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IN THE SPOTTED HYENA
(*CROCUTA CROCUTA*)

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By

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ABSTRACT

HORMONES, STRESS AND AGGRESSION IN THE SPOTTED HYENA (*CROCUTA CROCUTA*)

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In this dissertation, I used data from almost two decades of behavioral observations of wild spotted hyenas (*Crocuta crocuta*), coupled with non-invasive techniques to measure steroid hormone concentrations from these same individuals, to investigate the behavioral endocrinology of this unique species. Female spotted hyenas are morphologically and behaviorally masculinized. These traits make this species particularly interesting subjects to investigate the interaction between hormones and behavior, and how this interaction is influenced by an individual's social or ecological environment.

Our lab has measured concentrations of androgens, glucocorticoids, and now, estrogens present in fecal samples collected from free-living, individually recognized hyenas. I first use these data to investigate both naturally occurring and anthropogenic influences on stress physiology in spotted hyenas. From our longitudinal study, I demonstrated that spotted hyenas are well adapted to variation in their natural ecology, including seasonal fluctuations in prey and rainfall. However, unpredictable events, such as sudden periods of instability in the social hierarchy, do influence spotted hyena stress physiology. Interestingly, adult male hyenas experienced an impact of increasing human disturbance on their stress physiology. This result was strengthened by a cross-sectional comparison that demonstrated that this population of hyenas, which is now exposed to

high levels of human disturbance, had elevated stress hormone concentrations when compared to hyenas exposed to relatively low levels of human disturbance.

I next evaluated behavior likely to be mediated by steroid hormones in this species, focusing on aggressive behavior. Using careful behavioral rate calculations that controlled for variation in opportunity to behave aggressively, I showed that rates of aggression emitted by adult females toward all conspecifics are higher than those emitted by adult males, which is opposite mammalian norms. While rates of intrasexual aggression did not differ significantly between the two sexes, the quality of this aggression did, and attacks among females were of higher-intensity than those seen among males. My results also demonstrated that rates of aggression emitted by females as adults were positively correlated with variation in their prenatal androgen exposure, supporting the organizational hypothesis of steroid hormone control of behavior.

Finally, we investigated two functional hypotheses to explain a particular type of aggression observed among adult female hyenas. Unprovoked aggression describes attacks between individuals that are not related to maternal defense or control of resources. My results suggest that females use unprovoked aggression to test existing rank relationships. However, we did not find evidence supporting the notion that aggression is preferentially directed at females to suppress their reproductive efforts.

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GENERAL INTRODUCTION

In the natural world, animals are faced with an ever-changing environment that presents them with both predictable (*e.g.* seasonal changes in climate or daylight) and unpredictable events (*e.g.* predation or storms). An individual's success frequently hinges on its ability to perceive and adapt to these changes, often by altering its own behavior or physiology. Neuroendocrine and endocrine systems provide the basis for a fluid response mechanism whereby animals can respond with a certain amount of flexibility to environmental stimuli. Steroid hormones in particular play a key role in mediating behavioral responses to increase the responsivity of the individual to changes in their environment, and to increase the likelihood that the appropriate behavior is elicited in specific contexts. Thus a feed-back system exists in which hormones can influence the occurrence of a behavior, and in which that same behavior may also bring about a change in the animal's environment, which can then again influence the animal to affect its endocrine physiology (Nelson, 2005). The field of behavioral endocrinology therefore encompasses the study of both environment-endocrine interactions, and endocrine-behavior interactions.

In this dissertation, I present work done with my collaborators investigating aspects of both of these interactions in spotted hyenas (*Crocuta crocuta*) living in their natural environment. We investigated environmental influences on the physiology of steroid hormones secreted by the adrenals and the gonads, and we also evaluated behaviors likely to be mediated by these steroid hormones in this species. The majority of this dissertation work was focused on proximate level explanations of behavior.

However, the benefit of studying free-living animals is that this allows us to observe behavior within a relevant socio-ecological framework, which provides the opportunity to ask about the ultimate functions of the behavior. Therefore my overarching goal with this work was to incorporate answers to both "how" and "why" questions into our understanding of spotted hyena behavior.

Our current understanding of hormonal mediation of behavior has been developed by investigation of a large array of species, and it has been determined that the basic features of the endocrine system are highly conserved among all of the major vertebrate groups (Becker, et al., 2002; Nelson, 2005; Silver and Ball, 1989). Steroid hormones are the end products of a cascade of chemical events regulated by the central nervous system. Secretory neurons in the hypothalamus produce specialized releasing factors, which stimulate the release of pituitary hormones from the pituitary. These travel through the blood stream to act on peripheral endocrine glands, where they stimulate hormone production and release. Endocrine glands, such as the adrenals or gonads, respond by secreting steroid hormones into the circulatory system to act on target tissues both in the periphery and in the brain, ultimately influencing both physiology and behavior (Becker et al., 2002; Nelson, 2005).

The hypothalamus, as the bridge between the central nervous system and the endocrine system, integrates endogenous and exogenous information, including a vast amount of sensory input from the individual's environment (Kandel, et al., 2000). In this way, the environment, both social and ecological, can have a profound effect on endocrine physiology. This is most evident in the study of the endocrinology of stress, which examines how an animal perceives and responds to perturbations of their

physiological balance (Sapolsky, 2002). The impact of stressors is frequently measured as variation in circulating glucocorticoids (GC), the products of the hypothalamic-pituitary-adrenal (HPA) system. Acute activation of the HPA axis, and short term release of GC concentrations from the adrenals are adaptive parts of the "fight or flight" system; however, chronic elevation of GC concentrations can lead to pathology, including compromise of cardiovascular, immune, neural, and reproductive function (Sapolsky, 2002). Among group-living species, aspects of sociality may be perceived as stressors. For instance, among several cooperatively breeding carnivores such as the African wild dog (*Lycaon pictus*), dominants have higher GC concentrations than do subordinates, which may be associated with higher rates of aggression emitted by dominates to obtain and maintain their rank (Creel, et al., 1997; Creel, 2005; Creel, 1997). Ecological factors can also affect stress hormone physiology, for example, there is a negative correlation between food availability and GC concentrations among wild populations of elephants (*Loxodonta africana*) (Foley, et al., 2001) and chimpanzees (*Pan troglodytes*) (Muller and Wrangham, 2004). Fairly recently, researchers have been studying environmental influences on stress physiology to investigate the effects that human disturbance might have on wild populations (Walker, et al., 2005; Wingfield, et al., 1997).

In his textbook on behavioral endocrinology, Nelson (2005) introduces hormone-behavior interaction by pointing out that humans had an implicit understanding of the hormonal control of behavior long before hormones had been described by science. For instance, castration has been a useful tool to modulate animal behavior for millennia. We now know that castration, or gonadectomy in general, removes the endocrine glands responsible for the production of gonadal steroid hormones, and this removal, in turn,

causes the termination of certain behaviors. The results of castration represent an example of what Phoenix *et al.* (1959) called the "activational" control of steroid hormones on behavior. Yawning behavior among rhesus macaques (*Macaca mulatta*) provides another, more elegant, example of this activational control (reviewed by Graves and Wallen, 2006). Yawning is a sexually dimorphic behavior rarely exhibited by females, and frequently seen in post-pubertal males. Castration of males abolishes the behavior, whereas subsequent exogenous androgen administration restores the behavior (Phoenix, et al., 1973). Finally, females can be induced to yawn when administered exogenous androgen (Goy and Resko, 1972). This example also nicely outlines the experimental paradigm necessary to show that a behavior is influenced by "activational" effects of a hormone (Phoenix, et al., 1959). Whereas in this example, yawning is mediated by androgens, ovarian hormones, like estrogen and progesterone, can also activate behaviors, such as lordosis behavior in female rodents (Breedlove and Hampson, 2002; Nelson, 2005).

Relationships between hormones and behavior that support the activational hypothesis can also be observed without experimental manipulation. For instance, among seasonally breeding animals, such as the red deer (*Cervus elaphus*), males exhibit aggressive behaviors during the breeding season when circulating androgens are high; however, during the non-breeding season, the testes regress, and androgen production is reduced, as are rates of aggression (Lincoln, et al., 1972). Female reproductive cycles provide another predictable source of natural variation in hormone production. Maternal behaviors are tightly coupled to reproductive state in most species displaying maternal care, and the onset of these behaviors have been extensively studied with respect to

estrogen, progesterone, and the peptide hormone prolactin (reviewed by Lonstein and Gammie, 2002). Menstrual and estrus cycles also influence female behavior, obviously with respect to sexual behavior (Wallen and Zehr, 2004), but variation in gonadal hormones during these cycles has also been linked to variation in behaviors associated with memory and cognition (Farage, et al., 2008; Sherwin, 2003).

In contrast to these impermanent activational effects, which wax and wane with hormone titers, steroid hormones present during development may have more lasting effects on behavior. Hormone exposure early in life, particularly exposure to androgens, "organizes" the developing neural substrates of behavior in a permanent way. In their seminal paper, Pheonix et al. (1959) demonstrated that female guinea pigs exposed to prenatal androgens were unable to display normal female sexual behavior despite exogenous administration of estrogen and progesterone in adulthood. This work helped them formulate the concept of irreversible neural modification during development, which has become a central tenet of behavioral endocrinology. The sex differences seen in juvenile play in several mammalian species provide a good example of these organizing effects. Juvenile males engage in more vigorous forms of play than do juvenile females, and this sex difference is apparent before sexual maturity while the gonads are still quiescent with respect to hormone production. Juvenile sex differences in play behavior have been linked to prenatal, or in some cases neonatal, androgens in rodents (Meaney, et al., 1983), non-human primates (Goy, et al., 1988; but see Herman, et al., 2003), and humans (Berenbaum and Hines, 1992).

There are a growing number of experimental paradigms now in use whereby prenatal hormone environment may be altered to test predictions pertaining to the

organization of behavior. However, as in the observational studies used to corroborate activational effects, there is also natural variation in the prenatal hormone environment, and this variation can lead to individual differences in behavior (Goy et al., 1988; Groothuis, et al., 2005; Ryan and Vandenbergh, 2002; Wallen, 2005). Given the importance of prenatal hormone exposure on fetal development, it is useful to consider the variety of sources that can influence the prenatal steroid hormone environment. The predominant source is of course the fetus itself; the fetal testes produce androgens soon after gonadal differentiation, and these androgens are critical for masculinization of both morphology and behavior (Becker et al., 2002; Nelson, 2005). In litter-bearing species, intra-uterine position influences prenatal hormone environment, and the androgen exposure experienced by a female fetus situated between two males has profound effects on her behavior and fertility (Clark and Galef, 1995; Clark and Galef, 1998; Ryan and Vandenbergh, 2002). Although the placenta usually acts as a buffer between the mother and the fetus in mammals, maternally derived steroid hormones do influence the prenatal environment of the fetus, and this may provide a link between the fetal environment and the maternal environment. Maternal environmental stress is commonly studied with respect to its pathological consequences on fetal development (Weinstock, 2008). However, studies of maternal effects in oviparous species suggest that hormonal input from the mother may be adaptive for offspring development and survival (Groothuis et al., 2005), and this concept is now being investigated in mammals (e.g. Dloniak, et al., 2006b).

To summarize, the activational hypothesis predicts that hormone concentrations and the expression of the behavior should be positively correlated, and the organizational

hypothesis predicts that exposure to steroid hormones early in development will have long-lasting consequences with respect to behavior. Frequently, these two mechanisms work in concert, and both organization and activation are required for the full compliment of the behavior to be displayed (Phoenix et al., 1959). Although organizational and activational effects of steroid hormones were originally conceptualized to explain differences in sexual behavior, there is now a large literature using this heuristic framework to examine mechanisms underlying a variety of sexually dimorphic behaviors.

In this dissertation, I focus on a particular behavior, aggression, which has been studied extensively with respect to both organizational and activational effects of steroid hormones. Males are typically viewed as the more aggressive sex among mammals (Archer, 1988; Floody, 1983; Ostermeyer, 1983), and investigation of steroid hormone control of aggression among males has been productive (Bouissou, 1983; Harding, 1981). Intrasexual male aggression is often sexually driven; aggression among males increases when reproductive competition increases, be it during a breeding season, or simply in the presence of a fertile female (Nelson, 2005), and during these sexually charged situations, there is a strong relationship between circulating androgens and rates of the behavior ("Challenge Hypothesis," Wingfield, et al., 1990). Activating effects of androgens on aggressive behavior among males are well established (rodents, Buck and Barnes, 2003; primates, Cavigelli and Pereira, 2000; Zumpe and Michael, 1996; carnivores, Creel, et al., 1993; Creel, 2005; Dloniak, et al., 2006a; Goymann, et al., 2003; ungulates, Lincoln et al., 1972). There is also evidence in support of organizational control of aggressive behavior. For instance, in studies investigating effects of intrauterine position, male mice that developed between two male fetuses were found to have higher rates of intramale

aggression than males raised between two females; although, there does appear to be species specific variation in this mechanism of behavioral control (vom Saal, 1989). As I indicated previously, the investigation of organizational control of steroid hormones on behavior in oviparous species has advanced more quickly than in mammals, primarily because their prenatal environment, the egg, is easier to manipulate than that in placental mammals. For example, manipulated levels of testosterone in the eggs of the black-headed gull (*Larus ridibundus*) were positively correlated with aggressive behaviors after hatching (Groothuis et al., 2005; Muller, et al., 2006).

Despite this past research focus on aggression among males, aggression among females is reported to be prominent in a number of species (Floody, 1983), for instance, in species with cooperative breeding strategies, aggression is thought to play a role in maintaining the dominant status of breeders and the suppression of reproduction in subordinates (rodents, Bennett, 2000; carnivores, Moehlman, 1997; primates, Solomon and French, 1997). Female aggression is also a topic at the forefront of studies investigating species with female dominance. Although rare, the evolution of female social dominance over males has occurred independently in several different taxonomic groups (rodents, Clarke and Faulkes, 2001; primates, Drea, 2007; birds, Smith, 1980; carnivores, Kruuk, 1972), and the potential for steroid hormone control of female aggression exists among these species (Dloniak et al., 2006b; Drea, 2007; Glickman, et al., 1993; Sannen, et al., 2003; von Engelhardt, et al., 2000). For example, in the ringtailed lemur (*Lemur catta*) seasonal elevations in female aggression are positively correlated with circulating androstenedione and estrogen concentrations among females

(Drea, 2007). This study suggests that not only androgens, but also ovarian hormones might be mediating aggressive behavior among females.

The spotted hyena (*Crocuta crocuta*) is another species with well-documented female dominance. Spotted hyena society has a dominance structure based on maternal rank inheritance, more similar to that seen in cercopithecine primates than that in other social carnivores (Engh, et al., 2000; Frank, 1986; Smale, et al., 1993). Offspring slot into the social dominancy hierarchy right underneath their mothers, and the youngest sibling outranks older siblings from prior litters (Engh et al., 2000; Holekamp and Smale, 1993). Females are philopatric and males generally disperse between the ages of 2 and 5 years (Boydston, et al., 2005; Smale, et al., 1997). When males immigrate into a new clan, they slot into the lowest position in the social hierarchy, below all natal members of the group and all other adult immigrant males that arrived before them (East and Hofer, 2001; Engh, et al., 2002). Thus these reproductively mature males are socially subordinate to all natal members of the group (Kruuk, 1972; Smale et al., 1993).

All female hyenas in the social group are able to breed; however, there is strong reproductive skew, and higher-ranking females out-perform lower-ranking females on several measures of reproductive success (Holekamp, et al., 1996). Social rank determines priority of access to food in this species, and food availability is tightly coupled to reproduction (Holekamp et al., 1996; Holekamp, et al., 1999). This relationship has lead to the prevailing hypothesis to explain the evolution of female dominance in spotted hyena society, that selection has favored large, aggressive females who can dominate access to food (Frank, 1986; Gould, 1981).

In addition to this behavioral role-reversal, female spotted hyenas have masculinized external genitalia (Frank, et al., 1990; Kruuk, 1972; Matthews, 1939; Neaves, et al., 1980), which is unique even among species characterized by female dominance (Drea, 2007; Drea, et al., 1998). We have seen that androgens play a key role in shaping male-typical mammalian behaviors, such as aggression, but in most species they are also largely responsible for the development of male morphology and reproductive physiology (Jost, et al., 1973b), and when present at critical stages of development, androgens can masculinize the morphology and behavior of females (Beach, et al., 1982; Goy et al., 1988; Herman, et al., 2000; Jost, et al., 1973a; Phoenix et al., 1959). When compared to males, female spotted hyenas have high concentrations of circulating androstenedione (A4), an androgenic "prohormone" that can be converted either to testosterone or estrogen (Glickman, et al., 1992; Goymann, et al., 2001; Lindeque, et al., 1986). Primarily from an ovarian source, A4 concentrations rise during pregnancy and are converted to testosterone by the placenta, bathing the fetus in androgens during development (Licht, et al., 1998; Yalcinkaya, et al., 1993). Therefore, androgens would appear to offer a convenient mechanism by which selection might promote aggressive females able to dominate access to critical resources. The development of the female phallus was long thought to be a by-product of this androgen-mediated selection in spotted hyenas (Gould, 1981). However, investigation of urogenital development in this species has shown that the genital tissue is committed to phallic development even before fetal gonadal differentiation (Cunha, et al., 2005; Glickman, et al., 2005; Licht et al., 1998), and administration of anti-androgens during gestation does not prevent the development of the phallus in female fetuses (Drea et al., 1998). On the

other hand, there are important sex differences in the structure of the phallus (Frank et al., 1990; Glickman et al., 1992); for instance, the female phallus is much more elastic than the penis, and experimental administration of anti-androgens has shown that these sex-differences are dependent on androgen exposure during early development (Drea, et al., 2002; Drea et al., 1998). Researchers are now expanding their focus toward steroid-independent mechanisms to enhance our understanding of urogenital development among spotted hyenas.

Although it is apparent that androgens are not solely responsible for the masculinization of the external genitalia of female spotted hyenas, there is clear evidence that prenatal androgen exposure influences the behavior of spotted hyenas. Maternal androgen concentrations during pregnancy, measured in feces, are correlated with rates of mounting and aggressive behaviors in both male and female offspring when they are juveniles (Dloniak et al., 2006b). Offspring from mothers treated with anti-androgens during pregnancy exhibit a reduction in the postnatal sibling aggression typically seen in this species (C. Drea and S.E. Glickman, pers. communication). Taken together, these results suggest that prenatal androgen exposure may be organizing the neural structures underlying aggressive behaviors in both male and female spotted hyenas. However, little is known about the factors mediating aggressive behavior among adult hyenas.

There is evidence to suggest that aggressive behavior among spotted hyenas is under activational control of steroid hormones. Perhaps not surprisingly, circulating androgen concentrations among adult males are tightly correlated with aggression related to courtship and mate defense (Dloniak et al., 2006a; Goymann et al., 2003). The evidence suggesting that gonadal steroid hormones activate female aggression is less

straightforward. Among captive hyenas, juvenile ovariectomy appeared to reduce rates of inter-sexual aggression emitted by captive female hyenas as adults (Baker, 1990). However, gonadectomy had no effect on rates of intrasexual aggression among juveniles when compared to controls (Frank, et al., 1989). In this dissertation, I address the possibility that steroid hormones are mediating aggression in adult spotted hyenas living in the wild, and I investigate predictions of both the organizational and activational hypotheses of steroid hormone control of aggressive behavior in this species.

DESCRIPTION OF CHAPTERS

This work predominantly draws on data collected as part of a collaborative effort to monitor a single, large social group of hyenas living in the Masai Mara National Reserve. Begun in 1988 by Drs. Kay Holekamp and Laura Smale, this study has produced almost 20 years of continuous behavioral and ecological data, and over ten years of fecal hormone sampling. This longitudinal project was supervised by Dr. Holekamp, and data were collected by numerous graduate students and lab assistants under her training. It is only now, with 20 years of data, that I might begin to ask the sorts of questions I explore in the following chapters of my dissertation, and thus I will be using the first person plural as I write the data chapters ahead to indicate that this work was only possible as a collaborative effort.

In Chapter 1, I first present a validation for the measurement of estrogens excreted in spotted hyena fecal matter. Endocrine studies of free-living animals are plagued by difficulties associated with obtaining sufficient samples for analysis of hormones in blood. Mammalian carnivores, in particular, represent a taxonomic group in which

endocrine research advanced slowly until the advent of non-invasive hormone sampling (Brown, et al., 1996; Young, et al., 2004). Advances in non-invasive techniques, such as fecal steroid hormone analysis, offer an economical alternative to blood sampling for frequent collection of samples without disrupting the normal behavior of individuals or groups. Many species excrete multiple steroid metabolites that can be measured with immunoassays like those used to measure steroid hormones in blood.

Drs. Stephanie Dloniak and Jeffery French have already published similar validations for non-invasive measurement of both fecal androgens and fecal glucocorticoids in the spotted hyena (Dloniak et al., 2006a; Dloniak et al., 2006b; Dloniak, et al., 2003). The validation of a method for fecal estrogen analysis, presented here, adds to this arsenal of tools available for use in the investigation of hormonal mediation of behavior among spotted hyenas. In addition, having access to estrogen measures from females increases our ability to evaluate their reproductive condition. This work was published in *General and Comparative Endocrinology* (Van Meter, et al., 2008).

As a demonstration of the responsivity of hormone physiology to environment variability, in Chapter 2 we investigated both naturally occurring and anthropogenic influences on stress physiology in spotted hyenas. In this chapter we used both longitudinal data from a single social group, and cross-sectional data from multiple social groups to examine social, ecological, and anthropogenic factors as possible stressors in the lives of wild spotted hyenas. From our longitudinal study, we learned that spotted hyenas are well adapted to variation in their natural ecology, including seasonal fluctuations in prey and rainfall. However, unpredictable events, such as sudden periods

of instability in the social hierarchy do influence spotted hyena stress physiology. Finally, among adult male hyenas, we saw an impact of increasing human disturbance on stress physiology. This result was strengthened by our cross-sectional comparison, which demonstrated that this population of hyenas, which is now exposed to high levels of human disturbance, had elevated stress hormone concentrations when compared to hyenas exposed to relatively low levels of human disturbance. This cross-population study was made possible by the work of two students who received their Ph.D.'s under Dr. Holekamp's supervision. Dr. Joseph Kolowski expanded our understanding of hyenas living in the Masai Mara by intensively studying a second group living in the Mara. Also, Dr. Heather Watts began a parallel study of hyenas living in Amboseli National Park for her dissertation work. It was their effort and foresight that permitted the comparison presented in this chapter, and thus they are co-authors on this manuscript, which was published in *Hormones and Behavior* (Van Meter, et al., 2009).

The last two chapters in this dissertation primarily focus on aggressive behavior among adult spotted hyenas. In Chapter 3, I had two main goals. First, using 17 years of behavioral data collected from our longitudinal project, I endeavored to provide a comprehensive description of inter- and intrasexual aggression among adult spotted hyenas in a variety of social contexts. Second, I used these data along with 12 years of hormone measurements collected non-invasively, to test predictions stemming from both the organizational and activational hypotheses of steroid hormone action. Using careful behavioral rate calculations that controlled for variation in opportunity to behave aggressively, we show that rates of aggression emitted by adult females are clearly higher than those emitted by adult males, and that females are more aggressive to males than

males are towards females. While it appears that rates of intrasexual aggression do not differ significantly between the two sexes, aggression among females is of higher-intensity that that among males. I also showed that rates of intersexual aggression emitted by females as adults, were correlated with their prenatal androgen exposure, supporting the organizational hypothesis of steroid hormone control of behavior.

In the final data chapter, I examined a particular type of aggressive behavior exhibited by spotted hyenas, unprovoked aggression. I tested two functional hypotheses as possible explanations for this behavior among adult females. First, I investigated unprovoked aggression as a possible mechanism for reproductive suppression, like that seen in certain cooperatively breeding species (Solomon and French, 1997). The prediction here was that females receive higher rates of unprovoked aggression when they were reproductively vulnerable. Second, I hypothesized that females use unprovoked aggression to test the strength of rank relationships. There was no supporting evidence that aggression is preferentially directed toward females to suppress their reproductive efforts, as females did not receive higher rates of unprovoked aggression during early pregnancy or around the time of conception relative to when they were in other reproductive states. Females appear to use unprovoked aggression as a means for testing rank relationships among closely ranked individuals.

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CHAPTER 1

Van Meter, P. E., French, J. A., Bidali, K., Weldele, M. L., Brown, J. L., Holekamp, K. E., 2008. Non-invasive measurement of fecal estrogens in the spotted hyena (*Crocuta crocuta*). General and Comparative Endocrinology 155(2), 464-471.

CHAPTER 1

NON-INVASIVE MEASUREMENT OF FECAL ESTROGENS IN THE SPOTTED HYENA (*CROCUTA CROCUTA*)

INTRODUCTION

Knowledge about a female's reproductive state is essential to understanding aspects of her basic biology, including her fertility, behavior (Altmann et al., 2004; Maestripieri, 1999; Ramirez et al., 2004; Wise, 1974), nutritional requirements (Lambert et al., 2005; Schneider et al., 2000), and immune function (Gu et al., 2005; Hernandez-Gonzalez et al., 2006). Despite the importance of understanding reproductive cycles in females, there are many species for which we know virtually nothing about variation in reproductive status, particularly within free-living populations of large predatory mammals. Estrogen concentrations often vary predictably during female reproductive cycles, so the ability to measure estrogens offers one potential mechanism for monitoring ovarian status. Here our goal was to develop methods for non-invasive measurement of estrogens in free-living spotted hyenas (*Crocuta crocuta*).

Studies of the endocrinology of spotted hyenas to date have focused on androgenic hormones, so a great deal is known about circulating and excreted androgens in *Crocuta* (Dloniak et al., 2006; Dloniak et al., 2003; Glickman et al., 1998; Glickman et al., 1992; Goymann et al., 2001; Licht et al., 1992; Licht et al., 1998; Van Jaarsveld and Skinner, 1991). By contrast, little is known about estrogenic hormones in this species. In a study of captive spotted hyenas between infancy and sexual maturity, Glickman et al. (1992) found that plasma estrogen concentrations begin to rise in females by 23 months of age, which is also when they may conceive their first litters in nature (Holekamp et al.,

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1996). It was further shown that plasma estrogen concentrations are higher in adult females than in adult males, and that mean plasma estrogen concentrations are higher in adult than sub-adult females (Glickman et al., 1992). Additionally, pregnant females have higher plasma estrogen concentrations than non-pregnant females, and plasma estrogen concentrations peak during late pregnancy (Licht et al., 1992). However, we know very little about the female's estrous cycle in this species, including the cycle length, and whether ovulation is induced or spontaneous, nor is anything known about patterns of estrogen secretion among wild hyenas.

Gaining information about the reproductive condition of wild female hyenas is difficult. There are no external indicators of changing reproductive state, such as genital swelling, color change, or other visual signs of ovulation often used by researchers studying free-living mammals. Even late pregnancy is difficult or impossible to detect by visual inspection in this species because female body mass can change by up to 18 kg at a single meal (*e.g.* Henschel and Tilson, 1988). Sampling blood from hyenas for measurement of steroid hormone concentrations requires invasive and potentially dangerous procedures, including isolation and anesthesia (Glickman et al., 1992), so multiple samples from the same individual are difficult to obtain in both wild and captive settings. Recent advances in non-invasive sampling methods for hormone analysis offer opportunities for more rigorous investigation of the endocrine correlates and control of reproduction in *Crocuta*. Fecal steroid analysis, in particular, offers an attractive alternative to blood sampling in both wild and captive animals. For collecting samples frequently, non-invasively, and without disrupting normal behavior of individuals or groups, techniques have been developed to assay fecal estrogen concentrations in a

number of other mammalian carnivores (Brown et al., 1994; Monfort et al., 1997; Onuma et al., 2002; Shille et al., 1984; Young et al., 2001). A preliminary study in captive *Crocuta* detected measurable amounts of radiolabeled estrogen in fecal matter, and indicated that feces, rather than urine, should prove useful for sampling of excreted steroid hormones (Koretz, 1992).

Our aim here was to develop and validate an enzyme-immunoassay (EIA) for measurement of fecal estrogens (fE) in wild and captive spotted hyenas. First, we identified the immunoreactive estrogen metabolites in hyena feces using high-performance liquid chromatography (HPLC). We then investigated assay parallelism, examined possible procedural covariates of estrogen concentrations, and assessed the biological validity of our assay by monitoring changes in estrogen concentrations following treatment with luteinizing-hormone releasing hormone (LHRH) of wild and captive hyenas. Finally, we compared patterns of estrogen concentration between feces and plasma among classes of hyenas that were expected to exhibit significant variation in estrogen (*i.e.*, pregnant *vs.* non-pregnant females, mature *vs.* immature females).

METHODS

Field study site, subjects and sample collection

Field data were collected in the Talek region of the Masai Mara National Reserve, Kenya. Subjects were members of one large, stable social group ("clan") that defended a territory of 62 km^2 (Boydston et al., 2001). Talek hyenas have been closely monitored since 1988, and clan members are usually observed intensively on at least 23 days each month. All clan members were individually recognized by their unique spot patterns, and

sex was determined by the dimorphic glans morphology of the erect phallus (Frank et al., 1990). Ages of all individuals born into the clan since 1988 were known, and mother-offspring relationships were established on the basis of nursing associations. Age at first reproduction and full reproductive histories were known for all female members of the clan from 1988-2004 based on known dates of birth and weaning of cubs. Each female was assigned to one of the following three reproductive states each time she was observed:

Immature-Females were considered reproductively immature from birth until first conception. In the wild, we can confidently assign a date to each female's first parturition because of the extensive tearing and scarring of her phallus during the birth process (Frank and Glickman, 1994). Gestation in spotted hyenas lasts 110 days (Kruuk, 1972), so we could accurately estimate the date of first conception by counting backwards 110 days from the known date of first parturition.

Pregnant-Females were considered pregnant during the period between the birth of each litter and its estimated conception date. Birth dates were assigned based on the size, pelage, and behavior of cubs when they were first seen above ground, and were accurate to within ± 7 days (Holekamp et al., 1996). Pregnancy was further divided into trimesters to analyze variation in hormone concentrations among trimesters. Early pregnancy was the period between conception and day 37 of gestation. Middle pregnancy was day 38 through day 74, and late pregnancy was day 75 though the day of parturition.

