ANDROGEN AND SEROTONIN CONCENTRATIONS IN SPOTTED HYENAS (*CROCUTA CROCUTA*): PHYSIOLOGICAL PREDICTORS AND RELATIONSHIPS WITH THE SOCIAL ENVIRONMENT

By

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A DISSERTATION

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

Integrative Biology—Doctor of Philosophy
Ecology, Evolutionary Biology, and Behavior—Dual Major

2019

ABSTRACT

ANDROGEN AND SEROTONIN CONCENTRATIONS IN SPOTTED HYENAS (*CROCUTA CROCUTA*): PHYSIOLOGICAL PREDICTORS AND RELATIONSHIPS WITH THE SOCIAL ENVIRONMENT

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Animals display a wide range of social behaviors, and an individual's ability to express the 'right' behavior in the appropriate context is vital to its success. Neuroendocrine systems are key in allowing individuals to match their behavior to their current situation, as these systems affect behavior and are also responsive to changes in the environment and in an organism's internal state.

Serotonin (5-HT), a neuromodulator, and androgenic sex steroid hormones are key regulators of aggression and may play central roles in matching an organism's aggressive behavior to its social environment. In many species living in dominance hierarchies, an individual's social status affects both its ability to behave aggressively and its concentrations of 5-HT and androgens. Often, 5-HT inhibits aggression, whereas androgens facilitate it; accordingly, social dominance is often negatively correlated with 5-HT and positively correlated with androgens. Still, such patterns are not universal, and understanding how they're modulated remains a key question.

Here, I examine the physiological, demographic, and environmental correlates of serotonin and androgens in a despotic, female-dominant species, the spotted hyena. I focused on the relationship between social rank and serotonin/androgens, and its potential modulators. I first examined predictors of two androgens, testosterone (T) and androstenedione (A4), in pregnant female hyenas. I found a positive relationship

between both androgens and social rank in multiparous but not primiparous females, apparently due to an increase in maternal androgens in high- but not low-ranking females after the birth of their first litter.

I then examined the relationship between T and social status in adult females across reproductive states and social contexts, I found a positive correlation between T and social status in lactating and pregnant females, but not in nulliparous females; again, this was apparently due to an increase in high-ranking female T concentrations associated with their first breeding experience. In a comparison of T concentrations in lactating females during periods of social stability and instability, I found that T concentrations in low- but not high- ranking females increased during unstable periods, resulting in the disappearance of the positive correlation between social rank and T.

Finally, I examined predictors of 5-HT concentrations in male and female hyenas across ontogeny. 5-HT was negatively correlated with social rank in female but not male spotted hyenas. Social status at birth was particularly predictive of female 5-HT, as opposed to status at the time of physiological sampling, indicating early life social environment may have lifelong effects on serotonergic function.

Overall, I found that social status was an important predictor of androgen and serotonin concentrations, but that this relationship was dependent upon characteristics of the individual (e.g. sex, life history stage) and their social environment.

ACKNOWLEDGEMENTS

Many organizations contributed financial and logistical support to this project. This research was supported by National Science Foundation (NSF) grants to Kay Holekamp, and by an NSF Graduate Research Fellowship to me. I was also supported by grants from the Sigma Xi foundation and the College of Natural Sciences, the Graduate School, and the Ecology, Evolutionary Biology, and Behavior program at Michigan State University. I also thank the Kenya National Council for Science, Technology, and Innovation and the Kenya Wildlife Service, the Narok County Council, and the Senior Warden of the Maasai Mara National Reserve for the permissions to carry out this research.

This work would not have been possible without the contributions of individuals too numerous to list. To begin, I would like to thank my advisor Dr. Kay Holekamp for her mentoring and support through this process. She has been generous with her knowledge, time, and resources even while having many graduate students in her lab and other departmental responsibilities. She has fostered a collaborative lab atmosphere with many students engaging in independent research, and I consider myself lucky to have spent time in such a lab. I also thank my committee members, Drs. Laura Smale, Thomas Getty, and Mark Breedlove, for their support and intellectual contributions to this research. They likely do not know the positive impact on both my morale and my research even brief interactions with them have had, and I consider myself lucky to have had the opportunity to work with them. I also would like to thank Tracy Montgomery, Robert Burnett, Dr. Stephanie Watts, Dr. Jeffrey French, and Dr.

Jacinta Beehner, who were instrumental in the collection and analysis of the physiological data used in this dissertation.

Both past and present members of the Holekamp lab were integral to this research in terms of data collection and sharing, logistical and emotional support, and intellectual contributions. It is impossible to name them all, but Lily Johnson-Ulrich, Tracy Montgomery, Eli Strauss, Kenna Lehmann, Julia Greenberg, David Green, Julie Turner, Kevin McCormick, Katy Califf, Leslie Curren, Eli Swanson, Hadley Couraud, and Marc Wiseman have been important collaborators as well as people in my life. In particular, Tracy put in painstaking work to provide the data on testosterone used in this dissertation. Eli Strauss spent hours discussing my work with me and provided data on changes in dominance status. Both Tracy and Eli were also hugely instrumental in the preparation for my dissertation defense. Julia Greenberg spent an enormous amount of time editing multiple drafts of each chapter and discussing data analysis with me. She was a pillar of support for me throughout graduate school and I will always be grateful to her. Furthermore, John Graham, Julia, Tracy, and Eli housed me for weeks on end when I did not have a place to stay near the end of my dissertation. All of these people, in addition to Kenna, Lily, Kevin, Marc, and others spent hours discussing my research with me and offering food, (cough, Kevin), commiseration, and other support. I cannot emphasize enough how much support I have received from my friends and colleagues I met through this lab, and they are without a doubt among the best people I have had the privilege to meet, let alone work with.

I'd also like to thank members of our Kenyan team, including Joseph Kamaamia, Philomen Naigurian, Moses Naigurian, George Kilinyet, Jackson Kamaamia, Benson

Pion, for taking amazing care of all of us in the field. Their constant positive, fun, and welcoming presence has a made us all feel at home in the field, and I could not imagine our research group without them.

I also thank my colleagues and friends in the wider community. In particular Ash Zeminick has been an unwavering pillar of support. Ash's belief in me, and their willingness to include me in Project Biodiversify, along with Marjorie Weber and Alex Webster, has been a huge part of my personal and professional development. They, along with other friends, including Dennis Wayne (Cliff), Katherine Gleason, Emily Dittmar, Bethany Gross, and Siri and Issa Okamoto, have been a main source of social and emotional support during particularly difficult periods in my life. Finally, I thank Hayley Turner who was always there to make me a cup of tea should I need it, ensure that I ate, slept, and even on occasion had some fun. She has believed in me every step of the way and been more excited by my successes than even me. I also cannot resist thanking some dogs, including Bella, Alce, Juno, Iris, and, most importantly, Oli, who has been with me through thick and thin.

Finally, I want to thank my (human) family, Cindy Jones, Stephen Jones, Stephanie Jones, and our most recent addition, David Zee, for always being there for me and encouraging me to follow my own path and not to sell myself short. They have helped me in too many ways to list. Aside from the taking on the challenge of keeping me alive for this long, which I hear is a monumental task in itself, they are always a phone call away and ready to provide shoulders to cry on, jokes, care packages, life advice, etc. Stephanie and David have always been available with understanding ears and good advice, both having completed doctorate programs themselves, and

Stephanie was often the only person who was able to make me think this was doable. My mom and dad are two hugely generous, hard-working people that I strive to emulate. My dad can make people laugh in the direst of situations, and my mom can make someone feel loved even at their lowest. Both have made it possible for me to prioritize education through their overwhelming support, and any successes I have had or will have are due to this. Finally, my late grandfather, George Jones, provided me financial support, through financial resources left to me in his will, that was integral in helping me complete my dissertation in the final years. Without him and my grandmother's (Christine Jones) thoughtfulness, generosity, and love, the completion of this work might not have been possible. For all of this, thank you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

General Introduction

Introduction

Social behavior plays a crucial role in life; it impacts mating, parental care, access to resources and shelter, and thus has important fitness consequences in all animal species. However, the consequences to an individual for expressing various social behaviors will vary with both an individual's characteristics and its environment. For instance, in many group-living species, exhibiting aggression may often be costly for animals of low social status. However, if a low-ranking individual is faced with an opportunity to rise in social status, a rapid increase in aggression could allow them to defeat social rivals, and thus could be highly adaptive. In turn, the animal's own physiological state (e.g. physical condition or reproductive state) may influence the tradeoffs associated with high-risk behaviors such as aggression. It is clear then that the ability for social animals to fine-tune their expression of social behaviors to the social environment has considerable impacts on fitness. More generally, they must show behavioral plasticity, or the ability to adapt their behavior and physiology to match environment. This can happen via transient changes to behavior/physiology in response to shifts in the environment (activational plasticity) or long-term changes, often in response to the early life or even the maternal environment (developmental plasticity).

A central goal of behavioral ecology is to uncover the physiological mechanisms enabling such plasticity. Considerable work to date has identified the neuroendocrine system as a primary physiological system by which animals respond plastically to their environment (reviews include Kelly and Vitousek, 2017; Oliveira, 2009; Meylan et al., 2012; Wingfield, 2008). In this dissertation, we examine how two key regulators of social behavior, specifically aggression, vary with characteristics of both the individual

and the social environment in spotted hyenas, a social mammal living in despotic hierarchies, where matching one's (aggressive) behavior to one's social status likely has overwhelming impacts on individual fitness.

In social animals, repeated aggressive interactions among group members result in stable asymmetric relationships, resulting in the formation of a dominance hierarchy. Although the most dominant individuals are not necessarily the most aggressive, higher-ranking animals consistently defeat lower-ranking animals in agonistic encounters. While aggression is usually necessary for hierarchy formation, a stable, established hierarchy can suppress intense aggression and costly fights among group members over resources (Schjelderup-Ebbe, 1922). Nevertheless, the exertion of social control is necessary to prevent cheating (Taborsky and Taborsky, 2015); that is social rank often needs to be persistently reinforced with aggression emitted by dominant individuals. This can be especially true for animals living in despotic hierarchies, where the distribution of key resources among dominant and subordinate individuals is steep and asymmetrical (Theirry, 2013). Overall, aggressive behavior is often a critical component of social dominance, and an individual's ability to match its aggressive behavior to its dominance status, or alternatively, to take advantage of situations that allow it to move up the hierarchy or "cheat" (i.e. demonstrate behavioral plasticity) is of critical importance. (reviewed in Holekamp and Strauss, 2016).

Both the brain serotonin system and androgenic steroid hormones have been implicated in the neural mechanisms of behavioral plasticity. Specifically, they are thought to modulate social behaviors such as aggression and dominance by influencing the motivational state of an animal. Serotonin (5-hydroxytryptamine or 5-HT) is a

neuromodulator that plays a key role in inhibiting aggression. Studies across vertebrates find that long-term exposure to 5-HT decreases the likelihood that an individual will behave aggressively toward conspecifics (Carrillo et al., 2009). Defeat in social competition often increases brain concentrations of serotonin, and subordinate animals in many study systems have chronically elevated serotonin levels in the brain (Backström and Winberg, 2017; Kiser et al., 2012; Summers, 2002; Veenema, 2009; Winberg et al., 1991). On the other hand, androgens such as testosterone play a well-known facilitative role in expression of aggression (reviewed in Oliveira, 2009; Rosvall et al., 2019; Wingfield et al., 2001; Soma, 2006). For instance, they can prime individuals to aggressively respond to threat by modulating the processing of threat stimuli (reviewed in Montoya et al., 2012); in humans, administration of exogenous testosterone increased neural response towards angry faces in brain circuitry key to facilitating reactive aggression (Hermans et al., 2008).

Although the role of serotonin and androgens in mediating plasticity in social behavior has been extensively studied in lab settings (e.g. Maruska, 2015), natural studies relating functioning in neuroendocrine systems to the social environment and/or behavior are still lacking. Furthermore, the study of the physiological correlates of social competition is often conducted in males rather than females. In this dissertation, I examined socioecological correlates of serotonin and androgens in a wild population of spotted hyenas, a ‘sex role-reversed’ species in which females are socially dominant to and emit higher rates and intensities of aggression than do males (Szykman et al., 2003). Androgens are already thought to play a key role in the proximate mechanisms

of female dominance and aggression in this species (Dloniak et al., 2006; French et al., 2013; Holekamp et al., 2013)

Spotted hyenas live in highly despotic, female-dominant linear hierarchies in which high-ranking individuals are much more able to emit aggression, and therefore control access to limited/ephemeral food sources, than low-ranking animals. For females especially, social rank has important consequences on fitness, and aggression is important in terms of acquiring and maintaining one's status the social hierarchy. Because androgens and serotonin are thought to play a central role in mediating individual aggressive and dominance-related behaviors, I expected that androgen and serotonin levels would be linked to social status in this species. In addition to assessing the relationship between social status and androgens and serotonin, I also examined how this relationship is moderated by social context and/or individual physiology. Finally, I considered how serotonin and androgen concentrations vary with other aspects of the individual, including physiological state, sex and life-history stage. This work is organized as follows:

Summary of Dissertation

In the first chapter, I explore the determinants of the two primary circulating androgens in female spotted hyenas, androstenedione (A4) and testosterone (T), during pregnancy. Previously, studies in our system have found a positive relationship between a pregnant female's social rank and total fecal androgens (Dloniak et al., 2006). Here, I examined the relationship of T and A4 with variables, including social status, that are likely connected to social competition experienced by pregnant mothers. In light of

recent work showing that hormone-behavior relationships can depend on parity (reviewed in Bridges, 2016), I asked also whether determinants of maternal androgens vary with maternal reproductive experience (whether she is primiparous or multiparous). I found that maternal A4 and T were highly correlated and that neither were related to rates of emitted aggression, clan size, and prey density in the period of time leading up to fecal sample deposition. In primiparous females, there was no apparent relationship between social rank and maternal androgens; however, I found a positive correlation between social dominance and androgen concentrations in multiparous females. This pattern was apparently driven by an increase in maternal androgens after first parity for high- but not low-ranking females.

In Chapter 2, I explore the relationship between social dominance and testosterone in female hyenas across different reproductive states and social contexts. I first examined the relationship between rank and testosterone in nulliparous, lactating, and pregnant females. I then investigated whether social instability affects the relationship between testosterone and social rank in lactating females, the group for which I had the largest data set available.

I found a positive relationship between testosterone and social rank in lactating and pregnant, but not nulliparous females. Reflecting the results of chapter 1, this was apparently driven by an increase in testosterone in high-ranking females associated with their first breeding experience.

In lactating females, I found that the relationship between testosterone and rank disappeared during periods of social instability. This appeared to be largely driven by an

increase in testosterone concentrations of low-ranking females during periods of social instability.

I first placed the third chapter within the broader context of a review on the relationship between the serotonergic system, aggression, and social dominance across species. I then examined demographic and physiological correlates of serum serotonin, considered a biomarker for central serotonergic function (e.g. Nakatani et al., 2008), in female and male hyenas. Finally, I investigated serotonin's relationship with social dominance and individual aggression levels. I found that, while blood serotonin was relatively stable over individual lifetime, it did not correlate with individual lifetime average rates or intensities of aggression. I found a relationship between female but not male social rank and serotonin. Finally, I found that a female's social rank during early in life was a better predictor of her serotonin concentrations than her social rank at the time she was darted. This suggests that, like androgens (Holekamp et al., 2013), serotonin might be a modulator of developmental plasticity in females, preparing them for their approximate lifelong social status; however, more investigation into serotonin's effects on aggression and other dominance-related behaviors is needed.

This work has predominantly drawn 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 over 30 years of continuous behavioral and ecological data, and almost as many years of physiological data. This longitudinal project has been supervised by Dr. Holekamp, and data were collected by numerous graduate students and research assistants under her training. This research was only possible with the support of the

Kenyan members of our team, who were both involved in research and the logistics of keeping a field camp running. Finally, this work was only possible via collaboration and with the aid of other lab members and researchers, who have provided data and valuable input along the way. 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.

