, we did run a simple correlation between social rank and fGC values in Talek females assigned to our "unknown" category of reproductive state. In that analysis we found no relationship between social status and fGC concentrations.

The degree to which social support is available for subordinates is an important variable that predicts rank-related variation in GC levels in primates (Abbott et al., 2003). Female hyenas live in large stable groups with close kin as well as unrelated individuals. Female hyenas often do have access to social support from relatives, and this may explain a lack of a relationship between their dominance status and fGC concentrations. However, lower ranking females often have fewer potential allies than higher-ranking females, so we might expect higher ranking females to have lower fGC values if social support is important. In order to test this possibility it will be necessary to examine fGC concentrations in females of similar social rank with and without close relatives present in the clan. Alternatively, due to the fission-fusion nature of spotted hyena society, females may simply minimize psychosocial stress by avoiding other individuals as necessary.

In female spotted hyenas, we found that reproductive state influenced fGC concentrations. Pregnant females had higher fGC concentrations than did lactating females or females in which reproductive state was unknown. In Chapter 4, we showed that fGC concentrations increase at about mid-gestation in pregnant female spotted hyenas (Figure 4.2). A rise in glucocorticoids, beginning at mid-gestation and continuing through parturition, also occurs in

pregnant female primates, and is attributed to the development of the transitional zone of the fetal adrenal glands, which begin to synthesize cortisol at about mid-gestation (Coulter and Jaffe, 1998; Smith et al., 1999; Umezaki et al., 2001). In a previous study addressing the influence of female reproductive state on fGC concentrations in hyenas, Goymann et al. (2001) showed that, in general, lactating female spotted hyenas had higher fGCs than non-lactating females. However, these authors did not have a sufficient sample size to investigate whether fGCs were elevated in pregnant females. There might be an interesting difference between the two hyena populations, with Serengeti females having a "more stressful" time during lactation than Talek females. However, we suspect that part of the difference between studies may be explained by an influence of an age bias in the earlier study. Goymann and colleagues considered female hyenas to be adults at the age of 2, although many females do not start to reproduce for at least another year or two after this. Therefore, the "non-pregnant" category in that study may be biased towards young females, which tend to have lower fGC levels than older females regardless of reproductive state.

In our hyena study population, previous research has shown that an annual birth trough occurred approximately one gestation period after the phase of the annual cycle during which prey animals were least abundant in the home range of the study clan (Holekamp et al., 1999). Litters were least likely to be conceived when game was relatively scarce, indicating that prey availability has important effects on the timing of reproduction in Talek hyenas. Although we did

not find evidence of an influence of prey availability on female fGC concentrations in this study, it remains to be determined whether females who have higher concentrations of GCs while cycling have less chance of conceiving. Alternatively, the influence of prey availability on conception in female hyenas might be mediated by a mechanism other than one involving glucocorticoids.

Although we found no effect of prey availability on fGC concentrations in adult female hyenas, our results suggest that food deprivation has an important effect on fGC concentrations in males. All adult male hyenas are subordinate to all adult females. Therefore, when food becomes scarce, males may suffer the consequences more than females. The results presented here indicate that female dominance over males may indeed buffer females from effects of variation in the food supply.

Within limits, an increase in allostatic load represents an adaptive response to seasonal and other demands. Overall, spotted hyenas seem marvelously well-adapted to their environments, and they are extremely successful. We found no evidence of chronic social stress or periods with extreme allostatic load in the Talek hyenas. However, if additional stressors such as human disturbance are superimposed, allostatic load might still increase dramatically in this species. The results presented here suggest that periods of low prey availability may represent a time when male spotted hyenas might be particularly vulnerable to added pressures. By contrast, female spotted hyenas do not seem to be as strongly influenced by a relative decrease in prey availability. However, the fact that stress hormones increase during pregnancy

suggests that this may be a life history stage when female hyenas are more vulnerable to additional pressures. Future work will investigate whether individuals in these potential higher-risk categories do indeed suffer more from added disturbance.