Lactating-The lactation interval in *Crocuta* is highly variable in length (from 7 to 24 months), as it is affected by food availability, social rank, and litter composition (twin *vs.* singleton litters) (Holekamp et al., 1996; Holekamp et al., 1999). Termination of

lactation was determined here either by conception of the next litter, or by weaning or disappearance of the current litter, whichever came first. Wean dates were determined using behavioral observations of mother-infant conflicts over nursing, as described by (Holekamp et al., 1996). Wean dates for which mother-infant conflicts were not observed were calculated as the day halfway between the last time the cubs were seen nursing and the next time dam and cubs were seen together, if these could be estimated to ± 10 days.

Starting in 1990, each clan member was immobilized at least once for collection of physiological and morphological data. Individuals were anesthetized with Telazol (6.5 mg/kg body mass) administered in a plastic dart fired from a CO_2 rifle. All immobilizations took place between 0630 and 0900 hours, and were performed in accordance with Kenyan law and *NIH Guide for the Care and Use of Laboratory Animals.* Within 17 minutes of Telazol administration, a blood sample (10 ml) was taken from the jugular vein in a heparinized vacutainer tube. Blood samples were centrifuged, and plasma was stored in liquid nitrogen until it could be shipped on dry ice to Michigan State University (MSU), where it was stored at –80°C until radioimmunoassay.

Beginning in 1993, fecal samples were collected whenever a known hyena was observed to defecate. Fecal samples were collected either in the morning (0600-0900) or evening (1700-2000) into plastic bags within 30 minutes of excretion. Samples were maintained at room temperature after collection for up to 12 h, after which they were mixed and stored in liquid nitrogen until they were shipped on dry ice to MSU. (Dloniak, 2004). Dloniak (2004) found that variation in time elapsed between collection and freezing of fecal samples does not significantly affect concentrations of other steroid

hormones in the samples, and we assumed here this applied to fE as well. Samples were stored at MSU at minus 20°C until extraction and enzyme immunoassay.

Administration of exogenous LHRH, acting via intermediate effects on the pituitary, stimulates secretion of steroid hormones from the gonads of hyenas (Ensley et al., 1982; Holekamp and Sisk, 2003; Place et al., 2002). Nine parous wild females were tested during late lactation (7.9 - 13.3 months after parturition) for steroid production in response to LHRH injection. Additionally, three wild adult females were administered saline to serve as handling and injection controls. After immobilization, blood samples were taken at 5-minute intervals from the jugular vein for 45 minutes. Then, females were injected intravenously with LHRH (1 μg/kg LHRH, L-7134 Sigma Chemical CO., St. Louis, MO). Blood sampling then continued at 5-minute intervals for 120 minutes. Supplementary doses of Telazol were administered as necessary throughout sampling to maintain deep anesthesia.

Captive study site, subjects, and sample collection

Captive data were collected from *Crocuta* in the colony maintained at the Field Station for Behavioral Research of the University of California, Berkeley. Subjects were housed individually or in small groups, and fed a standard zoo carnivore diet supplemented with bone. All fecal samples were collected between 0800 and 1200 hours, mixed, and stored at -80°C until extraction and enzyme immunoassay.

Five gonadally intact adult captive hyenas (3 males and 2 females) were injected with LHRH to induce the release of gonadal steroids. On the day of LHRH challenge, subjects were isolated, and then immobilized with ketamine (6.0 mg/kg body mass) and xylazine (1 mg/kg body mass) administered by blow dart. Each subject was administered

an intravenous injection of LHRH, allowed to recover from anesthesia and released back into its home enclosure. Fecal samples were collected daily for 7–8 days prior to LHRH injection, on the day of injection, and then for 7–8 days following injection.

Extraction and immunoassay of fecal samples

A total of 871 fecal samples were collected from known female Talek hyenas. Extraction of steroid hormones has been described elsewhere (Dloniak et al., 2006; Dloniak et al., 2003). Briefly, frozen samples were lyophilized (Labconco Freeze-Dry System 10-269), ground to a fine powder, and shaken overnight in 100% ethanol. Samples were then boiled and centrifuged, and the resulting supernatant was poured off into culture tubes while the remaining fecal pellet was discarded. Finally, the supernatant was allowed to evaporate. Extracted samples were then reconstituted in 1.0 ml phosphate buffered saline (PBS; pH 5.0), sealed, and stored frozen at -20°C until assay.

The number and proportion of estrogen metabolites in *Crocuta* feces were determined by HPLC analysis of fecal extracts from one pregnant and one non-pregnant wild female hyena using modifications of methods described in Brown *et al.* (1994). Extracted samples were spiked with 7000 cpm of ^{3}H-estrone (E_1) and ^{3}H-estrone sulfate (E_1S), and air-dried. Samples were then reconstituted in 500 µl of PBS and vortexed. A C18 sample preparation cartridge (Spice™ Cartridge; Analtech, Inc., Newark, DE), was primed with methanol (MeOH) and distilled water, and loaded with the total volume of sample in PBS. Distilled water and MeOH were pushed through the cartridge and collected into test tubes. The MeOH portion was allowed to evaporate and the residue was resuspended in MeOH. HPLC was then conducted by injecting 0.05 ml of the

resuspended residue onto the column (Reverse Phase Microsorb^{TM} MV 100 C18, 5μm diameter particle size, Varian Analytical Instruments, Woburn, MA). Fecal extracts were recovered by a mobile phase gradient of 20% increasing to 80% MeOH in water over 80 minutes (1 ml/mm) at room temperature. A portion of each fraction (0.1 ml) was assayed for radioactivity to determine the retention times for the radiolabelled standards (E_1 and E_1S). The remainder of the fractions (0.9 ml) was allowed to evaporate until dry and reconstituted in 0.25 ml PBS. These samples were then assayed with an estradiol (E_2) EIA and an estrone conjugate (E_1C) EIA (French et al., 1996) to evaluate the immunoreactivity of the fractions.

Based on the results of these two assays (see Results), the E_2 EIA was chosen for use here, and all fecal samples were assayed for estrogens using a modified protocol previously described (Nunes et al., 2000). We used an E_2 antibody (R4972; Clinical Endocrinology Laboratory, University of California, Davis); other compounds cross-reactive with this E_2 antibody were estrone (3.3%), progesterone (0.8%), testosterone (1.0%), and androstenedione (1.0%). Absorbance was measured with a Dynex plate reader when optical density in B_0 wells reached 1.0. Precision of the assay was monitored by measuring two sets of hyena fecal extract pools. The intra-assay coefficients of variation for high and low pools were 3.66 and 3.30%, respectively. The interassay coefficients of variation were 11.96 and 14.71%, respectively (n = 25 plates). The 871 fecal samples were assayed over the course of two years. The correlation between 13 randomly chosen samples assayed repeatedly in each year was $r = 0.98$, demonstrating low inter-year variation.

Assay parallelism was tested by measuring fecal estrogen concentrations in serial dilutions of samples from three different individuals. The slopes of the resulting displacement curves were compared to that of the standard curve using a test of slopes available in the program Prism (GraphPad Software Inc.).

Immunoassay of plasma samples

Plasma samples collected from free-ranging hyenas in the Talek clan were assayed for estradiol in duplicate using coated tube I^{125} radioimmunoassay (RIA) kits (Diagnostic Products Corp.; Los Angeles, CA). Kit validation for use with *Crocuta* was accomplished by demonstrating parallelism between curves representing serial dilutions of pooled hyena samples, spiked samples, and standard curve calibrators included with the kit. The minimum detection limit for this assay was 7.4 pg/ml. The antibody is highly specific for estradiol and has minor cross-reactivity with other compounds: estriol 0.32%, estrone 10%, estrone-3-sulfate 0.58%, estradiol monosulfate 0.29%, estradiol propionate 0.70%, and DHEA, androstenedione, 5α-dihydrotestosterone, and testosterone all at less than 0.001%. The mean intra-assay coefficient of variation was 7.85% and interassay coefficient of variation was 12.14% (n = 23 assays).

Statistical analysis

Data were log-transformed before analysis if not normally distributed. Procedural covariates (sample collection time and hyena identity) were analyzed with a step-wise regression allowing for each predictor variable to be measured while controlling for reproductive state. Significant predictors were then further investigated using within subjects analyses. Differences among reproductive states (immature, pregnant, and lactating) were evaluated using one-way analysis of variance (ANOVA). Along with test

statistics, means and standard errors are presented. For further investigation of significant differences, two-tailed t-tests were used except when testing specific directional hypotheses based on expected differences in hormone concentrations between reproductive states, as when comparing reproductively immature and mature females, or pregnant and lactating females; in these cases one-tailed tests were used. To examine differences among trimesters of pregnancy, a post-hoc Tukey's analysis was performed for both plasma and fecal samples. When log-transformed data were not normally distributed, or sample size was very small (as in the case of the LHRH challenges), non-parametric tests were used. Significant differences were identified using $\alpha = .05$. All analyses were performed using SPSS for Windows (Version 12.0.0, SPSS Inc.).

RESULTS

High-performance liquid chromatography

Immunoreactive E_2 was detected in fecal extracts at levels three- to four-fold higher than immunoreactive E_1C in samples from both pregnant and non-pregnant females. We observed differences in immunoreactivity between samples for pregnant and non-pregnant females of roughly an order of magnitude (Figure 1.1). The highest concentrations of immunoreactive estrogen metabolites co-eluted with the ^{3}H-estrone standard (fractions 60-65), accounting for more than 74% of total immunoreactive estrogens. Also, a smaller peak in E_2 immunoreactivity was present in fractions 65-70, accounting for 14% of the total immunoreactivity. Thus, the E_2 antibody appears to detect multiple estrogen metabolites in the feces of female spotted hyenas, and may therefore be used as a measure of total fecal estrogens (fE).

Fecal EIA parallelism

Serial dilutions of three hyena samples were plotted along with standards as relative dose *vs.* percent bound. Fecal extract curves did not differ significantly from the standard displacement curve within the range of 20 – 80% antibody binding ($F_{3, 8} = 2.06$, $P = 0.18$; Figure 1.2). Of our assayed fecal samples (n = 871), 83% fell within these binding parameters, and samples falling outside this range were diluted accordingly and re-analyzed.

LHRH challenge experiments

Estradiol was measured in repeated plasma samples drawn from 9 free-living females challenged with LHRH administration during late lactation, and from 3 saline-treated control females. For each challenged individual, the sample with the highest concentration of E_2 prior to LHRH administration (pre-injection peak, 17.76 ± 2.44 pg/ml) was compared to its sample with the highest E_2 concentration after LHRH administration (post-injection peak, 21.71 ± 2.21 pg/ml). Peak E_2 concentrations were significantly higher after than before LHRH administration (paired $t_8 = -6.19$, $P < 0.001$; Table 1.1). By contrast, the three females injected with saline demonstrated no rise in plasma E_2 (Wilcoxon matched pairs test: $z_3 = -5.35$, $P = 0.59$). Thus, treatment with LHRH was followed by a significant rise in circulating E_2 in lactating female spotted hyenas.

Five captive hyenas (Table 1.2) were also challenged with LHRH. For each individual, the sample with the highest fE prior to LHRH administration (484.18 ±

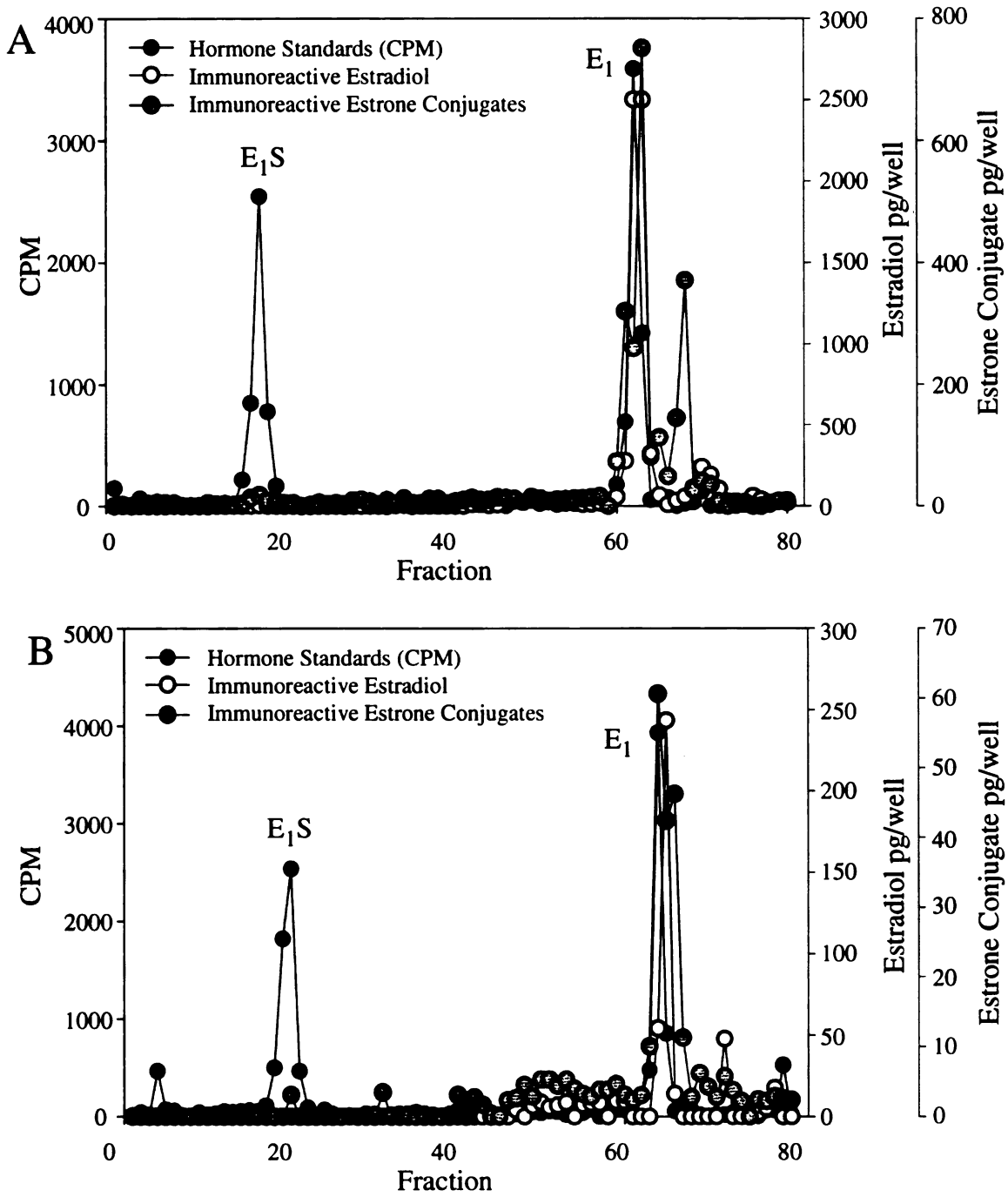

Figure 1.1. HPLC results from A) pregnant and B) non-pregnant female hyenas. Fractions were assayed for immunoreactivity with both estradiol (open circles) and estrone conjugate (gray circles) enzyme immunoassays, and immunoreactivity is expressed on the right-hand axes. Elution of radiolabeled standards (black circles), estrone (E_1) and estrone sulfate (E_1S), are expressed as counts-per-minute (CPM) per fraction on the left-hand axis.

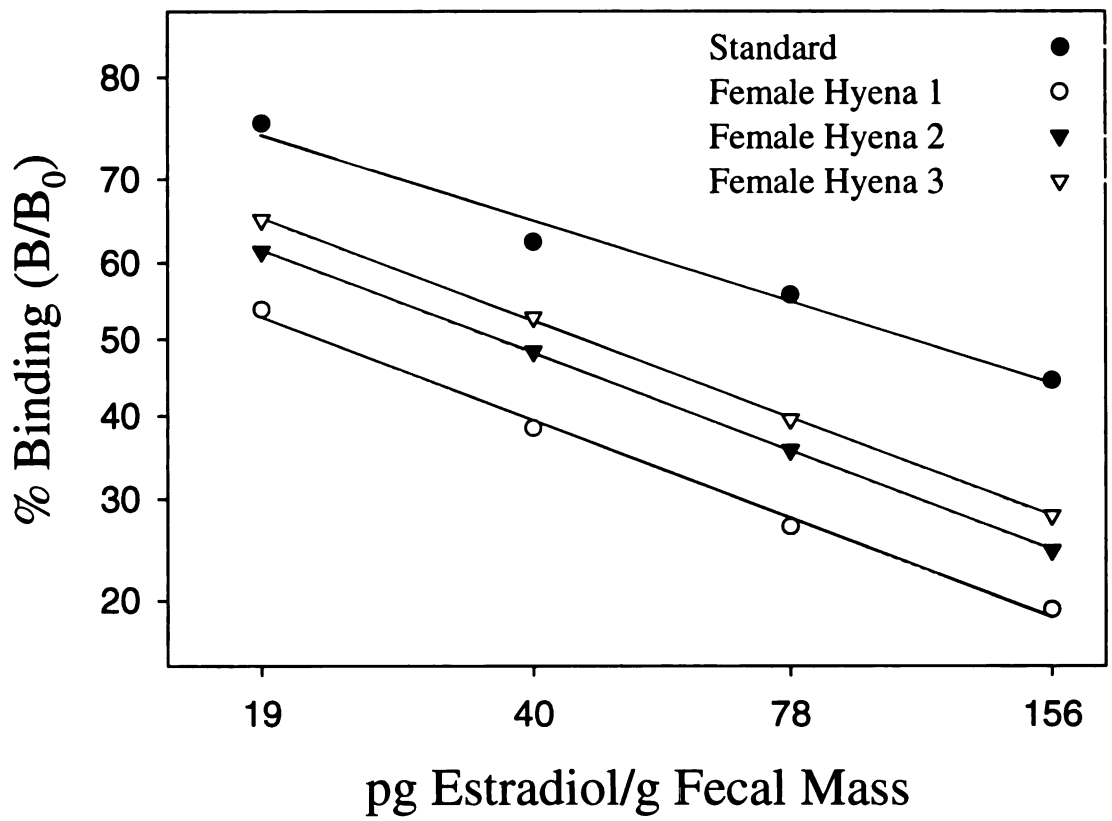

Figure 1.2. Relative dose *vs.* percent of antibody bound for standards and serial dilutions of samples from three hyenas. Slopes of all curves are parallel in this range (20–80% binding; $P = 0.18$).

Table 1.1.
Free-living hyena plasma estradiol (pg/ml): Individual and mean (± standard errors) response patterns to LHRH challenge

Hyena ID	Pre-Injection Peak	Post-Injection Peak	% Pre-Injection Peak	Minutes to Maximum
BM	7.8	14.23	182.40	58
COCH	26.3	29.51	109.70	24
DJ95	15.4	21.66	140.65	64
JAB	22.7	28.52	125.64	114
PT	18.4	19.27	108.42	67
SX	18.5	22.59	122.11	19
UA94	13.4	15.35	114.55	109
UA96	8.5	13.7	161.18	60
WHO	28.2	30.58	108.44	19
Mean	17.76 ± 2.44	21.71 ± 2.21	129.93 ± 8.87	59.33 ± 11.81

Table 1.2.
Captive hyena fecal estrogen (ng/g feces): Individual and mean (± standard errors) response patterns to LHRH challenge

Hyena ID	Pre-Injection Peak	Post-Injection Peak	% Pre-Injection Peak	Days to Maximum
Female 35	129.16	174.99	135.49	2
Female 49	1935.52	3480.34	179.81	2
Male 13	226.69	3175.10	1400.66	2
Male 45	84.06	191.82	228.18	3
Male 51	45.49	164.07	360.64	1
Mean	484.18 ± 364.09	1437.26 ± 773.30	460.96 ± 237.94	2.0 ± 0.31

364.09 ng/g) was compared to peak fE concentrations after LHRH administration (1437.26 ± 773.33 ng/g). Peak fE concentrations were significantly higher after than before LHRH administration (Wilcoxon matched pairs test: $z_5 = 2.02$, $P = 0.04$). Mean latency to peak fE post-challenge was 2.0 ± 0.31 days. Thus, as with plasma E_2, LHRH administration was followed by a measurable increase in fE, but with an average time lag of two days.

Comparison of plasma and fecal estrogen concentrations

Procedural covariates

To evaluate procedural covariates associated with our sampling technique and to avoid pseudoreplication, a step-wise regression was used to determine how much variation in fE concentrations could be explained by collection time (morning, 0600-0900, or evening, 1700-2000) and individual identity, after reproductive condition had been controlled. The full model yielded an $R^2 = .07$, $F_{3,536} = 14.09$, $P < 0.001$. Reproductive state (immature, pregnant, and lactating) explained a significant portion of the variance in fE ($P < .001$). Collection time was a significant predictor over and above the variance explained by reproductive state ($P = 0.009$), whereas hyena identity was not ($P = 0.22$). The effect of collection time was further examined by pairing morning and evening samples for 35 individuals. Concentrations of fE were significantly higher in morning than evening samples ($t_{34} = 4.19$, $P < .001$). Therefore all subsequent analyses utilized only morning samples.

Age

To examine how fE varies with age between 11 – 35 months, we assigned females that had not yet given birth to a specific four-month age interval, and calculated the

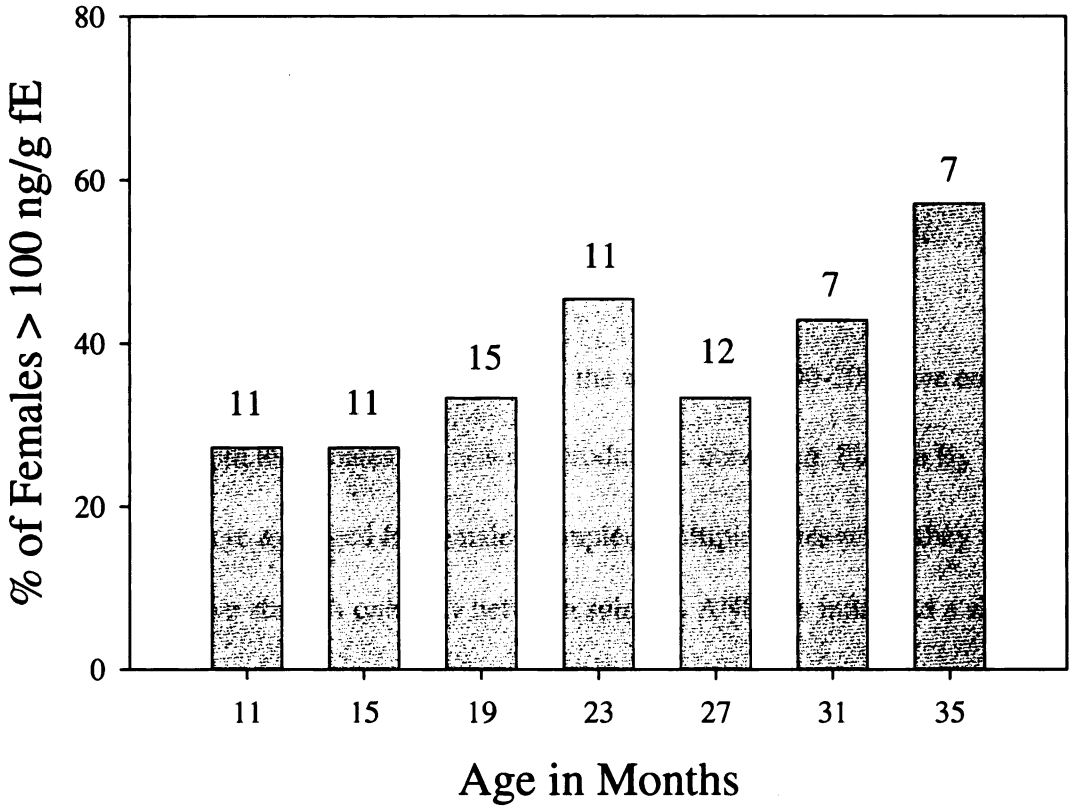

Figure 1.3 Percentage of nulliparous females sampled in each 4-month age interval whose mean fecal estrogen concentrations exceeded 100ng/g feces. Numbers over bars represent females sampled in each age group. Multiple samples collected from individual females within age interval were averaged.

percent of these females whose feces contained more than 100 ng/g fE within each interval (Figure 1.3). The threshold of 100 ng/g was chosen because it exceeded average fE concentrations from pre-reproductive females. Concentrations of fE were averaged for females sampled multiple times within a given age interval. These data suggest that fE begins to rise at approximately 23 months of age in free-ranging females.

Reproductive state

During immobilizations conducted in the wild from 1990-2004, we collected 136 blood samples from 89 females in known reproductive condition. Plasma E_2 concentrations were averaged for females sampled multiple times when they were in a single reproductive state. A one-way between subjects ANOVA indicated a significant effect of reproductive state (immature, pregnant, or lactating) on plasma E_2 ($F_{2,118}$ = 138.88, $P < 0.001$). Plasma E_2 concentrations were low in immature females (5.29 ± 0.42 pg/ml) and lactating females (6.44 ± 0.77 pg/ml), and highest in pregnant females (137.79 ± 36.88 pg/ml). We next looked within individuals by conducting paired t-tests on samples from females that were sampled multiple times when they were in different reproductive states. Plasma E_2 concentrations were significantly higher in females sampled after than before reproductive maturity (t_{23} = -3.53, $P < 0.001$; Figure 1.4.A), and concentrations were also higher during pregnancy than lactation (t_7 = 4.67, $P <$ 0.001; Figure 1.4.B). Within pregnancy, plasma E_2 increased across trimesters ($F_{2,21}$ = 15.53, $P < 0.001$) (Figure 1.5). A post-hoc Tukey's test (HSD = 0.20) showed that plasma E_2 concentrations were significantly higher in late pregnancy than during either the early or middle trimesters.

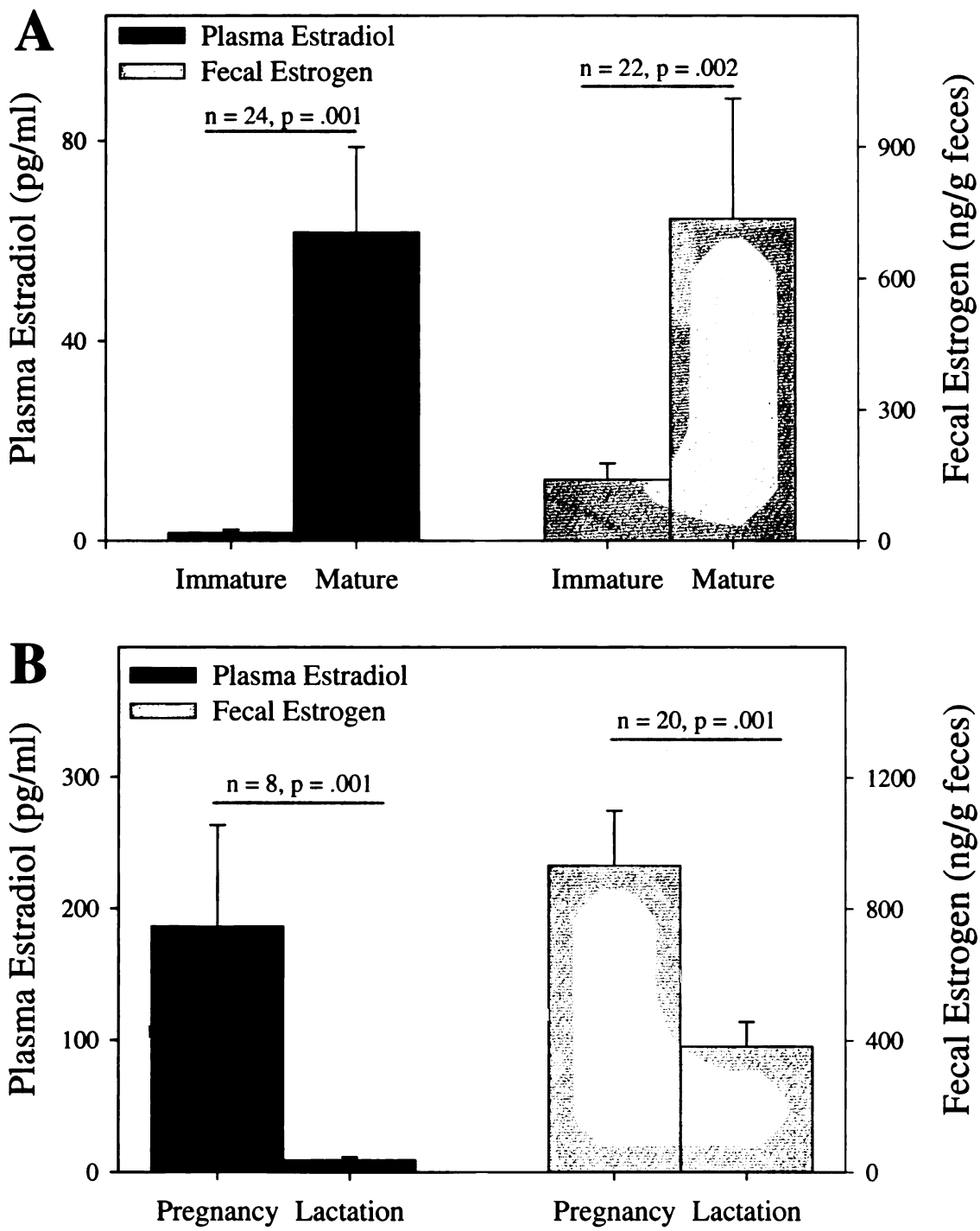

Figure 1.4 Within-female comparisons between A) reproductively immature and mature states, and B) pregnant and lactating states for plasma estradiol (black) and fecal estrogens (gray). Means and standard errors are presented, and significance was tested using paired t-tests. Note that the scales represented on the two y-axes differ between sampling techniques.