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CHAPTER 2

Socioecological and Physiological Correlates of Maternal Androgen Concentrations during Pregnancy in Spotted Hyenas (*Crocuta crocuta*)

Introduction

Hormone concentrations in breeding females vary widely among individuals and pregnancies and can have widespread effects on both the female's phenotype and that of her developing offspring (reviewed in Meylan et al., 2012). Understanding variation in hormones such as androgens is particularly important, as they have pleiotropic effects on behavior and physiology, and can play key roles in mediating life history trade-offs. In general, androgens enhance competition- and mating-related traits at the expense of self-maintenance (reviewed in Hau, 2007; Ketterson et al., 2009; e.g. DelBarco-Trillo et al., 2016; Smyth et al., 2016). Androgens can also have 'masculinizing' effects on phenotype due to their importance in the expression of male-typical secondary sex characteristics (reviewed in Ryan and Vandenbergh 2002; French et al., 2013). Their effects on phenotype are particularly consequential during sensitive periods of development, when hormones can have long-lasting and often irreversible organizational effects on neuroendocrine physiology and behavior. Early exposure to androgens can alter developmental trajectories (e.g. growth, sexual maturation), and have lifelong consequences for reproductive and aggressive behavior (Phoenix et al., 1959; reviewed in Ryan and Vandenbergh, 2002). For animals living in societies structured by dominance hierarchies, early androgen exposure can also affect adult social dominance status (reviewed in Ryan and Vandenbergh, 2002; Kelly and Vitousek, 2017; Houdelier et al., 2013). In both oviparous and viviparous species, maternal hormones can greatly influence the degree to which developing offspring are exposed to hormones during early development (reviewed in Meylan et al., 2012;

Stamps and Groothuis, 2010). It is thus useful to understand which factors contribute to variation in maternal androgen concentrations among individuals and breeding events.

Circulating hormone concentrations respond to both the physiological state of the individual animal and the animal's environment. In female mammals, androgen concentrations can vary with a variety of factors associated with their physiological state, including age (e.g. Davison et al., 2005; Pavitt et al., 2014; Carlsen et al., 2003), reproductive status (e.g. Gudermuth et al., 1998; Weizenbaum et al., 1979), past breeding experience (e.g. Schell et al., 2016; reviewed in Bridges, 2016), diet (e.g. Dantzer et al., 2011), and health (e.g., Smyth et al., 2016; Altmann et al., 2004). In pregnant mammals, circulating maternal androgen concentrations can further vary with offspring sex (e.g. Meulenberg and Hofman, 1991; Grant, 2007) and day of gestation (e.g. Weizenbaum et al., 1979). Parity has also been shown to influence hormones, including concentrations of circulating androgens, in both pregnant and nonpregnant female mammals (e.g. Musey et al., 1987; Toriola et al., 2011 (humans); Schell et al., 2016 (coyotes)).

Along with her physiological state, a range of environmental factors can influence a breeding female's androgen concentrations and the exposure of her developing young to maternal androgens. For example, in many avian species, maternal allocation of androgens to egg yolk varies with ecological factors such as food availability (e.g. Morosinotto et al., 2016), predator activity (e.g. Coslovsky et al., 2012), ectoparasite load and immune challenge (e.g. Lopez-Rull et al., 2010; Gil et al., 2006) and human disturbance (e.g. Guesdon et al., 2011). However, the direction of the causal

relationships between these factors and maternal androgens is variable and requires further study (reviewed in Groothuis et al., 2005; Bentz et al., 2016).

More consistently, the social environment has been linked to variation in maternal androgen concentrations. Conspecific competition in the social environment has been widely shown to stimulate androgen production in female vertebrates, including breeding females (reviewed in Oliveira, 2009; Kelly and Vitousek, 2017). Specifically, environments in which breeding females have frequent social interactions and opportunities to behave aggressively have been associated with higher maternal androgen concentrations deposited in yolk or circulating during pregnancy. For example, female birds have been shown to increase androgen deposition in the yolks of their eggs with increasing local breeding density, number of aggressive interactions with conspecifics, and social dominance status. (Tanvez et al., 2008; reviewed in Groothuis et al., 2005; Bentz et al., 2016). In pregnant mammals, researchers have found positive correlations between circulating androgens and population density (e.g. Meise et al., 2016), territorial intrusion (e.g. Schell et al., 2016), and social status (e.g. Davies et al., 2016; Dloniak et al., 2006). Overall, socioecological factors, particularly those reflecting social competition among mothers, may be key determinants of maternal androgens and the exposure of their offspring to these androgens. However, most research to date in this area has focused on oviparous species. We would benefit from further study of the socioecological determinants of circulating androgen concentrations in pregnant female mammals, and the extent to which their developing offspring are exposed to maternal androgens, in species with diverse social systems.

Here, we examine variation in maternal fecal concentrations of androstenedione (A4) and testosterone (T) in pregnant spotted hyenas (*Crocuta crocuta*), a species in which the organizational effects of androgens on both behavioral and morphological traits have been well documented. Previous research on pregnant hyenas in the same wild population investigated here (Dloniak et al., 2006) examined predictors of total fecal androgens, a measure which reflects variation in circulating androgens. Their results indicated that pregnant females with high social rank have higher fecal androgen concentrations during pregnancy than lower-ranking pregnant females. The study also demonstrated a relationship between maternal androgen concentrations and offspring behavior. Specifically, a mother's total fecal androgen concentrations during the second half of pregnancy predicted offspring rates of aggression and play mounting during the first few months of life (Dloniak et al., 2006). A follow-up study on female offspring by Holekamp et al. (2013) found that maternal androgen concentrations during pregnancy continued to predict female aggressive behavior into adulthood, particularly aggression directed against adult males. Overall, these studies suggest that maternal androgen concentrations are affected by the social environment, and that they predict important aspects of the social behavior of the female's future offspring.

We build on past research on spotted hyenas by investigating potential phycological and socioecological predictors of maternal fecal concentrations of two different androgens - androstenedione (A4) and testosterone (T). These are the two primary circulating androgens in female hyenas, but we were particularly interested in the predictors of maternal A4, as past research has indicated it might figure importantly in androgen-mediated variation in the morphology and behavior of spotted hyenas

(reviewed in French et al., 2013). Androstenedione, produced by the ovaries, is the primary circulating androgen in female spotted hyenas, and concentrations in females are higher than concentrations in either male hyenas or females of many other species (Glickman et al., 1987, 1992). A4 binds only weakly to androgen receptors and can be locally converted to more active sex steroids. Previous studies on captive pregnant hyenas reported that maternal androgens rise during pregnancy and that placental enzyme activity can convert A4 to more potent androgens, such as testosterone, or to estrogens. Changes in placental androgen metabolism across gestation appear to fine-tune the timing of exposure of developing offspring to high levels of androgens (primarily T) or estrogens via conversion of maternal A4 (Licht et al., 1992, 1998; Yalcinkaya et al., 1993; Conley et al., 2007). Because maternal A4 and T concentrations may vary differently across pregnancy, and because they may also differ in their effects on developing offspring (e.g. Hegyi et al., 2011), it was important to examine potential predictors of their concentrations separately here.

In this study, we investigated how maternal A4 and T vary with characteristics of the mother and the pregnancy ('maternal physiology') as well as her socioecological environment. First, we explored how maternal A4 and T vary with pregnancy stage, the sex of a female's developing offspring, and maternal parity. The relationship between maternal parity and androgen concentrations has not previously been investigated in our study system; however, a female's first pregnancy can have long-term consequences for not only her neuroendocrine systems, but also their physiological and behavioral correlates (e.g. Altmann et. al., 2004; Hussain et al., 2013; Wang and Buntin, 1999; reviewed in Bridges, 2016). Nevertheless, few studies on wild mammals have

been able to collect multiple samples from different breeding individuals longitudinally (see Schell et al., 2016), and they often pool primiparous and multiparous females for purposes of data analysis.

We also tested the hypothesis that maternal androgens reflect the competitive environment – that is, that they increase in contexts in which a female is more likely to express aggression. To test this hypothesis, we examined how maternal A4 and T concentrations vary with several aspects of the female's socioecological environment. These included a female's social status, prey availability within her territory, and the number of adult females within her social group around the time of fecal collection. Hyenas experience wide variation in both food availability and social group size, and the ability to successfully compete for sporadically occurring food sources with conspecifics, specifically with other adult females, can greatly improve females' fitness and alter their physiology (reviewed in Smith et al., 2017). We therefore predicted that pregnant females living in groups with numerous adult females and/or during periods of limited prey availability would have higher androgen concentrations than females in less "competitive" social environments. We also predicted that high-ranking females, who are most able to aggress without risk of retaliatory attack, would have relatively higher androgen concentrations than their subordinate counterparts. A positive relationship between social rank and androgens has been found by past research on spotted hyenas (Dloniak et al., 2006) and several other mammalian species with similar social structures (e.g. Davies et al., 2016; reviewed in French et al., 2013; Clutton-brock, 2007). We also tested for a relationship between fecal androgens and maternal rates of aggression emitted in the two weeks before fecal sample collection, to ascertain

whether those individuals displaying the most aggression around the time of fecal deposition would have the highest concentrations of fecal androgens.

Materials and Methods

Study Populations and Subjects

This research focused on a large social group, or “clan”, of free-living spotted hyenas whose behavior and demography have been monitored intensively since 1988, including the period during which fecal samples used in this study were collected, which was between February 1993 and October 2004. This clan, referred to as the Talek clan, defends a large territory in the Masai Mara National Reserve (MMNR) in southwestern Kenya. Hyena clans are fission-fusion societies consisting of multiple matrilines of adult females, their offspring, and one or more adult immigrant males (reviewed in Smith et. al., 2017). Hyenas live in a linear social hierarchy characterized by youngest ascendancy and strict maternal rank ‘inheritance,’ in which offspring enter the dominance hierarchy immediately below their mothers but above their older siblings (Holekamp and Smale, 1991; Engh et al., 2000). Hyena clans are also female dominant; immigrant males join their new clan at the very bottom of its social hierarchy, and forever remain below all adult females and their offspring (Kruuk, 1972; Frank, 1986; Henschel and Skinner, 1987). Clan members here were individually recognized by their spot patterns and ear damage and sexed based on the dimorphic glans of the erect phallus (Frank et al., 1990).

Reproductive Status

Female spotted hyenas are promiscuous and breed year-round, generally giving birth to litters containing only 1-2 cubs (Holekamp et al., 1999; Kruuk, 1972). Mother-offspring relationships were established here on the basis of nursing associations and genotyping. Based on cubs' appearance when first seen, birthdates (+/- 7 days) are assigned to them (Holekamp et al., 1996). Gestation in hyenas lasts 110 days, so we subtracted this number from each estimated cub birthdate\ to determine a conception date for each litter (Kruuk, 1972). This method allowed us to estimate the day of gestation for a pregnant female at the time of her fecal sample collection. However, during some periods in our long-term study, cubs were not seen until some months after birth due to poor visibility at den sites; in these cases, the accuracy of estimated birthdates, and associated conception dates, was less reliable. Here we only consider birthdates to be reliable for cubs who were first seen within 30 days of their estimated birthdates, totaling 33 cubs from 23 litters. Therefore, our analysis of variation in maternal androgens across gestation was limited to the fecal samples (n = 26) from females who were pregnant with these cubs.

For most pregnant females, we were able to sex the cubs in her litter within a few months of birth by observing their erect phalluses; however, some cubs died before we could sex them. If cubs died before we saw them above ground, we were nevertheless able to determine whether or not a female had given birth because of changes to the female's external genitalia. Female spotted hyenas give birth through an elongated clitoris, which tears at first parturition, leaving a vertical band of pink scar tissue on its posterior surface. By recording any sighting of a torn phallus on a young adult female

and looking for evidence of maternal behavior (e.g. spending time near a single den-hole), we were able to track full reproductive histories of our study females even when their cubs died very young.

Social Rank and Clan Size

Social ranks of adult females in the clan were determined from a matrix of outcomes of dyadic aggressive and submissive interactions (Strauss & Holekamp, 2019). Here, females were considered to be sexually mature, and were assigned their own ranks, starting at 24 months of age or at their age at first conception, whichever came first (Glickman et al., 1992; Holekamp et al., 1996). The hierarchy was updated annually to accommodate demographic change within the clan. Ranks were standardized and centralized to account for temporal variation in clan size, with the highest-ranking female in the clan assigned a rank of 1 and the lowest ranking assigned a rank of -1. Clan size, also updated annually, was defined here as the number of adult females present in the clan, which varied between 10 and 28 females over the course of this study.

Rates of Aggression

All aggressive acts emitted by the hyenas during all observation sessions were recorded using all-occurrence sampling (Altmann, 1974). For a subset of females, we calculated an average rate of aggression (# aggressive acts / # hours observed in the presence of potential targets) for the two weeks before each fecal sample was collected. Aggression rate was only calculated for females that were observed for at least one hour in the presence of one or more potential targets within this 2-week

interval. Due to the rarity of aggressive acts directed up the hierarchy, any adult hyena in the clan with a lower rank than the focal female was considered to be a potential target. Secondly, observation sessions in which the hyena was observed for less than ten minutes were not included in our calculations. This ensured that each hyena in the data set had a reasonable chance of being observed while engaging in aggression.

Prey Density

Prey abundance in the clan's territory was assessed every two weeks by counting all ungulates within 100m of two 4km transects in the territory of the Talek clan (see Cooper et al. 1999; Holekamp et al., 1997). Transect counts were used to calculate the mean prey density (# prey animals/ km^2) within the territory during the month before each fecal sample was collected.

Fecal Sampling and Immunoassays

Fecal samples were collected from known hyenas immediately after deposition whenever possible. Samples were then mixed, aliquoted, and stored frozen in liquid nitrogen until shipment to Michigan State University, where they were stored in a -80-degree freezer until analysis (Van Meter et al., 2008). All samples analyzed here (n=76) were collected from 32 individual pregnant females, with multiple fecal samples collected from most of these females both within and across 59 different pregnancies.

Frozen fecal samples were lyophilized and extracted in ethanol, as described previously (Dloniak et al., 2004; Van Meter et al., 2008). Fecal samples were reconstituted in 1.0 ml methanol, aliquoted and stored frozen at 20˚C until assay. Aliquots underwent separate procedures for A4 assay at the Endocrine Bio services

Laboratory, University of Nebraska at Omaha (UNO), and T assay at the Core Assay Facility at the University of Michigan (UM).

At UNO, a second extraction specific for A4 was conducted prior to assay. We extracted 200 µl of the reconstituted samples in 6.0 ml of an ethyl acetate: hexane (60:40) in 16 x 125 mm culture tubes. Samples were vortexed for 20 sec and the phases were allowed to separate at room temperature for 5 min. We then transferred 5.0 ml of the ethyl acetate: hexane portion (upper phase) to a 12 x 75 mm culture tube. This solution was evaporated under compressed air in a warm-water bath and samples were reconstituted in 2.5 ml of phosphate-buffered saline (PBS). Prior to assay, samples were further diluted 1:30 with PBS and 500 µl of solution was taken to assay in duplicate. We then used an androstenedione radioimmunoassay (RIA) kit (MP Biomedicals; 07-109202) to measure fecal A4 levels in the hyena fecal extracts. Cross-reactivity of the kit antibody with other steroids was as follows: androstenedione: 100%; dehydroepiandrosterone-sulfate: 4.4%; dehydroepiandrosterone: 3.5%; estrone: 1.8%; testosterone: 0.6%; progesterone: 0.07%; estradiol: 0.02%; All other steroids tested: < 0.01%. Serial dilutions of a quality control pool produced a displacement curve that was parallel to the standard curve. The assay's sensitivity was 0.10 ng/ml. A pooled sample of hyena fecal extract was measured in each assay run (n = 3). Intra- and interassay coefficients of variation based on this pool were 6.2% and 9.5%, respectively.

At UM, fecal extracts were assayed for T in duplicate at a 1:5 dilution with a testosterone radioimmunoassay kit (MP Biomedicals ImmuChem Double Antibody Testosterone ^{125}I RIA Kit). Cross-reactivity of the kit antibody with steroids was as follows: testosterone: 100%; 5α-dihydrotestosterone: 3.40%; 5α-androstane-3β, 17β-

diol: 2.20%; 11-oxotestosterone: 2.00%; 6β-hydroxytestosterone: 0.95%; 5β-androstane-3β, 17β-diol: 0.71%; 5β-dihydrotestosterone: 0.63%; androstenedione: 0.56%; and epiandrosterone: 0.20%. All other steroids tested: < 0.01%. Linearity, accuracy, and precision were assessed and demonstrated as suggested in Brown et al., 2011. The intraassay coefficient of variation was 11.7%, and the interassay coefficient of variation was 14.8% (n= 15 assays).