CHAPTER 6

CONCLUSIONS

The original motivation for studying the socioendocrinology of spotted hyenas was sparked by their complex social system, and unusual characteristics such as female dominance and behavioral masculinization. As stated in the introduction, the goal of socioendocrinology is to understand the links among the social environment, hormones, and behavior in order to know how they modulate the reproductive success of individuals living in social groups (Bercovitch and Ziegler, 1990). Evolution has led to individuals with physiological flexibilities that enable them to respond to their social surroundings and environment in ways that maximize reproductive success, and socioendocrine factors can mediate ontogenetic trajectories that span the entire period between embryonic development and death. A socioendocrine perspective provides a unique framework for connecting reproductive endocrinology with evolutionary biology, thereby integrating proximate and ultimate levels of analysis of behavior. I was inspired to investigate how various endocrine mechanisms may shape or mediate the behavior of individuals within hyena society, both to determine whether hyenas might exhibit unique mechanisms compared to other vertebrates, and to test the generality of certain recent theories in behavioral endocrinology that have been developed by studying various other mammalian and avian species. A focus on the individual, and on the social relationships among individuals, ultimately leads to an understanding of social systems in

terms of the adaptive strategies of their constituent members (Clutton-Brock and Harvey, 1976).

The work presented in this dissertation demonstrates that spotted hyenas can be considered a model system for the study of socioendocrinology, in addition to behavioral ecology and development (Holekamp and Smale, 2000; Holekamp and Smale, 1998). Due to long-term studies of known individual hyenas in a few populations over the last twenty years, we now have enough data from this species to be able to tie together such diverse aspects of their lives as behavior, reproduction, life history, and ecology, in order to answer numerous fundamental questions of interest in behavioral ecology. In addition, with the development of both traditional and non-invasive endocrine techniques for use in this species, we can now integrate yet another facet of hyena biology, their socioendocrinology. This multifaceted approach allows us to thoroughly understand the particular system of interest, and perhaps more importantly to put it all together to address questions at multiple levels to provide a foundation for behavioral ecology that constantly produces interesting new areas for further exploration (Dugatkin, 2001).

The strength of such studies lies in direct proportion to the extent that they both put existing concepts to the test, and generate new models and ideas; on the one hand we can use empirical study of a model system to test well-established theories and on the other we can develop new hypotheses (Dugatkin, 2001). In addition to the integration of various fields and disciplines, model systems are thus often springboards for new directions. As large

carnivores living a social existence more similar to that of baboons than that of wild dogs or lions, spotted hyenas pose many interesting questions about the factors forcing them to live in groups that are structured remarkably differently from the other social carnivores, as well as questions about the consequences of group living on behavior and physiology (Creel, 2001). Thus, fundamental questions about ecology, hormones, and behavior can be addressed by investigating spotted hyenas, with the hope of also generating new theories and questions at the same time.

In this dissertation, I believe that I have successfully shown that spotted hyenas are indeed both a fascinating and useful species in which to study socioendocrinology. This is particularly true when we consider the hyena as representative of certain patterns, but at the same time somewhat unique. For example, the spotted hyena is a typical polygynous mammalian species in which males live within a dominance hierarchy, mate with multiple females, and provide no paternal care to offpsring. However, spotted hyenas are also quite unusual, in that all adult females dominate all adult males and males do not engage in much direct aggressive competition over females, perhaps due to the high degree of female control over mating. By determining whether there are similarities in hormone-behavior relationships across closely related species that differ remarkably in behavioral and social parameters, or across distantly related species that are more similar, we can determine whether these parameters indeed shape or are shaped by hormone-behavior relationships within a given system.