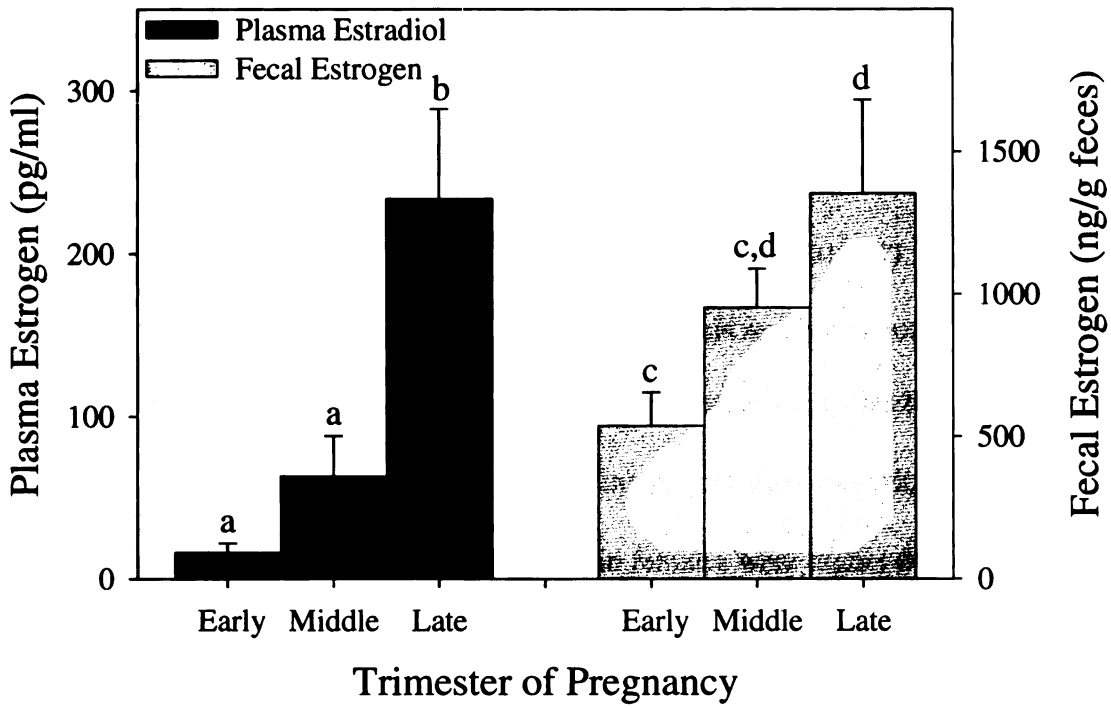

Figure 1.5 Estrogen concentrations in plasma (black) and fecal material (gray) across trimesters of pregnancy, averaged across females. Significant differences among mean (±SEM) plasma estradiol and fecal estrogen concentrations are indicated by different letters, and were tested using Tukey's post-hoc test. Note that the scales represented on the two y-axes differ between sampling techniques.

Variation in fE among reproductive states resembled that seen in plasma E_2. From 1993-2005, 295 morning fecal samples were collected from 56 females. A one-way between subjects ANOVA indicated a significant effect of reproductive state on fE ($F_{2,294} = 56.57$, $P < 0.001$). Fecal estrogen concentrations were lowest in immature females (150.27 ± 32.95 ng/g), higher in lactating females (368.91 ± 37.25 ng/g), and highest in pregnant females (1235.73 ± 229.24 ng/g). This result was confirmed by paired t-tests within females sampled in multiple reproductive states. Females had significantly higher fE concentrations after than before reaching reproductive maturity ($t_{21} = -6.49$, $P < 0.001$; Figure 4a), and fE concentrations within females were higher during pregnancy than lactation ($t_{19} = 3.23$, $P = 0.002$; Figure 4b). Finally, fE rose during the course of gestation ($F_{2,75} = 3.22$, $P = 0.045$; Figure 5). A post-hoc Tukey test ($HSD = 362.13$) comparing trimesters revealed that late trimester fE concentrations were significantly higher than early fE concentrations, but middle trimester fE concentrations were not significantly different from either early or late trimester concentrations.

DISCUSSION

The aim of this study was to validate a non-invasive technique for measuring estrogens in spotted hyenas. Our results show that biologically significant variation in fecal concentrations of estrogen metabolites can be assessed using the E_2 EIA in both wild and captive hyenas. Furthermore, estrogen concentrations in feces show the same patterns of variation as those in plasma. Fecal hormone analysis offers a means for collecting samples repeatedly and frequently from individuals without disturbance of

normal behavior, and should therefore help future researchers to answer a number of questions about the developmental and reproductive biology of this species.

For the EIA developed here, we chose an antibody specific for E_2 because immunoreactivity of fractions from female hyenas was three- to four-fold higher when assayed with the E_2 antibody than with an antibody specific for E_1C. Use of the E_1C antibody in the immunoassay would have rendered a large proportion of our samples below detectable limits, whereas all of our samples run with the E_2 EIA yielded measurable fE. Although E_2 was not run as a radiolabeled standard in our HPLC analysis, HPLC results from several felids, the taxonomic group closest to the hyenids (Bininda-Emonds et al., 1999) from which we have HPLC data from feces, reveal that elution of E_2 often overlaps with that of E_1 (Brown et al., 1994), the largest immunoreactive peak in fecal samples from both pregnant and non-pregnant hyenas seen here. Additionally, radiolabeled estrogens in the domestic cat are predominantly excreted as unconjugated E_2 and E_1 (Brown et al., 1994; Shille et al., 1984), and we presume because of their close taxonomic relationship to hyenas, these particular estrogens may also be important immunoreactive metabolites in hyena feces. All of these factors suggest that an E_2-specific antibody represents an acceptable choice for measuring estrogen in hyena feces. With the understanding that other estrogens, and perhaps also novel metabolites, may be cross-reacting with this antibody in *Crocuta* samples, our assay permits measurement of total fecal estrogens, rather than fecal estradiol *per se*.

We assessed parallelism by comparing the slopes of curves generated by several diluted samples to the slope of the curve generated by the E_2 standards. Although these

slopes were parallel only when antibody binding was between 20 and 80%, this assay should nevertheless be informative as long as this limitation is considered. The large majority of our samples fell within this range, and samples outside of this range could be assayed after appropriate dilution.

Circadian rhythms in fecal and urinary steroid hormones have been documented in many mammals (Bos et al., 1993; Sousa and Ziegler, 1998), so we examined time of day here as a procedural covariate. We found that, like fecal glucocorticoids (Dloniak, 2004), but unlike fecal androgens (Dloniak et al., 2003), concentrations of fecal estrogens in *Crocuta* samples collected in the morning are higher than in samples collected in the evening. This source of variation, once identified, was easily controlled by restricting analysis to morning samples.

The biological tests we conducted *in vivo* further validated our assay. After excluding evening fecal samples from our analyses, the same variation seen in plasma estradiol among age classes and reproductive states was observed in fE values. As in plasma, fE concentrations remained low until reproductive maturity, were highest during the final trimester of gestation, and were relatively low during lactation. Furthermore, although this requires further testing, our fE data offer a preliminary indication that estrogen concentrations in wild spotted hyenas began to rise late in the second year of life, as is also true in captive females, based on measurements of plasma estrogen (Glickman et al., 1992).

Our data suggest that, like plasma estrogens, fE concentrations rise in response to LHRH challenge, but not after saline injection. A previous study had shown that plasma LH, testosterone, and androstenedione concentrations in both males and females rise in

response to LHRH administration (Place et al., 2002), but estrogen was not among the steroid hormones measured in that work. In the brown hyena (*Parahyaena brunnea*), a species closely related to the spotted hyena, non-invasively measured estrogens similarly appeared to rise after LHRH challenge in females (Ensley et al., 1982). Here captive hyenas of both sexes showed significant increases in fE concentrations after LHRH challenge, despite having highly variable baseline values. The variable responses observed in the two parous females may have been a consequence of differing stages of the ovulatory cycle at the time of the LHRH challenge. Endogenous hormone levels and ovarian status can greatly affect responses of the HPG axis to LHRH (Edwards et al., 1963; Hamilton and Armstrong, 1991; Jeffcoate, 1992), and Place et al. (2002) showed that the response of plasma LH to LHRH challenge was more variable in female than male *Crocuta*. Here, the three captive males, whose average baseline fE concentrations were lower than those in females, had post injection fE concentrations roughly equal to those in the females. Estrogens in male mammals are produced in small amounts by the testes, and may also be aromatized from circulating testosterone in various other peripheral tissue (Nitta et al., 1993). However, the source of estrogens is not known for male spotted hyenas.

Use of the assay developed here for analysis of fecal estrogen should contribute to our understanding of reproduction and genital development in the female spotted hyena. There has been a keen interest in the endocrinology of this species and its ties to the unique characteristics of female dominance and genital masculinization; however, conventional methods of blood sampling for hormone analysis cannot be repeated with the frequency needed to explore these issues. Glickman et al. (unpublished data) have

obtained preliminary data suggesting that estrogens may be involved in formation and development of the phallus in this species. Furthermore, we currently know virtually nothing about estrous cyclicity in any hyena species. By allowing for repetitive sampling from individuals in a non-invasive manner, it is our hope that availability of an assay for fecal estrogens will help answer these and many other questions about these fascinating animals.

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CHAPTER 2

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CHAPTER 2

FECAL GLUCOCORTICOIDS REFLECT SOCIO-ECOLOGICAL AND ANTHROPOGENIC STRESSORS IN THE LIVES OF WILD SPOTTED HYENAS

INTRODUCTION

Whereas endocrine studies have traditionally been used in conservation to monitor reproduction in rare or threatened species, hormones are now being used to monitor stress physiology as an indicator of how animals cope with social and ecological challenges in their daily lives (Millspaugh and Washburn, 2004; Walker, et al., 2005a; Wingfield, et al., 1997). In this context, fluctuating glucocorticoid (GC) concentrations, released from the adrenal glands in response to stressful stimuli, are often measured as an expression of how animals perceive and respond to both predictable (e.g. seasonal changes in food availability) and unpredictable (e.g. encounters with predators) changes in their environment (Walker et al., 2005a; Wingfield et al., 1997). In the short term, circulating GC concentrations released in response to an acute stressor help mobilize energy reserves, and suppress non-essential processes like growth and digestion. However, chronic elevation of GC concentrations can lead to pathology, including compromise of cardiovascular, immune, neural, and reproductive function (Sapolsky, 2002).

In recent years, our understanding of responses by free-living animals to potential stressors has been greatly enhanced by the development of methods for non-invasive hormone sampling (Keay, et al., 2006; Millspaugh and Washburn, 2004; Walker et al., 2005a; Wasser, et al., 2000), mainly because more traditional methods of blood sampling can themselves be stressful (e.g. Sapolsky, 1985). Fecal steroid analysis, in particular, offers an economical alternative to drawing blood for frequent collection of samples

without disrupting the normal behavior of individuals or groups. Many species excrete multiple steroid metabolites, which can be extracted and measured with immunoassays like those used to measure circulating steroid hormones (Millspaugh and Washburn, 2004; Wasser et al., 2000).

Here we investigated endocrine responses to a number of potential stressors experienced by wild African carnivores, using spotted hyenas (*Crocuta crocuta*) as model animals. Spotted hyenas offer an excellent model because of their behavioral plasticity: they occur in a wide array of ecosystems throughout sub-Saharan Africa, can be active night or day, breed year round, and can survive on foods ranging from termites to elephants. As this plasticity far exceeds that observed in other large African carnivores, responses observed in spotted hyenas may represent conservative indicators of how more specialized carnivores might respond to the same potential stressors.

We inquired about likely sources of stress experienced by spotted hyenas. First, seasonal variation in ecological factors, including local prey abundance and rainfall, might affect their stress physiology. Second, competition and intra-guild predation occur among carnivores exploiting similar ecological niches (Caro and Stoner, 2003). In most African ecosystems, lions (*Panthera leo*) are the most important interspecific competitors for spotted hyenas; lions not only compete with hyenas for access to live prey and carcasses, but they also represent a significant source of hyena mortality (Honer, et al., 2002; Kruuk, 1972; Watts and Holekamp, 2009). Thus, lions may represent important stressors for hyenas. Third, many gregarious carnivores, including spotted hyenas, also experience intra-specific social stress (Creel, 2005; Goymann, et al., 2001; Young, et al., 2006). Unlike other gregarious carnivores, which are predominantly cooperative

breeders, all adult spotted hyenas have the opportunity to breed despite status-related reproductive skew occurring in both sexes (Holekamp, et al., 1996; Engh et al., 2002). Spotted hyena society is structured by strict hierarchical rank relationships like those found in troops of cercopithecine primates, and it appears that, as in these primate societies (Abbott, et al., 2003; Creel, 2001), there is no relationship between rank and fecal GC concentrations among male hyenas (Dloniak, 2004; Goymann, et al., 2003). Although one study demonstrated a weak relationship between rank and fecal GC concentrations among female hyenas that were neither pregnant nor lactating (Goymann et al., 2001), these results were not confirmed in our study population (Dloniak, 2004). Rare events such as the overthrow of the alpha matriline have been found to increase GC concentrations among wild baboons (Engh, et al., 2006; Sapolsky, 1983). Such events, and the ensuing periods of social instability, could potentially affect animals in any hierarchical society, so we asked whether social instability might affect stress hormone concentrations among spotted hyenas.

Finally, burgeoning human populations near many African wildlife reserves have put increasing pressure on protected lands and resources in recent decades (Wittemyer, et al., 2008). Humans now represent an important mortality source for hyenas and other carnivores living inside protected areas (Kolowski and Holekamp, 2006; Watts and Holekamp, 2009; Woodroffe, 2000; Woodroffe and Ginsberg, 1998). In addition, recent work has suggested that anthropogenic activity, particularly that occurring in the form of pastoralists and grazing livestock within the boundaries of protected areas, alters the behavior of spotted hyenas and forces them to make energetic compromises not observed where pastoralists are absent (Boydston, et al., 2003b; Kolowski and Holekamp, 2008).

Measures of tourist activity have also been shown to influence stress physiology in vertebrates (e.g. Creel, et al., 2002; Pereira, et al., 2006; Walker, et al., 2005b). Therefore, pastoralist activity, and perhaps also seasonal variation in visitation by tourists, might be expected to affect hyena stress physiology.

We analyzed both longitudinal data from a single large hyena group monitored continuously for many years, and cross-sectional data collected from shorter-term studies of multiple groups in two different protected areas, to inquire about these potential stressors in the lives of wild hyenas. We utilized non-invasive sampling of fecal glucocorticoid (fGC) concentrations, which have previously been shown to accurately reflect stress responses in spotted hyenas (Dloniak, 2004; Dloniak et al., 2006b; Goymann et al., 2003; Goymann et al., 2001; Goymann, et al., 1999). We used the longitudinal dataset to determine whether variation in fGC concentrations among spotted hyenas was associated with seasonal variation in rainfall and prey abundance, temporal variation in the presence of lions, periods of social instability, and tourism and pastoralist activity as two forms of anthropogenic disturbance. The study groups used for the cross-sectional study were selected to take advantage of natural variation among the study areas relating to ecological and anthropogenic factors. Specifically, these study groups varied markedly with respect to climate, prey abundance, local lion density, and anthropogenic disturbance, thus allowing us to test specific hypotheses about the effects of these variables on fGC concentrations.

MATERIALS AND METHODS

Study populations and subjects

Four social groups, or "clans," of free-living spotted hyenas were monitored, two in each of two different national parks in southern Kenya, the Masai Mara National Reserve (MMNR), and Amboseli National Park (ANP) (Figure 1). Both the MMNR and ANP are areas of open grassland grazed year-round by several different ungulate species (Kolowski, et al., 2007; Western, 2007). Each of the four study clans contained multiple matrilines of adult females, their offspring, and several immigrant males that had joined the clan as adults. All clan members were individually recognized by their unique spot patterns, and sex was determined by the dimorphic glans morphology of the erect phallus (Frank, et al., 1990). Members of each clan defended a stable group territory. Clans were observed daily in the morning from 0600 to 1000 and in the evening from 1600 to 2000 hours.

We monitored fGC concentrations among adults of both sexes. Whereas female hyenas remain with their natal clan throughout their lives, most males disperse at 2 to 5 years of age and immigrate into a new clan (Henschel and Skinner, 1987; Smale, et al., 1997). As adult males who have not yet dispersed differ from immigrant males with respect to both their behavior and physiology (Dloniak, et al., 2006a; Holekamp and Sisk, 2003; Smale et al., 1997), here we included only immigrant males in our analyses.

Female spotted hyenas are promiscuous and breed throughout the year (Holekamp, et al., 1999; Kruuk, 1972). Reproductive histories were known here for female members of all study clans. Ages of all individuals born into each clan during the study period were known, and mother-offspring relationships were established on the

basis of nursing associations. Birth dates (± 7 days) were assigned based on appearance and behavior of cubs when they were first seen (Holekamp et al., 1996). Female spotted hyenas reach reproductive maturity at around 2 years of age (Glickman, et al., 1992; Holekamp et al., 1996; Van Meter, et al., 2008), and were considered here to be adults at 24 months. Duration of gestation in spotted hyenas is 110 days (Kruuk, 1972), so, counting backward from parturition dates, we could determine conception dates. We classified adult females as "nulliparous" from 24 months until their first known conception; first parturition could be detected by the extensive tearing of the phallus that occurs then, even when the cubs do not survive (Frank and Glickman, 1994).

Glucocorticoid concentrations vary with reproductive state among female hyenas (Goymann et al., 2001), and are highest during pregnancy (Dloniak et al., 2006b); therefore, we treated pregnant and non-pregnant females separately throughout the study. Females were categorized as "pregnant" for the 110 days prior to the known birth of a litter, and were considered to be "non-pregnant" from the day they gave birth until their next litter was conceived. We further assigned our non-pregnant females as either lactating or non-lactating. Females were considered to be lactating from the day they gave birth until weaning of their litter or disappearance of the cubs, whichever came first. Weaning dates were assigned as described by Holekamp et al. (1996). Females were classified as non-lactating during the gap between weaning one litter and conceiving the next. Little is known about the timing or length of the estrus cycle in spotted hyenas, so females that were neither pregnant nor lactating here may have been cycling.

Longitudinal study of the Talek clan

Longitudinal data were collected from a single clan that defended a large territory along the northeastern border of the MMNR in the Talek region (Figure 1). The Talek clan had been intensively monitored since 1988, but data analyzed here were collected between January 1993, when fecal sample collection began, and December 2005.

Socio-ecological variables: We investigated two ecological variables that vary seasonally in the MMNR. Daily rainfall was measured (mm) using a standard plastic rain gauge. We calculated the mean rainfall during the 30 days before collection of each fecal sample. Abundance of prey animals within the Talek home range was assessed biweekly by counting all ungulates within 100 m of two 4 km transects. These transect counts were used to calculate the mean prey availability during the 30 days before collection of each fecal sample.

To examine the effect of lions on spotted hyena fGC concentrations, the presence of lions was recorded when one or more lions were present within 200 m of any Talek hyena. Fecal samples reflect a general pattern of circulating hormone concentrations depending on the internal clearance time of the hormone (Brown, et al., 1994; Wasser et al., 2000). Captive spotted hyenas administered an adrenocorticotrophic hormone (ACTH) challenge, which increases circulating GC, exhibited elevated fGC concentrations from 1 to 8 days after the challenge (Dloniak, 2004; Goymann et al., 1999). Acute stressors, such as caged transport, also produced measurable elevations in fGC concentrations for several days after the incident (Goymann et al., 1999). Therefore, we identified fecal samples from individuals present with lions up to one week prior to sample collection. We coded lion presence as a binary predictor variable such that each

fecal sample was or was not associated with the presence of a lion during the preceding week.

Whereas the social hierarchy of the Talek clan was generally stable and unchanging over time (Frank, 1986; Holekamp, et al., 1993), during the course of our longitudinal study two events occurred that resulted in brief periods of social instability within the clan. First, in May of 1999, the highest-ranking (alpha) female died after having occupied that rank since the mid 1980s (Holekamp et al., 1993). For some months after her death, the daughters of the alpha female fought vigorously for position, and observers noted an increase in serious wounding among them. Here we considered the six months following the alpha's death to be a period of social instability. Second, in 2000, the Talek clan split into two smaller clans; by 2001, contact between the two groups resulting from this fission event was rare, and restricted to only a single individual (J.E. Smith, unpublished data). Therefore we considered the last six months of 2000 to represent a second period of social instability. For the purpose of analysis we coded social stability as a binary predictor variable indicating stable or unstable monthly periods.

Anthropogenic disturbance: Since the mid-1990s, increasing anthropogenic activity in the Talek area has led to altered use of dens and territory, altered temporal patterning of activity, and frequent human-hyena conflict (Boydston et al., 2003b; Kolowski and Holekamp, 2006, 2008; Kolowski et al., 2007). Although illegal, the once rare presence of livestock and herders inside the reserve was a daily occurrence by 2005 (Kolowski and Holekamp, 2006, 2008). The Talek region now supports a high density of pastoralist settlements along the northern border of the MMNR (Reid, et al., 2003), and

thus along the northern edge of the Talek clan territory. Kenyan census data indicated that the human population in the Talek area has approximately doubled every 15 years since 1950 (Lamprey and Reid, 2004). To investigate the effect of this burgeoning human population along the border of the home range of the Talek clan, we asked whether or not fGC concentrations increased over the 12-year duration of our study, measured in samples collected during twenty-six 6-month intervals.

Tourism also brings spotted hyenas into contact with humans, although tourists travel in vehicles whereas pastoralists travel on foot. Since the 1980s, the MMNR has been one of the top safari destinations in the world; tourist visits to the MMNR peaked in the early 1990s and have remained high over the past decade (Okello, et al., 2005). Tourist visitation to the MMNR is highly seasonal, with peaks between June and October, and also in December (Heath, 2008), collectively treated here as the "high season" for tourism. The remaining six months of the year were considered the "low season" for tourism. To evaluate seasonal tourism as a possible stressor among spotted hyenas, we coded the high and low seasons as a binary predictor variable.

Cross-sectional comparison among clans

To compliment our longitudinal data, we investigated variation in fGC concentrations among several clans in a cross-sectional study. Starting in September 2002, a second clan within the MMNR was monitored, the Mara River clan. The territory of the Mara River clan was located deep within the Reserve (Figure 2.1), and too far from pastoralist settlements for livestock grazing. However, the closest border of the territory of the Mara River clan was only 6 km from the Talek clan's territory. Also, from October 2003 through July 2005, two hyena clans were monitored that occupied adjacent

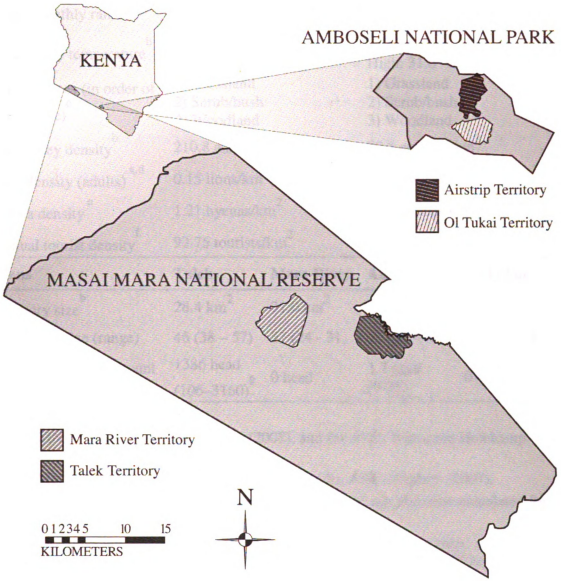

Figure 2.1 Map of parks and clan territories.

Table 2.1. Ecological features of parks and clans.

Park	**Masai Mara (MMNR)**		**Amboseli (ANP)**	
Mean monthly rainfall[a]	84.9 mm		23.0 mm	
Mean daily temperature[b]	Low: 13.8 °C High: 28.3 °C		Low: 15.8 °C High: 31.8 °C	
Habitat types (in order of abundance)[c]	1) Grassland 2) Scrub/bush 3) Woodland		1) Grassland 2) Scrub/bush 3) Woodland	
Mean prey density[b]	210.8 animals/km^2		90.5 animals/km^2	
Lion density (adults)[a,d]	0.15 lions/km^2		0.06 lions/km^2	
Hyena density[e]	1.21 hyenas/km^2		1.65 hyenas/km^2	
Annual tourist density[f]	92.75 tourists/km^2		361.18 tourists/km^2	
Clans	**Talek**	**Mara River**	**Airstrip**	**Ol Tukai**
Territory size[b]	28.4 km^2	31.0 km^2	28.0 km^2	26.4 km^2
Mean clan size (range)	46 (38 – 57)	27 (24 - 31)	51 (42 – 64)	39 (32 – 48)
Mean livestock per count (range)	1386 head (106–3160)[g]	0 head	1.7 head (0-75)	0 head

[a] Watts and Holekamp (2008).

[b] For MMNR, Kolowski and Holekamp (2008), and for ANP, Watts and Holekamp (2008).

[c] For MMNR, Kolowski and Holekamp (2008), and for ANP, Western (2007).

[d] Lion density values represent adults older than 2 years of age and were calculated for both the MMNR and ANP for 2005.

[e] Hyena densities are based on monthly mean clan sizes and territory sizes, and were calculated for 2003-2005 for the MMNR; ANP data are from Watts and Holekamp (2008).

[f] Okello et al. (2005).

[g] Kolowski and Holekamp (2008).

territories in ANP, the Ol Tukai clan and the Airstrip clan (Figure 2.1). In our cross-sectional study we restricted the study period in Talek to correspond with the onset and duration of the studies conducted on the other three clans. Therefore, Talek data in this analysis were limited to 2002 through 2005. In all clans we used identical methods for continuous collection of demographic, behavioral, and ecological data.

The population densities of hyenas living in the MMNR and ANP during the period of the cross-sectional study were similar (Kolowski et al., 2007; Watts and Holekamp, 2008) (Table 2.1); however, other ecological differences between ANP and the MMNR might potentially affect the stress physiology of resident hyenas. First, abundance of prey did not differ significantly between the territories of Talek and Mara River clans (Kolowski et al., 2007), but prey density in the MMNR was over twice that in ANP (Table 2.1). Second, the MMNR typically received more rain on average than ANP, and had slightly lower average daily temperatures during the study period than did ANP (Table 2.1). Finally, the lion density in ANP was less than half that in the MMNR (Watts and Holekamp, 2008).

The four clans also varied in their exposure to anthropogenic disturbance. Similarly large numbers of tourists visit both ANP (141581 ± 1331 tourists) and the MMNR (139127 ± 7864 tourists) each year (Okello et al., 2005), although the smaller size of ANP generates a much higher tourist density than that in the MMNR (Table 2.1). Within the MMNR, researchers encountered tour vehicles 4.8 times more frequently while observing hyenas in Talek than in the Mara River territory (Kolowski et al. 2007). Although comparable data were not available from the ANP clans, the territories of both

of these clans were located close to tourist lodges, and tourist visitation to both ANP territories more closely approximated that in Talek than that in the Mara River territory.

In contrast to the Talek clan, none of the other three study clans experienced any significant exposure to pastoralist activity within their territories (Table 2.1). In ANP, livestock and herders were seen only once during this study, within the Airstrip clan's territory (herd size = 75 head), and were never seen within the Ol Tukai territory (n = 22 months). Likewise, livestock were never seen in the territory of the Mara River clan (n = 25 months) (Kolowski et al., 2007). In the Talek territory, we monitored the presence of livestock accompanied by herders and conducted detailed head counts of livestock. The mean number of livestock counted in the Talek territory was 1386 ± 181 head (Kolowski and Holekamp, 2008). Thus the Talek clan had a much greater exposure to pastoralist activity than did either Mara River or ANP hyenas.

The similarities and differences among our four study clans permitted us to develop the four sets of predictions shown in Table 2.2. If ecological variables, such as prey availability or rainfall, act as stressors (H1), then we expected to see significant differences in fGC concentrations between the two parks, with higher fGC concentrations among hyenas living in the harsher ANP conditions. However, if local lion density affected the stress physiology of spotted hyenas (H2), we predicted fGC concentrations would be higher in both clans in the MMNR than those in ANP, because MMNR has a higher lion density. If hyenas find tourist visitation stressful (H3), then fGC concentrations among hyenas in the Mara River clan should be markedly lower than in any of the other three clans. Finally, if anthropogenic disturbance in the form of pastoralist activity represents an important stressor to wild hyenas (H4), then we expected

Talek hyenas to have higher fGC concentrations than hyenas in any of the other three study clans, regardless of the park in which they were located.

Fecal sample collection, extraction, and immunoassay

A fecal sample was collected whenever a known hyena was observed to defecate. Samples were collected into plastic bags within 30 minutes of excretion. Within 12 hours of collection, samples were mixed and frozen in liquid nitrogen for storage until they could be shipped to Michigan State University (MSU) for analysis. In an analysis of methodological covariates Dloniak (2004) examined sample collection time, time between collection and freezing, and time between collection and assay, to show that only sample collection time influences fGC concentrations among spotted hyena fecal samples. We also analyzed these covariates with our expanded dataset.

Frozen fecal samples were lyophilized and extracted in ethanol as described previously (Dloniak, et al., 2003; Van Meter et al., 2008). Extracts were further diluted 1:20 in steroid diluent, and assayed with a corticosterone radioimmunoassay kit (ImmuChem Double Antibody Corticosterone ^{125}I RIA Kit, MP Biomedicals (formerly ICN), 07-120102). The corticosterone antibody displays a high affinity to the major glucocorticoid metabolites present in the feces of a wide variety of vertebrates (Wasser et al., 2000), including spotted hyenas (Goymann et al., 1999). The minimum detection limit for this assay was 10 ng of glucocorticoid per gram hyena feces. Precision of the assay was monitored by assaying serial dilutions of pooled hyena fecal extracts. The intra-assay coefficient of variation was 4.79 ± 2.76%, and the interassay coefficient of variation was 10.12% (n = 17 assays). Serial dilutions of pooled samples produced

displacement curves parallel to that produced by the corticosterone standards from the kit.