Statistical Analysis

Analyses of T(ng/g) and A4(ng/g) concentrations were performed in R version 3.1.3 (R Core Team, 2015). For the purposes of analysis, T and A4 fecal concentrations (ng/g dry weight of fecal matter) were logged to normalize their distributions. To test for a relationship between T and A4 concentrations, a Pearson's correlation test was performed on values of these hormones obtained from the same sample. Subsequently, in two separate linear mixed models, we used the hormone values as the response measures and tested possible predictors of T and A4 concentrations. For these models, we used R packages lme4 (Bates et al., 2015) and lmertest (Kuznetsova et al., 2016). All continuous predictor variables were scaled and centered for ease of analysis and interpretation. Model comparison was used to determine which variables to include in final models, and was performed using Akaike's information criterion, adjusted for small sample sizes (AICc), with R package AICcmodavg (Mazerolle, 2016). All models within two AICc points of the best fit model ($dAICc \leq 2$) were considered to be of equal goodness of fit to the data (Burnham and Anderson, 2002). The restricted maximum likelihood method of estimation was used to

evaluate model parameters, the data met all assumptions for tests used, and alpha was set at 0.05. Models were evaluated for outliers using R package influence.ME (Nieuwenhuis et al., 2012), with data points having a Cook's Distance (CD) of > 4/n considered to be potential outliers (Van der Meer et al., 2010).

Because females were often sampled more than once over the course of multiple pregnancies, a random effect structure was included in all models. For many individuals, multiple samples were collected within the same pregnancy, so each pregnancy was assigned a unique identifier (ID). Three possible random effect structures were considered: a random intercept of pregnancy ID, a random intercept of individual female, and a random intercept of pregnancy nested within each individual female. Parameter estimates and significance of fixed effects were similar across all three possible random effect structures. In our final model we chose to include only a random intercept of pregnancy; this was determined via model comparison and an examination of marginal and conditional R^2 values (R package piecewiseSEM; see Lefcheck, 2015; Nakagawa and Schielzeth, 2013) from models that included all fixed effects (see below) and one of the three possible random effect structures (see Appendix, Table A2.1).

Fixed effects in the final models included predictors controlling for methodological variables; these were time of day when the fecal sample was collected (morning (am) or evening (pm)) and the number of years between fecal sample collection and assay (see van Anders et al., 2014; Khan et al., 2002). Final models also included the following fixed effects: the sampled female's parity (primiparous vs multiparous), standardized social rank (ranging from lowest ranking, -1, to highest ranking, 1), social group size (#

of adult females in the clan), and the mean prey density (#/km^2) within the Talek territory at the time of sampling. Finally, an interaction between parity and social rank was included. Other potentially important predictors of maternal androgens, including day of gestation, litter sex, and maternal rates of aggression, were examined in separate models, discussed below.

Preliminary data analysis led us to exclude both maternal age (months), and maternal parity, represented as a continuous integer (i.e. first pregnancy = 1, second pregnancy = 2, etc.), from the above models. These two variables were highly correlated (Pearson's correlation test, n = 76, df = 74, t= 18.08, r = 0.903, $p < 0.0001$), and including them both resulted in highly multicollinear models (VIF (variation inflection factor) scores ~ 12 for both fixed effects). We therefore used model comparison to decide between models that included maternal age, parity as an integer (# pregnancy), parity as a binomial factor (primiparous vs multiparous), or both parity as a binomial factor and maternal age. Based on AICc values, the model that included only parity as a binomial factor was best fitting for both A4 and T, followed by the next best model including only parity as an integer (A4 model: ΔAICc = 4.0; T model: ΔAICc = 2.9). Thus, we did not include maternal age in our final models, and parity was included as a binomial factor, indicating whether a female has previously given birth (multiparous) or not (primiparous). Note that models including maternal age and parity as a binomial factor yielded similar results, and indicated that maternal age had no significant effect on maternal T ($t = 0.31$, $p = 0.76$) and A4 ($t = 0.87$, $p = 0.39$)

Model comparison was also used in preliminary data analysis to determine which, if any, interaction(s) to include in the model. Two- and three-way

interactions between clan size, social rank, and prey density were considered, as well as interactions between parity and each of the three socioecological variables (see Appendix, Table A1.2). To maximize power and for purposes of parsimony, only one interaction (two- or three- way) was included in each candidate model. Results from all top-ranked models are included and discussed in our Appendix (Tables A1.3, A14). For both A4 and T, the model without an interaction and the model including an interaction between parity and social rank were the two top-ranked (all < 1 dAICc) (Appendix, Table A1.2). For purposes of this paper, we focused on the model that included an interaction between parity and rank.

Post-hoc analysis of the parity by rank interaction was performed for A4 and T models using R packages “interplot” and “interactions.” Conditional coefficients ($\pm$ simulated 95% confidence intervals (CI)) were calculated from the results of linear mixed regression models and visualized using package interplot (Solt and Hu, 2018). Johnson-Neyman intervals and associated test statistics were obtained via simple slopes analysis (R package interactions; Long, 2019), and adjusted for false discovery rates as suggested by Esarey and Sumner (2017).

Separate models were used to investigate the effects of maternal rates of aggression (n = 61), litter sex composition (n = 42), and gestation day (n = 26) on maternal A4 and T concentrations. Information on these three variables was only available for a subset of samples due to the limitations described earlier.

We examined the effect of these three variables on maternal fecal A4 and T by running reduced models with an additional fixed effect (litter sex composition, gestation day, or maternal rate of aggression) on the relevant subset of data. The reduced models

included a random effect of pregnancy and any significant fixed effects from the full A4 and T models described above.

Results

Outlier Analysis

For our main models (Table 2.1), one point was removed from the dataset after outlier analysis (see Appendix, Table A1.5). Regarding main effects, there were no significant differences between the results of the models before outlier removal (see Appendix, Table A1.6) and models presented here (Table 2.1). The interaction between parity and rank, though significant for both A4 and T models after outlier removal (Table 2.1), was non-significant in the T model ($p = 0.25$, see Appendix, Table A2.6B) and marginally significant in the A4 model ($p = 0.08$, see Appendix, Table A2.6A) before removal of the outlying point. Results of models before outlier removal are discussed further in the Appendix.

Relationship between T(ng/g) and A4 (ng/g)

A4 was present in significantly higher concentrations (ng/g), by roughly one order of magnitude, than T (paired t test on logged values, $t = 16.2005$, $df = 74$, p-value < 0.0001), but T and A4 concentrations from the same samples were highly correlated (Figure 2.1, $n = 76$, $r = 0.88$, $p < 0.0001$)

Because of the high positive correlation between T and A4 concentrations in our fecal samples, results of T and A4 models were very similar, and discussion of both models are combined in most sections below. To differentiate between the results of

the models for fecal T(ng/g) concentration and fecal A4(ng/g) concentration, we used subscripts T and A4 (e.g. t-test statistics/p-values from A4 and T models were reported as T_{A4} / p_{A4} and T_T /p_T , respectively).

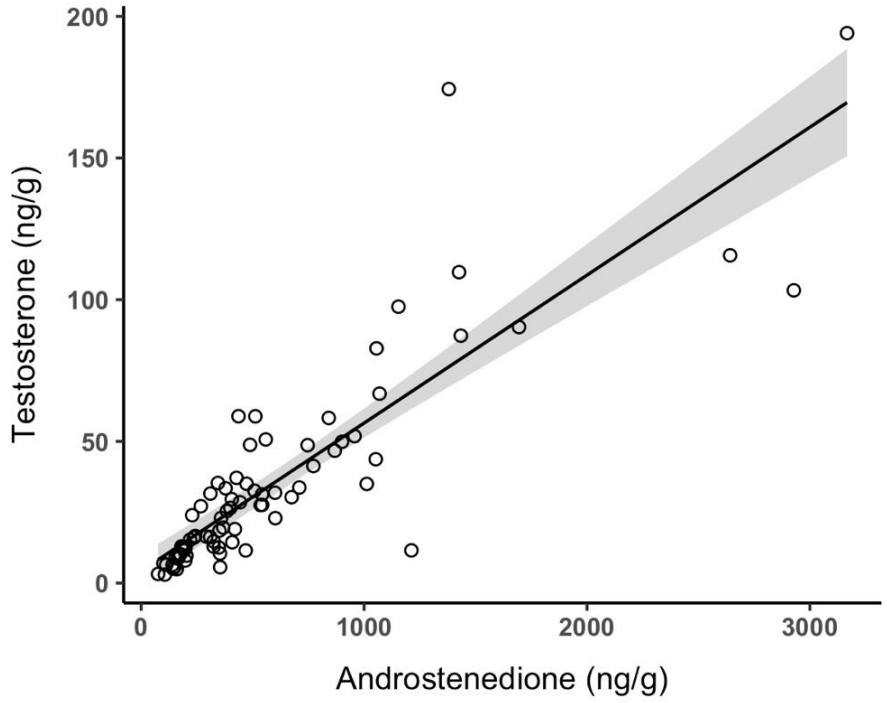


Figure 2.1. Correlation (±95% interval) between testosterone (T) and androstenedione (A4) concentrations in pregnant hyena fecal samples. Extracts from each fecal sample were aliquoted and assayed for T and A4 via separate RIA's. Concentrations of both hormones are expressed as ng/g of dry weight of fecal material. A Pearson's correlation test on logged concentrations showed a strong positive correlation between logged concentrations of the two hormones (n = 76 samples, t = 16.2, r = 0.88, p < 0.0001).

Maternal Physiology

Litter Composition and Gestation Day

To investigate the influence of fetal sex on maternal androgens, a reduced model, with a random effect of pregnancy and fixed effects of litter sex composition (all male, all female, or mixed sex), rank, parity, and a rank by parity interaction, was run on the subset of data for which we had information on litter sex composition (n = 42

samples). We found that litter sex composition had no effect on maternal androgens (likelihood ratio test; $x^2_{A4} = 1.48$, $p_{A4} = 0.48$; $x^2_T = 3.51$, $p_T = 0.17$).

To investigate the influence of gestation day on maternal androgens, we used another reduced model, with a random effect of pregnancy and fixed effects of social rank and gestation day. All samples used in this analysis were collected from multiparous females (n=26). We found that both A4 and T concentrations rose from early to late gestation (n = 26 samples, $t_{A4}= 4.00$, $p_{A4} = 0.004$, $t_T =2.34$, $p_T = 0.05$).

Maternal Parity

We found that, for females in the upper portion of the social hierarchy, both maternal A4 and T increase after a first pregnancy, with multiparous females having higher androgen concentrations than primiparous females. Specifically, post-hoc analysis of the interaction between social rank and parity ($T_{A4} =-2.12$, $p_{A4} = 0.04$, $T_T= -2.01$, $p_T = 0.05$, Table 2.1) showed that parity's effect on A4 and T was significant only for females with standardized social ranks of > 0.28 (approximately the upper third of the hierarchy) and > 0.51 (approximately the upper fourth of the hierarchy), respectively (Figure 2.2).

Table 2.1. Predictors of maternal fecal concentrations of (A) androstenedione (A4) (ng/g) and (B) testosterone (T) (ng/g) in fecal samples collected from pregnant spotted hyenas (n = 75 samples). Concentrations of both hormones are expressed as ng/g of dry weight of fecal material. We present parameter estimates and associated values (standard errors (SE), degrees of freedom (df), t-statistic (t)) from linear mixed models predicting logged hormone concentrations. Models included a random effect of pregnancy and fixed effects of social rank (1 being highest rank and -1 lowest), mean prey density (# prey items/km^2), clan size (#adult females in clan), maternal parity (multiparous vs primiparous, with primiparous as the reference level), years to assay (# of years between fecal collection and assay), and fecal collection time (am vs pm, with pm as the reference level). Continuous predictor variables are centered and standardized. Statistically significant effects are shown in bolded font.

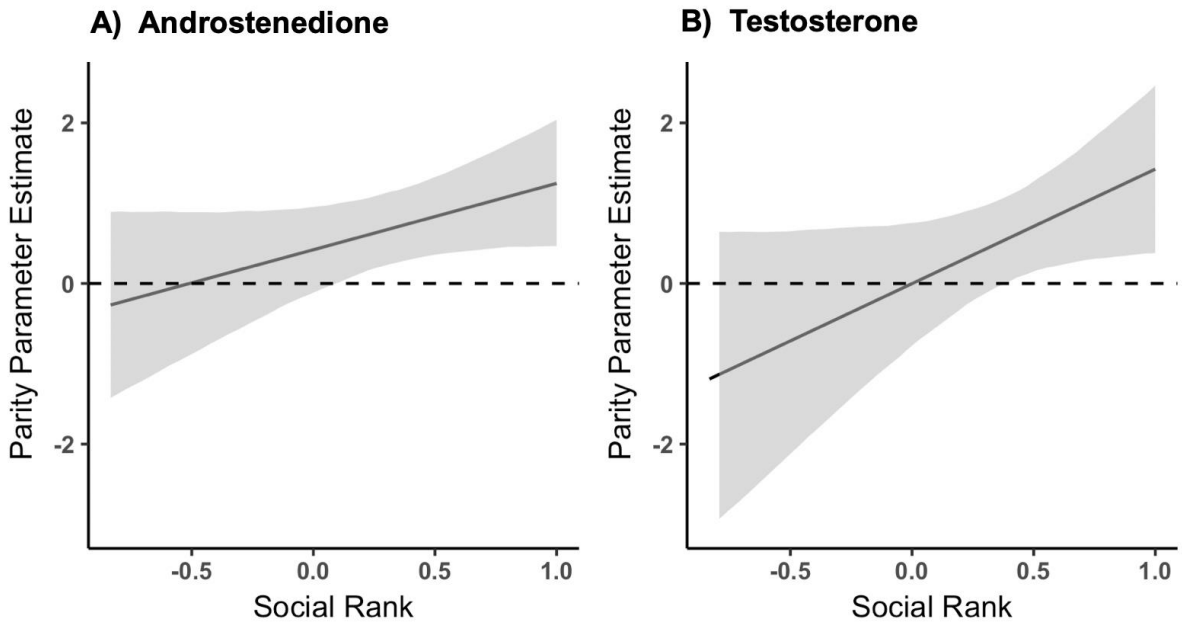
A) Androstenedione

Fixed effect	**Estimate**	**SE**	**df**	**t**	***p*-value**
Intercept	6.08	0.128	63	47.32	**< 0.0001**
Social Rank	0.601	0.175	52	3.43	**0.001**
Prey Density	0.194	0.096	66	2.01	**0.050**
Clan Size	-0.110	0.123	58	-0.892	0.380
Parity	-0.205	0.325	59	-0.631	0.530
Years to assay	-0.038	0.119	51	-0.317	0.752
Collection time	-0.138	0.169	60	-0.815	0.418
Parity*Rank	-1.278	0.603	62	-2.118	**0.038**

B) Testosterone

Fixed effect	**Estimate**	**SE**	**df**	**t**	***p*-value**
Intercept	3.24	0.153	62	21.22	**< 0.0001**
Social Rank	0.581	0.207	47	2.81	**0.007**
Prey Density	0.187	0.115	65	1.63	0.109
Clan Size	-0.109	0.146	55	-0.747	0.458
Parity	0.013	0.386	57	0.033	0.974
Years to assay	-0.125	0.141	46	-0.888	0.379
Collection time	-0.288	0.203	60	-1.417	0.162
Parity*Rank	-1.443	0.719	61	-2.008	**0.049**

Figure 2.2. Parameter estimates for the effect of parity on fecal concentrations (ng/g) of maternal A) androstenedione (A4) and B) testosterone (T) as they vary with standardized female social rank. Plots show changes in the coefficient estimate of parity conditional on female social rank (± 95% confidence intervals, represented by grey shaded area) calculated from the results of the linear mixed regression A4 and T models (see Table 2.1) using R package interplot (Solt and Hu, 2018). Social rank is standardized and centralized, with the highest-ranking hyena in the clan having a social rank of 1 and the lowest of -1. The parameter estimate for parity was significant for animals with social ranks of > 0.28 and > 0.51 in the A4 and T models, respectively.



Socioecological Environment

Both A4 and T concentrations in pregnant female hyenas were positively correlated with social rank (T_{A4} = 3.43 , p_{A4} = 0.001, T_T = 2.81, p_T = 0.007), but unrelated to clan size (T_{A4} = -0.89, p_{A4} = 0.38 , T_T = -0.75, p_T = 0.46) (Table 2.1). Prey density had no significant effect on T concentrations (T_T = 1.63, p_T = 0.11, Table 2.1B), but had a significant positive effect on A4 concentrations (T_{A4} = 2.01, p_{A4} = 0.05, Table 2.1A). However, the effect on A4 appeared to be driven largely by one datapoint and disappeared upon its removal (T_{A4} = 1.31, p_{A4} = 0.19, see Appendix, Figure A2.5)

Overall, our analysis indicated that both maternal A4 and T increase with social rank in pregnant spotted hyenas (T_{A4} =3.43, p_{A4} = 0.001 , T_T= -2.81 , p_T = 0.007, Table 2.1). Social rank appeared to be predictive of maternal androgens independently of prey density and clan size, which themselves had no clear, robust impact on maternal androgens, even when considering possible interactions among these three socioecological variables.