In Chapter 3, we attempted to test the 'challenge hypothesis' (Wingfield et al. 1993; 2000) in spotted hyenas. Although originally proposed in birds, recent work has suggested that the challenge hypothesis may be applicable to mammals as well. Our goals were to determine the best predictors of elevated androgen levels in adult male spotted hyenas, and to elucidate whether sexually motivated aggression against other males or interactions with females had a strong relationship with fecal androgen concentrations. Whereas male-male competition is predicted in most polygynous species, male aggression towards other males is surprisingly low in *Crocuta* (Kruuk, 1972; Frank, 1986; Szykman et al., 2003; Chapter 3). In addition, all adult males are socially subordinate to adult females and females have extraordinary control over reproduction due to their dominance status and masculinized external genitalia. Due to these characteristics, spotted hyenas do not appear to fit neatly within the challenge hypothesis framework, which has a lack of predictive power for species with males that show low levels of male-male aggression and no paternal care (Wingfield et al., 2000). However, if we consider interactions with attractive but socially dominant females to be an important "challenge" for male hyenas (as opposed to male-male competition), we are better able to predict male androgen levels: males interact with females at high levels, and they should respond to these challenges with elevated androgen levels during periods characterized by high levels of association. Our results do seem to support the challenge hypothesis if we consider interacting with female hyenas to be the key challenge for male hyenas. Thus our work sheds light on how the challenge hypothesis can

be applied to complex social mammals. We have in effect tested an hypothesis proposed to be generally applicable across taxa and found some support, but suggest that the hypothesis may need to be altered or re-defined somewhat depending on certain characteristics of a given social system.

The work presented in Chapter 4 also attempted to determine whether another model developed in birds may be applicable to a social mammal, suggesting it could be a more widespread, important phenomenon that deserves further study. The evolution of maternal effects is a fascinating new area of research. These effects can occur whenever variation among maternal phenotypes provides a source of variation among offspring phenotypes (Mousseau and Dingle, 1991). In birds, population density during the breeding season, social rank, and aggressive interactions have all been shown to influence the amount of androgen allocated to eggs. Nestlings hatching from eggs with relatively high androgen concentrations exhibit increased growth rates, accelerated embryonic development, and enhanced social rank (see Chapter 4). We set out to determine whether maternal effects of androgens were important mediators of behavioral development in spotted hyenas. High-ranking female hyenas begin breeding at younger ages, are more frequently able to support pregnancy and lactation concurrently, experience shorter intervals between litters, and their offspring are more likely to survive to adulthood than are those of lower-ranking females. We show here that higher-ranking females also have greater fecal androgen concentrations during pregnancy than lower-ranking females.

Many studies have shown the effects of prenatal androgens on the organization of behavior in mammals. However, these studies have been in the context of attempting to understand the origins of sexually dimorphic behavior and sexual differentiation of morphology. Few studies have even suggested that natural variation in prenatal androgen exposure could be adaptive in mammals (vom Saal and Bronson, 1980, Zielinski et al., 1992), and no study has yet investigated whether a mother's social rank or her social environment might influence prenatal androgen exposure of offspring, thus influencing behavioral development of her offspring, in an adaptive fashion. Here we present the first strong mammalian support for this hypothesis, and we suggest a potentially new mechanism for transfer of social status-related traits, via prenatal hormone exposure.

While we have contributed to an extensive body of research indicating that the prenatal hormonal environment is extremely important, we have extended it. By studying this phenomenon in hyenas, we have extended avian work to another taxon, showing that the maternal effects of androgens might potentially be having very important effects on the evolution of all sorts of behavior in a large variety of species. This study increases our knowledge of the long-term evolutionary processes behind the hyena's social system, which presumably might also be operating in other social mammals. For this species in particular, we have shown the first definitive link between androgens and aggressive behavior in spotted hyenas, suggesting support for the epigenetic mechanism of female masculinization proposed by Yalcinkaya et al. (1993). The spotted hyena

may prove to be a particularly useful model for determining any far-reaching effects of prenatal androgen exposure. This potential mechanism of selection is just now being studied in a small number of vertebrate species, and we have made a provocative contribution that will hopefully lead to more investigations. This link between physiological mechanism and function may be a new and very helpful way to study the evolution and maintenance of social systems.