Statistical analysis

Analyses were performed with SPSS 15.0.0 (SPSS) and Statistica 6.1 (StatSoft). Analysis of quantile plots and frequency histograms of the distribution of our data indicated that fecal glucocorticoid data were normally distributed after log transformation (base 10). Although transformed values were used in data analysis, we used untransformed fGC values for graphical representation of the data. A significant difference was identified when $\alpha < .05$.

Longitudinal analysis of the Talek clan: We used general linear mixed models (GLMM) to determine whether our independent variables explained variation in fGC concentrations in the longitudinal data. Hyena identity was entered into all models as a random factor to control for the unequal numbers of samples obtained from different individuals, and models were fitted with a heterogeneous co-variance structure. First, we investigated whether methodological factors explained any variation among our samples. We tested males and females separately for sample collection time (morning or evening), time from collection to freezing (minutes), and time from collection to assay (years); in the female model we also included reproductive state (nulliparous, pregnant, or non-pregnant).

Based on the results of our methodological models, we further separated models into groups based on sex and reproductive condition (immigrant males, nulliparous females, non-pregnant females, and pregnant females) to test our variables of interest along with group-specific covariates previously shown to influence fGC concentrations.

Table 2.2. Summary table showing relative ecological and anthropogenic differences among parks and clans, and the respective sets of predictions generated for our nested cross-sectional analysis. The first prediction was based on differences in the ecological conditions experienced by hyenas in each clan and park (H1), the second was based on variation in lion density among clan territories (H2), the third was based upon variation in tourism (H3), and the fourth was based on variation in pastoralist activity (H4). Only the last of these predictions was confirmed, as shown in Figure 2.

		MMNR		ANP	
		Talek	Mara River	Ol Tukai	Airstrip
H1	Rainfall	High	High	Low	Low
	Prey	High	High	Low	Low
	Prediction 1: fGC	Low	Low	High	High
H2	Lions	High	High	Low	Low
	Prediction 2: fGC	High	High	Low	Low
H3	Tourism	High	Low	High	High
	Prediction 3: fGC	High	Low	High	High
H4	Pastoralist Activity	High	Low	Low	Low
	Prediction 4: fGC	High	Low	Low	Low

We controlled for lactation state among non-pregnant females (lactating or non-lactating) (Dloniak, 2004; Goymann et al., 2001), and day of gestation for pregnant females (Dloniak et al., 2006b). We examined the main-effects of each parameter as well as interactions with covariates.

We used both multimodal inference (Burnham and Anderson, 2002) and hypothesis testing to draw conclusions about the relative importance of our variables of interest. All terms and interactions were included in the initial model and were sequentially removed to minimize the Akaike's information criterion, adjusted for small sample sizes (AICc). Following Burnham and Anderson (2002) we considered all models within 3 AICc units of the model with the lowest AICc score to be equally parsimonious, and we included a discussion of these models in our results. We then chose the most inclusive model that contained all terms from among the set of equally parsimonious models to use for hypothesis testing. We used the restricted maximum likelihood method of estimation to evaluate the parameters in our models; for our chosen models we present the parameter estimates, degrees of freedom, Sattherwaite's F test, and the corresponding p-value.

Cross-sectional comparison among clans: For the cross-sectional comparison, a nested ANOVA was used to test for significant differences among clans in fGC concentrations. Before performing the nested ANOVA we investigated the procedural variables that were found to have significant effects in the longitudinal dataset. Males and females, and morning and evening samples were equally represented in each study group. However, we had an unequal distribution of pregnant females among clans, so samples from pregnant females were excluded from the nested analysis. For the nested ANOVA,

clan (Talek, Mara River, Ol Tukai, or Airstrip) was nested within park (MMNR or ANP) to assess the main effects of park and clan. A post hoc analysis was performed using Fisher's LSD test; means and standard errors are presented.

RESULTS

Longitudinal analysis of the Talek clan

A total of 811 fecal samples were collected from adult hyenas of known identity and reproductive condition; we collected 232 samples from 39 immigrant males, 59 samples from 26 nulliparous females, 83 samples from 34 pregnant females, and 437 samples from 44 non-pregnant adult females; of these last 437 samples, 378 came from lactating females and 59 came from females that were neither pregnant nor lactating.

Methodological variables: Table 2.3 shows the results of the methodological analysis. For immigrant males, only sample collection time explained a significant portion of the variation among fGC concentrations, indicating that samples collected in the morning were higher than those collected in the evening. The same was true in the model for adult females; here reproductive state also significantly affected fGC concentrations. We controlled for these variables in all subsequent models.

Immigrant males: Terms included in the model for immigrant males were sample collection time, study duration time, tourism, and social instability (AICc = 230). The further removal of study duration time (AICc = 227), tourism (AICc = 227), or social instability (AICc = 227) generated equally parsimonious models, so we present the parameter estimates for the model containing all terms together (Table 2.4). Whereas

Table 2.3. Models (GLMM) investigating methodological sources of variation in fGC concentrations. Males and females were tested in separate models; all variables included in the models are shown. Individual identity was entered as a random variable.

	Estimate	df	F	p-value
Immigrant males	Wald z = 1.41, p = 0.16			
Intercept	1.93	68.95	329.90	<0.001
Collection time	-0.178	112.64	21.56	<0.001
Time collection to assay (years)	-0.031	100.22	0.56	0.46
Time collection to freezing (mins.)	0.000	101.46	0.72	0.40
Adult females	Wald z = 2.61, p = 0.01			
Intercept	1.71	276.63	631.99	<0.001
Collection time	-0.115	482.16	40.22	<0.001
Time collection to assay (years)	-0.000	261.12	0.00	0.98
Time collection to freezing (mins.)	0.000	496.91	2.54	0.11
Reproductive state	0.119	492.47	26.51	<0.001

Table 2.4. Parameter estimates from the selected models (GLMM) explaining fGC concentrations among each group. Individual identity was entered as a random factor in all models.

	Estimate	df	F	p-value
Immigrant males	Wald z = 2.31, p = 0.02			
Intercept	1.68	108.66	216.386	<0.001
Collection time	-0.18[a]	183.82	46.22	<0.001
Duration of study	0.02[b]	156.23	3.86	0.05
Tourism	0.04[c]	177.95	2.14	0.14
Instability	-0.05[d]	184.26	1.35	0.23
Nulliparous females	Wald z = 1.76, p = 0.07			
Intercept	1.82	36.92	468.96	<0.001
Collection time	-0.07	46.51	2.34	0.13
Instability	-0.19	47.82	5.69	0.02
Non-pregnant females	Wald z = 2.27, p = 0.02			
Intercept	2.23	368.72	263.89	<0.001
Collection time	-0.12	368.83	34.53	<0.001
Lactation status (LS)	-0.14[e]	378.99	3.60	0.06
Instability	-0.20	372.92	2.41	0.12
LS X instability	0.01	374.08	1.67	0.20
Pregnant females	Wald z = 0.29, p = 0.77			
Intercept	2.00	50.19	359.75	<0.001
Collection time	-0.09	76.92	5.09	0.03
Day of gestation	0.004[f]	63.94	7.86	0.01
Instability	-0.11	69.43	2.70	0.12

a The negative estimate for collection time indicates that morning samples had higher fGC concentrations than evening samples.
b The positive estimate for duration of study indicates fGC concentrations increased as our study progressed.
c The positive estimate for tourism indicates that fGC concentrations were higher during months of peak tourism.
d The negative estimate for instability indicates that samples from periods of instability had higher fGC concentrations than those from stable periods.
e The negative estimate for lactation status indicates that samples from non-lactating females had higher fGC concentrations than those from lactating females.
f The positive estimate for day of gestation indicates that fGC concentrations increased during gestation.

only sample collection time and study duration were significant variables in this model, the model estimates also indicated that fGC concentrations increased among immigrant males during months of heavy tourism, increased over the course of our study, and were relatively high during periods of social instability.

Nulliparous females: Only sample collection time and social instability remained in the model for nulliparous adult females (Table 2.4). Here, although the parameter estimate for sample collection time indicated that morning samples contained higher fGC concentrations than evening samples, the estimate was not significant in the model. Fecal samples collected during periods of social instability contained significantly higher fGC concentrations than those collected during stable periods.

Non-pregnant females: Terms included in the model for non-pregnant females were sample collection time, lactation status, social instability, and an interaction between lactation status and instability (AICc = 395). The removal of lactation status (AICc = 395), stability (AICc = 395), or the interaction between these two variables (AICc = 393) generated equally parsimonious models, so we present the estimates for the model containing all terms together, although only sample collection time was significant in this model (Table 4). Model estimates indicated that females tended to have higher fGC concentrations when not lactating than during lactation. Samples collected during periods of social instability generally had higher fGC concentrations than those collected during stable periods, and the interaction term indicated that this difference was more pronounced among non-lactating females.

Pregnant females: The model including sample collection time and day of gestation (AICc = 86) was equally parsimonious with the model that included these two

terms plus social instability (AICc = 87; Table 4). Significant terms in the model included sample collection time and day of gestation, which indicated that fGC concentrations increased during the course of gestation. Samples collected during periods of social instability had higher fGC concentrations than those collected during stable periods, although this term was not significant in the model.

Cross-sectional comparison among clans

In this dataset, we found no effect of sample collection time, sex, or reproductive state after pregnant females were excluded. Results from our nested ANOVA revealed an overall significant model ($F_{1,152} = 2744.84$, $p < 0.001$). There was no effect of park ($F_{1,152} = 0.70$, $p = 0.40$), but there was an effect of clan ($F_{2,152} = 3.86$, $p = 0.02$). Further investigation of this clan effect with a post hoc Fisher's LSD test (Fig. 2) showed that there was no significant difference in mean fGC concentrations between the two ANP clans (Ol Tukai, 103.46 ± 14.31 ng/g; Airstrip, 123.46 ± 21.60 ng/g) and that fGC concentrations in these clans did not differ from those in the Mara River clan (105.91 ± 25.08 ng/g). Hyenas in the Talek clan had the highest mean fGC concentrations (185.30 ± 19.13 ng/g), and these were significantly higher than those of Mara River ($p = 0.01$) or Ol Tukai hyenas ($p = 0.01$). The Airstrip clan had mean fGC concentrations that were lower than those in the highly disturbed Talek clan, but higher than those in the other two undisturbed clans, though these differences were not statistically significant. These results reflect the pattern of predictions made under H4 in Table 2, and support the hypothesis that anthropogenic disturbance, in the form of pastoralist activity, increases fGC concentrations.

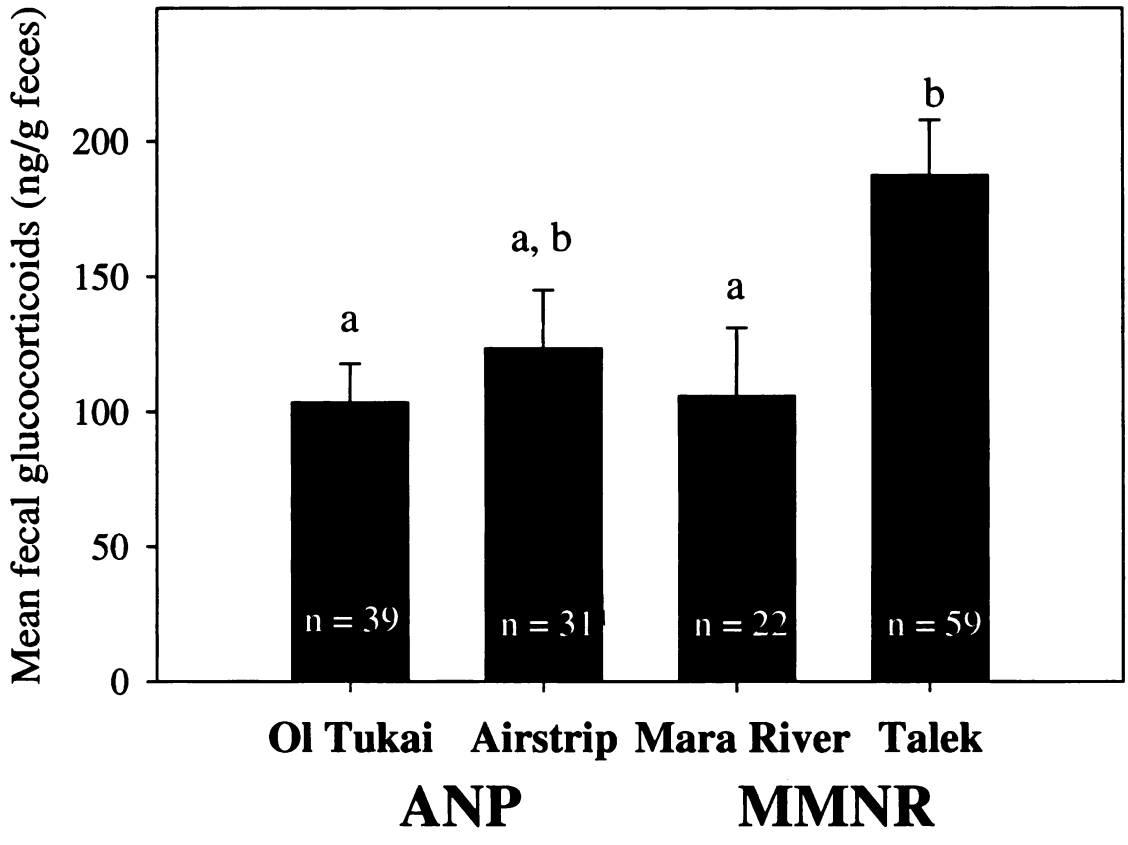

Figure 2.2 Mean (± SEM) morning fGC concentrations for adults from four hyena clans. A nested ANOVA indicated a significant effect of clan. Different letters above bars reflect significant differences indicated by the Fisher's LSD analysis.

DISCUSSION

Our aim here was to identify natural and anthropogenic influences on the stress physiology of wild spotted hyenas using non-invasive hormone sampling techniques. Fecal hormone sampling has proven to be a valuable tool for monitoring wild populations with regard to their physiology, and for evaluating variation in physiological factors in response to variable environmental conditions. The value of fecal hormone analysis as such a tool increases when we evaluate potential variation in hormone concentrations introduced by collection and storage conditions in the field. In our study, samples were frozen fresh in the field, which is the best option for reducing the influence of handling and storage on hormone concentrations (Millspaugh and Washburn, 2004). Nevertheless, we examined three potential sources of methodological variability among our samples: minutes between collection and freezing, years between freezing and assay, and sample collection time of day. Fecal glucocorticoid concentrations in our samples were robust to slight delays between collection and freezing, and even to long-term storage. Like Dloniak (2004), we did see a strong relationship between time of day of sample collection and fGC concentrations; this relationship most likely reflects a circadian rhythm in circulating GC concentrations.

Socioecological variables

In our longitudinal study, natural ecological variation was not found to be a significant stressor in the lives of spotted hyenas. Seasonal variation in prey availability and monthly rainfall did not explain variation in fGC concentrations among hyenas. Nor did our data support the notion that the immediate presence of lions elevates fGC concentrations among hyenas. Even large carnivores like hyenas experience competition

from intra-guild predators (Caro and Stoner, 2003); lions and hyenas compete for access to both carcasses and live prey (Cooper, 1991; Honer et al., 2002; Kruuk, 1972). Watts and Holekamp (2009) found that lions cause the majority of known deaths among adult hyenas in the Talek clan, but that varying levels of competition with lions could not explain patterns of mortality among adult hyenas. Here we were unable to detect a physiological stress response, measured as elevated fGC concentrations, among individuals that had encountered lions 1 to 7 days before fecal sample collection. Although lion-hyena encounters were common, violent interactions between lions and hyenas were rare, and it may be only the latter type of interaction that elicits a stress response measurable in hyena fecal samples. However, like our longitudinal analysis, our cross-sectional data comparing fGC concentrations among clans exposed to dramatically different lion densities (H2; Table 2.2) also failed to suggest that lion presence represents a chronic stressor to hyenas. Although the lion density in the MMNR was twice that in ANP, there was no indication that park influenced hyena fGC concentrations, and there were no significant differences in fGC concentrations between the ANP clans and the Mara River clan.

In carnivore societies structured by linear dominance hierarchies, patterns of social stress vary among dominant and subordinate members of a group (Creel, 2005), but this does not seem to be true among hyenas living in the MMNR (Dloniak, 2004; Dloniak et al., 2006b). However, here we examined instability in the social hierarchy as a predictor of social stress; such instability could potentially affect members of any hierarchical society. Our data indicate that nulliparous females experienced elevated fGC concentrations in response to social instability, and, although not significant, we observed

trends in the same direction among pregnant and non-pregnant females, and among immigrant males. Social instability was the only predictor, other than sample collection time, to explain any variation in fGC concentrations among nulliparous females. Juvenile spotted hyenas gain their rank by "inheriting" their mother's position in the dominance hierarchy, but it is not until their second year of life that their ranks finally become established relative to those of older clan members (Engh, et al., 2000; Smale, et al., 1993). The recency of their rank acquisition may account for the heightened response to social instability apparent among nulliparous females.

A study investigating acute social stress among hyenas living in the Serengeti National Park, demonstrated that lactating females who participated in severe fighting 48 hours prior to sample collection showed elevated fGC concentrations (Goymann et al., 2001). However, we seldom observed severe fighting among females in our study populations, and we collected too few samples after major fights to perform a comparable analysis with our own data. Goymann et al. (2001) also reported that lactating females in the Serengeti had higher mean fGC concentrations than did non-lactating/non-pregnant females. By contrast, fGC concentrations did not vary significantly with lactation state in our study. Lactating females in the Serengeti may be more energetically challenged than those in the MMNR (Trinkel, et al., 2006) because most Serengeti females routinely commute long distances to forage (Hofer and East, 1993) whereas MMNR females feed near their dens (Boydston, et al., 2003a; Watts and Holekamp, 2008). Lactating females in the Serengeti must return frequently from distant foraging sites to their communal den in order to nurse their cubs. The difference in energy budgets between Serengeti and

MMNR females may account for the difference in fGC concentrations seen between lactating and non-lactating females in our two study populations.

Anthropogenic variables

Tourists have been present in large numbers in both the MMNR and ANP for more than 3 decades (Okello et al., 2005). Within the MMNR, the Talek and Mara River territories differed very little except with respect to the intense daily exposure of Talek hyenas to the presence of tour vehicles and livestock (Kolowski et al., 2007). The Mara River territory was visited by tourists at lower rates than were the territories of any of our other study clans. Therefore, had tourist visitation been generally stressful to hyenas, we expected to observe lower fGC concentrations among Mara River hyenas than among hyenas in any of the other three study clans (H3; Table 2.2). Tourists in vehicles pose no direct threat to hyenas, and in both parks, hyenas are well habituated to vehicle presence and do little to avoid them. However, data from our longitudinal analysis suggest that immigrant males may not be as well habituated to tour vehicles as are the natal members of the Talek clan.

In contrast to tourists, pastoralist herdsmen represent a direct threat to hyenas; they are an important source of mortality for the Talek population, second only to lions (Watts and Holekamp, 2009). Additionally, hyenas appear to perceive herdsmen as threats because hyenas often flee from guarded cattle herds whereas cattle left unattended by herders are not avoided (Kolowski and Holekamp, 2008). Our longitudinal data indicate that fGC concentrations among immigrant male hyenas have increased since the early 1990s; this increase has been correlated with the increasing size of the human population living along the border of the Talek clan's home range (Lamprey and Reid,

2004). Although our study did not directly test proximate mechanisms linking human population growth with hyena stress physiology, a recent study comparing the behavior of Talek and Mara River hyenas indicated that it was the presence of pastoralists and their livestock, not tourism, that accounted for altered behavior among Talek hyenas relative to that observed in the undisturbed Mara River clan (Kolowski et al., 2007).

Variation in pastoralist activity, but not tourism or ecological variables, was also consistent with the patterns of variation we observed among clans with respect to fGC concentrations. A study of Serengeti hyenas living inside and outside the Ngorongoro Crater found lower fGC concentrations among females (Goymann et al., 2001) but not males (Goymann et al., 2003) living within the Crater. The authors attributed the lower fGC concentrations to ecological differences experienced by the two populations, and the lower energetic demands enjoyed by females inside the Crater. In our own cross-sectional study, we observed no effect of park on fGC concentrations in our samples, suggesting that the differences in ecology experienced by hyenas living in ANP and the MMNR did not influence hyena stress physiology (H1; Table 2.2). Furthermore, differences in pastoralist activity among monitored clans (H4; Table 2.2) better predicted elevated fGC concentrations than did any natural ecological variables. Growing human populations adjacent to protected areas, as has been described here for the Talek region, are common in many African and Latin American countries (Wittemyer et al., 2008). Whereas this form of anthropogenic disturbance has clear ties to reduction in resources available to protected carnivores, here our data suggest that growing human populations along borders of protected areas may also have direct physiological consequences for the animals living within these areas. Together the results of our longitudinal and cross-

sectional studies suggest that increasing activity of local pastoralists in the Talek region represents a source of stress to Talek hyenas.

Whereas many studies have documented behavioral and demographic changes in wild populations caused by anthropogenic disturbance, relatively few have measured its effects on stress physiology (summarized by Walker et al., 2005a). Nevertheless, our study adds to a growing number of others demonstrating an increase in mammalian stress responses, measured as elevated GC concentrations, caused by various forms of anthropogenic disturbance (e.g. Creel et al., 2002; Millspaugh, et al., 2001; Pereira et al., 2006). It seems reasonable to expect that the previously documented effects of anthropogenic disturbance on spotted hyena behavior (Boydston et al., 2003b; Kolowski et al., 2007), and the physiological correlates demonstrated here, may presage negative changes in demography and fitness within the Talek clan. Although adaptive in the short term, it is well documented that chronic elevation of GC concentrations can have negative effects on fertility in both male and female vertebrates (Sapolsky, 2002; Wingfield and Sapolsky, 2003). Future work should inquire whether or not elevated fGC concentrations predict significant demographic change in wild hyena populations. Spotted hyenas are unusually adaptable animals, so their responses to the natural and human stressors assessed here might predict exacerbated stress responses in more specialized and endangered species, such as cheetahs or African wild dogs.

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CHAPTER 3

INTER- AND INTRASEXUAL AGGRESSION AMONG ADULT SPOTTED HYENAS

INTRODUCTION

In most mammalian species males are socially dominant to females, and direct aggression toward conspecifics at much higher rates than do females (Archer, 1988; Floody, 1983; Walters and Seyfarth, 1987). However, females also exhibit aggressive behavior toward conspecifics. For instance, defense of young is one context in which female aggression has been well studied, and much is known about both the proximate mechanisms and ultimate functions of this behavior (Lonstein and Gammie, 2002). In addition, females emit aggressive behavior as they compete among themselves for access to scarce resources such as food, territory, and to a lesser extent, mates (Floody, 1983; Koenig, 2002; Silk, 1993). There is a growing recognition that social conflict and competition experienced by females has important consequences for both their reproduction and sociality (Silk, 1993; Smuts, 1987; Walters and Seyfarth, 1987; Wrangham, 1987).

Researchers are now examining the neural, endocrine, genetic, and environmental control of aggressive behavior (Nelson, 2006), and steroid hormones, particularly androgens, feature prominently in the investigation of sex differences in aggression (Bouissou, 1983; Harding, 1981). Exposure to steroid hormones early in life may "organize" the developing neural substrates of aggressive behavior (Beach, et al., 1982; Clark and Galef, 1998; Ryan and Vandenbergh, 2002; vom Saal, 1989). Maternal stress translated to the developing fetus via glucocorticoids may also influence aggressive

behavior in offspring (maternal stress, Kaiser and Sachser, 2005; Marchlewska-Koj, et al., 2003). Circulating androgens among adult males can "activate" the expression of aggressive behavior in specific contexts (Wingfield, et al., 1990), and this has been well documented in a number of mammalian taxa (rodents, Beach and Holz, 1946; Buck and Barnes, 2003; primates, Cavigelli and Pereira, 2000; carnivores, Creel, et al., 1997; Creel, et al., 1993; Dloniak, et al., 2006a; Goymann, et al., 2003). Strong evidence demonstrating activating effects of steroid hormones and aggression among females has been more elusive, but some studies suggest that female aggressive behavior may covary with concentrations of androgens (Beehner, et al., 2005; Drea, 2007) or ovarian hormones, such as estrogen and progesterone (Davis and Marler, 2003; Kapusta, 1998).

In a small number of species, a reversal of status from mammalian norms is evident, such that females are socially dominant to males. The evolution of female dominance apparently has occurred independently among taxonomic groups that are only distantly related (e.g., carnivores, Kruuk, 1972; rodents, Clarke and Faulkes, 2001; and primates, Drea, 2007). The leading functional explanation in the literature for female dominance is that it represents an adaptation to high reproductive costs for females occurring in conjunction with intense intraspecific competition for food (Drea, 2007; Frank, 1986; Jolly, 1984); therefore, selection has favored females able to out-compete males such that females can secure access to scarce resources for themselves and their young. There has been much attention paid to the potential for steroid hormone control of female aggression among female-dominated species (Dloniak, et al., 2006b; Drea, 2007; Glickman, et al., 1993; Goymann, et al., 2001a; Sannen, et al., 2003; von Engelhardt, et al., 2000). In this study we investigate aggression and its possible mediation by steroid

hormones in the spotted hyena, (*Crocuta crocuta*), a gregarious carnivore species in which females are socially dominant to males.

Spotted hyena society is structured by a rigid linear dominance hierarchy based on "inheritance" of maternal rank between generations (Engh, et al., 2000; Frank, 1986; Smale, et al., 1993). Rank determines priority of access to food in this species (Frank, 1986; Kruuk, 1972; Tilson and Hamilton, 1984), and although all females breed, high social rank confers a large reproductive advantage, as higher-ranking females out-perform lower-ranking females on most measures of reproductive success (Hofer and East, 2003; Holekamp, et al., 1996). Females are philopatric, but males generally disperse between the ages of 2 and 5 years (Holekamp et al., 1996; Smale, et al., 1997). When males immigrate into a new clan, their social rank is not determined by fighting ability, size or other common indicators of male-male competition; instead, they slot into the lowest position in the social hierarchy, below all natal members of the group and all other adult immigrant males that arrived before them (East and Hofer, 2001; Engh, et al., 2002). Thus adult immigrant males are socially subordinate to all natal members of the group (Frank, 1986; Kruuk, 1972; Smale et al., 1993; Tilson and Hamilton, 1984).

Female spotted hyenas have the reputation of being particularly aggressive; although, whether or not this reputation is warranted remains somewhat controversial. Although they have usually based their assessments on anecdotal rather than quantitative information, observers of spotted hyena behavior are largely in agreement that females attack conspecifics more frequently than do males (Boydston, et al., 2001; East, et al., 2003; East and Hofer, 2002; Frank, 1986; Frank, et al., 1989; Glickman, et al., 1997; Hamilton, et al., 1986; Hofer and East, 1993; Kruuk, 1972; Tilson and Hamilton, 1984),

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which is the opposite of mammalian norms. Many researchers have suggested that female aggressiveness in this species is one of many traits favored by selection to promote female dominance over males, and secure access to food in highly competitive feeding situations (Frank, 1986; Frank, et al., 1995; Glickman et al., 1993; Hamilton et al., 1986; Holekamp and Smale, 1999; Tilson and Hamilton, 1984). Others (East and Hofer, 2002; Goymann et al., 2001a) have suggested that the aggressiveness exhibited by female spotted hyenas has been overstated, and that females only appear to be highly aggressive because rates of aggression emitted by males are very low. These authors correctly point out that, were data available to compare aggressive acts emitted by female spotted hyenas with those emitted by females of other species, we might find that female spotted hyenas are in fact not unusually aggressive (or "hyperaggressive" to use the terminology of Goymann *et al.* (2001a)). Unfortunately, such comparative data are very sparse in the existing literature. An alternative way to assess the aggressive behavior of female spotted hyenas would be to directly compare their behavior with that of male spotted hyenas. Szykman et al. (2003) demonstrated that while female hyenas direct aggression toward both male and female conspecifics, males rarely direct aggression toward adult females. Here, our goal was to expand on those results to provide a quantitative and qualitative comparison of the aggressive behavior exhibited by adult male and female spotted hyenas. Specifically, we directly compare rates and intensities of intrasexual aggression among adult females with those of intrasexual aggression among adult males, and we also compare the sexes with respect to rates and intensities of inter-sexual aggression.

In addition to their apparent role-reversed patterns of social dominance and aggression, female spotted hyenas also differ from mammalian norms in that they have

masculinized external genitalia, which is unique even among species characterized by female dominance (Drea and Weil, 2008; Frank, et al., 1990; Matthews, 1939). Our current understanding of sexual differentiation in mammals assumes that androgens are necessary during development for masculinizing both morphology and behavior (Jost, et al., 1973b). When they are present at critical stages of development, androgens have even been found to masculinize the morphology and behavior of females (Beach et al., 1982; Goy, et al., 1988; Herman, et al., 2000; Jost, et al., 1973a; Phoenix, et al., 1959). The spotted hyena, however, does not appear to conform to the traditional Jostian model of steroid-dependent masculinization, and investigations into the urogenital development in this species are now refocusing on androgen-independent mechanisms to explain the masculinization of the female's external genitalia (Drea, et al., 1998; Glickman, et al., 2005). However, we are still building our understanding of the role that steroid hormones play in shaping behavior in this species. Androgen concentrations sampled from pregnant spotted hyenas are higher than those measured in non-pregnant females, and this difference has been demonstrated in concentrations measured in both blood (Dloniak, et al., 2003; Glickman, et al., 1992; Goymann et al., 2001a; Licht, et al., 1992) and fecal samples (Dloniak et al., 2003). Several studies have shown that androgen concentrations are highest during late gestation among females (Dloniak et al., 2006b; Licht et al., 1992), and that these concentrations are well within the range of those measured in reproductively mature male hyenas (Dloniak et al., 2003; Licht et al., 1992). These androgenic hormones are produced by the maternal ovary (Glickman et al., 1992; Lindeque, et al., 1986) and are transferred to the developing fetus by the placenta (Licht

et al., 1992; Yalcinkaya, et al., 1993); this prenatal androgen exposure may have lasting effects on spotted hyena behavior.