Interestingly, the positive relationship between social rank and maternal androgens was specific to multiparous females. Post-hoc investigation of the social rank by parity interaction (T_{A4} =-2.12, p_{A4} = 0.04, T_T= -2.01, p_T = 0.05, Table 2.1) revealed that the relationship between social rank and fecal A4 and T concentrations was significant for multiparous (t_{A4} = 3.43, p_{A4} = 0.001, t_T =2.81, p_T =0.01) but not primiparous (t_{A4} = -1.19, p_{A4} =0.24, t_T = -1.27, p_T = 0.21) females (Figure 2.3, 2.4). This may be due to the fact that high-ranking but not low-ranking females showed a significant increase in fecal A4 and T concentrations after their first pregnancy.

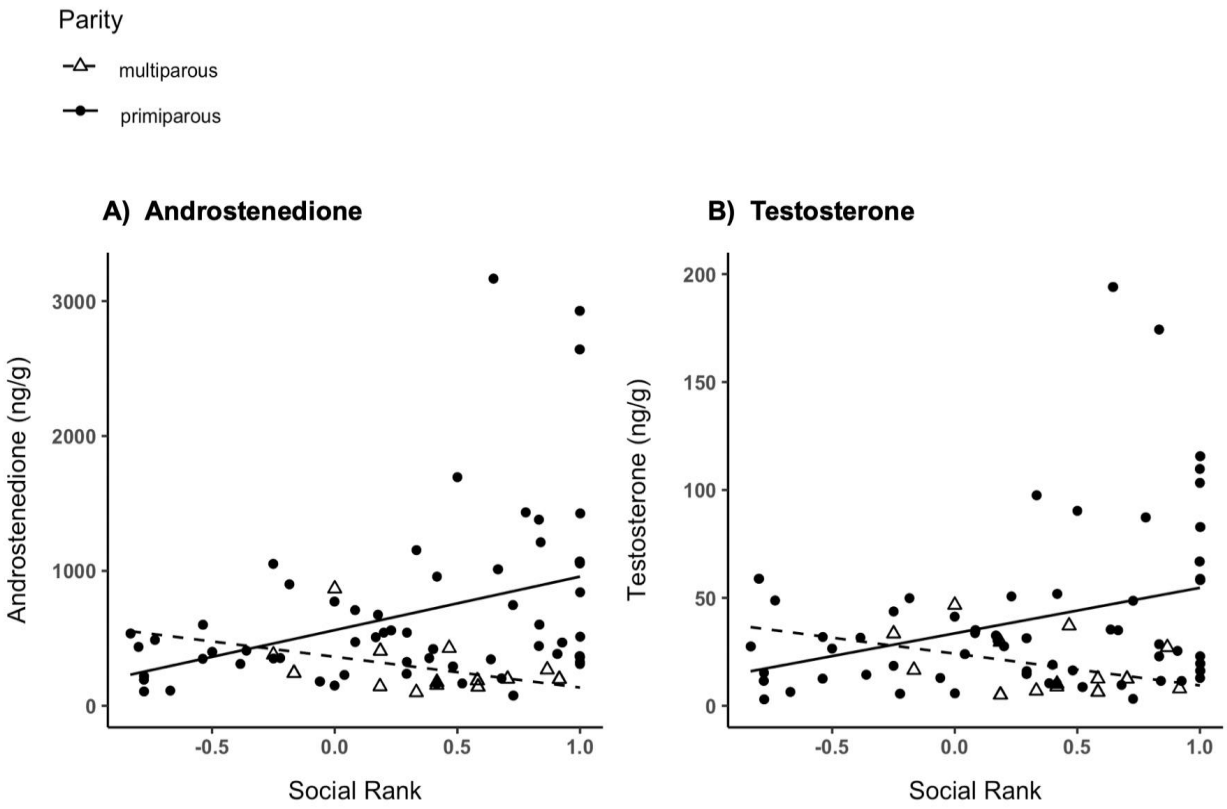


Figure 2.3. Relationship between social status and fecal concentrations (ng/g) of A) androstenedione (A4) and B) testosterone (T) in pregnant multiparous (n = 61 samples) and pregnant primiparous (n = 14 samples) females. Adult females who had not yet given birth at the time of fecal collection were considered primiparous. Social rank is standardized and centered, with the highest-ranking hyena in the clan having a social rank of 1 and the lowest of -1. The relationship between social rank and fecal androgen concentrations was significant for multiparous ($t_{A4} = 3.43$, $p_{A4} = 0.001$, $t_T = 2.81$, $p_T = 0.01$) but not primiparous ($t_{A4} = -1.19$, $p_{A4} = 0.24$, $t_T = -1.27$, $p_T = 0.21$) females.

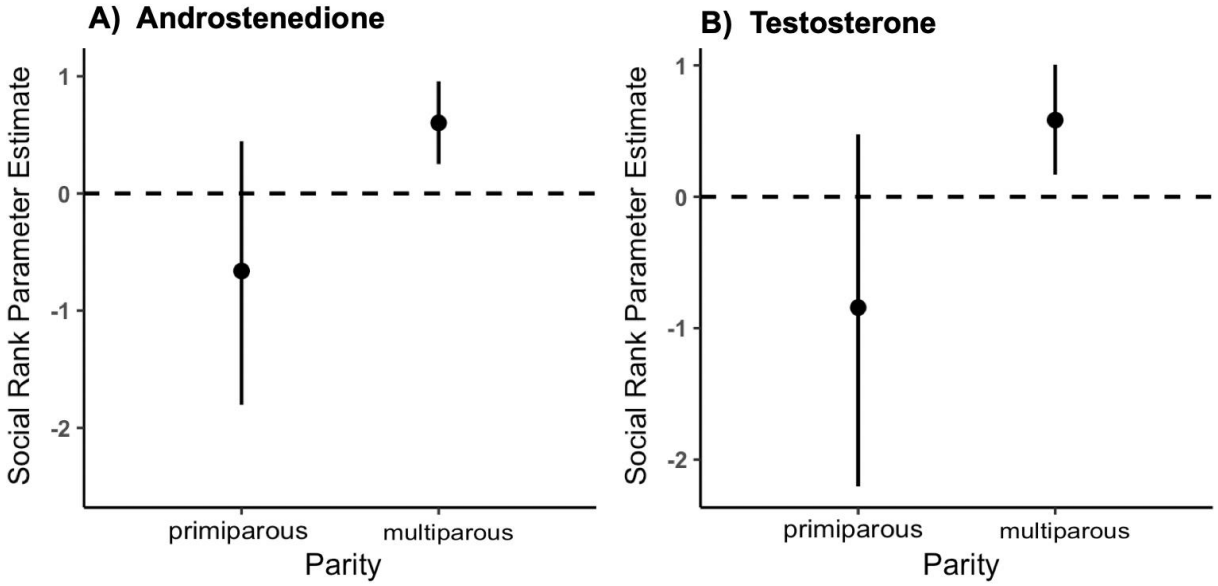


Figure 2.4. Parameter estimates for the effect of social rank on fecal concentrations (ng/g) of maternal A) androstenedione (A4) and B) testosterone (T) in pregnant multiparous (n =61 samples) and primiparous (n = 14 samples) spotted hyenas. Plots show changes in the coefficient estimates of social rank conditional on maternal parity ($\pm$ simulated 95% intervals) calculated from the results of A4 and T linear mixed regression models using R package interplot (Solt and Hu, 2018). The relationship between social rank and fecal androgen concentrations was significant for multiparous (t_{A4} = 3.43, p_{A4} = 0.001, t_T =2.81, p_T =0.01) but not primiparous (t_{A4} = -1.19, p_{A4} =0.24, t_T = -1.27, p_T = 0.21) females.

Maternal Behavior

A reduced model with a random effect of pregnancy and fixed effects of maternal rates of aggression, rank, parity, and a rank by parity interaction was run on the subset of females for which we had calculated maternal rates of aggression (n = 61 samples). Maternal rates of aggression (the average rate of aggression emitted by each pregnant hyena in the two weeks before her fecal sample deposition) were predictive of neither maternal A4 (p = 0.90, t = -0.13) nor T (p = 0.55, t = 0.61) concentrations.

Discussion

Our results extend previous findings by showing a positive relationship between social rank and two separate androgens, T and A4, and by demonstrating that this relationship may depend upon female parity, appearing only in multiparous females. The positive correlation between social rank and fecal androgens in multiparous females represents some support for the hypothesis that maternal androgens are relatively heightened in contexts in which females are most likely to emit aggressive behavior. However, we also found that neither social group size nor prey density affected maternal androgen concentrations. Also, and in agreement with previous research (Dloniak et al., 2006), there was no apparent relationship between maternal rates of aggression emitted around the time of sample collection and fecal androgen concentrations.

One basic aim of this study was to examine how maternal concentrations of A4 and T, hormones previously not analyzed separately in our study population, vary with maternal physiology. In agreement with previous research on females from captive populations (e.g. Glickman et al., 1987, 1992), A4 was present in significantly higher concentrations than T, and the two hormones were highly correlated (Goymann et al. (2001). In agreement with previous research on total androgen concentrations (Dloniak et al., 2006), we found that neither androgen was influenced by litter sex composition, but both A4 and T concentrations increased across gestation. Our results differ slightly from those of Licht et al., 1992's study of five captive spotted hyenas, which indicated a progressive rise in T but relatively stable A4 concentrations during pregnancy.

Finally, for high-ranking females, we found that multiparous females had significantly higher fecal A4 and T concentrations than primiparous females. Although there is relatively little information available regarding the effect of maternal parity on maternal androgens across different mammalian species, several studies on humans and one on coyotes have found a significant decrease in maternal androgens after first pregnancy, with multiparous individuals having significantly lower concentrations of androgens during pregnancy than primiparous ones (Schell et al., 2016; Toriola et al., 2011; Musey et al., 1987). Interestingly, our findings indicate the opposite pattern in spotted hyenas, with multiparous females having higher androgen concentrations during pregnancy than primiparous females. Furthermore, the fact that this pattern is rank-dependent, appearing only in high-ranking females, lends support to the idea that more attention should be paid to a female's experience, including social and past breeding experience, in analyses of maternal hormones (as suggested by Altmann et al., 2004 and Müller et al., 2002).

Overall, the relationship between various aspects of maternal state and a female's circulating androgen concentrations is highly variable across species, and the mechanisms mediating this variation remain poorly understood (Setchell et al., 2015; Groothuis et al., 2005). More comparative studies on the determinants of maternal androgens among mammals can shed light on this issue, particularly long-term studies collecting data from females across pregnancies. Ideally, such studies would be able to consider variables relating to maternal physiology that we were unable to include. For example, nutritional factors and health influence androgens in a wide range of vertebrates (e.g. Smyth et al., 2016; Muriel et al., 2015; Koren and Geffen, 2009; Tobler

et al., 2007a; Dantzer et al., 2011), and future investigations of their influence on maternal androgens in spotted hyenas would aid in the interpretation of our results. We could not obtain reliable indices of maternal body condition around the time of fecal sampling, a challenge common to many field studies (Barnett et al., 2015).

The main aim of our study was to investigate the impact of the socioecological environment on maternal androgens; we predicted that socioecological factors associated with relatively high levels of conspecific competition would be linked to a rise in maternal androgen concentrations. Specifically, we predicted that females living in environments characterized by low prey density and/or numerous social competitors would have relatively high androgen concentrations. We also predicted that socially dominant females, who are most likely to engage in agonistic behavior (Yoshida, 2012) would show higher fecal androgen concentrations than low-ranking females. In support of our prediction, we found that high-ranking pregnant females had higher fecal concentrations of A4 and T than their subordinates. However, we did not find that prey density or clan size were predictive of A4 or T. The lack of predictive power of clan size and prey density may also be due to the ability of hyenas to behaviorally mitigate resource competition in the face of changing ecological factors (reviewed in Holekamp and Dloniak, 2010). For instance, they can easily switch between hunting and scavenging, and are known to adjust their diets according to what is most readily available in their environment (reviewed in Jones et al., 2015). Because they live in fission-fusion societies, they can also avoid high levels of conspecific resource competition by spending more time alone and traveling long distances outside of their territory to forage (Smith et al., 2008; Green, 2015; Hofer and East, 1993). Due to such

flexibility in foraging behaviors, prey density and clan size may be superseded by social rank with respect to their long-term impact on a hyena's social environment and, in turn, the regulation of socially modulated hormones such as androgens.

Though animals could potentially behaviorally mitigate their involvement in agonistic interactions over resources, we also found that a potentially more direct measure of competition, rates of aggression emitted in the two weeks prior to fecal deposition, was unrelated to maternal androgens. Therefore, the patterns seen here may not be due to a more direct relationship between emitted aggression and social rank than between emitted aggression and prey density/clan size in the weeks leading up to sample deposition. Still, we cannot rule this hypothesis out, as we cannot account for interactions an individual engaged in outside of observational sessions.

Several other studies of hierarchically organized female mammals have also found a positive correlation between social status and circulating androgen concentrations, but have not identified the mechanisms mediating this relationship (e.g. Davies et al., 2016 (meerkats, *Suricata Suricata*); Lutermann et al., 2013 (Natal, *Cryptomys hottentotus natalensis*, and Damaraland mole-rats, *Fukomys damarensis*); Grant et al., 2011 (Barbary macaques, *Macaca sylvanus*); reviewed in Staub and de Beer, 1997; Setchell et al., 2015 (primates)). In many despotic hierarchies, socially dominant and subordinate animals experience different social environments, specifically in the nature and outcomes of agonistic interactions. This can cause their steroid profiles to diverge over time, possibly due to social phenomena such as winner-loser effects (Rose et al., 1971; Clinard et al., 2016; reviewed in Oliveira, 2009). Aside from long-term differences in their social environment, factors such as rank-related variation

in diet, nutritional status, or disease might also drive the positive association between androgens and social status.

Our finding of rank-related variation in maternal androgens is especially interesting in light of the organizational effects of androgens on dominance-related traits in vertebrates, including hyenas and other mammals (e.g. Wewers et al. 2005; French et al., 2013). Depending on species-specific reproductive physiology, rank-related variation in the androgen profiles of breeding females can be reflected in their offspring's prenatal androgen exposure. Indeed, many studies on social birds and mammals, including spotted hyenas, have found that offspring of dominant females are exposed to higher levels of prenatal androgens than are the offspring of subordinates (Nelson et al., 2010; Tanvez et al., 2008; Howlett et al., 2015; Correa et al., 2013); in turn, exposure to high concentrations of prenatal androgens is associated with a range of physiological and behavioral traits associated with social dominance (reviewed in Ryan and Vandenbergh, 2002; Kelly and Vitousek, 2017; Houdelier et al., 2013). Findings such as these have led many researchers to suggest that rank-related variation in prenatal exposure to maternal androgens contributes significantly to the patterning of social structure in female vertebrates, particularly for species living in despotic hierarchies characterized by maternal rank inheritance. In such species, rank-related variation in maternal androgens might provide a means by which pregnant females can "prepare' their offspring for their future social position via organizational effects (Howlett et al., 2015; Nelson et al., 2010; Dloniak et al., 2006; Davies et al., 2016; Tanvez et al., 2008; reviewed in Holekamp and Dloniak, 2009). The relationship between maternal androgens and dominance in spotted hyenas, and their correlation

with offspring aggression in both infancy and adulthood (Dloniak et al., 2006; Holekamp et al., 2013), supports this hypothesis. Finally, if maternal androgens in spotted hyenas function to prepare offspring for their future social environment, we would not expect a strong correlation between maternal androgens and factors such as prey density and clan size. These factors, particularly prey density, can change rapidly within a few days, and are thus relatively poor predictors of upcoming environmental challenges. A focus on the organizational effects of A4 and T on a suite of dominance-related traits (i.e.. growth, time to reproductive maturity or dispersal), could provide interesting avenues for future research in our study system and shed light on the idea of maternal androgens functioning in preparing individuals for their future social environment.

Finally, it is important to keep in mind that, in our study, the relationship between maternal androgens and social status is apparent only in multiparous hyenas, possibly because dominant females show an increase in maternal A4 and T after their first litter, whereas subordinate females do not. The mechanisms driving this pattern deserve further exploration, but our finding lends support to the idea that a female's first pregnancy can alter not only neuroendocrine systems, but also their behavioral and physiological determinants and effects. (e.g. Altmann et. al., 2004; Hussain et al., 2013; reviewed in Bridges, 2016). Future studies on the costs and benefits of prenatal androgen exposure (e.g. Packer et al., 1995), and how these might vary with maternal traits like parity and social status, could help elucidate the ultimate causes of this phenomenon.