The two chapters of this dissertation concerning the roles of androgens within spotted hyena society thus have both tested and extended hypotheses originally developed in other model systems. Chapters 3 and 4 are therefore important contributions towards understanding the general applicability of broad theories by testing their fit in this unique system. Although mainly descriptive in nature, Chapter 5 also leads to new hypotheses to test. Our goal in Chapter 5 was to determine what factors predict glucocorticoid (GC) levels in wild spotted hyenas, in order to determine the relative influence of social status and other factors on the HPA axis in this species. We found no evidence of chronic social stress or periods with extreme allostatic load in the Talek hyenas. Our results in male hyenas thus support a growing body of evidence that GC levels are not primarily influenced by social status in species with low frequencies and intensities of agonistic interactions among males (reviewed in Sapolsky, 2002).

Although we found no effect of prey availability on GC concentrations in adult female hyenas, our results suggest that food deprivation has an important effect on GC concentrations in males. All adult male hyenas are subordinate to all adult females. Therefore, when food becomes scarce, males may suffer the

consequences more than females. The results presented here indicate that female dominance over males may buffer females from effects of variation in the food supply. Due to female dominance over males, when males are spending a lot of time courting females, they may not be eating as much. Female interactions may directly influence GC levels in males, or food deprivation may mediate an increase in GCs. We can now investigate these possibilities.

Studying new species is important in an obligately comparative discipline such as socioendocrinology, for in this field variety is informative. A logical extension of my thesis work, for example, might be to extend this line of research to other female-dominated species in order to determine whether male-female interactions are associated with increased androgens in these species as well as in *Crocuta*, and to determine whether the challenge hypothesis is truly widely applicable or whether female-dominated species represent an exception to the general 'rule' described by this hypothesis. Lastly, little work has been done investigating the normal roles of androgens in female mammals, and new research should certainly investigate how widespread maternal effects of androgens may be within this large taxonomic group. Although the female spotted hyena is quite unique, it is surely possible that variation in androgens could be playing subtle, but important roles in the development of offspring and/or the influence of dominance status on reproductive success in many other species.

Although we have been able to address many interesting questions regarding the socioendocrinology of the spotted hyena, we have only conducted

observational experiments thus far, and our results are mainly correlational. While purely observational research provides a critical test of the adaptive significance of natural behaviors in the real world, more detailed manipulative experiments offer another way forward. More work needs to be done to investigate cause and effect now. For example, it would be illuminating to give exogenous androgens to males and females to determine whether elevated androgens do indeed cause increased rates of behavior. In order to determine the direct effects of androgens on offspring development we could do experiments giving androgens to pregnant females of various social ranks to determine the extent of the effect of prenatal androgens on offspring development. This field remains wide open, and investigating relationships between hormones and behavior in more diverse taxa will allow us to determine what is unique to each species and what general patterns emerge across many species, telling us more about the evolution of these mechanisms.

Only by benefit of a long-term study program such as the Mara Hyena Project are we able to generate the right questions in order to focus our efforts in the most useful ways when it comes to manipulative field experiments. All of the data presented in this dissertation represents the work of many people during 15 years of intensive research. Socioendocrinology often requires intensive field work with known individual subject animals, so it requires a lot of time and effort. By determining correlations and testing certain hypotheses with non-invasive methods, we can continue in ways that will best focus our efforts. As more studies involve scioendocrinology of diverse taxa, social systems, mating

systems, and behavioral repertoires, we will begin to be able to employ an even stronger comparative method for testing whether various hormone-behavior patterns are common to diverse species living in similar systems. The spotted hyena is a fascinating creature, and a wonderful model for socioendocrine research. This dissertation is but a beginning to the vast amount of work left to do, however I hope that it is a strong foundation. We have answered some important questions, but have generated many, many more. With luck, the hyena project will continue for many more years and data will continue to be collected in order to answer them.

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