Maternal androgen concentrations during hyena pregnancy, measured in feces, are correlated with mounting and aggressive behavior in both male and female offspring when they are 2 to 6 months old (Dloniak et al., 2006b). Offspring of mothers treated with anti-androgens during pregnancy exhibit reductions in both the postnatal sibling aggression typically seen in this species, and in the aggression directed by females in adulthood toward adult males during feeding competition (C. Drea and S.E. Glickman, pers. communication). Taken together, these results suggest that prenatal androgen exposure might be organizing the neural structures underlying aggressive behavior in both male and female spotted hyenas. There is also some evidence to suggest that aggressive behavior among spotted hyenas might be under activational control of steroid hormones. Among adult male hyenas, circulating androgen concentrations are tightly correlated with aggression related to courtship and mate defense (Dloniak et al., 2006a; Goymann et al., 2003). Furthermore, juvenile ovariectomy appears to reduce rates of inter-sexual aggression emitted by captive female hyenas as adults (Baker, 1990). On the other hand, gonadectomy had no effect on rates of intrasexual aggression among captive juveniles when compared to controls (Frank et al., 1989). However, aggressive behavior among spotted hyenas has never been systematically studied when both observation time and number of potential target animals have been controlled. Furthermore, no one has yet investigated the relationship between aggression and steroid hormones among adult female hyenas living in the wild.

Here we documented sex differences in rates and intensities of aggressive behavior among free-living adult hyenas, and used natural variation in steroid hormone concentrations to test predictions about organizational and activational influences on aggressive behavior in wild spotted hyenas. We used non-invasive hormone sampling techniques to measure fecal steroid hormones, including fecal androgens (fA), fecal estrogens (fE), and fecal glucocorticoids (fGC).

METHODS

Study site and subjects

This study was conducted in the Masai Mara National Reserve, Kenya. We monitored a single large social group, or clan, of spotted hyenas from 1988 through 2005. The clan defended a large territory in the Talek region as described previously (Boydston et al., 2001; Frank, 1986; Holekamp, et al., 1993). All clan members were individually identified by their unique spot patterns, and their sex was determined by the dimorphic glans morphology of the erect phallus (Frank et al., 1990). Spotted hyena clans are fission-fusion societies, and clan members spend their time in small subgroups, the composition of which may change several times a day (Kolowski, et al., 2007; Smith, et al., 2008). Females are generally more gregarious than are males, and social rank affects the gregariousness of individuals of both sexes (Smith et al., 2008; Smith, et al., 2007); the mean subgroup in which females are found in this study population contains 3.9 individuals, while mean subgroup size for males is 3.1 (Smith et al., 2008).

Our study clan was comprised of multiple matrilines of adult females, their offspring, and several immigrant males that had joined the clan as adults. Ages of all

individuals born into the clan during the study period were known (to ± 7 days) (Holekamp et al., 1996), and reproductive histories were known for all females. Birth dates were assigned based on appearance and behavior of cubs when they were first seen (Holekamp et al., 1996). Female spotted hyenas reach reproductive maturity at approximately two years of age (Glickman et al., 1992; Holekamp et al., 1996; Van Meter, et al., 2008), and we considered females to be adults at 24 months. Gestation in this species is 110 days (Kruuk, 1972), so counting backward from known parturition dates, we could estimate dates of conception. To compare rates of behavior among phases of gestation, pregnancy was divided into trimesters of 37 days each. Lactation may last up to 24 months in this species (Holekamp et al., 1996), and each female was considered to be lactating from the day she gave birth until the weaning of that litter or her next conception, whichever came first. Incomplete lactation intervals caused by litter death were not considered here. We assigned wean dates (± 10 days) based on observed nursing conflicts between mothers and cubs, and the cessation of nursing, as has been previously described (Holekamp et al., 1996; Holekamp, et al., 1999).

Adult natal males, who have not yet dispersed, differ from immigrant males with respect to both their behavior and their physiology (Dloniak et al., 2006a; Holekamp and Sisk, 2003; Smale et al., 1997). Therefore we included only immigrant males in our analyses. Immigrants queue for position in the intrasexual dominance hierarchy and only move up in rank as males who arrived before them die or secondarily disperse (East and Hofer, 2001; Engh et al., 2002; Van Horn, et al., 2003). Therefore, the immigrant male with the longest tenure in the clan is the highest-ranking of the immigrants, in a rank

position immediately below that of the lowest ranking natal animal in the clan's overall dominance hierarchy.

Behavioral observations

Most adult members of the clan were fitted with radio-collars that could be tracked from our vehicle, and researchers observed clan members daily in the morning (0530 – 0900 hours) and again in the evening (1700 – 2000 hours). We collected behavioral data during observation sessions on groups of two or more hyenas that were separated from other hyenas by at least 200 m, and identities of all hyenas in each group were recorded. Sessions lasted at least 15 minutes (mean ± standard error = 43.28 ± 0.003 minutes), and ended when we moved away from the subgroup. We observed adult hyenas for a total of 5051 hours during 9863 observation sessions. All aggressive interactions among adults were recorded as critical incidents (Altmann, 1974). For each aggressive interaction we recorded the aggressor, behavior, recipient, response, and if possible, the context in which the aggression occurred. Aggressive interactions among spotted hyenas can involve one actor and one recipient, termed dyadic aggression, or multiple actors engaging in coalitionary aggression. Here we considered only dyadic aggression. Social ranks of adult females in the clan were determined by creating a matrix of outcomes of dyadic aggressive and submissive interactions (Frank, 1986), and this dominance hierarchy was updated annually. Offspring joined the dominance hierarchy below their mothers but above their older siblings (Engh et al., 2000; Holekamp and Smale, 1991). By convention, the highest-ranking adult individual of each sex was assigned a rank of 1. Our ethogram of aggressive behaviors included head-wave, displacement, lunge, push, stand over, rush, chase, bite, and bite-shake. We defined biting

contact as high intensity aggression and all other aggressive behaviors were considered to be of low-intensity. Responses to aggression included appeasement behaviors (head bob, carpal crawl, squeal, back-off), and counter-attacks.

Aggressive behavior by both adult males and females occurred in multiple contexts, classified as one of the following: feeding, "pesky," unprovoked, "other," and unknown. The sex-specific contexts of mating and maternal defense were collectively demarcated as "other" in this study. The feeding context referred to aggression directly related to a food source, and was restricted to scenes at which a carcass was present. The "pesky" context indicated aggression directed toward conspecifics that were clearly annoying the aggressor, often inadvertently. Unprovoked aggressions occurred when the actor aggressed against the recipient without provocation, and usually there were no interactions of any sort within the dyad immediately preceding the attack. "Unprovoked" was not the same as the "unknown" context, which was assigned when the context was simply not clear to observers.

Quantification of aggression

An hourly rate of aggression was calculated for each individual present in a given observation session as:

$$\frac{(\text{number of aggressive acts emitted by that individual} \div \text{number of targets present})}{\text{number of hours in that observation session}}$$

This rate calculation controlled for variation among sessions with respect to opportunities for attack available to each aggressor. The aggression rates calculated for each individual in each session were then averaged together over his/her entire lifetime in the clan as

adults, or over shorter periods of time, as when females were in specific reproductive states. These mean aggression rates therefore included sessions in which each aggressor was seen with one or more potential "targets," but did not attack. In this way, the mean rates of aggression for individuals who were frequently seen with targets, but did not attack, were weighted in comparison to individuals who were seen less often.

The definition of "targets" was determined by the question being asked in each specific analysis. First we wished to inquire about sex differences in aggression emitted by adults toward other adults and toward juveniles. Thus lifetime aggression rates toward adults were calculated using all sessions in which the aggressor was present with one or more adults, and lifetime aggression rates toward juveniles were calculated using all sessions in which the aggressor was present with one or more natal animals less than 24 months. Next, we wished to evaluate sex differences in aggression directed by adults of each sex toward other adults of the same or opposite sex. We refer to aggression directed by adults toward adults of the opposite sex as inter-sexual or between-sex aggression. We had two categories of intrasexual aggression indicating whether the subject was directing aggression toward same-sex individuals who were higher-ranking than themselves, or up the hierarchy, or instead toward same-sex individuals who were lower-ranking than themselves, or down the hierarchy.

Testing hypotheses suggesting steroid hormone mediation of female aggressive behavior

If steroid hormones activate female aggression, then naturally occurring steroid hormone concentrations should be positively correlated with rates or intensities of aggressive behavior. As concentrations of circulating (Goymann et al., 2001a; Licht et al., 1992; Van Jaarsveld and Skinner, 1991) and excreted (Dloniak et al., 2003;

Goymann, et al., 2001b; Van Meter et al., 2008; Van Meter, et al., 2009) steroid hormones are higher during pregnancy than during lactation among spotted hyenas, we predicted that rates of aggression should be higher when females are pregnant than when they are lactating. Additionally, since steroid hormone concentrations are highest late in gestation (Dloniak et al., 2006b; Licht, et al., 1998; Van Meter et al., 2008), aggression rates should also increase as gestation progresses. We used repeated measures analyses to investigate changes in rates of aggression within individuals during the various stages of each female's reproductive cycle. We examined both rates of inter- and intrasexual aggression in this manner.

We used fecal samples collected from females to investigate whether steroid hormone concentrations directly predicted their rates of aggression. We controlled for reproductive condition by comparing concentrations of fA, fE, and fGC measured in samples collected only from females during the second half of pregnancy, and evaluated these hormone concentrations in relation to the mean rate of aggression emitted by each female during this reproductive state.

We also tested the hypothesis that the organizational effects of androgens on aggressive behavior, described for juvenile hyenas by Dloniak *et al.* (2006b), continue to be expressed in adulthood. Here we quantified rates of aggression emitted by 14 adult females for whom excreted steroid hormone concentrations could be measured in their mothers while those female offspring were *in utero.* Like Dloniak *et al.* (2006b), we also compared maternal fecal hormones measured during only the second half of gestation, with the rates of aggression of their female offspring as adults (n = 9 females). Prior studies have demonstrated that fE (Van Meter et al., 2008) and fGC concentrations

(Dloniak, 2004; Van Meter et al., 2009) vary with the time of day (morning vs. evening) of fecal sample collection. Therefore we investigated this factor here, but did not find significant effects of sample collection time in our dataset, so we used both morning and evening samples. From two females, we collected two samples within 10 days of each other, and in these cases we calculated a mean maternal hormone value for that pregnancy. We used maternal hormone concentrations as linear predictors of rates of offspring aggression as adults toward a) all adults, b) lower-ranking females, and c) immigrant males.

Fecal sample collection, extraction, and immunoassay

Fecal samples were collected into plastic bags within 30 minutes of excretion whenever a known hyena was observed to defecate. Within 12 hours of collection, samples were mixed, aliquoted, and frozen in liquid nitrogen for storage until they could be shipped to Michigan State University for analysis.

Frozen fecal samples were lyophilized and extracted in ethanol as described previously (Dloniak et al., 2003; Van Meter et al., 2008). Methods for assay of fecal glucocorticoids (fGC) (Goymann, et al., 1999), fecal estrogens (fE) (Van Meter et al., 2008), and fecal androgens (fA) (Dloniak et al., 2003) have been previously validated. For the analysis of fGC, extracts were assayed in duplicate with a corticosterone radioimmunoassay kit (ImmuChem Double Antibody Corticosterone ^{125}I RIA Kit, MP Biomedicals (formerly ICN), 07-120102), which displays a high affinity to the major glucocorticoid metabolites present in the feces of spotted hyenas (Dloniak, 2004; Goymann et al., 1999). Concentrations of fE were assessed with a modified enzyme-immunoassay protocol using an estradiol antibody (R4972; Clinical Endocrinology

Laboratory, University of California, Davis) shown to cross-react with the major immunoreactive estrogen metabolites in hyena feces (Van Meter et al., 2008). Spotted hyena fA concentrations were analyzed with a modified enzyme-immunoassay using a testosterone antibody (R156/7; Clinical Endocrinology Laboratory, University of California at Davis), shown to display a high affinity for the major immunoreactive androgen metabolites in hyena feces (Dloniak et al., 2003). The precision of each assay was monitored by assaying serial dilutions of pooled hyena fecal extracts. The intra-assay coefficients of variation for fGC, fE and fA were 4.79%, 3.48%, and 6.18% respectively, and the interassay coefficients of variation were 10.12% (n = 17 assays), 13.34% (n = 25 assays), and 12.05% (n = 51 assays).

Statistical analysis

All statistical testing was performed using SPSS 16.0.0 (SPSS) and graphs were made with Sigma Plot 8.0 (StatSoft). In our behavioral data, a value of zero was often a meaningful datapoint, indicating that an individual failed to attack conspecifics when it had opportunities to do so. Many of our datasets contained enough zero values that the distributions of the data were highly skewed, so did not meet basic parametric test assumptions, even after transformations were applied. Therefore, we used nonparametric statistical tests to assess the behavioral data. We used within-individual comparisons to evaluate within-sex variation in rates or intensity of aggression directed toward animals in various target groups (e.g., comparing behavior of the same females when they were in different reproductive states and individuals among different contexts of aggression). We used a Friedman ANOVA omnibus test to compare more than two dependent groups, and when this was significant, we used Wilcoxon signed-ranks tests for post-hoc

comparisons. To make comparisons between the sexes we used tests for independent data. When making comparisons among multiple independent groups, as with our assessment of sex differences in intensity of aggression directed toward various targets, we used a Kruskal-Wallis test omnibus test, then used Mann-Whitney U tests for post-hoc comparisons. All tests were two-tailed ($\alpha = 0.05$) and we corrected for multiple testing using the sequential Bonferroni adjustment (Rice, 1989), and report and interpret only adjusted p-values. We present means ± standard errors throughout.

We used linear regression to determine whether fecal steroid hormones explained variation in aggression rates. Here, we could log-transform the aggression rates for these analyses to approximate normal distributions (assessed by looking at the qq-plots generated by these data). We found moderate correlations in our data among the three fecal hormones ($R^2 \geq 0.10 \leq 0.76$), and to avoid issues of multicollinearity we entered each hormone into the model one at a time (Keppel and Wickens, 2004).

RESULTS

Distribution of aggression among contexts for adult males and females

We first evaluated the contexts in which adults of both sexes exhibited aggression toward other adults. The percent of total attacks emitted varied significantly among contexts for both males (Friedman ANOVA $X^2_{4,50} = 64.42$, $p < 0.001$) and females ($X^2_{4,72} = 58.89$, $p < 0.001$) (Figure 3.1). We found no significant sex differences in percent of attacks in feeding (Mann-Whitney $U = 1732.00$, $p < 0.510$), unprovoked ($U = 1573.50$, $p < 0.458$), "other" ($U = 1622.00$, $p < 0.533$), or unknown contexts ($U =$

1847.00, $p < 0.941$); however, females directed significantly more aggression toward pesky individuals than did males (Mann-Whitney $U = 751.00$, $p = 0.005$).

Sex differences in rates of aggression

To compare rates of aggression directed by adults of both sexes toward conspecifics, we calculated a rate of aggression emitted by each adult, controlled for opportunity, directed toward either juvenile or adult conspecifics (Figure 3.2). Adult females (n = 73) directed aggressive behavior at significantly higher rates than did immigrant males (n = 58) toward both adults (Mann-Whitney $U = 767.00$, $p < 0.001$) and juveniles ($U = 24.00$, $p < 0.001$). Both females (Wilcoxon $Z = 2.69$, $p = 0.007$) and males ($Z = 6.00$, $p < 0.001$) were significantly more aggressive toward adults than they were to juveniles.

We next used within-female comparisons to assess variation in rates of aggression directed by adult females (n = 71) toward their various adult targets: higher-ranking females, lower-ranking females, and males (Figure 3.3). There was one female, the highest-ranking female, who was never present with a higher ranking individual and one female, the lowest-ranking female, who was never present with a lower ranking female, and both females were excluded from this analysis. Rates of aggression emitted by adult females varied significantly among target types (Friedman ANOVA $X^2_{2,70} = 64.42$, $p <$ 0.001). Females directed significantly higher rates of aggression toward males than toward females lower ranking (Wilcoxon $Z = 2.03$, $p = 0.042$) or higher ranking than themselves ($Z = 7.18$, $p = 0.003$). Females directed the lowest rates of aggression toward females higher ranking than themselves ($Z = 7.27$, $p = 0.003$).

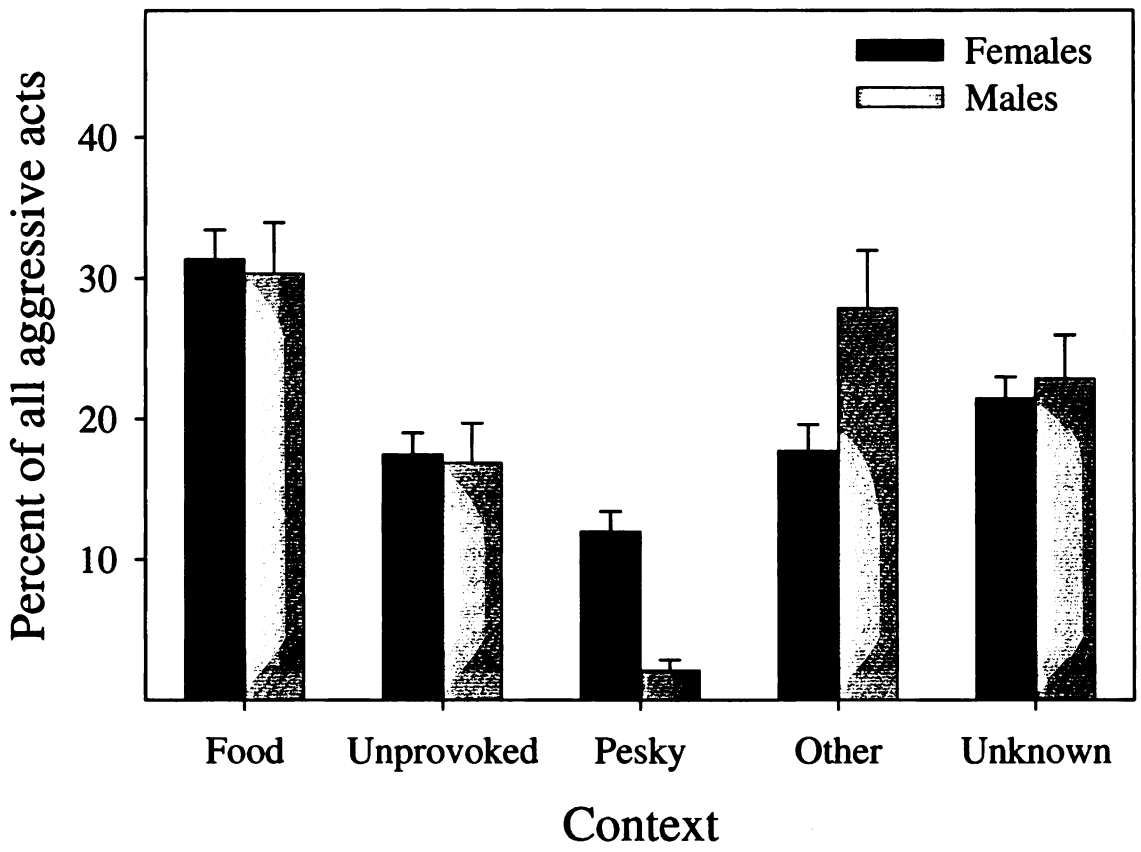

Figure 3.1. Mean (± standard error) percentage of all attacks (n = 10098) directed toward all adult conspecifics by adult females (n = 73; black bars) and males (n = 51; gray bars) observed in various contexts. The "other" context refers to sex-specific situations like mating and maternal defense. The sexes only differed significantly with respect to aggression directed toward "pesky" conspecifics (p = 0.005).

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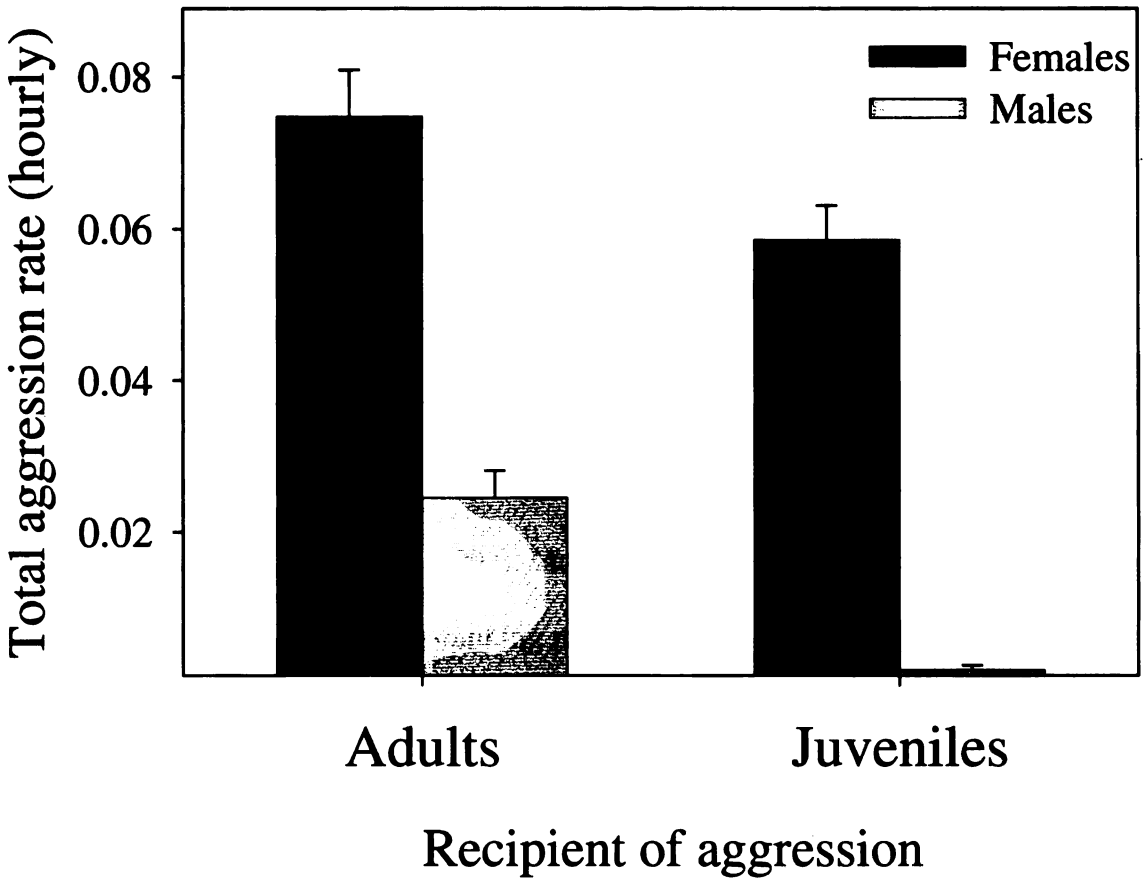

Figure 3.2. Mean (± standard error) lifetime aggression rates, controlled for numbers of available targets in each observation session, directed by adult females (n = 73; black bars) and males (n = 58; gray bars) toward adult and juvenile conspecifics. Adults of both sexes were significantly more aggressive to adults than to juveniles, and adult females were significantly more aggressive to both adults and juveniles than were adult males.

Similarly, we used within-male comparisons to assess variation in rates of aggression directed by adult males (n = 57) toward their various adult targets: higher-ranking males, lower-ranking males, and adult females (Figure 3.3). There was one male (the highest-ranking male at the beginning of the study) who was never present with a higher-ranking male, so he was omitted. As with females, rates of aggression emitted by adult males varied significantly among target types (Friedman ANOVA $X^2_{2,56} = 45.65$, $p < 0.001$). Males directed aggression at significantly higher rates toward males lower ranking than themselves than toward either higher-ranking males (Wilcoxon $Z = 5.72$, $p = 0.003$) or females ($Z = 5.59$, $p = 0.003$). Males directed significantly more aggression toward females than toward high-ranking males ($Z = 2.48$, $p = 0.013$).

Next, we directly compared rates of aggression emitted by females with those emitted by males (Figure 3.3). Females were significantly more aggressive toward higher-ranking targets (Mann-Whitney $U = 1584.00$, $p < 0.045$) and toward members of the opposite sex ($U = 321.50$, $p < 0.001$) than were males; however, males and females were equally aggressive toward same-sex targets lower-ranking than themselves ($U = 1844.00$, $p < 0.389$). Females were significantly more aggressive toward males than males were to each other ($U = 1605.00$, $p < 0.045$).

As a qualitative measure of sex differences in aggression, we compared the sexes with respect to the intensities of the aggressive acts they emitted. Figure 3.4 shows the percent of aggressive acts of high intensity directed by adult males and females toward different target types (Kruskal-Wallis $H = 47.14$, $p < .001$). Severe aggression occurred more often among female-female dyads than among dyads of any other type (for all comparisons Mann-Whitney $U < 640.5$, $p < 0.006$).

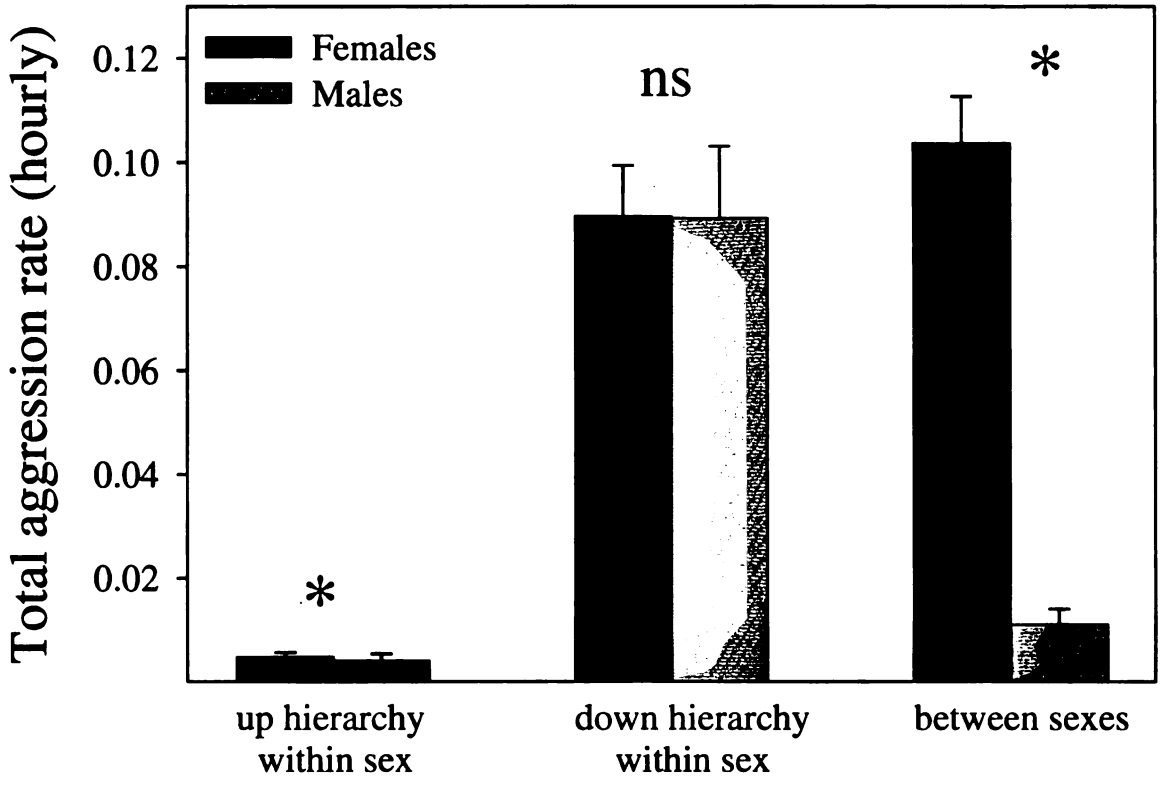

Figure 3.3. Mean (± standard errors) rates of aggression directed toward adult conspecifics by adult male (gray bars) and female (black bars) spotted hyenas. Rates of aggression emitted by females (n = 71) varied significantly among target types (females higher-ranking, females lower-ranking, and males). Rates of aggression emitted by males (n = 57) also varied significantly among target types (males higher-ranking, males lower-ranking, and females). Asterisks indicate significant sex differences in rates of aggression directed toward target types.

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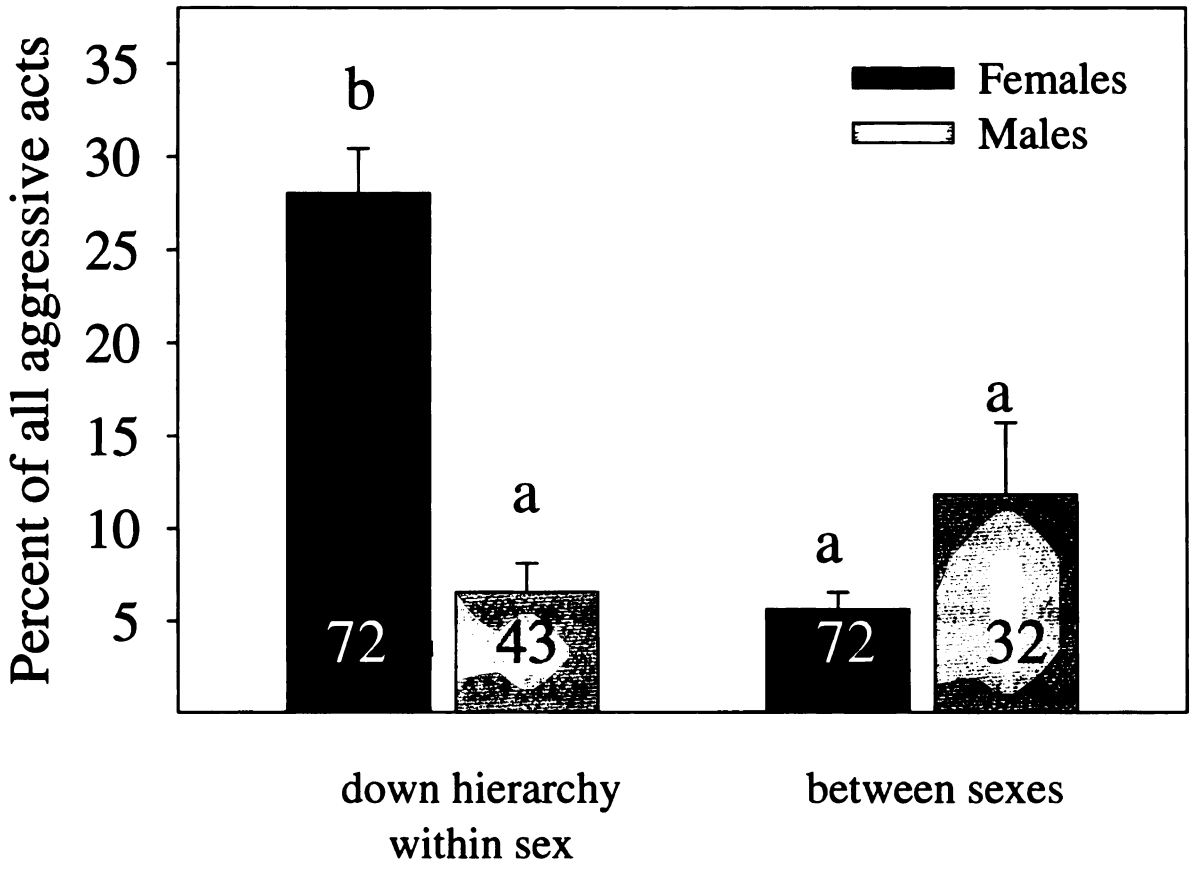

Figure 3.4. Mean percent (± standard errors) of all aggressive acts that were of high-intensity (n = 1232). Not all males were seen to emit high-intensity aggressions, and the numbers under the bars indicate number of individuals used in this analysis. Letters above the bars indicate significant differences among groups.