In many avian species, mothers appear to adjust androgen deposition in eggs according to the relative costs and benefits of high androgen exposure to both

themselves and their offspring, given the current environment (e.g. Morosinotto et al., 2016; Muriel et al., 2015). They can even adjust androgen deposition within a clutch based on laying order, and in doing so, may either strengthen or weaken sibling hierarchies (e.g. Gil 2008, Kuzlowski and Ricklefs, 2010). Though this idea has not been applied in studies of mammals, it is possible that variation in maternal androgens across litters influences the social hierarchy among siblings. Hyenas have a strict system of youngest ascendency, with a female's youngest offspring assuming the dominance rank just below her, but above its older siblings. It is thus possible that the benefits of maternal androgen exposure are greater in later litters, as they could help give younger siblings a competitive advantage over their older siblings.

The costs of androgen exposure may vary across litters as well, given that organizational effects of androgens often enhance competition-related traits (e.g. fast growth, aggression) at the expense of self-maintenance (e.g. immune health), parental care, and for females, fertility (reviewed in Hau, 2007; Ketterson et al., 2009; e.g. delBarco-Trillo et al., 2016; Smyth et al., 2016). In spotted hyenas, high concentrations of maternal androgens may even interfere with the ability of a female to carry a pregnancy to term (Glickman et al., 1987). For first-time hyena mothers, whose offspring are less likely to survive than offspring born to females that have successfully reared previous litters (Drea et al., 2002), the costs of prenatal androgen exposure to their offspring may outweigh the potential advantages. For high-ranking females, the costs of androgen exposure to offspring may decline after the first pregnancy, but for low-ranking hyenas, who must contend with sparse resources and whose offspring may not benefit from high levels of aggression, the advantages of relatively high prenatal

androgen exposure may never outweigh the costs. Finally, the costs of androgens to the mothers themselves, including costs to immunity and body condition (e.g. Gil et al., 2006; Tschirren et al., 2016; Smyth et al., 2016), may vary with factors such as social rank and parity; an investigation of these costs may provide further insight regarding the drivers of individual variation in maternal androgens.

APPENDIX

APPENDIX

Random Effects

Three possible random effect structures were considered: a random intercept of pregnancy ID, a random intercept of individual female (“mother”), and a random intercept of pregnancy nested within mother. According to AICc comparison, A4 and T models with only mother or only pregnancy as the random effect did not substantially differ in fit, but both were considered better fits to the data than the nested model (> 2 dAICc, Table A2.1). Examination of marginal and conditional R^2s indicates that the proportion of variance explained by adding the random effect structure differed between models, with the random effect of mother explaining the least amount of variation (A4: 11.2% T: 12.1%), and the nested random effect explaining roughly the same amount of variation (A4: 18.2%; T: 16.0%) as the more parsimonious model with a random intercept of pregnancy (A4: 18.6%, T: 16.8%) (Table A2.1). Comparison of the intraclass correlation coefficients (ICC) reinforced this finding, with the random effect of pregnancy explaining the greatest proportion of variance (ICC_{A4} =0.25, ICC_T = 0.21), followed closely by the nested random effect structure ((ICC_{A4} = 0.24, ICC_T = 0.19) (Table A2.1). Because pregnancy explained a good amount of variation in the model on its own and provided a more parsimonious random effect structure than the nested effect, we report results from the pregnancy model for the analyses in this chapter.

Table A2.1. Comparisons of A4 (A) and T (B) models with varying random effect structures, including a random intercept of pregnancy ID ("Pregnancy"), a random intercept of female hyena ID ("Mother"), and a model with a random effect of pregnancy ID nested within female ID ("Pregnancy: Mother"). Data analyzed (n = 76 samples) came from 32 individual females over the course of 59 pregnancies. Models also included all main fixed effects (collection time, years to assay, parity, prey density, clan size, and social rank). The difference between each model's AICc score (dAICc) and that of the best model (dAICc = 0) are listed. Marginal R^2s represent the proportion of variance in the model explained by the fixed effects alone and conditional R^2 represents the proportion of variance explained by both fixed and random effects. The difference ("Difference') in conditional and marginal R^2s can be viewed as variation explained by adding the random effect structure to the model. Intraclass correlation coefficients (ICC) associated with the random effects of each model are also included. Variance components were estimated via maximum likelihood.

A) Androstenedione

Model A4	**Pseudo R^2**			**dAICc**	**ICC**
	Marginal	**Conditional**	**Difference**		
Pregnancy	0.248	0.434	0.186	0.0	0.2470
Mother	0.235	0.347	0.112	0.0	0.1462
Pregnancy: Mother	0.238	0.420	0.182	2.5	0.2384

B) Testosterone

Model T	**Pseudo R^2**			**dAICc**	**ICC**
	Marginal	**Conditional**	**Difference**		
Pregnancy	0.182	0.350	0.168	0.3	0.206
Mother	0.170	0.291	0.121	0.0	0.146
Pregnancy: Mother	0.170	0.330	0.160	2.7	0.193

Model Comparison: Interactions

According to AICc comparison of models including different interactions, top (dAICc < 2) A4 and T models included the one with no interactions ($dAICc_{A4}$ = 0.9, $dAICc_{T}$ = 0.0) and the model with an interaction between parity and rank ($dAICc_{A4}$ = 0.0,

$dAICc_T$ = 0.6) (Table A.2.2). For T, the model with an interaction between clan size and prey density was also among the top models ($dAICc_T$ = 1.1, Table A.2.2).

Table A2.2. Comparison of linear mixed models predicting logged hormone concentrations with differing interactions between fixed effects. Response variables are logged maternal fecal concentrations of androstenedione (A4) (ng/g) or testosterone (T) (ng/g).). Data analyzed (n = 76 samples) came from 32 individual females over the course of 59 pregnancies. All models included a random effect of pregnancy and fixed effects of relative social rank, mean prey, clan size, parity, years between fecal sample collection and assay, and sample collection time. Models differ in whether or not they included an interaction between fixed effects, and if so, which variables were part of the interaction (left column). For all models, we present the difference in AICc score (dAICc) between that model and the "best" model (dAICc = 0) for the corresponding androgen. dAICc scores for models within 2 AICc of the best model for each androgen are marked with a "*".

Model Interactions	**dAICc**	
	A4	**T**
No interactions	0.9*	0.0*
Parity x Rank	0.0*	0.6*
Clan x Prey Density	3.4	1.1*
Parity x Clan	4.0	2.2
Parity x Prey Density	3.4	2.3
Prey Density x Rank	4.3	4.1
Clan x Rank	5.3	4.0
Prey Density x Clan x Rank	14.9	13.1

Other Top-Ranked A4 Models

The results of top-ranked A4 models (Table A2.2), not presented in main chapter manuscript, are summarized below (Table A2.3).

Table A2.3. Predictors of maternal fecal concentrations of androstenedione (A4) (ng/g) in fecal samples (n = 76) collected from pregnant spotted hyenas. Hormone concentrations are expressed as ng/g of dry weight of fecal material. We present parameter estimates and associated values (standard errors (SE), degrees of freedom (df), t-statistic (t)) from a linear mixed model predicting logged A4 concentrations. Models included a random effect of pregnancy and fixed effects of social rank (1 being highest rank and -1 lowest), mean prey density (# prey items/km^2), clan size (#adult females in clan), maternal parity (multiparous (n = 61) vs primiparous (n = 15), with primiparous as the reference level), years to assay (# of years between fecal collection and assay), and fecal collection time (am vs pm, with pm as the reference level). Continuous predictor variables are centered and standardized. Statistically significant effects are shown in bolded font.

Fixed effect	**Estimate**	**SE**	**df**	**t**	***p*-value**
Intercept	6.096	0.129	65	47.09	**< 0.0001**
Social Rank	0.463	0.164	55	2.83	**0.007**
Prey Density	0.178	0.098	69	1.824	0.073
Clan Size	-0.062	0.123	59	-0.504	0.616
Parity	-0.677	0.227	53	-2.979	**0.004**
Years to assay	-0.047	0.119	53	-0.392	0.697
Collection time	-0.083	0.169	62	-0.493	0.623

Other Top-Ranked T Models

The results of top-ranked T models (Table A2.2), not presented in main chapter manuscript, are summarized below (Table A2.4).

Table A2.4. Predictors of maternal fecal concentrations of testosterone (T) (ng/g of dry weight of fecal material) in fecal samples (n = 76) collected from pregnant spotted hyenas from linear mixed models with A) no interaction between fixed effects and B) an interaction between prey density and clan size. We present parameter estimates and associated values (standard errors (SE), degrees of freedom (df), t-statistic (t)) from linear mixed models predicting logged T concentrations. Models included a random effect of pregnancy and fixed effects of social rank (1 being highest rank and -1 lowest), mean prey density (# prey items/km^2), clan size (#adult females in clan), maternal parity (multiparous (n = 61) vs primiparous (n = 15), with primiparous as the reference level), years to assay (# of years between fecal collection and assay), and fecal collection time (am vs pm, with pm as the reference level). Continuous predictor variables are centered and standardized. Statistically significant effects are shown in bolded font.

A) No Interaction Model

Fixed effect	**Estimate**	**SE**	**df**	**t**	***p*-value**
Intercept	3.24	0.154	64	21.05	**<0.0001**
Social Rank	0.461	0.194	51	2.37	**0.022**
Prey Density	0.176	0.116	68	1.51	0.135
Clan Size	-0.052	0.147	57	-0.36	0.724
Parity	-0.569	0.269	49	-2.11	**0.040**
Years to assay	-0.156	0.142	49	-1.10	0.277
Collection time	-0.214	0.203	61	-1.05	0.297

B) Clan size by prey density model

Fixed effect	**Estimate**	**SE**	**df**	**t**	***p*-value**
Intercept	3.124	0.162	62	19.31	**<0.0001**
Social Rank	0.442	0.187	47	2.36	**0.02**
Prey Density	-0.031	0.156	59	-0.20	0.84
Clan Size	0.031	0.148	53	0.21	0.84
Parity	-0.621	0.260	45	-2.39	**0.02**
Years to assay	-0.084	0.140	42	-0.60	0.55
Collection time	-0.155	0.205	62	-0.76	0.45
Clan Size*Prey Density	0.268	0.137	57	1.96	**0.05**

Post-hoc Analysis of Clan Size by Prey Density Interaction

Post-hoc analysis was performed to further investigate the interaction between clan size and prey density from the T model presented in Table A2.4B ($p = 0.05$). The parameter estimate for clan size increased with mean prey density within a female's territory around the time of sampling; however, the effect size ($\pm$95% confidence interval) was never clearly above or below zero, regardless of prey density (Figure A2.1). The same could be said when examining the parameter for prey density at different clan sizes (Figure A2.2). It is likely the significant interaction between clan size and prey density was the result of an uneven distribution of data among different clan sizes and prey densities, with data at high levels of prey density and low clan sizes both being relatively rare.

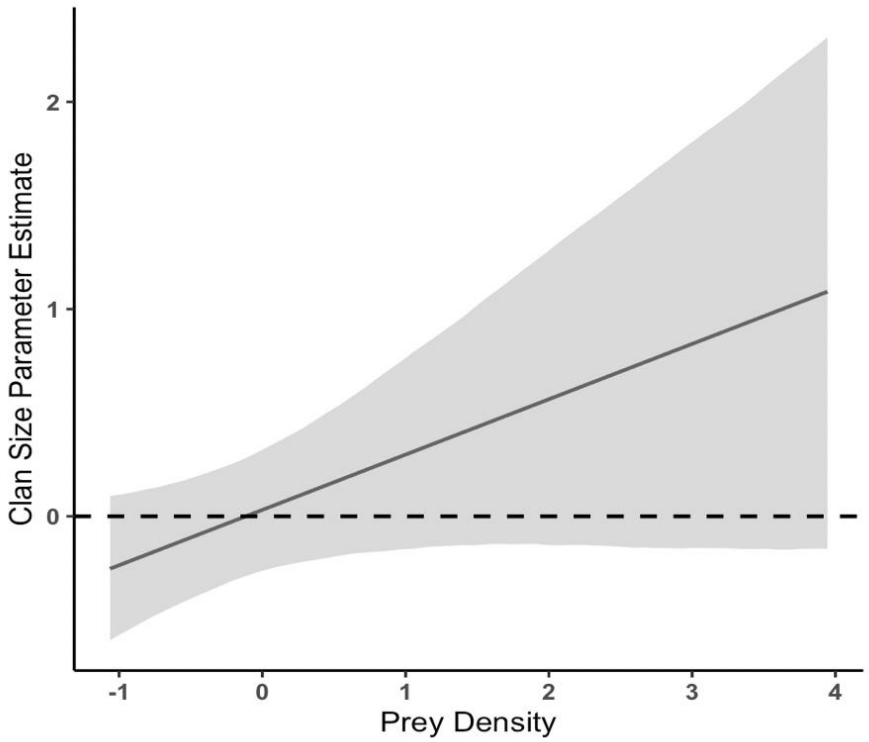


Figure A2.1. Parameter estimate for the effect of clan size on maternal T fecal concentrations (ng/g) (n = 76 samples) depending on mean prey density in a female's territory around the time of sampling. Plot shows changes in the coefficient estimate of

clan size conditional prey density (± simulated 95% intervals, represented by grey shaded area) calculated from the results of linear mixed regression T model (see Table A2.4B) using R package interplot (Solt and Hu, 2018). Clan size and prey density are standardized and centered in the model.

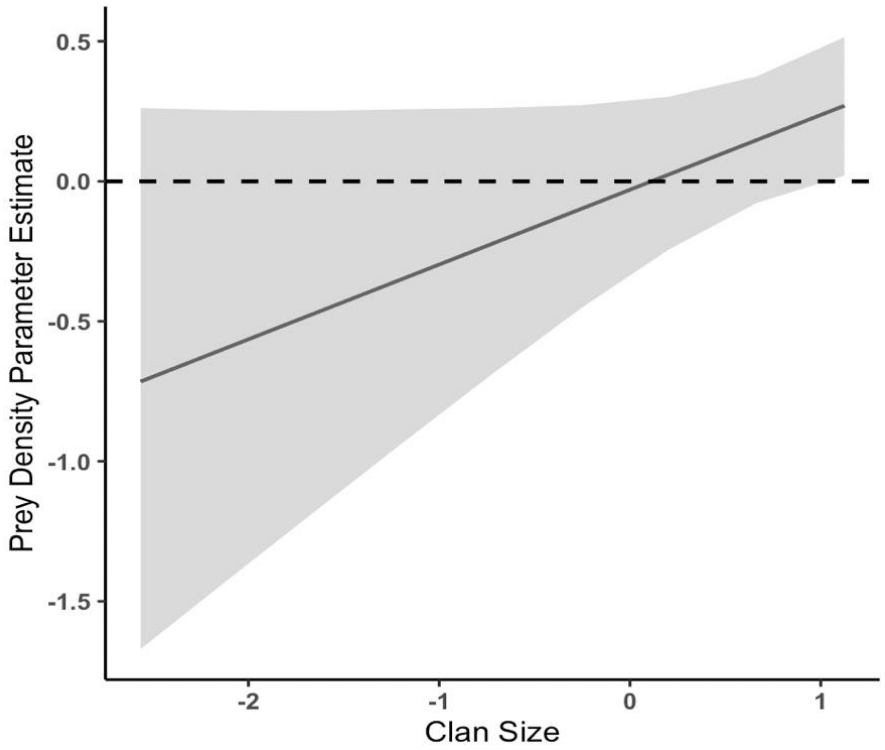


Figure A2.2. Parameter estimate for the effect of prey density on maternal T fecal concentrations (ng/g) (n = 76 samples) depending on the number of adult females (clan size) in a female's social group. Plot shows changes in the coefficient estimate of prey density conditional clan size (± simulated 95% intervals, represented by grey shaded area) calculated from the results of linear mixed regression T model (see Table A2.4B) using R package interplot (Solt and Hu, 2018). Clan size and prey density are standardized and centralized in the model.

Outlier Analysis for Main A4 and T Models

Potential Outliers

Here we present the results of outlier analysis performed on the final (Parity x rank) A4 and T models (n = 76). Datapoints with a Cook's Distance > 0.053 (4/n, n = 76 fecal samples) were considered potential outliers (Table A2.5).