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We next compared the sexes with respect to their rates of intrasexual aggression using only aggression directed down the hierarchy within each aggressive context (Figure 3.5.A). Rates of intrasexual aggression did not differ here between the sexes within the contexts of feeding ($U = 1698.00$, $p = 0.216$), unprovoked ($U = 1891.00$, $p = 0.519$) or "other" ($U = 1613.00$, $p = 0.129$). However, females were significantly more aggressive toward pesky targets than were males ($U = 1209.00$, $p < 0.001$). We also evaluated sex differences in rates of inter-sexual aggression within each aggressive context (Figure 3.5.B), and found that females consistently directed significantly more aggressive acts toward males than did males toward females ($p \leq 0.005$ for all comparisons).

Finally, intrasexual aggression was of higher intensity among females than among males in all contexts (feeding, $U = 537.50$, $p = 0.004$; unprovoked, $U = 368.00$, $p = 0.004$; other, $U = 409.00$, $p = 0.004$) (Figure 3.6). Although we saw a few instances of low-intensity attacks directed by males toward other pesky males, males were never seen to direct high intensity aggression toward pesky conspecifics.

Activating effects of hormones in adult females

We used the natural fluctuation in steroid hormone concentrations among reproductive states to investigate whether rates of aggression emitted by adult females covaried with these hormone concentrations. First, we asked if rates of aggression emitted by females were higher when they were pregnant than when they were lactating, corresponding to the natural variation in steroid hormone concentrations. In a paired analysis (n = 40 females) (Figure 3.7.A), rates of female aggression directed at males did not vary with reproductive state (Wilcoxon $Z = 1.57$, $p = 0.116$), and rates of aggression by females toward lower-ranking female targets were significantly higher during lactation

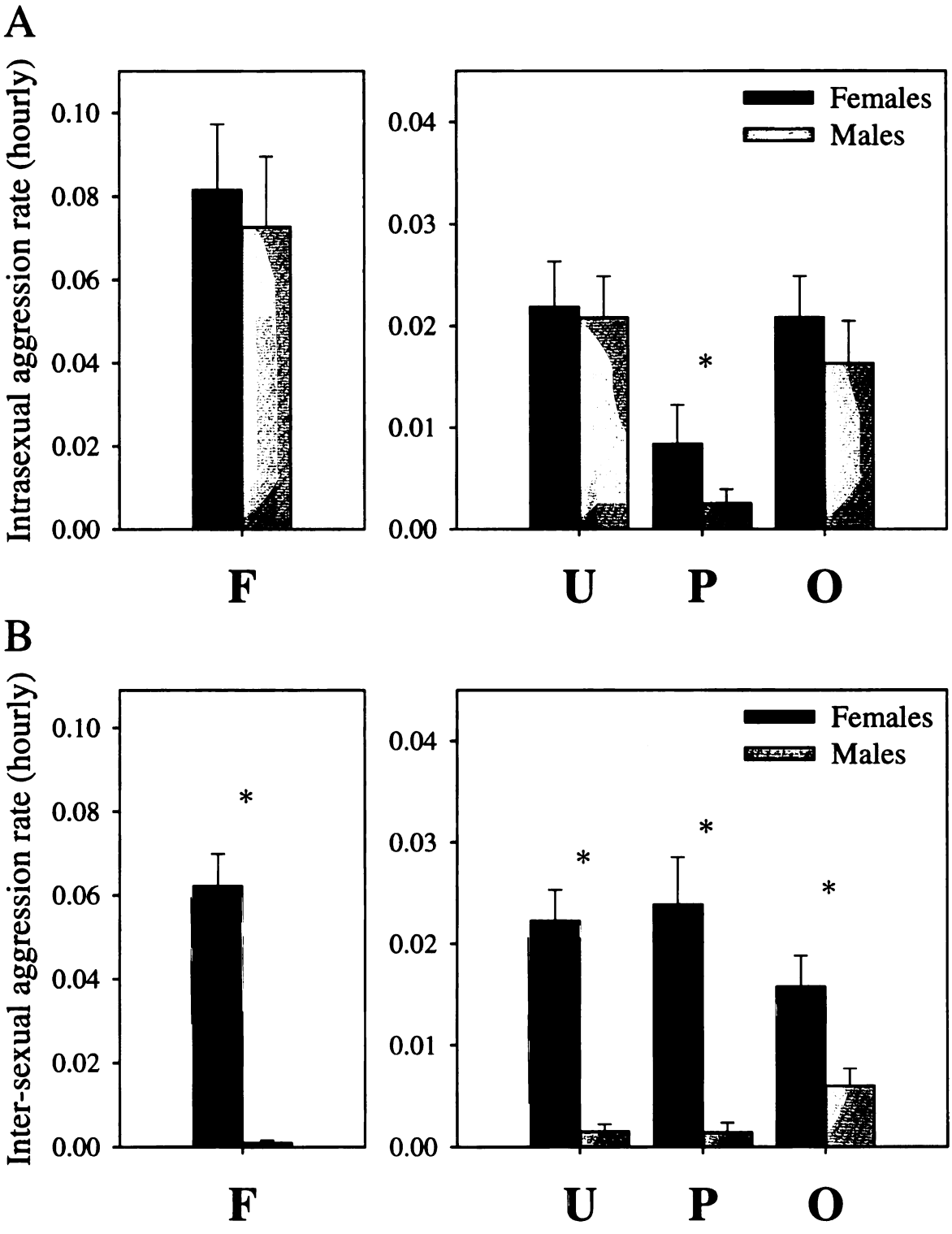

Figure 3.5. Mean (± standard errors) rates of (A) intrasexual aggression toward lower-ranking conspecifics, (B) and inter-sexual aggression. Because feeding aggression could only occur when food was present, rates were calculated for this context on a different scale than for all other contexts. Asterisks indicated significant differences. (Contexts are abbreviated such that: F = feeding, U = unprovoked, P = pesky, and O = "other").

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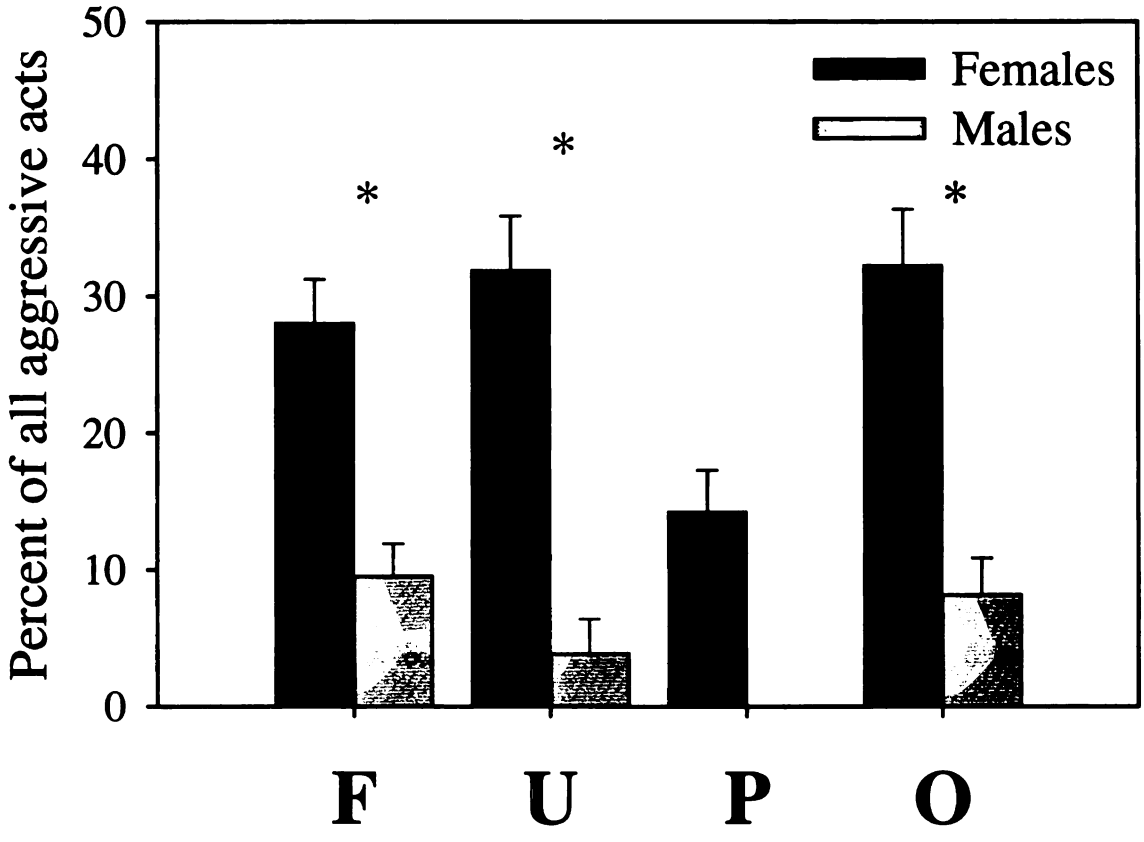

Figure 3.6. Mean percent (± standard errors) of all intrasexual aggressive acts that were of high-intensity (n = 925). Aggression among females was of significantly higher intensity in every context. (Contexts are abbreviated such that: F = feeding, U = unprovoked, P = pesky, and O = "other").

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than during pregnancy ($Z = 4.12$, $p = 0.004$), which is opposite to the pattern seen in steroid hormone concentrations during these two states. That is, androgen concentrations are substantially higher among pregnant than lactating females. Females were significantly more aggressive toward males than toward females during both pregnancy ($Z = 3.49$, $p = 0.004$) and lactation ($Z = 5.72$, $p = 0.004$). Next, we compared rates of aggression emitted by females among the trimesters of pregnancy, since steroid hormone concentrations are highest during the last trimester of pregnancy (Figure 3.7.B). We found no effect of trimester on rates of female aggression directed toward either lower-ranking females (Friedman ANOVA $X^2_{2,27} = 0.09$, $p = .958$) or males (Friedman ANOVA $X^2_{2,27} = 2.85$, $p = 0.482$). Females were more aggressive toward males than toward females during all three trimesters; however, after Bonferroni adjustment, this difference was significant only during early pregnancy (Wilcoxon, $Z = 3.19$, $p = 0.003$).

Finally, we inquired whether or not steroid hormone concentrations measured in feces sampled from individuals directly predicted their concurrent rates of aggression. To control for reproductive state, we focused here only on females in the last trimester of pregnancy (n = 16). Fecal concentrations of three steroid hormones measured in adult females during their third trimester of pregnancy failed to explain any variation in their rates of aggression toward females (fA, $R^2 = 0.00$, $p = 0.978$, fE, $R^2 = 0.03$, $p = 0.478$, and fGC, $R^2 = 0.03$, $p = 0.556$) or toward males (fA, $R^2 = 0.001$, $p = 0.908$, fE, $R^2 = 0.07$, $p = 0.305$, and fGC, $R^2 = 0.06$, $p = 0.380$).

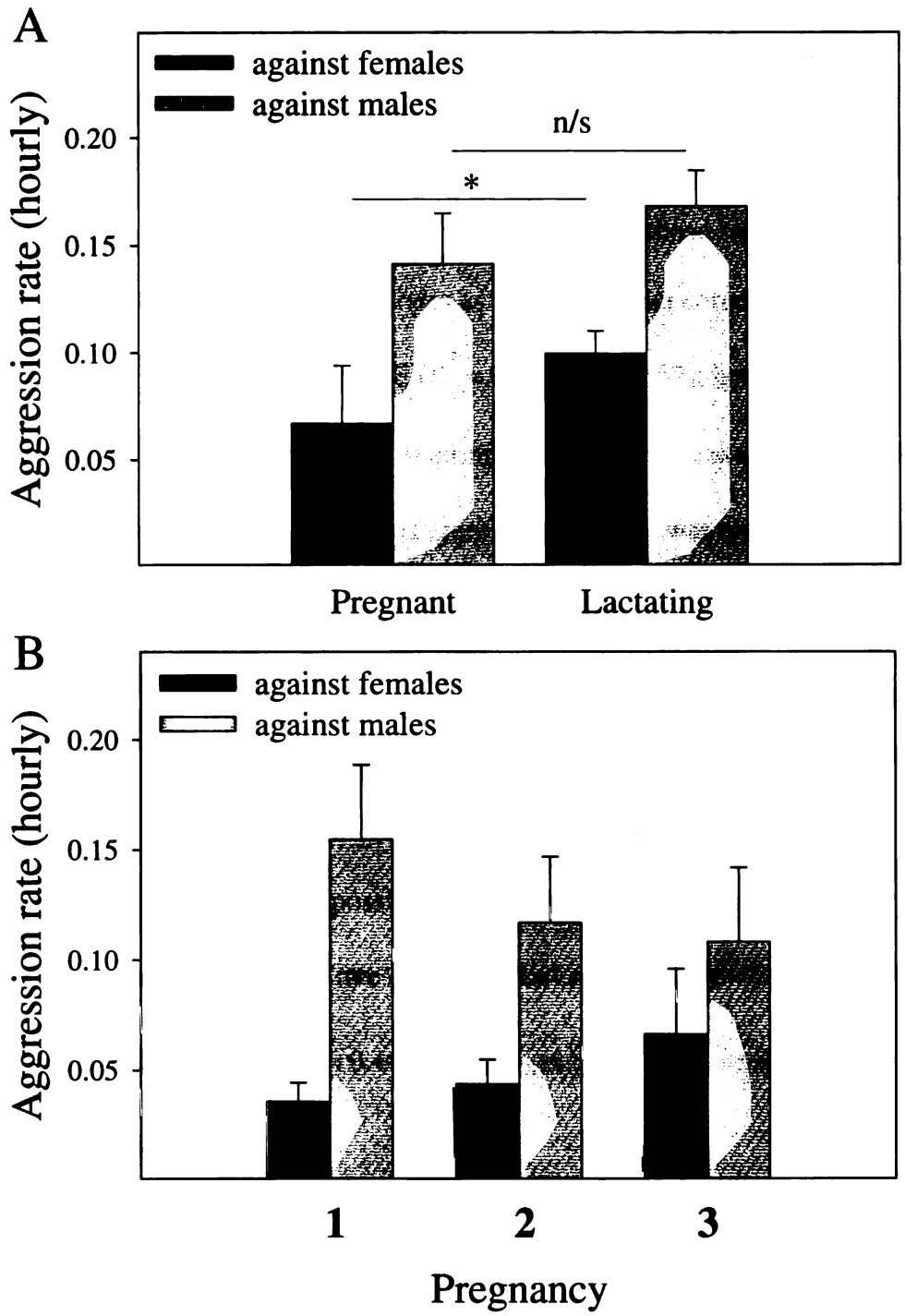

Figure 3.7. Mean (± standard error) rates of aggression directed by females toward lower-ranking female targets, and adult males (A) during pregnancy and lactation (n = 40 females), and (B) during successive trimesters of pregnancy (n = 28 females). Females were significantly more aggressive toward other adult females during lactation than during pregnancy ($p = 0.004$). During pregnancy, rates of aggression emitted by females did not vary among successive trimesters of pregnancy. Females were more aggressive toward males than toward females during all trimesters of pregnancy, although this difference was significant only for the first trimester ($p = 0.003$).

Organizing effects of hormones in adult females

We evaluated the rates of aggression emitted by 14 female subjects as adults toward other adults in relation to fA, fGC, and fE concentrations measured in samples collected from their mothers during the pregnancies in which those same subjects had been in the womb. Offspring aggression rates as adults were not correlated with either maternal fGC ($R^2 = 0.14$, $p = 0.191$) or fE ($R^2 = 0.003$, $p = 0.852$); however, there was a significant positive correlation between offspring aggression rates as adults and maternal fA concentrations ($R^2 = 0.84$, $p < 0.001$) (Figure 3.8.A). There was one data point that proved to be an outlier in both x-space (assessed with leverage) and y-space (assessed with Cook's distance), but the relationship between offspring aggression as adults and maternal fA during pregnancy was still significant even after removal of this point ($R^2 = 0.65$, $p = 0.001$). We assessed day of gestation, maternal rank during gestation, and average offspring rank as adults as possible covariates, but none of these factors significantly influenced aggression rate (day of gestation, R^2 partial = 0.001, $p = 0.930$; maternal rank, R^2 partial = 0.01, $p = 0.460$; and offspring rank, R^2 partial = 0.01, $p = 0.467$).

Next, we examined the relationship between maternal fA and rates of aggression directed by adult offspring toward immigrant males and also that directed toward lower-ranking females. Maternal fA remained positively correlated with rates of offspring aggression toward males ($R^2 = 0.71$, $p < 0.001$), and neither day of gestation, maternal rank, nor average offspring rank explained any additional variation (Figure 3.8.B). However, maternal fA was not correlated with offspring aggression toward lower-ranking

females ($R^2 = 0.005, p = 0.813$) (Figure 3.8.C). Thus rates of inter-sexual aggression, but not intrasexual aggression emitted by females, were correlated with maternal fA concentrations. To make our results strictly comparable to those of Dloniak *et al.* (2006), we restricted our analysis to females who survived into adulthood for which we had hormone data from samples collected from their mothers only during the second half of gestation (n = 9 females). As with the larger dataset, there was a significant positive correlation here between maternal fA and offspring rates of aggression directed at males ($R^2 = 0.52, p = 0.028$), but not with rates of aggression towards females ($R^2 = 0.02, p = 0.746$).

Our data revealed a strong sex difference in the intensity of aggressive acts emitted by adult hyenas; however, we found no relationship between maternal fA and the proportion of high intensity aggression directed by adult offspring toward adult females ($R^2 = 0.017, p = 0.957$). Although we had hoped to investigate the relationship between maternal fA and rates of aggression received by female offspring from adult males, because dyadic aggression directed by adult males toward adult females was so rare, we did not have sufficient data for this analysis; only four of the 14 females, for which we had hormone data, ever received any aggression from an adult male.

Our data indicated that rates of aggression emitted by adult females varied with the reproductive state of the aggressor, so we thought it important to control for reproductive state in our analysis. We calculated rates of aggression from females only while they were lactating, and these rates were significantly and positively correlated with maternal fA concentrations ($R^2 = 0.86, p < 0.001$).

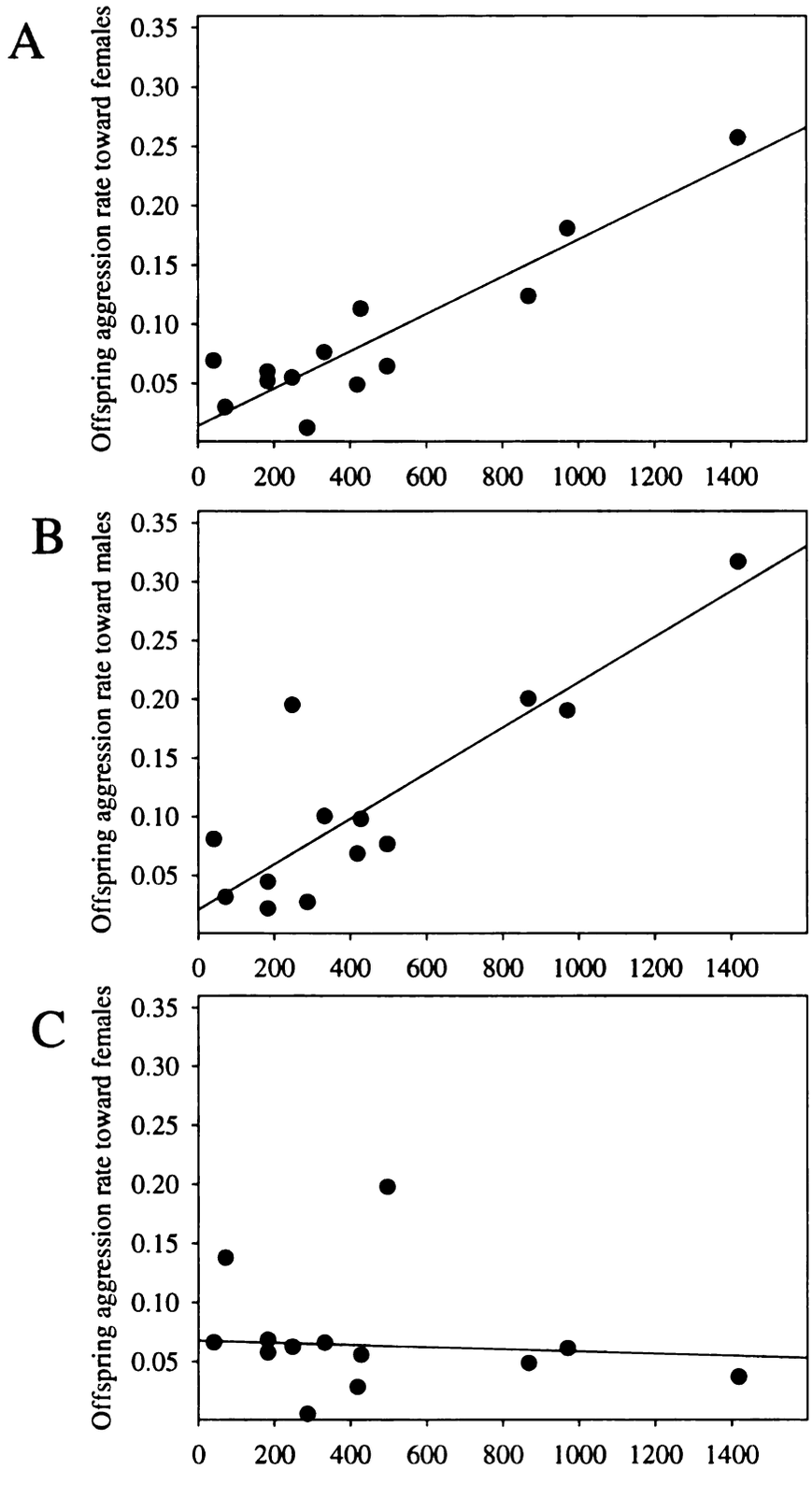

Figure 3.8. Relationship between maternal fA during gestation and rates of offspring (n = 14) aggression in adulthood toward (A) all other adults, (B) adult males, and (C) adult females. All offspring included here were adult females.

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DISCUSSION

Aggressive behavior serves different functions in different contexts. Although the form of an aggressive act may look very similar when an aggressor attacks a conspecific in the presence of food to when it attacks in the presence of a potential mate, these different contexts suggest different functions. In species in which males gain reproductive advantages through competition with each other, aggression among males is often frequent and intense. However, there is a growing recognition that competition among females is critical in shaping dominance relationships that determine access to key resources, such as food (Sterck, et al., 1997; Walters and Seyfarth, 1987), and the functions of aggressive behavior directed toward conspecifics often differ between males and females. While winning conflicts may convey reproductive advantages, aggression carries with it obvious costs, and the expression of aggressive behavior is often constrained by social factors, such as the dominance relationship between the attacker and the recipient, and the immediate social setting (Mason and Mendoza, 1993). In the majority of mammalian societies, one sex tends to be dominant to the other, making it difficult to compare males and females with respect to aggressive behavior directed toward conspecifics of the opposite sex. With this chapter, we demonstrate that significant sex differences in aggression exist among adult spotted hyenas even after controlling for the sex and rank of the attacker in relation to the sex and rank of the targets available in the immediate social group, as well as the aggressive context in which the attack occurred. We then also provide evidence indicating that androgens may play a role in regulating the expression of aggressive behavior among adults in this species.

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Sex differences in aggression among adult spotted hyenas

In agreement with anecdotal field reports from many hyena researchers (Boydston et al., 2001; East et al., 2003; East and Hofer, 2002; Frank, 1986; Frank et al., 1989; Glickman et al., 1997; Hamilton et al., 1986; Hofer and East, 1993; Kruuk, 1972; Tilson and Hamilton, 1984), we have shown that overall rates of aggression directed by females toward conspecifics are almost three times higher than rates of aggression emitted by males. Like the results reported by Szykman *et al.* (2003), we found that rates of inter-sexual aggression emitted by females were significantly higher than rates of inter-sexual aggression emitted by males, and this was true in every aggressive context observed. Dyadic aggression emitted by males toward females occurs very rarely in this species; instead males seem to rely on coalitions with other males to attack female hyenas. The function of this coalitionary aggression among males is poorly understood, as females do not submit to, and frequently counterattack their male attackers (Szykman, et al., 2003). In the current study we also found that females directed aggression toward males at significantly higher rates than toward other adult females. By contrast, Szykman *et al.* (2003) reported no difference in rates of aggression emitted by females toward male and female targets, but did show a trend in the opposite direction from what was demonstrated here. Male spotted hyenas are less gregarious than females (Smith et al., 2008; Smith et al., 2007), and the ratio of adult females to adult males present simultaneously in this clan was close to 2 to 1, so nearly twice as many adult females as adult immigrant males were present at any given time (Kolowski et al., 2007; Watts and Holekamp, 2008). Szykman *et al.* (2003) did not control for these important sex differences in sociality in their earlier analysis, but when the opportunities available to

each aggressor to attack conspecifics of each sex are controlled, as we have done here, we see that females frequently attack males that are present with them while being observed. These data suggest that there is very little risk to females when attacking adult males, and that it may be more risky to attack other females, even those who are lower-ranking than the aggressor.

We also directly compared rates of intrasexual aggression emitted by the two sexes and showed that these rates are not significantly different after controlling for the number of opportunities available in which to attack. However, the intensity of intrasexual attacks is consistently higher when emitted by female than male hyenas. This sex difference is also reflected in patterns of wounding, which indicate that adult females experience higher rates of injury than do males (Holekamp, *unpublished data*). Overall, our results suggest that female spotted hyenas emit aggressive acts more frequently than do immigrant males, and that they fight with greater intensity than do male conspecifics. These sex differences in aggressive behavior may be important for the maintenance of female dominance over males in spotted hyena society.

Goymann *et al.* (2001a) suggested that a comparative study should investigate whether female spotted hyenas are more or less aggressive than females of other species with similar social systems. Few studies have investigated rates of aggression emitted by females of species living in the wild to the extent we do here; however, we can make a few comparisons between spotted hyenas and well-studied primate species that also live in multi-male, multi-female groups. Ehart and Overdorff (2008) made a simple comparison of rates of intrasexual aggression among females for several species of wild lemurs, monkeys, and apes by using available data from the literature. For each species,

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the authors divided the total number of observed attacks among females by the number of females included in the study, further divided by the total number of observation hours in the study. Using this exact protocol we calculated the hourly rate of intrasexual aggression among female hyenas in our study, and found that it falls well within the range of rates Ehart and Overdorff (2008) reported for female primate species (Figure 3.9). This suggests that female spotted hyenas are not "hyperaggressive" relative to female primates. However, spotted hyenas differ from most primate species in that rates of inter-sexual aggression emitted by female spotted hyenas are significantly higher than those emitted by males. Among adult primates, males generally enjoy physical superiority and social dominance over females, and females rarely direct aggression toward adult males (Campbell, 2003; Kuester and Paul, 1996; Smuts, 1987; Smuts and Smuts, 1993). Therefore, while rates of intrasexual aggression among females of both hyenas and primates are similar, female hyenas can frequently aggress against socially subordinate males, but female primates cannot freely direct aggression against higher-ranking males. Finally, female primates and hyenas also differ with respect to the quality of the aggression emitted. There is an almost universal tendency for aggression among male primates to be more intense than that among females (reviewed by Smuts, 1987), and fighting can even lead to death (e.g., Campbell, 2006; Watts, 2004), indicating that the situation in hyenas is highly unusual insofar as aggression among females is usually of higher intensity than inter-sexual aggression or aggression among males.

Some earlier hyena researchers (East and Hofer, 2002; Goymann et al., 2001a) have suggested that the aggressive behavior of males in this species has been reduced by natural or sexual selection, and that reduced aggressiveness among males merely makes

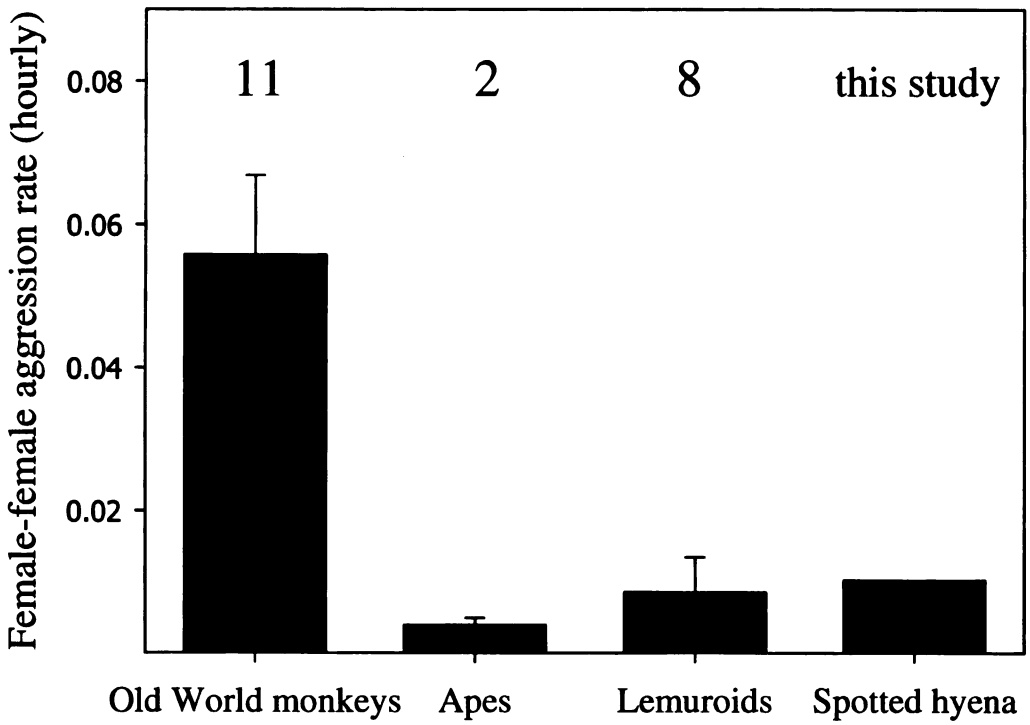

Figure 3.9. Mean (± standard error) rates of intrasexual aggression emitted by adult females. Rates are calculated as total aggressive acts among females, divided by the number of study subjects, over the total observation time for the study period. Primate data were adapted from Erhart and Overdorff (2008), and the numbers over each bar indicates the number of species represented in each group.

females appear "hyperaggressive" by comparison. Others have suggested that natural selection has favored the evolution of traits among females, including enhanced aggressive behavior, that have promoted female dominance in this species (Dloniak et al., 2006b; Engh et al., 2002; Frank, 1986; Frank, 1996; Glickman et al., 1993; Kruuk, 1972; Muller and Wrangham, 2002; Tilson and Hamilton, 1984). Surprisingly, our data show that male and female spotted hyenas direct similar rates of aggression toward same-sex targets. From these data alone we cannot determine whether selection has favored enhanced aggression among females, reduced aggression among males, or both (Frank, 1986; Frank et al., 1995; Glickman et al., 1993; Hamilton et al., 1986; Holekamp and Smale, 1999; Tilson and Hamilton, 1984). However, our results indicating that the intensity of aggressive acts directed by males towards other males generally tends to be quite low, suggest that selection may indeed have acted to reduce fight intensity among males. Among adult males in most other mammalian species, fight intensity tends to be very high (Bernstein, et al., 1983; Campbell, 2006; Clutton-Brock, et al., 1979; Creel et al., 1997; Lincoln, et al., 1972; Scott, et al., 2005; Smuts, 1987). On the other hand, aggression appears to have unusually important fitness consequences for adult female hyenas (Hofer and East, 2003; Holekamp and Smale, 1999; Holekamp et al., 1996), so selection may have favored enhanced aggressiveness among females in addition to reduced aggressiveness among males in this species.

Many observers have commented on the low frequency with which attacks are seen among male spotted hyenas (East et al., 2003; East and Hofer, 2001; Engh et al., 2002; Frank, 1986; Henschel and Skinner, 1987; Holekamp and Smale, 1998; Kruuk, 1972); however, we suggest that this perception might be related, in part, to the fact that

males are less gregarious than females (Kolowski et al., 2007; Smith et al., 2008; Smith et al., 2007). East *et al.* (2003) suggest that, in this female-dominated society, males have little to gain by competing among themselves for mates, and that selection should instead favor submissive, non-aggressive males in this species. Our results here show clear overall sex differences in aggression, suggesting that females are the more aggressive sex; however, our data also show that females do not corner the market on aggressive behavior in this species, as males do compete among themselves in a variety of contexts. Some studies have demonstrated that male hyenas compete aggressively, albeit at low intensity, for access to females (Dloniak et al., 2006a; Goymann et al., 2003; Szykman et al., 2003). Also, males participate vigorously in attacking prospective immigrants and other male intruders (Boydston et al., 2001). Finally, prior to dispersal, adult males maintain positions in the dominance hierarchy immediately below those of their mothers, as do their female siblings (Engh et al., 2000; Holekamp and Smale, 1993; Smale et al., 1993), allowing adult natal males to dominate and direct aggression toward females lower-ranking than their mothers. Taken together, these data indicate that adult males are fully competent aggressors. Dispersal has been shown to have profound effects on both the behavior and physiology of male spotted hyenas (Dloniak et al., 2006a; Holekamp and Sisk, 2003; Holekamp and Smale, 1998). We speculate that dispersal may be associated with induction of an inhibitory mechanism functioning to dampen the aggressive behavior of males. One of us (K.E.H.) has observed that immigrant males sometimes behave erratically when they have been administered an anesthetic, but before the drug has taken full effect. Under these circumstances, males may attack "inappropriate" individuals, including adult females and young cubs. This inappropriate

behavior suggests the disinhibition of aggression while the hyena is under the influence of the drug. Whether or not this is the case, further investigation of the proximate mechanisms controlling aggressive behavior, in both males and females, should yield greater insight into the mediation of sex differences in aggression in this species.

Steroid hormones and aggressive behavior by females

As we were unable to directly manipulate steroid hormone concentrations in our wild spotted hyena study population, we used the natural fluctuations that are known to occur among reproductive states to investigate variation in aggressive behavior within individuals. We found no evidence that rates of aggressive behavior were high during reproductive states when steroid hormone concentrations were also high. Moreover, individual variation in rates of aggression among pregnant females was not correlated with fA, fE, or fGC concentrations. Thus our data failed to provide support for activational effects on aggressive behavior emitted by adult female spotted hyenas by the steroid hormones we are currently able to measure in hyena fecal samples.

On the other hand, our results add to a growing body of evidence in support of an organizational role for androgens in the mediation of aggressive behavior among spotted hyenas. We showed that rates of aggression emitted by adult females were positively correlated with the prenatal androgen concentrations to which these females were presumably exposed *in utero*. This builds on work done by Dloniak et al. (2006b), who showed that maternal fA concentrations sampled during gestation were positively correlated with offspring behavior, including aggression, in juveniles aged 2 to 6 months. It thus appears that prenatal androgens may be organizing the neural substrates controlling aggressive behavior and that this organization has long-lasting consequences

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for offspring behavior among spotted hyenas. Dloniak et al. (2006b) also showed that maternal fA concentrations during pregnancy were positively correlated with maternal rank. These results echo those obtained in studies of maternal effects in many bird species. Maternal effects occur when maternal phenotype influences offspring phenotype independent of offspring genotype, and maternal effects in regard to androgen exposure have been suggested to enhance offspring fitness among several bird species (Groothuis, et al., 2005; Groothuis and Schwabl, 2008). Spotted hyenas experience intense feeding competition at kills, and because reproductive success among females is strongly tied to their ability to secure food for themselves and dependent young (Holekamp et al., 1996; Holekamp et al., 1999), it is reasonable to suggest that enhanced aggressive behavior, mediated by prenatal androgen exposure, might be adaptive among females of this species.

An adaptive function for prenatal androgenization of females does not preclude the possibility that males might also benefit from prenatal androgen exposure. As males disperse out of their natal clans after puberty (Holekamp et al., 1996; Smale et al., 1997), we do not have comparable behavioral data from male offspring when they are adults. Nevertheless, we would not predict that the aggressive behavior of adult males would mirror the relationship between maternal fA concentrations and aggression seen among adult female offspring. Low social rank among adult male hyenas in their new clans after dispersal inevitably constrains their ability to behave aggressively. However, prenatal androgen exposure does appear to influence the sexual behavior of male hyenas, at least when they are juveniles. Rates of mounting behavior in juveniles of both sexes were positively correlated with maternal fA, and this relationship was much more robust

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among males (Dloniak, et al., 2006b). Mating is uniquely challenging for the male spotted hyena who must overcome both behavioral (female dominance) and physical (masculinized external genitalia of female) obstacles, and it would be interesting to determine whether variation in prenatal androgen exposure is related to male reproductive success.

When we controlled for the sex of the target of aggression in the current study, we found that maternal fA concentrations were much better predictors of inter-sexual aggression than intrasexual aggression emitted by females. This result raises the possibility that adult females exposed to high concentrations of androgens during gestation are treated differently later in life by adult males than are other females. We did not see any support for this idea in our aggression data, as males did not direct substantial amounts of aggression toward females exposed to either high or low concentrations of prenatal androgens; however, these females might still be responding to some other behavior emitted by males that we did not measure in this study. Although female hyenas are more aggressive than males overall, we have clearly shown that adult males do not lack the ability to behave aggressively. We have also presented evidence from the primate literature suggesting that female aggression in species with matrilineal dominance hierarchies need not be associated with female dominance. Therefore, it is possible that androgens function uniquely in the prenatal environment experienced by spotted hyenas to enhance female aggressiveness toward males, and thereby achieve and maintain social dominance over males. Alternatively, the relationship between maternal fA and offspring aggression directed toward males may reflect aggressive behavior in the absence of social constraints. As noted above, female hyenas have very little risk of

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retribution when directing aggression toward adult males, whereas the social context might impact their decision to attack another female, even a lower ranking one.

We emphasize that the occurrence of organizational androgen effects on adult aggressive behavior by female spotted hyenas does not by any means rule out effects in hyenas that might be induced by any of the myriad variables known to mediate aggressive behavior in females of other species. For instance, we cannot currently measure progesterone in hyena feces, yet progesterone has been linked to female aggression in other species (Davis and Marler, 2003). There are also several steroid-independent mechanisms that might be acting in spotted hyenas to mediate female aggressive behavior. Maternal aggression has received little attention in this species, and the rates of aggression we observed here during lactation may suggest an excitatory role for prolactin in this species, as occurs in some other mammals (e.g., Gammie and Lonstein, 2006; Lonstein and Gammie, 2002). There is also growing evidence demonstrating that aggression in some species is influenced by serotonin and serotonin-receptor distribution in the central nervous system. For instance, metabolites of serotonin measured in the cerebrospinal fluid are thought to reflect serotonin turnover in the brain, and low cerebrospinal fluid concentrations of serotonin metabolites are positively correlated with high-intensity aggression among male (Higley, et al., 1996; Higley, et al., 1992) and female macaques (Westergaard, et al., 2003). The possibility that one or more of these additional factors influences expression of aggressive behavior among spotted hyenas must await the attention of future investigators.

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CHAPTER FOUR

FUNCTIONS OF UNPROVOKED AGGRESSION AMONG FEMALE SPOTTED HYENAS

INTRODUCTION

The benefits associated with group living are well established (Cheney, et al., 1986; Wrangham, 1987), and gregariousness itself may convey direct fitness benefits to individuals (Silk, 2007; Silk, et al., 2003). However, group living is also associated with costs, especially those related to competition for resources (Sterck, et al., 1997; van Schaik, 1989), and this competition often involves aggressive interactions (Silk, 1993; Silk, 2002; Walters and Seyfarth, 1987). Aggression frequently occurs among conspecifics in the context of acquisition of resources, such as food, territory, or mates (Archer, 1988; Silk, 1993; Walters and Seyfarth, 1987), in defense of young (Hrdy, 1979; Silk, 1993; Walters and Seyfarth, 1987) or it may be a product of fear or frustration (Archer, 1988; Lorenz, 1970; Wingfield, et al., 2006). However, here we are particularly interested in the occurrence of aggression in the apparent absence of causal factors in the individual's immediate environment, termed unprovoked aggression. Unprovoked aggression has been investigated as a manifestation of disease or pathological anti-social behavior, as among domestic dogs (Dodman, et al., 1996; Velden, 1979; Velden, et al., 1976) and humans (Reidy, et al., 2008). However, unprovoked attacks, particularly among female conspecifics, have been described to occur frequently in a number of primate species (Silk, 2002; Smuts, 1987; Walters and Seyfarth, 1987), as well as among spotted hyenas (*Crocuta crocuta*) (Engh, et al., 2000; Smale, et al., 1993; Wahaj and Holekamp, 2006). These findings suggest that unprovoked attacks are not merely the

result of pathology. Indeed unprovoked aggression might have important fitness benefits. In this chapter we describe the unprovoked aggressive behavior that frequently occurs among adult female spotted hyenas, and examine hypotheses suggesting possible adaptive functions for this behavior.

Spotted hyenas live in large, multi-male, multi-female social groups, called "clans." Like those of many cercopithecine societies (Drea and Frank, 2003; Watts and Holekamp, 2007), the society of the spotted hyena is structured by a rigid linear dominance hierarchy based on transfer of rank among generations via maternal rank "inheritance" in which daughters slot into the hierarchy just below their mothers (Engh et al., 2000; Frank, 1986; Smale et al., 1993). Females are philopatric, but males generally disperse between the ages of 2 and 5 years (Boydston, et al., 2005; Smale, et al., 1997), and adult immigrant males are socially subordinate to all natal members of the group (East and Hofer, 2001; Engh, et al., 2002; Frank, 1986; Kruuk, 1972; Smale et al., 1993; Tilson and Hamilton, 1984).

In contrast to mammalian norms, female spotted hyenas are more aggressive to conspecifics than are adult immigrant males (Szykman, et al., 2003), and we showed in Chapter 3 that intrasexual aggression among female hyenas occurs just as frequently as does intrasexual aggression among males in a number of different contexts, including unprovoked aggression. Unprovoked attacks among spotted hyenas are characterized by seemingly spontaneous interactions in which the aggressor and the recipient have had little or no contact in the minutes preceding the attack. Both juveniles (Smale et al., 1993; Wahaj and Holekamp, 2006) and adults (Engh et al., 2000) direct unprovoked aggression

towards conspecifics, and given its frequency in the natural habitat, we think it is unlikely to reflect pathological behavior among adult females.

In cercopithecine primate societies, unprovoked aggression among females has been proposed as one mechanism for teaching rank relationships. Adult females preferentially direct unprovoked aggression toward the infants of lower-ranking females, which facilitates rank acquisition among the infants (Berman, 1980; Horrocks and Hunte, 1983). However, there was no support for this hypothesis among spotted hyenas, as rates of unprovoked aggression received by cubs do not vary with ranks of the adult female aggressors (Engh et al., 2000). Therefore, the function of unprovoked aggression in spotted hyenas, particularly among adult females, remains unexplained. Here we tested predictions of two hypotheses suggesting functional explanations for unprovoked aggression among female spotted hyenas.

Hypothesis 1: Unprovoked aggression among female hyenas functions to suppress reproduction in other females. Among female spotted hyenas, reproductive success varies with social rank (Hofer and East, 2003; Holekamp, et al., 1996). While all females bear young, higher-ranking females have longer reproductive lifespans, experience shorter inter-birth intervals, and produce more surviving offspring than do lower-ranking females (Holekamp et al., 1996). Much of this variation may be explained by access to food; for instance, inter-birth intervals among low-ranking females are longer during times of low prey availability than during periods of prey abundance (Holekamp et al., 1996; Holekamp, et al., 1999). However, in addition to differential food access, it is possible that high-ranking females may directly suppress reproductive function in subordinate females via unprovoked aggression. The eusocial naked-mole rat is an example of a

species displaying this kind of socially-mediated reproductive suppression; despotic reproductive skew is controlled in these rodents by aggression emitted by queens toward subordinate females (Bennett, 2000; Faulkes and Abbott, 1997). Moreover, targeted female aggression serving as a mediator of reproductive suppression has been shown to occur in a number of callitrichid primate species (reviewed by Solomon and French, 1997), as well as in some social carnivores (Creel, et al., 1997; McLeod, et al., 1996). Reproductive suppression mediated by coalitionary attacks has also been demonstrated in the yellow baboon, a species exhibiting sociality and reproductive skew similar to those of spotted hyenas (Wasser and Starling, 1988). If unprovoked aggression similarly represents a mechanism for reproductive suppression among spotted hyenas, then females should be the recipients of aggression most often when they are in reproductively vulnerable states (Wasser and Starling, 1988). That is, females should be targets of unprovoked aggression most frequently in early pregnancy, when the health of the mother and fetus may be compromised, and/or around the time of ovulation, when the probability of successful conception may be reduced.

Hypothesis 2: Females use unprovoked aggression to test existing rank relationships. The simple rules of rank inheritance in both cercopithecine and hyena societies provide great stability in dominance relationships over long periods of time. Given the substantial fitness advantage enjoyed by higher-ranking hyenas, improving one's rank should be highly beneficial for females. Rank reversals do occasionally occur in both cercopithecine and hyena societies (Chapais, 1985; Holekamp, et al., 1993; Mills, 1990; Smuts, 1987; Walters and Seyfarth, 1987), supporting the notion that opportunities for social climbing exist even in these largely stable societies. We propose that female

hyenas may be employing alternative strategies for testing the stability of social relationships when directing unprovoked aggression toward either lower- or higher-ranking females. Females may use unprovoked aggression directed toward lower-ranking conspecifics as a low cost reminder of the attacker's dominant status (Silk, 2002). Alternatively, individuals might use unprovoked aggression directed toward higher-ranking conspecifics to test the stability of their social relationships and thereby assess opportunities for rank advancement (Ehardt and Bernstein, 1986). This type of opportunistic testing has been proposed as a precursor to matrilineal overthrows among captive rhesus macaques (Ehardt and Bernstein, 1986). If this type of testing also occurs among hyenas, then we would expect females to direct unprovoked aggression toward targets who are close in rank to themselves, as interaction between individuals most alike in fighting ability or social standing would most likely provide opportunities for improvement in the attacker's social status (Ehardt and Bernstein, 1986; Forkman and Haskell, 2004).

Here we compare the frequency and intensity of unprovoked attacks directed toward higher- and lower-ranking targets and examine factors, such as rank and composition of the immediate subgroup (Silk, 2002), which might influence the likelihood of attack. We predict that, when unprovoked attacks occur in the presence of bystanders, females directing aggression toward lower-ranking females should do so in groups containing other higher-ranking individuals, who might provide support to the attacker (Engh, et al., 2005; Smith, et al., 2009). Alternatively, the likelihood of attacking a higher-ranking female should be positively correlated with the number of females

lower-ranking than the attacker, as these females also have something to gain, and might provide aid when challenging dominant individuals.

METHODS

Study site and subjects

A single large clan of spotted hyenas living in the Talek region of the Masai Mara National Reserve was the subject of a longitudinal study of hyena behavior and ecology. Here we used data collected between July 1988 and December 2005; during this period all clan members were individually recognized by their unique spot patterns, and were sexed based on penile morphology (Frank, et al., 1990). Females over 2 years of age were considered adult members of the dominance hierarchy, which was updated annually. During the study period, the number of adult females in the clan ranged from 16 to 34 (mean = 23.2) individuals. Offspring joined the dominance hierarchy below their mothers but above their older siblings (Engh et al., 2000; Holekamp and Smale, 1991). By convention the highest-ranking female in the clan was assigned a rank of 1.

Reproductive histories were known here for all adult females, and only parous females were used in this study. Birth dates (± 7 days) were assigned based on appearance and behavior of cubs when they were first seen (Holekamp et al., 1996). Gestation in this species is 110 days (Kruuk, 1972), so counting backward from dates of parturition, we could estimate dates of conception. To compare rates of behavior among phases of gestation, pregnancy was divided into trimesters; early pregnancy (P1), middle pregnancy (P2), and late pregnancy (P3). Duration of lactation is highly variable in this species and can range from 7.5 to 24 months, and lower-ranking females take longer to

wean litters than do higher-ranking females (Holekamp et al., 1996). Lactation intervals began at birth, and lasted until weaning of the litter or the conception of the next litter, whichever came first; incomplete lactation intervals caused by litter death were not considered here. We assigned wean dates (± 10 days) based on observed nursing conflicts between mothers and cubs, and the cessation of nursing, as has been previously described (Holekamp et al., 1996; Holekamp et al., 1999). We partitioned each lactation cycle (L) into thirds, and since many females conceive their next litter prior to weaning the last (Holekamp et al., 1996), we omitted the last third of lactation, in this way we controlled for the possibility that females had begun cycling during this time. Very little is known about ovulation in this species, but it is estimated to occur in a 14 day cycle (Kruuk, 1972; Lindeque, 1981; Matthews, 1939). We focused on the 30 days prior to a known conception as a reproductive period during which females were potentially cycling (C).

Behavioral observations

Spotted hyenas live in a fission-fusion social system, and clan members spend the majority of their time in small subgroups, the size and composition of which changes many times a day (Kolowski, et al., 2007; Smith, et al., 2008). Therefore, it is important to control for the variation in availability of social partners available with which an individual might interact. We collected daily behavioral data during morning (0530 – 0900 hours) and evening (1700 – 2000 hours) observation sessions on groups of two or more adult female hyenas that were separated from other hyenas by at least 200 m. Identities and social ranks of all hyenas in each group were known and recorded, and a location was assigned to each session (communal den, kill scene, or "other" location). Sessions lasted at least 15 minutes and ended when we moved away from each subgroup.

We observed groups containing two or more adult females for a total of 4823 hours during 6222 observation sessions. All aggressive interactions among hyenas were recorded as critical incidents (Altmann, 1974). For each aggressive interaction we recorded the aggressor, behavior, recipient, response, and, if possible, the context in which the aggression occurred. Here we considered only dyadic aggression, occurring between one aggressor and one recipient, in an unprovoked context. Observers categorized an attack as unprovoked when the aggression occurred in absence of any apparent contested resource, and when there was little or no interaction between the aggressor and recipient leading up to the attack that would indicate provocation by the recipient. Our ethogram of aggressive behaviors included head-wave, displacement, stand over, and point as low-intensity aggressive acts; lunge, push, rush, and chase, we included as medium-intensity behaviors, and any biting attack was categorized as high-intensity. Responses to aggression included appeasement behaviors (head bob, carpal crawl, squeal, back-off) and counter-attacks.

To test the reproductive suppression hypothesis, we compared rates of unprovoked aggression received by females among different reproductive states using a repeated measures within-female design. Rates of aggression received were calculated for each female during each trimester of pregnancy (P1, P2, and P3), early lactation (L), and the period one-month prior to conception (C). Since conception dates are estimated from birth dates, we also calculated rates of aggression received for the two weeks prior and two weeks post conception. All females included in this analysis were observed in all these reproductive states, and we included only those observation sessions in which

females were seen with at least one potential attacker. Hourly rates of aggression received were calculated for every female in each session as:

$$\frac{\text{(number of unprovoked attacks acts received} \div \text{number of females present that were higher-ranking than recipient)}}{\text{number of hours in the observation session}}$$

This rate calculation thus controlled for variation in number of adult female hyenas present in a given observation session that might have attacked the target female, as well as for the amount of time each female was observed in that session. Aggression rates were averaged over all sessions during which a female was in a particular reproductive state, including those sessions when the female was seen with potential aggressors but did not receive any attacks. In this way, the mean rates of aggression received by individuals who were frequently seen with potential aggressors, but did not receive aggression, were weighted in comparison to individuals who were seen less often.

We refer to aggressive acts by a lower-ranking aggressor toward a higher-ranking recipient as aggression directed "up the hierarchy," and aggressive acts emitted by a higher-ranking aggressor toward a lower-ranking recipient, as attacks "down the hierarchy." To examine the rank relationships among aggressors and recipients involved in unprovoked aggression directed both up and down the hierarchy, we calculated the rank distance for all interacting dyads in two ways. First, we used an absolute rank distance, which was the difference between the aggressor and the recipient's overall rank position in the current female dominance hierarchy. Second, we calculated a relative rank distance, which was the difference in rank between the aggressor and recipient relative to

the other adult female hyenas present in the immediate subgroup at the time of the attack. We used multivariate techniques to investigate whether rank and subgroup composition influenced the likelihood of attack. To assess the influence of subgroup composition, we analyzed all observation sessions in which both members of an interacting dyad were present and asked whether the number of adult females higher- or lower-ranking than the aggressor influenced the likelihood of an attack occurring within the dyad. We predicted that higher-ranking bystanders would positively influence the likelihood of attacking down the hierarchy, while lower-ranking bystanders would positively influence the likelihood of attacking up the hierarchy.

Statistical analyses

We used a repeated measures design to assess changes in rates of aggression received when females were in each reproductive state (P1, P2, P3, L, and C) and to compare unprovoked aggression directed up and down the hierarchy. Since a value of zero was often a meaningful datapoint, indicating that an individual was present with potential aggressors but was not attacked, our dataset contained enough zero values that data distributions were highly skewed, and therefore did not meet basic parametric test assumptions. Therefore, we assessed these data with nonparametric statistical tests using SPSS 15.0.0 (SPSS). We first used a Friedman ANOVA omnibus test, then used Wilcoxon signed-ranks tests for post-hoc comparisons. All tests were two-tailed (α = 0.05) and we corrected for multiple testing using the sequential Bonferroni adjustment (Rice, 1989); we report only adjusted p-values. We investigated the influence of aggressor rank, controlling for number of targets, on the rank distance within dyads with

an analysis of covariance (ANCOVA). We present means ± standard errors throughout; all graphs were made with Sigma Plot 8.0 (StatSoft).

To investigate whether the composition of the immediate subgroup influenced the likelihood of an attack within interacting dyads we used generalized estimating equations (GEE), a technique for fitting generalized linear models to clustered data (Faraway, 2006; Liang and Zeger, 1986). All observation sessions in which the interacting dyads were seen represented the clustered units, and we categorized the occurrence of an attack as a binomial response (attack occurred = 1, attack did not occur = 0). These models were fit using geepack in R 2.4.1 (R Development Core Team).

RESULTS

As has been previously described (Chapter 3) aggression among adult female spotted hyenas occurs in a number of different "provoked" contexts, such as feeding, defense of young, or in response to "pesky" behavior by conspecifics; contexts for interactions were classified as "unknown" when it was unclear due to poor visibility. Aggressive acts were only classified as unprovoked when they occurred under conditions of good visibility, in the absence of contested resources, and with no interaction between the aggressor and the recipient leading up to the attack. Frequently the recipient of unprovoked aggression was sleeping, resting or nursing their cubs prior to the attack. Hyenas engaging in unprovoked attacks were commonly new arrivals to the subgroup, and they had therefore had no opportunity for interaction with the recipient before the attack.

We witnessed a total of 3762 aggressive acts among adult females, of which 614, or 16.3%, were classified as unprovoked. We found that the large majority of unprovoked attacks occurred at dens (Figure 4.1), which are communal locations where females raise their young, and serve as social centers for clan members (Holekamp and Smale, 1998; White, 2007). The communal den is a complex of burrows used by mothers to house young cubs. The narrow diameter of the burrow opening allows entry to cubs, but not to adult hyenas, and mothers frequently lie in the den opening to nurse. Therefore we considered the possibility that the den itself might serve as a limited resource and thus provoke conflict. As the only females who use den entrances for nursing have young cubs (Kruuk, 1972; White, 2007), we compared rates of aggression emitted and received by females at the den when they did or did not have a cub aged less than 3 months. Using a within-female comparison (N = 41 females), we found no significant difference in rates of aggression emitted (Wilcoxon $Z = -1.69$, $p = 0.091$) or received ($Z = -0.72$, $p = 0.471$) by females at the den when they did or did not have a young cub. We concluded that this aggressive behavior occurred without provocation, and in the absence of obvious competition for resources including den access.

Testing the reproductive suppression hypothesis

To address this hypothesis, we compared the rates of unprovoked aggression received by 31 females observed in every reproductive state (P1, P2, P3, L, and C) (Figure 4.2). There was an overall significant effect of reproductive state on aggression received by these females (Friedman ANOVA $X^2_{4,29} = 33.98$, $p < 0.001$). This hypothesis predicted that females should receive higher rates of aggression earlier rather than later in gestation, when the fetus is most vulnerable; however, a Friedman ANOVA comparing

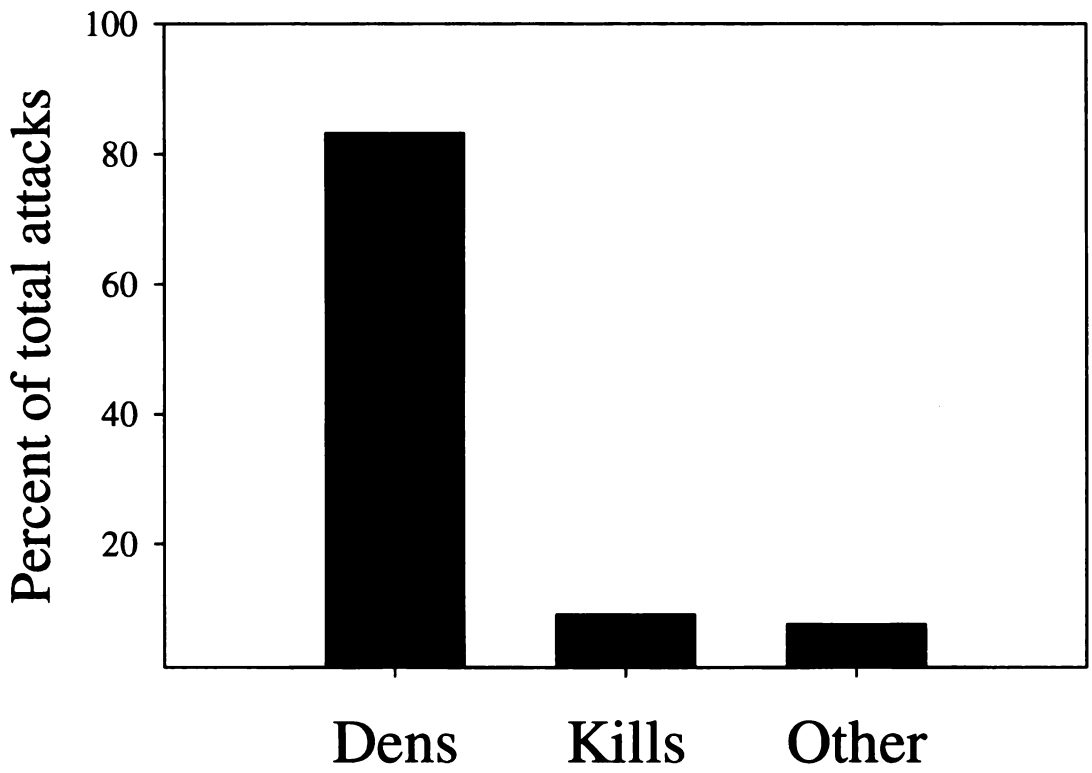

Figure 4.1. Percent of all unprovoked aggressive acts (n = 614) observed at each location.