Results presented in the main manuscript (Table 2.1, Figures 2.2, 2.3, 2.4) and Figure A2.5 were from A4 and T models *after* the removal of one outlying data point. (observation #10, Table A2.5) (See next Appendix sections for model results before the removal of this outlier.)

The removed data point (observation #10, Table A2.5) had the highest Cooke's distance for both the A4 (Cooke's distance = 0.05) and T model (Cooke's distance = 0.19) and was well above the cut off value for consideration as a potential outlier (4/n). It was associated with a fecal sample of a primiparous female early in gestation (22 days after her estimated conception date). After the removal of this data point, overall sample size went from n = 76 (including n = 15 samples collected from 12 primiparous females) to n = 75 (including n = 14 samples from 11 primiparous females).

Table A2.5. Potential outlying observations according to outlier analysis on the original parity*rank A) A4 and B) T models including all datapoints (n = 76 samples). Data points considered potential outliers (Cooke's distance > 4/n) are listed (Observation (#)) along with their exact Cook's distance.

A) Androstenedione

Observation (#)	Cook's Distance
48	5.73e-2
4	6.48e-2
69	6.62e-2
76	7.14e-2
10	8.46e-2

B) Testosterone

Observation (#)	Cook's Distance
4	5.30e-2
57	5.76e-2
69	8.35e-2
10	1.86e-1

Results of Parity x Rank Models before Removal of Potential Outlier

Presented below are the results of the models presented in the main chapter 2 manuscript (see Table 2.1) before outlier removal.

Regarding main effects, there were no significant differences between the results of the models before outlier removal (Table A2.6) and models presented in the main manuscript (after removal of observation #10). The interaction between parity and rank, though significant for both A4 and T models after removal of observation #10 (Table 2.1), was non-significant in the T model ($p = 0.25$, Table A1.6B) and trending toward significance in the A4 model ($p = 0.08$, Table A2.6A) before removal of observation #10.

Table A2.6. Results of models predicting logged maternal fecal concentrations of A) androstenedione and B) testosterone (T) (ng/g of dry weight of fecal material) before removal potential outlying data points. Data analyzed (n = 76 samples) came from 32 individual females over the course of 59 pregnancies. 15 samples came from 12 primiparous females. Parameter estimates and associated values (standard errors (SE), degrees of freedom (df), t-statistic (t)) are presented. The models included a random effect of pregnancy and fixed effects of social rank (1 being highest rank and -1 lowest), mean prey density (# prey items/km^2), clan size (#adult females in clan), maternal parity (multiparous vs primiparous, with primiparous as the reference level), years to assay (# of years between fecal collection and assay), fecal collection time (am vs pm, with pm as the reference level), and an interaction between social rank and parity. Continuous predictor variables are centered and standardized. Statistically significant effects are shown in bolded font.

A) Androstenedione

Fixed effect	**Estimate**	**SE**	**df**	**t**	***p*-value**
Intercept	6.068	0.128	64	47.32	**< 0.001**
Social Rank	0.589	0.175	52	3.36	**0.001**
Prey Density	0.196	0.097	68	2.03	**0.047**
Clan Size	-0.090	0.122	59	-0.734	0.466
Parity	-0.422	0.264	59	-1.60	0.115
Years to assay	-0.061	0.118	52	-0.516	0.608
Collection time	-0.107	0.167	62	-0.639	0.525
Parity*Rank	-0.829	0.459	62	-1.809	0.075

B) Testosterone

Fixed effect	**Estimate**	**SE**	**df**	**t**	***p*-value**
Intercept	3.22	0.155	62	20.84	**< 0.001**
Social Rank	0.56	0.210	47	2.66	**< 0.05**
Prey Density	0.19	0.117	67	1.63	0.108
Clan Size	-0.07	0.147	56	-0.50	0.618
Parity	-0.37	0.318	56	-1.16	0.249
Years to assay	-0.17	0.141	47	-1.17	0.246
Collection Time	-0.23	0.203	61	-1.14	0.258
Parity*Rank	-0.65	0.553	61	-1.17	0.245

Post-hoc Analysis of Interaction Term before Outlier Removal

The only substantial difference between the models including all datapoints and the models presented in the main manuscript (after the removal of observation #10) was the significance of the social rank by parity interaction terms. For comparative purposes, we show plots of conditional coefficients from A4 and T models before outlier removal here. Despite the differences in the statistical significance, this analysis revealed the same pattern for both T and A4 as discussed in the main manuscript, though the pattern is weaker for T. (Figures. A2.3, A2.4).

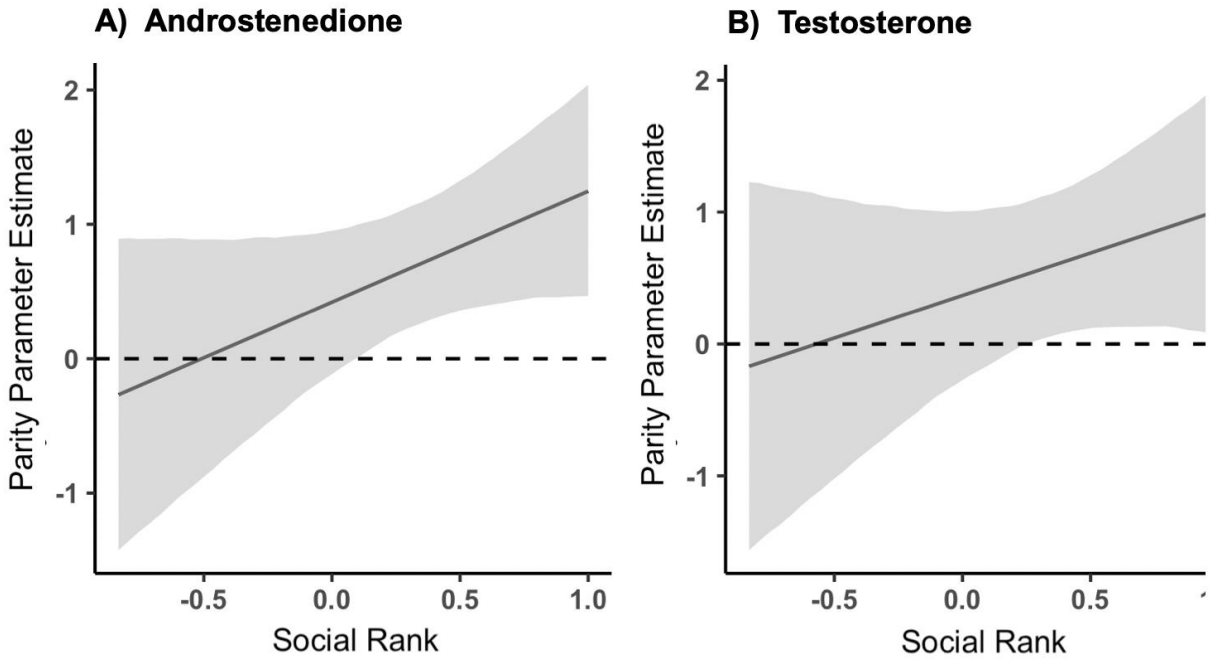


Figure A2.3. Parameter estimates for the effect of parity on fecal concentrations (ng/g) (n = 76 samples) of maternal A) androstenedione and B) testosterone (T) as they vary with standardized female social rank. Plot shows changes in the coefficient estimate of parity conditional on female social rank (± simulated 95% intervals, represented by grey shaded area) calculated from the results of the linear mixed regression A4 model (see Table A2.6) using R package interplot.(Solt and Hu, 2018). Social rank is standardized and centralized, with the highest-ranking hyena in the clan having a social rank of 1 and the lowest of -1.

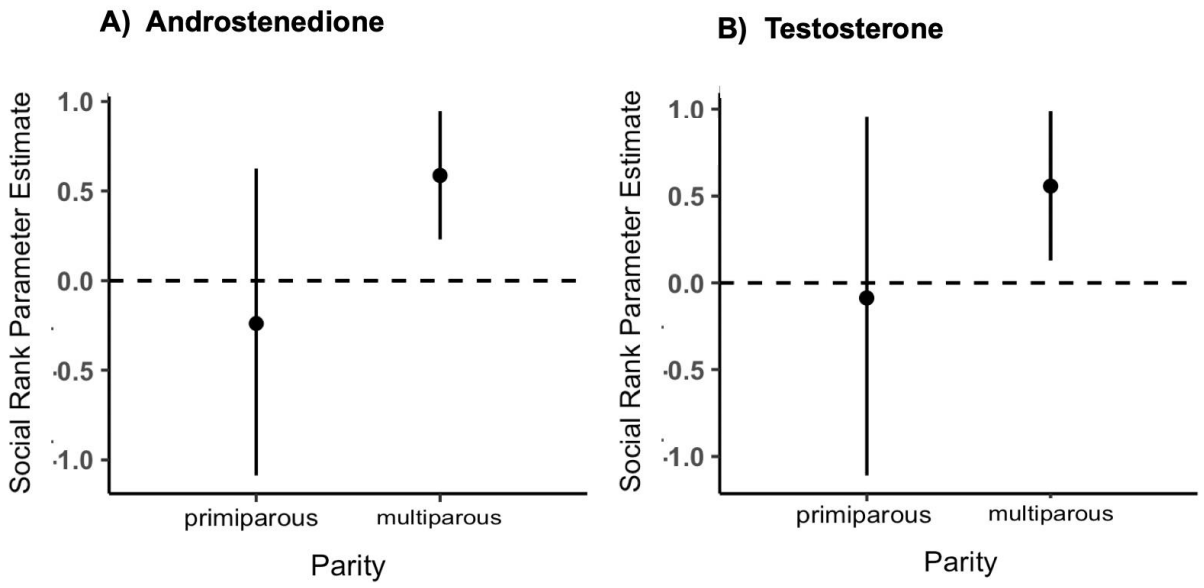


Figure A2.4. Parameter estimates for the effect of social rank on fecal concentrations (ng/g) (n = 76 samples) of maternal A) androstenedione (A4) and B) testosterone (T) in pregnant multiparous (n = 61) and primiparous (n = 15) spotted hyenas. Plot shows changes in the coefficient estimate of social rank conditional on maternal parity (± simulated 95% intervals) calculated from the results of the linear mixed regression models using R package interplot (Solt and Hu, 2018).

Maternal A4 and Prey Density

Below is further discussion of results presented in Table 2.1 of the main chapter 2 manuscript.

The relationship between A4 and prey density was significant in our final model ($T_{A4} = 2.01$, $p_{A4} = 0.05$, Table 2.1); however, this effect appeared to be driven by one data point (point A, Figure A2.5) from a high-ranking female, and the effect was no longer significant upon its removal ($T_{A4} = 1.31$, $p_{A4} = 0.19$).

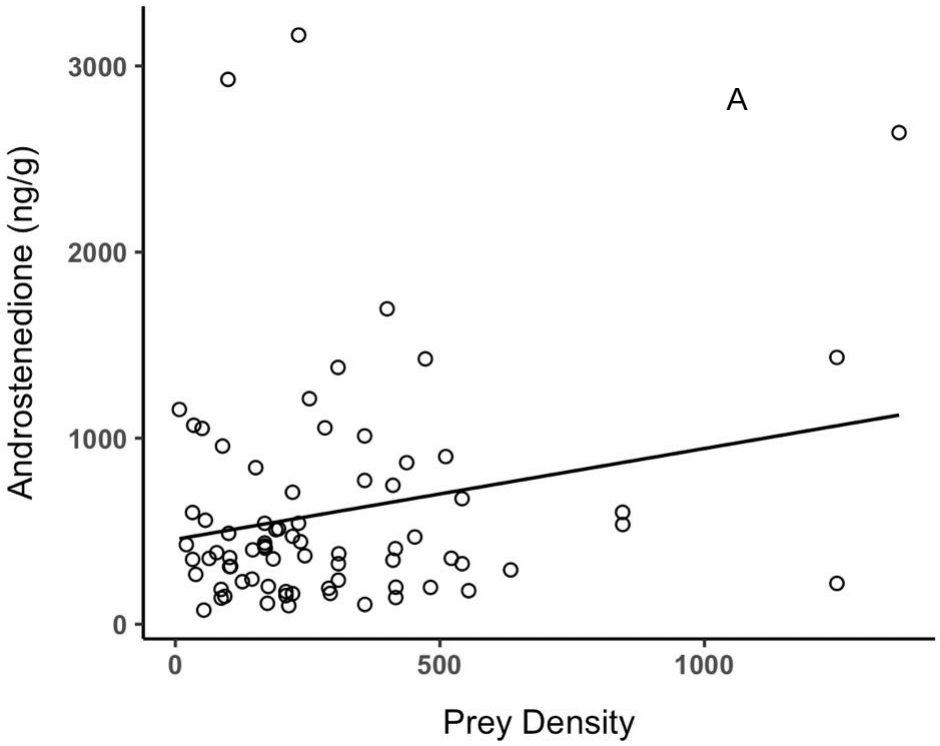


Figure A2.5. Relationship between prey density (# animals/km^2) and maternal fecal concentrations of androstenedione (A4) (ng/g). Linear mixed models indicated a significant effect of prey density on A4 concentrations (t = 2.01, p = 0.05, n = 75 samples, Table 2.1), but this effect was no longer significant after the removal of the data point labeled “A”. Prey is density centered and standardized.

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LITERATURE CITED

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CHAPTER 3

Social Instability and Reproductive State as Modulators of the Relationship between Social Dominance and Testosterone in Female Spotted Hyenas

Introduction

Across vertebrate taxa, androgens both respond to social competition and play a key role in modulating competition-related physical traits and behaviors (reviewed in Oliveira, 2009; Soma, 2006). In social species with dominance hierarchies, increased exposure to androgens is often associated with higher social status and more frequent dominance- related behaviors (i.e. those related to maintaining or obtaining dominance status), including aggression (reviewed in Oliveira, 2009; de Almeida et al., 2015). Testosterone (T), in particular, has been linked to social dominance in male vertebrates, and it is the most commonly studied status-related hormone in humans and other animals (Hamilton et al., 2015). The relationship between testosterone and social status is often bidirectional. On the one hand, testosterone can increase an individual's ability to enhance or maintain its social status via activational effects. On the other hand, changes in social status can influence an individual's testosterone concentrations, with rises or falls in dominance being associated with corresponding heightened or suppressed testosterone concentrations (reviewed in Oliveira, 2009; Kelly and Vitousek, 2017)

A significant positive relationship between testosterone and social status has been found in a wide range of social vertebrates, including fish (e.g. Oliveira et al., 1996), reptiles (e.g. Greenberg and Crews, 1990), and mammals. A large percentage of these studies are on male mammals, most often nonhuman primates (Gesquiere et al., 2011; reviewed in de Almeida 2015) but also canids (Johnston et al. 2007; van Kesteren et al. 2012), rodents (Williamson et al., 2017), and others (e.g. Nubian ibex (Shargal et al., 2008)). Nevertheless, findings have been mixed, with many studies failing to find a significant positive relationship between circulating testosterone concentrations and

social dominance (reviewed in Hamilton et al., 2015; Williamson 2017). These mixed results suggest that the relationship between testosterone and social status is far from straightforward, and that it is likely modulated by various factors, including sex, individual neuroendocrine status (e.g. levels of cortisol), social context, or the nature of the social hierarchy (reviewed in Setchell et al., 2015; Hamilton et al., 2015; Dekkers et al., 2019).

The role of social context in modulating androgens and their effects, in particular, has been put forward as an explanation for the variability in findings relating social status to testosterone concentrations. One commonly explored potential modulator is hierarchy stability. The majority of these studies focus on populations of male primates and compare testosterone concentrations during periods of social stability and instability. Hierarchies are considered unstable when they are disrupted, ranks are actively disputed (often via high rates of overt aggression), and a relatively high number of rank reversals occur among group members. On the other hand, stable hierarchies are associated with very few or no rank reversals; dominance status is maintained by social inertia and thus *relatively* low rates of overt aggression are often observed (e.g. Sapolsky 1993; Higham et al., 2013; Mendonça-Furtado et al., 2014). In a number of species, positive relationships between social rank and testosterone appear to be specific to periods of social instability (e.g. Higham et al., 2013; reviewed in Setchell et al., 2008)). For instance, Sapolsky (1993) found that in wild olive baboons, dominant males had elevated testosterone concentrations and higher levels of aggression relative to subordinate males during a period of social instability (specifically, after the death of an alpha male, when the alpha position was contested). However, testosterone was

unrelated to dominance during more stable periods. Studies in other animal groups have produced similar results (e.g. (fish) Oliveira et al.1996; (birds)Wingfield et al. 1990). Nevertheless, recent studies have questioned the universality of this pattern. For instance, some find that male rank and testosterone are correlated regardless of social instability (e.g. (wild chimpanzees) Muehlenbein et al., 2004; Muller and Wrangham, 2004), or that dominant males have higher but also more variable testosterone levels during unstable periods (e.g. (rhesus macaque) Hingham, et al., 2013). A few have found that T and rank are inversely correlated during times of intensive mating competition (bonobos) or social instability (e.g. (gelada baboons) (Pappano and Beehner, 2014)).

Researchers have cited other factors related to the nature of the social hierarchy to explain variation in the relationship between testosterone and dominance. For example, the level of despotism can vary greatly across social hierarchies according to sex, species, and social group. Highly despotic hierarchies, are steep, asymmetrical hierarchies, often characterized by high rates of aggression directed down the hierarchy. Animals living in such hierarchies might show a stronger relationship between rank and androgen concentrations than those living in relatively egalitarian ones. For instance, socially suppressed subordinate male mice (*Mus musculus*) living in hierarchies with highly despotic alpha males had relatively lower testosterone concentrations compared to the alpha; however, subordinate males living in less despotic hierarchies, where all animals engaged in high rates of competitive interactions, had significantly elevated testosterone compared to subordinates of despotic hierarchies, and there was no relationship between testosterone and status

(Williamson et al., 2017). This concept is supported by some comparative studies on primates living in hierarchies with varying levels of despotism (e.g. Kalbitzer et al., 2015), but evidence is still mixed (see Setchell et al., 2015 for discussion).

Similarly, the relationship between status and testosterone can depend on how rank is acquired or maintained in a specific hierarchy. Testosterone and other androgens are expected to vary less with rank when animals queue for, or inherit, their status rather than compete for it, or when status is maintained through cooperative as opposed to competitive means (Hamilton 2015). For instance, female mandrills, who live in a stable social hierarchy characterized by maternal rank inheritance and little serious dyadic conflict, show no consistent rank relationship with androgen (Setchell et al, 2015). On the other hand, male mandrills, who contest their rank positions via physical aggression and whose ranks can change dramatically in a short period of time, show a positive relationship between social rank and testosterone concentrations (Setchell et al., 2008). Other aspects of the social system, such as fission fusion dynamics or the length of alpha tenure, have also been proposed as modulators of the relationship between testosterone and social rank.

Overall, researchers have cited many ways in which social hierarchies vary in order to explain both between- and within-species variation in the relationship between androgens, usually testosterone, and social dominance. These proposals are not mutually exclusive to one another; they generally reflect the idea that a relationship between androgens and social rank is most apparent when individuals maintain or obtain high social rank via aggression. Nevertheless, some authors point out that even after accounting for how aggressively individuals contest rank, significant unexplained

variation in androgen-dominance relationships remains, especially in females (reviewed in Setchell et al., 2015; de Almeida et al, 2015)

A positive relationship between androgens and social status has been found in females in several taxa (Rosvall et al., 2019), including mammals with particularly intense female competition (Davies et al., 2016; French et al., 2013) and several primate species (e.g. hybrid baboons (Beehner et al., 2005), Barbary macaques (Grant et al., 2011). However, generally fewer studies on females than males find a positive relationship between dominance and androgens (reviewed in de Almeida et al., 2015; Rosvall et al., 2019). This can be attributed to several factors, including lower levels of aggression in many female than male hierarchies. Furthermore, the physiological mediation of agonistic behavior can vary with sex, and female aggression may be more closely correlated to levels of non-androgenic hormones (reviewed in Soma 2006), Finally, low levels of androgens (particularly for testosterone (T), which is often at undetectable levels), in females relative to males, and their fluctuation across female reproductive states, can make it difficult to detect a relationship between their concentrations and dominance.

One particular challenge for the study of females is that hormone concentrations can vary both in their determinants and their relationship with behavior across female reproductive states (reviewed in Bridges, 2016). In many female-dominant mammals for instance, the relationship between androgens and social rank is either only present during, or amplified by, pregnancy (Davies et al, 2016; French et al., 2013); this relationship can vary even further with parity (see chapter 1) and stage of pregnancy (Dloniak et al., 2006).

Unfortunately, in studies of natural populations, females of different reproductive states are often treated as one group, primarily due to small sample sizes or a lack of data on individual reproductive status. This can present a confounding factor in studies examining the relationship between androgens and social status. It also presents a lost opportunity to gain a better understanding of the functional relationship between female androgens and social status; androgens, particularly testosterone (Hau, 2007), can have pleiotropic organizational and activational effects, the strength and nature of which are often dependent upon a female's reproductive state (Kelly and Vitousek, 2017).

Here, we investigate the relationship between testosterone and social rank in female spotted hyenas across different reproductive and social contexts. Spotted hyenas present a fruitful study system with which to examine the relationship between testosterone and female dominance, as previous research has already established a central role for androgens in the physiological mediation of female competition This includes work that has documented rank-related variation in androgen concentrations during pregnancy, and linked this to variation to the aggressive behavior of the resulting offspring (Dloniak et al., 2006; Holekamp et al., 2013). Nevertheless, there are, as yet, no comprehensive studies on the relationship between social status and androgens in non-pregnant female hyenas.

Spotted hyenas also provide a useful comparison to the primate literature, as they live in a social system similar to that of many cercopithecine primates. As in those systems, females live in a linear, stable hierarchy shaped by a learning process called maternal rank 'inheritance'. However, in contrast to most mammals, female spotted hyenas are socially dominant to males, and they emit higher rates and intensities of

aggressive behavior (Van Meter, 2012). This provides an interesting contrast to primate studies. For example, Setchell et al. (2015) suggested that female mandrills show no relationship between fecal androgens and rank because they live in stable social hierarchies in which females inherit their rank and emit low levels of overt aggression. Female spotted hyenas live in similarly structured hierarchies; however, overt aggression is commonly used to maintain the status quo (Van Meter, 2012). Due to the despotic nature of the female hierarchy, we might therefore expect a relationship between social rank and testosterone in this system. However, this relationship may be modulated by the relative stability of the social hierarchy.

Here, we exploit a unique opportunity to assess the relationship between testosterone and female social status across both reproductive states and social contexts by utilizing a large longitudinal data set available to us. We performed two main analyses, asking whether the relationship between testosterone and social dominance varies with either reproductive state ("reproductive state*social rank" analysis) or social instability ("social instability*social rank" analysis). For our "reproductive state*social rank" analysis, we used a dataset of fecal T concentrations from adult females that were either nulliparous, lactating, or pregnant when sampled. For our "social instability*social rank" analysis, we used a subset of samples from lactating females taken during periods in which the hierarchy was known to be relatively stable versus other periods characterized by rapid change and instability.

Materials and Methods

Study Populations and Subjects

This research focused on a large social group, or “clan”, of free-living spotted hyenas whose behavior and demography have been monitored intensively since 1988, including the period during which fecal samples used in this study were collected (between February 1993 and June 2016). This clan, referred to as the Talek clan, defends a large territory in the Masai Mara National Reserve (MMNR) in southwestern Kenya. Hyena clans are female-dominant, fission-fusion societies consisting of multiple matrilines of adult females, their offspring, and one or more adult immigrant males (reviewed in Smith et al., 2017). Hyena social structure is characterized by youngest ascendancy and strict maternal rank ‘inheritance’ with youngest ascendency, a system in which offspring enter the dominance hierarchy immediately below their mothers but above their older siblings (Holekamp and Smale, 1991). Immigrant males join their new clan at the very bottom of its social hierarchy, and forever remain below all adult females and their offspring (Kruuk, 1972; Frank, 1986). Clan members here were individually recognized by their spot patterns and ear damage and sexed based on the sexually dimorphic glans of the erect phallus (Frank et al., 1990). Ages for all natal animals were known and determined based on their appearance when first seen, as described previously (Holekamp et al., 1996). Maternity was established based on genotyping and observations of cubs nursing (Holekamp and Smale, 1993)

Reproductive State

Female spotted hyenas, which reach reproductive maturity at around 24 months of age, are promiscuous and breed year-round, usually giving birth to litters containing

only 1-2 cubs (Holekamp et al., 1999; Kruuk, 1972). They give birth through an enlarged clitoris (or “pseudopenis”), which tears at first parturition, leaving a vertical band of pink scar tissue on its posterior surface. Thus, the date of a female’s first conception can be estimated relatively accurately, regardless of whether or not her litter survived, from recording the appearance of fresh pseudopenis scarring (Frank and Glickman, 1994).

Reproductively mature females (> 24 months) were considered nulliparous until the date of their first conception. Females were categorized as pregnant for the 110 days prior to the known birth of a litter (Kruuk, 1972), as 110 days is the length of the gestation period in this species. Females were considered to be lactating from the day they gave birth to a litter until the first of three milestones was reached: (1) the date of conception of the next litter, (2) the date of weaning of the last litter, or (3) the disappearance of the last litter (Holekamp and Smale 1996). Weaning dates were assigned as described by Holekamp et al. (1996).

All samples analyzed here (n = 1023) were collected from 162 individual females, all of whom were reproductively mature (>24 months) and known to be nulliparous, pregnant, or lactating at the time of sampling.

Social Rank

Social ranks of adult females in the clan were determined from a matrix of outcomes of dyadic aggressive and submissive interactions (Strauss & Holekamp, 2019). Here, females were assigned their own ranks when they reached 24 months of age or their first conception, whichever came first (Glickman et al., 1992; Holekamp et al., 1996). The female hierarchy was updated annually to accommodate demographic change within the clan. Ranks were standardized and centered to account for temporal

variation in clan size, with the highest-ranking female in the clan assigned a rank of 1 and the lowest-ranking female assigned a rank of -1.

Social Instability

Whereas the social hierarchy of the Talek clan was generally stable and unchanging over time (Frank, 196; Holekamp et al., 1993), we witnessed four brief periods of social instability during the course our longitudinal study. Two of these periods of instability were brought on by the death of the clan's highest-ranking (alpha) female at the time, and two others were associated with clan fission events. Specifically, in May of 1999, the alpha female died after having occupied that rank since the mid-1980s (Holekamp et al., 1993). For some months after her death, her daughters and other females fought vigorously for position, and observers noted an increase in serious wounding among them. The daughter who took over the alpha position during this time occupied it until her death in April 2011, at which point a similar period of social upheaval occurred. After Van Meter, 2009, we consider the six months following the death of these alpha females to be periods of social instability.

Periods of social instability were also observed leading up to two clan fission events, during which animals living in the Talek territory split into two distinct social groups. One fission occurred in 2001 and another in 2015. By early January 2001 and May 2015, respectively, contact between the two groups resulting from each fission was rare and usually aggressive (e.g., Holekamp et al., 1993). Following Van Meter et al., 2009, we considered the six months before the completion of a fission event (January of 2001 and May 2015) to represent periods of social instability. For the purpose of analysis, we considered both (1) binning samples associated with the death of an alpha

female with those associated with a clan fission together into one “unstable” category or (2) treating them as separate categories (“alpha death” and “fission”).

For the majority of our 31-year long longitudinal study, the Talek hierarchy was relatively stable. We designated “matched” stable periods of time to compare with our unstable periods in terms of duration and season. In other words, we used samples corresponding to the same months as those from the unstable periods, in order to control for seasonal variation in resource competition but collected in different years. All samples included in the ‘stable’ group were collected between July 1997 and May 2013.

These control periods included June-November 1998 (corresponding to our period of social instability after the first alpha death , but a year before it), April--September 2010 (corresponding to our period of social instability after the second alpha death , but a year before it) , July—December 1997 (corresponding to our period of social instability before the first clan fission, but 3 years before it), and December 2012 – May 2013 (corresponding to our period of social instability before the second clan fission, but 2 years before it). We chose the years for the control periods with the following criteria in mind: a) periods should come well before (for fissions) or after (for alpha deaths) periods of social instability, b) periods should come well before or after the period of time around a mass poisoning event in 2014 that killed many members of the clan, and c) all samples included in the analysis were collected within the same decade. Samples collected during the chosen control periods were binned together into one “stable” group.

Overall, our analysis on social instability included 142 samples collected from 51 lactating females. Of these, 67 samples were collected during designated stable periods

and 75 samples were collected during designated unstable periods. Samples from unstable periods are about equally distributed between “alpha death” (n = 39) and “fission” (n = 36) periods.

Fecal Sampling and Immunoassays

Fecal samples were collected from known hyenas immediately after deposition whenever hyenas were observed defecating. Samples were then mixed, aliquoted, and stored frozen in liquid nitrogen until shipment to Michigan State University, where they were stored in a -80-degree freezer until analysis (Van Meter et al., 2008). Frozen fecal samples were lyophilized and extracted in ethanol, as described previously (Dloniak et al., 2004; Van Meter et al., 2008). Fecal extracts were reconstituted in 1.5 ml methanol, aliquoted and stored frozen at 20˚C until assay. Aliquots were then assayed for T at the Core Assay Facility at the University of Michigan (UM). Fecal extracts were assayed in duplicate at a 1:5 dilution with a testosterone radioimmunoassay kit (MP Biomedicals ImmuChem Double Antibody Testosterone ^{125}I RIA Kit). Cross-reactivity of the antibody with steroids was as follows: testosterone: 100%; 5α-dihydrotestosterone: 3.40%; 5α-androstane-3β, 17β-diol: 2.20%; 11-oxotestosterone: 2.00%; 6β-hydroxytestosterone: 0.95%; 5β-androstane-3β, 17β-diol: 0.71%; 5β-dihydrotestosterone: 0.63%; androstenedione: 0.56%; and epiandrosterone: 0.20%; all other steroids tested: < 0.01%. Linearity, accuracy, and precision were assessed and demonstrated as suggested in Brown et al., 2011. The intra-assay coefficient of variation was 11.7%, and the inter-assay coefficient of variation was 14.8% (n= 15 assays). Three separate researchers performed these fecal assays, and we included a fixed effect of researcher (“assay_by”) in our models to account for minor methodological differences in assays.

Specifically, one researcher, used smaller solutions of fecal extract (a larger dilution) for performed assays.

Statistical Analysis

Fecal T concentrations (ng/g) were logged to normalize the distribution, and their predictors were examined in linear mixed effect models, using R packages lme4 (Bates et al., 2015) and lmertest (Kuznetsova et al., 2016). The restricted maximum likelihood method of estimation was used to evaluate model parameters. All continuous predictor variables were scaled and centered for ease of analysis and interpretation.

Two main analyses were performed: (1) an analysis on reproductive state as a modulator of the relationship between testosterone and rank ("reproductive state*social rank analysis"), including samples from nulliparous, pregnant, and lactating females and (2) an analysis of social instability as a modulator of the effect of social dominance on female T concentrations ("social instability*social rank analysis"). To further assess the results of the initial social instability*social rank model, a follow up analysis was performed by running the same model on females from only the upper and lower thirds of the social hierarchy (excluding mid-ranking females), with social rank coded as a binary categorical variable ("high" vs "low").

Because individual females were often sampled multiple times, a random intercept of individual ID was included in all models. Models included an interaction between social rank (either categorical or continuous) and the modulator of interest: reproductive state ("nulliparous" vs "pregnant" vs "lactating") or social instability ("stable" vs "unstable"). For the "social instability*social rank" model, the main results did not depend on whether social instability was included as a categorical variable with 3 levels

("alpha death" vs "fission" vs "stable") or two ("stable" vs "unstable") (although see Appendix for details). For ease of interpretation, we present the latter here.

For both analyses, we also included the following control variables as fixed effects: (1) the researcher who performed the relevant fecal assay (referred to as "assay by") (see previous section and Appendix for details) , (2) female age (in months). For the social instability*social rank analysis, we also included a binomial variable ("litter") designating whether a female was nursing her first or a subsequent litter. This was included to control for the female's prior breeding experience, which can affect concentrations of other steroid hormones (see chapter 2; reviewed in Bridges, 2015).

Likelihood ratio tests (using maximum likelihood method of estimation) were used to evaluate the significance of categorical variables with more than two levels (e.g. reproductive state) and associated interactions. Post-hoc analysis of pairwise comparisons for categorical variables were conducted with Tukey-adjusted p-values using package emmeans (Length, 2018). Post-hoc analysis of interactions involving a continuous and categorical variable were performed using R packages jtools and interactions (Long, 2019). Conditional regression coefficients ($\pm$ simulated 95% confidence intervals (CI)) were calculated from the results of linear mixed regression models and visualized using package interactions (Long, 2019). Associated test statistics were obtained via simple slopes analysis (R package interactions; Long, 2019), and adjusted for false discovery rates as suggested by Esarey and Sumner (2017).