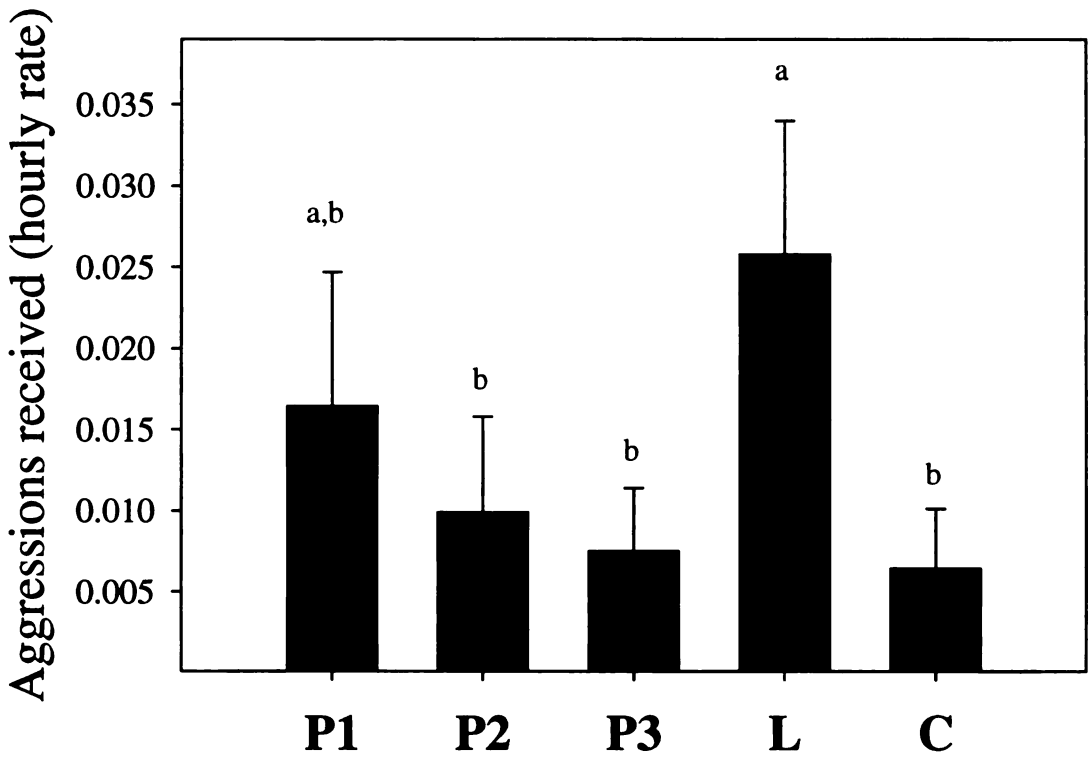

Figure 4.2. Mean (± standard error) rates of unprovoked aggression received by females (n = 31) when they were in each reproductive state, where P1, P2, and P3 refer to successive trimesters of pregnancy, L refers to early lactation, and C indicates the month prior to a known conception. Different letters above the bars represent significant differences among the states.

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P1, P2, and P3 was not significant ($X^2_{2,29} = 2.33$, $p = 0.311$), indicating that there was no significant variation in rates of aggression received among the three phases of pregnancy. It also predicted that females should receive high rates of aggression while cycling when conception might be disrupted; however, rates of aggression received prior to conception did not differ from those received during any other reproductive state except lactation, and here aggression received during lactation was significantly higher than rates received one month prior to conception (Wilcoxon $Z = -3.34$, $p = 0.004$). We also measured rates of aggression received for the period ± 2 weeks from our estimated conception dates; these were also significantly lower than rates received during lactation (Wilcoxon $Z = -4.08$, $p < 0.001$), but were not significantly different from rates measured during the month prior to conception (Wilcoxon $Z = -0.357$, $p = 0.721$). As these results were inconsistent with predictions of the reproductive suppression hypothesis, it could be rejected.

Females use unprovoked aggression to test existing rank relationships

The aggressor was higher-ranking than the recipient in 576, or 93.8% of 614 unprovoked attacks, and lower-ranking than the recipient in only 38, or 6.19% of these attacks. We compared the intensity of unprovoked aggressive acts emitted by females directed up the hierarchy with those directed down the hierarchy (Figure 4.3). In this analysis we used aggressive acts emitted by 14 females who attacked females both higher- and lower-ranking than themselves. The distribution of aggressive acts among our different intensity levels varied significantly regardless of whether they were directed up the hierarchy (Friedman ANOVA $X^2_{2,13} = 9.67$, $p = 0.008$) or down the hierarchy (Friedman ANOVA $X^2_{2,13} = 12.29$, $p = 0.002$). The majority of attacks both up and down

the hierarchy were of low intensity; however, females directed significantly more aggressive attacks of high intensity towards lower-ranking females than toward higher-ranking females (Wilcoxon $Z = 2.81$, $p = 0.015$). The rarity and relatively low intensity of unprovoked attacks directed up the hierarchy suggests that this behavior may produce more severe consequences for the attacker than when attacks are directed down the hierarchy.

We first examined the 38 attacks directed up the hierarchy, which occurred within 26 dyads. The absolute rank distance among 14, or 51.85%, of these dyads was within 5 rank places, so, the majority of the aggression directed up the hierarchy occurred among closely ranked females (Figure 4.4.A). Likewise, the rank distance of the dyads calculated using only females present in the immediate subgroup at the time of the attack indicated that the majority of unprovoked aggression occurred among females that were closely ranked within the observation session (members of 19 dyads, or 70.37% were within 2 relative rank places) (Figure 4.4.B). We were interested to see if the composition of the immediate social group influenced the likelihood of attack. Both the number of females lower-ranking ($R^2 = 0.39$, $p = 0.042$) and higher-ranking ($R^2 = 0.83$, $p = 0.001$) than the aggressor were positively correlated with the total number of females present at the time of the attack. Surprisingly, the relationship between higher-ranking females and total females present was much more robust, and indeed, there was always at least one higher-ranking female other than the interacting dyad present at the time of the attack. However, the likelihood of an attack toward a higher-ranking recipient was not significantly influenced by either the number of lower-ranking bystanders (Estimate:

0.02, Wald statistic: 0.03, $p = 0.859$) or the number of higher-ranking bystanders (Estimate: 0.04, Wald statistic: 0.29, $p = 0.593$) present in the immediate social group.

We next examined the 576 attacks directed down the hierarchy, which occurred within 444 different dyads. The absolute rank distance among 186, or 41.89%, of these dyads was within 5 rank places, so, the majority of the aggression directed down the hierarchy occurred among closely ranked females (Figure 4.5.A). Likewise, the rank distance within the dyads calculated using only the females present in the immediate subgroup indicated that the majority of the aggression occurred among females that were closely ranked within the observation session (313 dyads, or 70.49% were within 2 relative rank places) (Figure 4.5.B). Again, we wanted to see if subgroup size or composition influenced the likelihood of attacking a lower-ranking female. As with attacks directed up the hierarchy, we very rarely saw an attack down the hierarchy when the interacting dyad was alone; 14 out of 576 unprovoked attacks, or 2%, occurred when the dyad was alone. Both the number of females lower- ($R^2 = 0.68$, $p < 0.001$) and higher-ranking than the aggressor ($R^2 = 0.63$, $p < 0.001$) were positively correlated with the total number of females present at the time of the attack. We found that the likelihood of attacking a lower-ranking female significantly increased with the number of higher-ranking females present (Estimate: 0.07, Wald statistic: 8.94, $p = 0.003$), and significantly decreased with the number of lower-ranking females present (Estimate: -0.16, Wald statistic: 34.45, $p < 0.001$). These results support the prediction that females directing aggression toward lower-ranking females do so when the opportunity for support from other higher-ranking females is high.

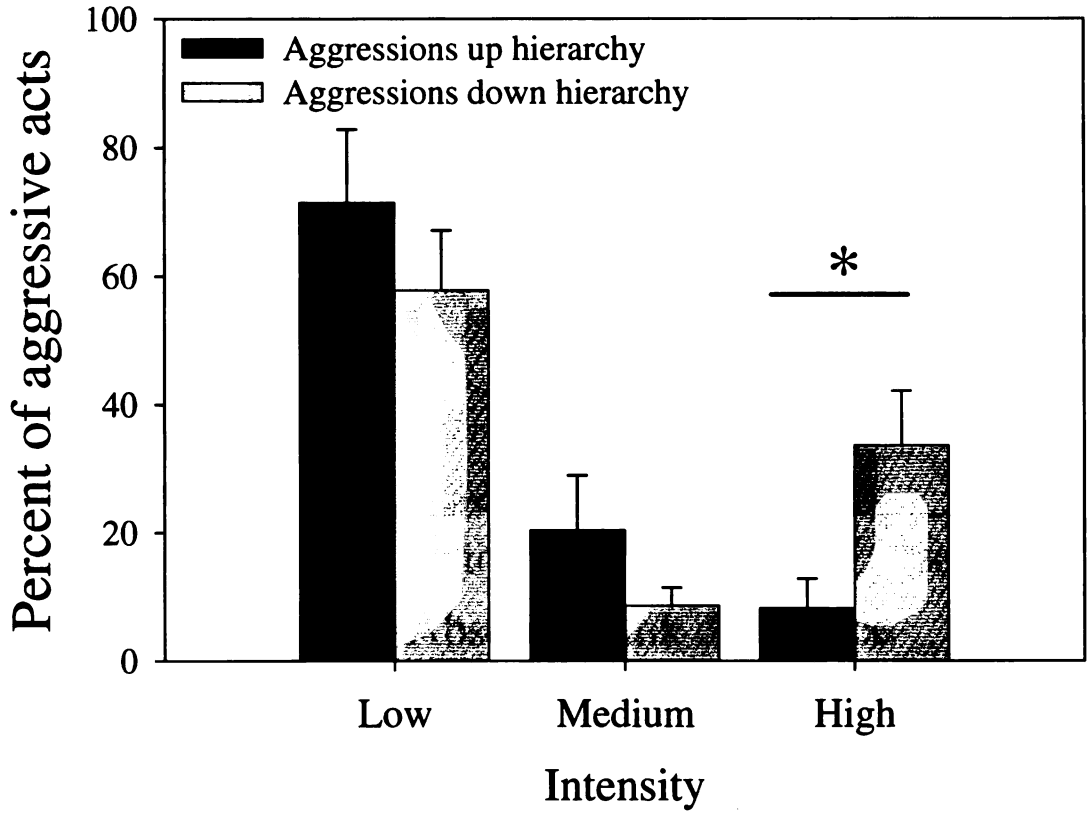

Figure 4.3. Percent of total aggressive acts in each intensity category directed by females (n = 14) towards higher-ranking recipients (black bars) or towards lower-ranking recipients (gray bars). Asterisk indicates a significant difference (p = 0.015).

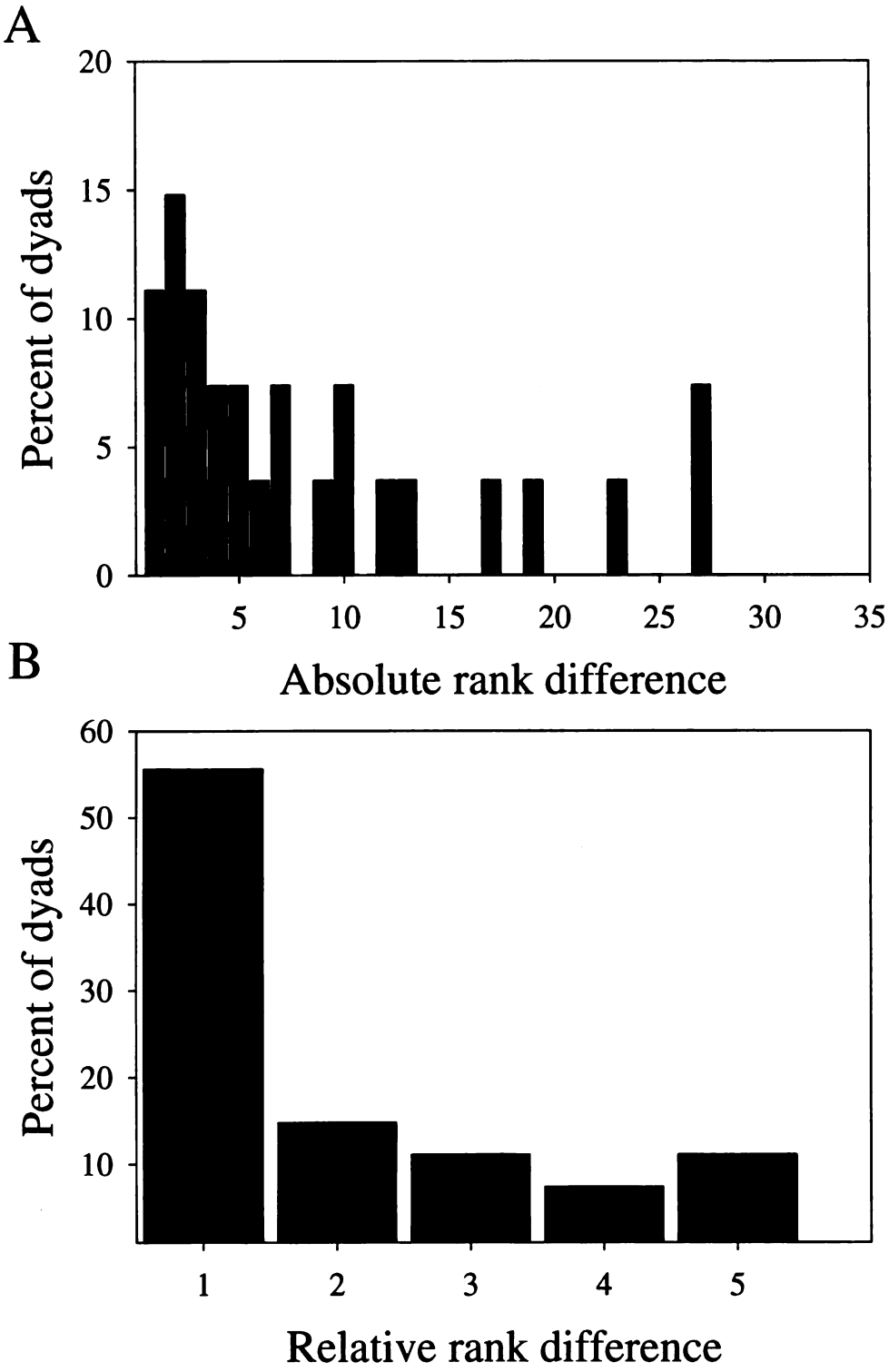

Figure 4.4. Frequency distribution showing rank distances within 26 dyads in which the aggressor was lower-ranking than the recipient. Rank distance was calculated for each dyad as the difference between aggressor and recipient in a) absolute rank in the clan, and b) relative rank among females present in the immediate subgroup.

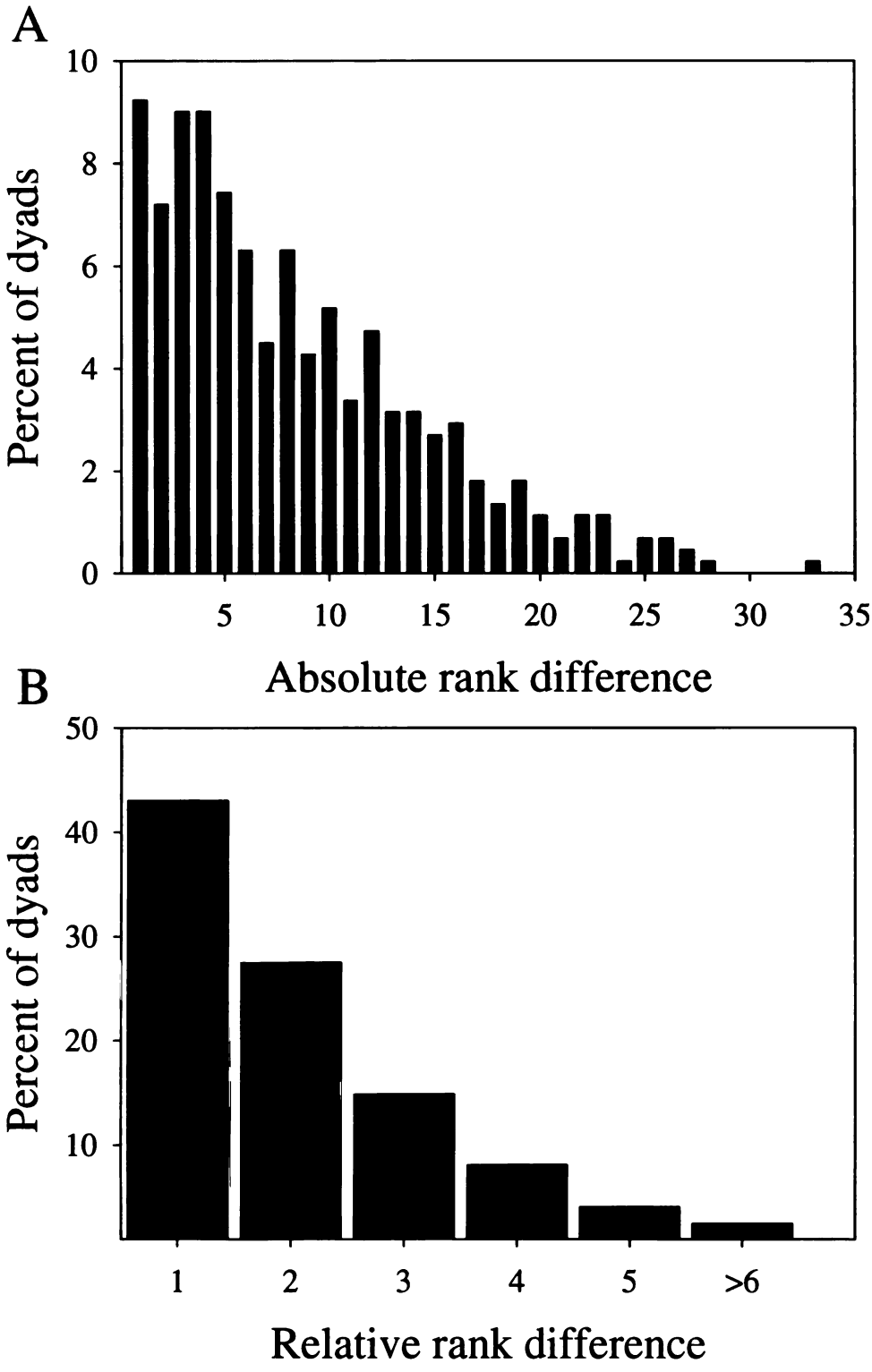

Figure 4.5. Frequency distribution showing rank distances within 444 dyads in which the aggressor was higher-ranking than the recipient. Rank distance was calculated for each dyad as the difference between aggressor and recipient in a) absolute rank in the clan, and b) relative rank among females present in the immediate subgroup.

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We used an ANCOVA to inquire whether the social rank of the aggressor influenced the absolute rank distance between the aggressor and the recipient (whole model $R^2 = 0.08$, $F = 618.93, 22592.16$, $p < 0.001$). Rank of the aggressor was a significant predictor in the model such that high-ranking females attacked females ranked farther from them in the hierarchy than did low-ranking females ($F = 17.90, 653.19.16$, $p < 0.001$). The number of targets available to the aggressor was included as a marginally significant covariate, so there was a trend for rank distance to increase as number of available targets increased ($F = 2.97, 108.41$, $p = 0.086$). However, the interaction between aggressor rank and number of targets was not significant ($F = 2.55, 93.23$, $p = 0.111$); therefore, after controlling for targets available to the aggressor, the rank distance within the dyads was not influenced by the rank of the aggressor.

DISCUSSION

A substantial proportion of aggressive acts among adult female spotted hyenas cannot be immediately related to conflict over resources such as food or dens, defense of young, or any other provocative behavior emitted by the recipient prior to the attack. Nearly one fifth of all aggressive acts emitted by females are unprovoked, which is second only to fights over food. Here we tested predictions of two hypotheses suggesting functional explanations for the occurrence of unprovoked aggression among adult females. We did not find any support for the hypothesis suggesting females were using unprovoked aggression to suppress the reproduction of subordinates, but our data were consistent with the hypothesis suggesting that this behavior functions to test existing rank relationships.

Reproductive suppression hypothesis

An important cost associated with group living is the increase in competition for resources necessary for reproduction (Sterck et al., 1997; van Schaik, 1989). Females may be able to derive fitness benefits for themselves and their offspring by suppressing the reproductive efforts of others (Creel and Wasser, 1997; Wasser and Barash, 1983; Wasser and Starling, 1988). Social harassment is one behavioral mechanism known to suppress reproduction among conspecifics in cooperatively breeding species (Creel et al., 1997; Faulkes and Abbott, 1997; McLeod et al., 1996). In contrast to most other social carnivores (Moehlman, 1997), spotted hyenas are not cooperative breeders, but they do experience reproductive skew associated with social rank. As higher-ranking female spotted hyena out-perform lower-ranking females on several measures of fertility (Hofer and East, 2003; Holekamp et al., 1996), we looked for evidence suggesting that females might use unprovoked aggression to hinder the reproductive efforts of lower-ranking individuals. However, we found no evidence that females in reproductively vulnerable states were preferentially receiving unprovoked aggression from dominants. Wasser and Barash (1983) suggested that reproductive physiology is easiest to disrupt early in the reproductive effort; therefore, the reproduction suppression hypothesis predicted that females would receive higher rates of aggression early, rather than late in pregnancy, but rates did not vary among trimesters. Among baboons, cycling females were shown to receive the highest rates of aggression (Wasser and Starling, 1988); however, among female spotted hyenas rates were not elevated around the time of conception, when females were presumed to be cycling. As reproductive success is strongly connected to social rank in this species (Holekamp et al., 1996), and as rank determines priority of

access to food (Frank, 1986; Holekamp, et al., 1997b; Kruuk, 1972; Tilson and Hamilton, 1984), energy availability (Bronson, 1989) is likely a sufficient explanation for the lower reproductive output seen among lower-ranking female hyenas, and additional suppression of reproduction via unprovoked aggression is unnecessary. Consistent with this idea is the fact that the study clan experiences an annual peak in conceptions that coincides with an influx of prey species into the Masai Mara during the annual wildebeest migration (Holekamp et al., 1999).

Challenging the status quo

As we have discussed, high social rank conveys a reproductive advantage associated with access to resources in this species; therefore, females should be motivated to elevate their position in the dominance hierarchy if presented with an opportunity to do so (Smuts, 1987; Walters and Seyfarth, 1987). We proposed that unprovoked aggression might serve as a tool for assessing such opportunities among adult females, and examined the frequency and intensity with which adult females attacked higher-ranking females. Female dominance relationships within this clan have been extremely stable over many years (Engh et al., 2000; Frank, 1986; Holekamp et al., 1993), and by 2 years of age, a female's position in the dominance hierarchy is well established (Holekamp and Smale, 1993; Smale et al., 1993). Thus, it is perhaps not surprising that we recorded very few cases of attacks directed up the hierarchy, and these attacks were of relatively low-intensity. This result is consistent with prior work demonstrating that aggression directed up the hierarchy occurs infrequently in this species (Chapter 3; Engh et al., 2005), suggesting that there are severe risks involved in attacking higher-ranking individuals. The large majority of unprovoked attacks directed up the hierarchy occurred among

females close together in rank, suggesting that, if rank reversals within the hierarchy did occur, they would most likely occur between females ranked close together in the clan's hierarchy. Although we did not see an influence of subgroup composition on the likelihood of attacking a higher-ranking conspecific, we believe it would be premature to conclude that females ignore the subgroup composition when attacking higher-ranking individuals. The non-significant results obtained here may simply reflect inadequate statistical power, and larger sample sizes may be required to confirm or reject this idea.

Rank reversals have been reported among adult female spotted hyenas living in the wild (East and Hofer, 2002; Henschel and Skinner, 1987; Hofer and East, 2003; Holekamp et al., 1993; Mills, 1990; Tilson and Hamilton, 1984; White, 2007), but due to the rarity of these reversals, the factors contributing to them remain unclear. Rank reversals are also reported anecdotally in the cercopithecine primate literature. Reversals among female baboons have been associated with the advanced age of the unseated female (Hausfater, et al., 1982), suggesting that elderly females may not be able to maintain their dominance over would-be usurpers. Several primate studies report that rank reversals occur after the loss of a key figure in the deposed female's social network (Chapais, 1985; Ehardt and Bernstein, 1986; Hausfater et al., 1982; Raper, et al., 2006). These scenarios suggest that even in these societies in which social rank is "inherited," and dominance hierarchies are relatively stable, stochastic events may promote weaknesses in the dominance hierarchy, and a tool for probing for weakness would be valuable. Unprovoked aggression might function as such a tool.

Maintenance of the dominancy hierarchy

Our results suggest that most unprovoked aggression serves the same function among adult spotted hyena females as it serves in many cercopithecine primate societies (Silk, 1993; Silk, 2002; Smuts, 1987; Walters and Seyfarth, 1987), which is to maintain existing dominance relationships. Among adult female spotted hyenas, most unprovoked aggression occurs downward in the existing dominance hierarchy, and among individuals close together in rank, suggesting that females are using unprovoked aggression to reinforce the status quo. Despite the fact that higher-ranking females have a wider range of targets available to them, when we controlled for opportunity we saw that both high- and low-ranking females attack conspecifics that are close to themselves in rank. This suggests that closely ranked females may pose the biggest threat with respect to rank-reversals, and that these relationships therefore need the most maintenance and attention (Forkman and Haskell, 2004).

In the fission-fusion society of spotted hyenas, lower-ranking females are less gregarious and spend more time alone than do higher-ranking females (Boydston, et al., 2003; Holekamp, et al., 1997a; Smith et al., 2008; Smith, et al., 2007), and it is difficult to assess the frequency with which particularly solitary clan members interact. Holekamp *et al.* (1993) describe several events in which females returned to the clan's home range after small absences of one to three months with little or no penalty; however, clan members that returned after absences of six months or more experienced a drop in rank and elevated rates of aggression. Therefore, mechanisms for maintaining the dominance hierarchy might be particularly critical in this dynamic social system, and hierarchy

maintenance appears to be a life-long necessity (Silk, 1993; Silk, 2002; Smuts, 1987; Walters and Seyfarth, 1987).

In addition to understanding how the relationship between the aggressor and the recipient influences unprovoked aggression, we were also interested in how the immediate social environment might affect the likelihood of attack. Only 2% of unprovoked attacks down the hierarchy occurred when the interacting dyad was alone, which suggests that the audience of an unprovoked attack may be nearly as important as the recipient of the attack (Zajonc, 1965; Zuberbuhler, 2008). An example of this type of audience effect was demonstrated in a group of wild chimpanzees (*Pan troglodytes*). Victims of aggression enhanced their recruitment screams when in the presence of an individual higher-ranking than their attacker, as intervention by a third party generally occurs in support of the victim among chimpanzees (Slocombe and Zuberbuhler, 2007). However, among spotted hyenas, females tend to intervene on behalf of the higher-ranking female (Engh et al., 2005; Smith, et al., 2007) indicating that higher-ranking bystanders usually represent allies for the attacker rather than the recipient. We also found that females are more likely to attack down the hierarchy when the presence of lower-ranking females, other than the recipient, is low, suggesting that lower-ranking bystanders might be potential allies for the attacked female. The positive relationship between the probability of attack and the number of higher-ranking bystanders seen here in dyadic aggression is opposite to that seen during coalitionary aggression, in which two or more hyenas attack a single target animal. In coalitionary aggression the likelihood of a female joining an on-going dyadic fight decreases as the number of higher-ranking bystanders increases (Smith et al., 2009). It may be that higher-ranking bystanders

perceive dyadic aggression toward lower-ranking females as situations in which intervention is unnecessary, whereas coalitionary aggression might represent escalated aggression in which benefits accrue to higher-ranking females that intervene to "police" or terminate the aggressive interaction (Slocombe and Zuberbuhler, 2007).

Silk (2002) suggests that recipients of unprovoked aggression cannot predict when attacks will occur, how long they will last, or when the threat has ended. This creates continuous uncertainty among subordinate individuals, and this uncertainty may represent a form of social stress. Social stressors may be potent stimulators of the hypothalamic-pituitary-adrenal (HPA) axis, often measured as an increase in circulating glucocorticoids (GC) (Sapolsky, 1982; Sapolsky, 2002; Sapolsky, 2005). Acute activation of the HPA axis, and short term release of GC concentrations from the adrenals is an adaptive part of the "fight or flight" system; however, chronic elevation of GC concentrations can lead to pathology, including compromise of cardiovascular, immune, neural, and reproductive function (Sapolsky, 2002). If the hypothesis that unprovoked aggression negatively influences the stress physiology of recipients is true (Silk, 2002), we might predict that subordinates would have higher GC concentrations than would dominant individuals. While there does not appear to be a strong relationship between social rank and individual variation in GC concentrations among female hyenas, a study has demonstrated that fecal GC concentrations were elevated among females after a serious fight (Goymann, et al., 2001). Although these authors did not indicate who won these conflicts, it seems reasonable to assume that recipients of unprovoked aggression might experience acute activation of the HPA access. To conclude, we find that

unprovoked aggression is a useful means for testing the stability of social relationships, with minimal risk for the attacker, and a potentially large cost to the recipient.

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