Model diagnostics were performed to ensure the data met test assumptions, and alpha was set at 0.05. Models were evaluated for outliers using R package

influence.ME (Nieuwenhuis et al., 2012), with data points having a Cook's Distance (CD) of > 4/n considered to be potential outliers (Van der Meer et al., 2010). Removal of potential outliers resulted in no changes in the significance of our main variables of interest.

Results

Reproductive State* Social Rank Analysis

Our analysis indicated the presence of a significant interaction between reproductive state and social rank on female fecal T concentrations (LRT test, chisq = 7.76, df = 2, p = 0.02). Post-hoc testing using simple slopes analysis (Figure 3.1) revealed that T concentrations increased with social rank in lactating (t = 4.84, p < 0.0001) and pregnant (t = 3.21, p = 0.001), but not nulliparous, females (t = -0.043, p = 0.97). The appearance of a rank relationship in lactating and pregnant females seems to be driven largely by an increase in fecal T concentrations in high-ranking females at some point after the conception of their first litter (Figure 3.1).

There was also an overall significant effect of reproductive state (LRT, chisq = 100.18, df = 2, p < 0.0001). Nulliparous females had significantly lower T concentrations than lactating (t = 3.52, p = 0.0012) and pregnant females (t = 9.23, p < 0.001); pregnant females also had higher fecal T concentrations than lactating females (t = -8.71, p < 0.0001).

Table 3.1. Results from the “reproductive state*social rank” model predicting logged testosterone (T) concentrations (ng/g) in fecal samples collected from nulliparous (n =162 samples), pregnant (n = 163 samples), and lactating (n = 698 samples) spotted hyenas. Parameter estimates and associated values (standard errors (SE,), t-statistic (t), and p-values) are presented. The linear mixed effects model included a random effect of individual and fixed effects of ‘assay by’ (designating the researcher who conducted the assay), age (months), social rank ("Rank"), and reproductive state (“Repro.State”; categories included nulliparous (n), pregnant (p), and lactating as the reference level). Statistically significant effects are shown in bolded font. A likelihood ratio test (LRT) revealed a significant reproductive state*social rank interaction (chisq = 7.76, df = 2, p = 0.02).

Parameter	**Estimate**	**SE**	**t**	***p*-value**
Intercept	3.20	0.074	42.82	**< 0.0001**
Assay by (1)	-0.660	0.081	-8.20	**< 0.0001**
Assay by (2)	-0.070	0.081	-0.864	0.388
Age	0.179	0.035	5.11	**< 0.0001**
Social Rank	0.337	0.070	4.84	**< 0.0001**
Repro. State(n)	-0.242	0.085	-2.84	**0.005**
Repro State(p)	0.603	0.075	8.04	**< 0.0001**
Repro State.(n)*Rank	-0.341	0.127	-2.69	**0.007**
Repro State(p)*Rank	0.020	0.112	0.176	0.860

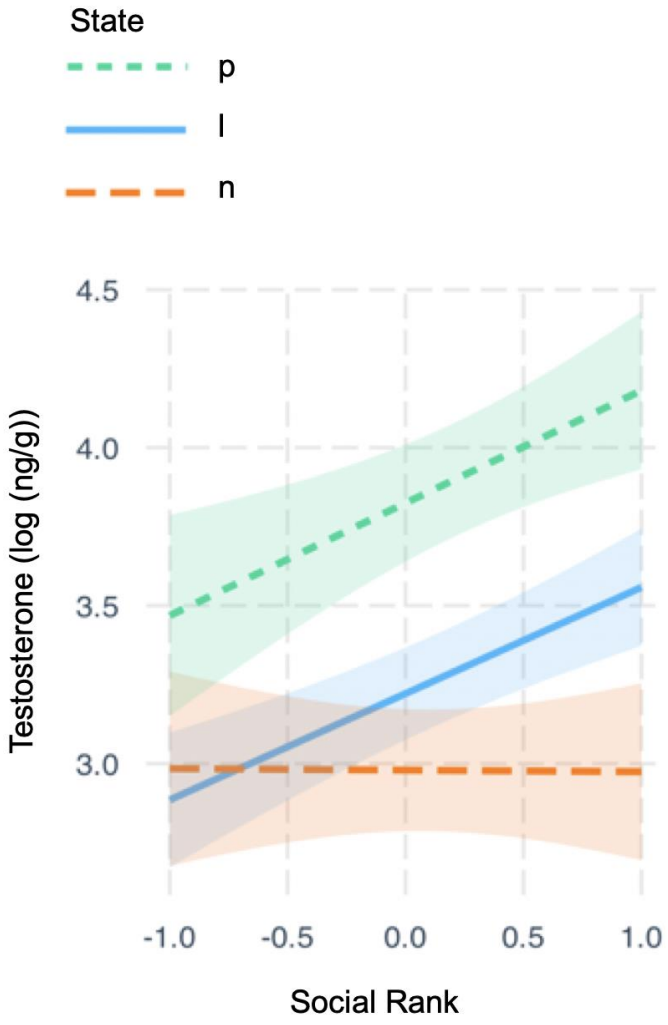

Figure 3.1. Plot of regression lines (±95% confidence intervals) relating logged fecal testosterone (T) concentrations (ng/g) to social rank for nulliparous (n = 162 samples), lactating (n = 698 samples) and pregnant females (n = 163 samples). Social rank is standardized and centered, with the highest-ranking hyena in the clan having a social rank of 1 and the lowest of -1. Effects of social rank on fecal T concentrations were significant in lactating ($t = 4.84$, $p < 0.0001$) and pregnant ($t = 3.21$, $p = 0.0014$) but not nulliparous females ($t = -0.0433$, $p = 0.966$). Regression coefficients and associated statistical estimates are obtained via simple slopes analysis, calculated from the results of the linear mixed effects model (see Table 3.1) model using R package "jtools" (function interact_plot) (Long, 2019).

Social Instability* Social Rank Analysis

Our analysis indicated the presence of a significant interaction between social instability and social rank in their effects on female fecal T concentrations ($t = -2.446$, p

= 0.016, Table 3.2). Post-hoc testing using simple slopes analysis indicated that fecal T concentrations in lactating females increased with social rank during periods of social stability ($t = 3.10$, $p = 0.003$), but not periods of instability ($t = -0.201$, $p = 0.84$) (Figure 3.2). It is unclear whether the lack of a relationship between T concentrations and rank during periods of instability was due to a) an increase in fecal T concentrations in low-ranking females, b) a decrease in fecal T concentrations in high-ranking females, or c) both. (Figure 3.2)

The results of a follow up analysis comparing females in the upper and lower thirds of the hierarchy, but omitting mid-ranking females, revealed a similar pattern, with a significant difference between high- and low-ranking animals during stable ($t = -2.90$, $p = 0.027$) but not unstable times ($t = 0.602$, $p = 0.93$) (Figure 3.3). Low-ranking animals had significantly higher fecal T concentrations during unstable periods than during stable ones ($t = 2.85$, $p = 0.028$, Figure 3.3).

Table 3.2. Results from the “social instability*social rank” model predicting logged testosterone (T) concentrations (ng/g) in fecal samples collected from lactating spotted hyenas (n =142 samples from 51 individuals collected during socially stable (n = 67 samples) and unstable (n = 75 samples) periods. Parameter estimates and associated values (standard errors (SE), t-statistic (t), and p-values) are presented. The linear mixed effects model included a random effect of individual and fixed effects of ‘assay by’ (designating the researcher who conducted the assay), age (months), social rank (“Rank”), and social instability (“Instability”) (reference level is stable). Statistically significant effects are shown in bolded font.

Fixed effect	**Estimate**	**SE**	**t**	***p*-value**
Intercept	2.97	0.257	11.59	**< 2e-16**
Assay by (1)	-0.451	0.220	-2.06	**0.042**
Assay by (2)	-0.166	0.236	-0.705	0.483
Age	0.109	0.091	1.20	0.233
Litter	0.140	0.185	0.756	0.452
Social Rank	0.530	0.171	3.10	**0.003**
Instability	0.237	0.133	1.78	0.076
Rank* Instability	-0.567	0.232	-2.45	**0.016**

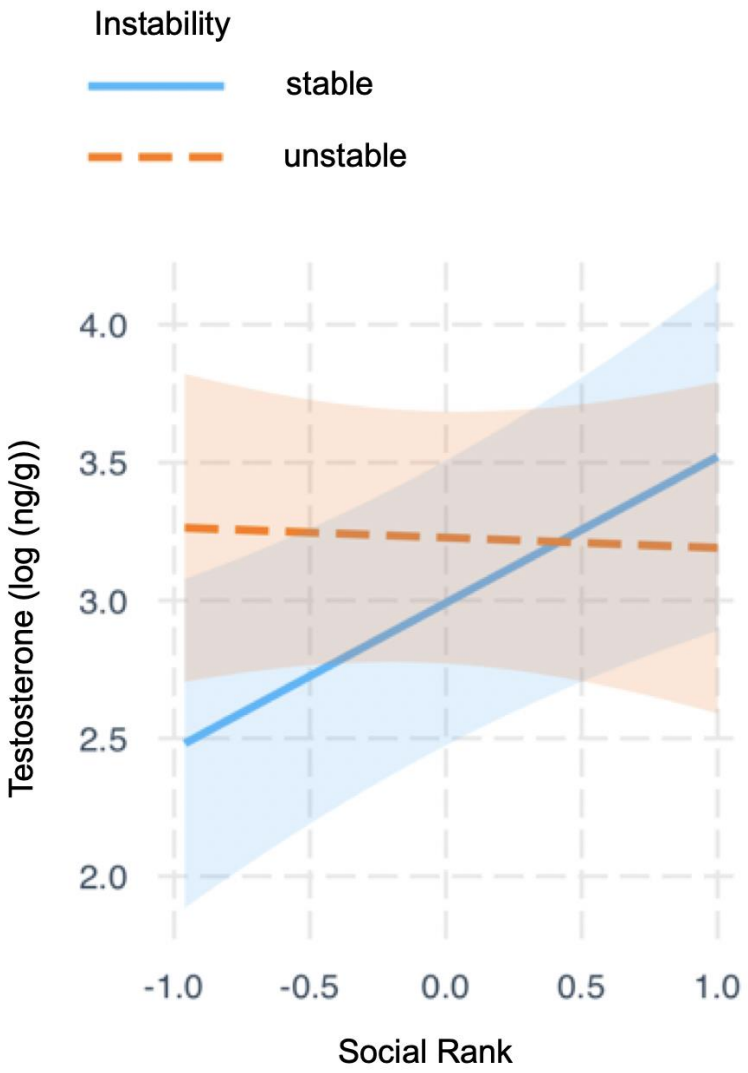

Figure 3.2. Plot of regression lines (±95% confidence intervals) relating logged fecal testosterone (T) concentrations (ng/g) to social rank in lactating females during unstable (red dashed, n = 75 samples) and stable (blue solid, n = 67 samples) periods. Social rank is standardized and centralized, with the highest-ranking hyena in the clan having a social rank of 1 and the lowest of -1. Effects of social rank on fecal T concentrations in lactating females are significant during periods of social stability ($t = 3.10$, $p = 0.003$), but not periods of instability ($t = -0.201$, $p = 0.84$). Regression coefficients and associated statistical estimates are obtained via simple slopes analysis, calculated from the results of the linear mixed effects model (see Table 3.2) model using R package "jtools" (function interact_plot) (Long, 2019).

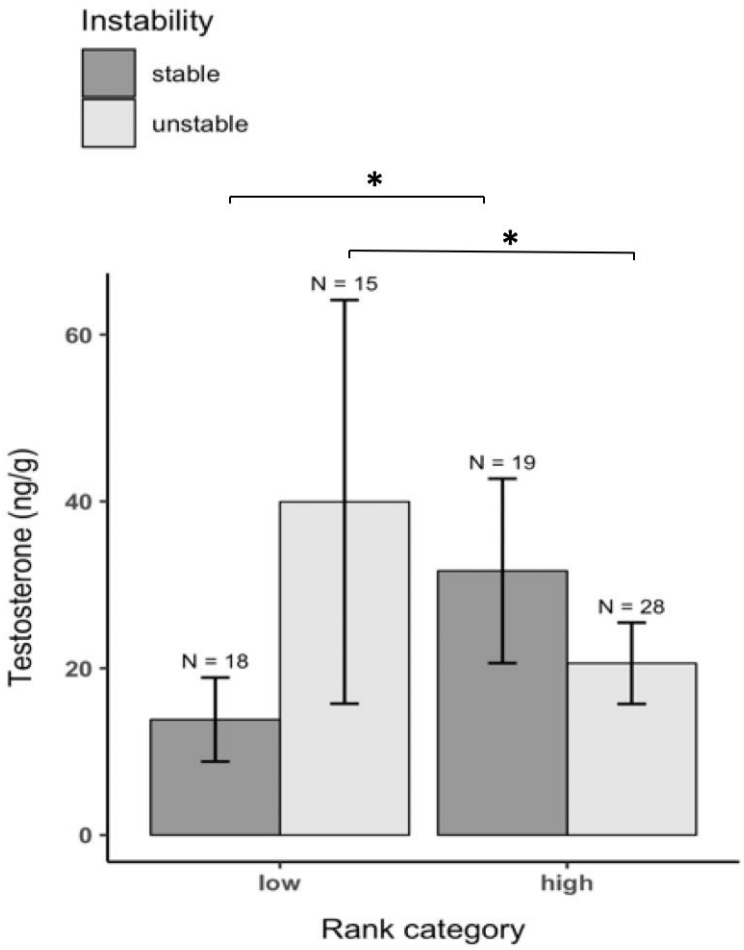

Figure 3.3. Average fecal testosterone (T) concentrations (ng/g) for high- (top third of the hierarchy) and low- (bottom third of the hierarchy) ranking lactating females when the social hierarchy is stable or unstable. Overall sample size is n = 93 fecal samples from 40 individuals, and the number of fecal samples associated with each group are indicated on the plot. Linear regression revealed a significant interaction between social rank and hierarchy stability (LRT, chisq = 8.4179, p = 0.004). Statistically significant differences between groups (Tukey-adjusted p-value < 0.05) are indicated by asterisks.

Discussion

Here we show that the relationship between testosterone and social status in female spotted hyenas varies with both reproductive state and social context. As seen in many despotic social hierarchies marked by intense competition, a positive relationship between social status and testosterone was apparent; high-ranking females

had higher fecal T concentrations than low-ranking ones. However, this relationship was apparent only in breeding females living in a stable social hierarchy. Specifically, a positive relationship between T concentrations and social status was present in lactating and pregnant females, but not in nulliparous females. In a further analysis on lactating females, there was no significant relationship between T concentrations and social rank during times of social instability.

Our results support the idea that social instability can modulate the relationship between testosterone and social status; however, our finding of a positive relationship between testosterone concentrations and rank during socially stable but not unstable times differs from the results of many studies on male primates. These studies often find the opposite pattern, with relationships between rank and testosterone appearing only during times of social instability (e.g. Sapolsky, 1982, 1993; Hingham et al., 2013; Mendonca-Furtado et al., 2014). These results from other species have often been explained in terms of the challenge hypothesis (Wingfield et al., 1990), which suggests that transient changes in androgen levels adjust the expression of androgen-dependent aggressive behaviors to the social context, thus avoiding costs associated with maintenance of chronically elevated testosterone levels. This hypothesis has been applied inconsistently in primate studies, but some researchers suggest that androgens should rise in response to social instability (representing a social 'challenge'), and that this rise should be particularly evident in dominant animals, who must maintain/obtain their rank via aggression (e.g. Mendonça-Furtado et al., 2014). However, Pappano and Beehner (2014) call attention to the fact that, while useful, the challenge hypothesis alone is insufficient to explain variation in androgenic responses to social instability.

This idea is consistent with our results, as the challenge hypothesis fails to explain our finding of a diminished relationship between testosterone and social status during periods of social instability. Furthermore, this is seemingly driven by an increase in testosterone in subordinate individuals and, at least partially, a decrease in testosterone among dominant individuals during unstable periods relative to stable ones. Several other studies on male primates also show that the effect of social instability on testosterone is far from predictable (e.g. Muehlenbein et al., 2004; Muller and Wrangham 2004). One such study on gelada baboons (*Theropithecus gelada*) compared testosterone concentrations in bachelor males, which do not have access to females, and harem-holding males, the dominant individuals in harem units comprised of mostly or all females. Although for most of the year, harem-holding males had higher T concentrations than bachelors, this pattern was reversed during the annual take-over period, when bachelor males challenged harem-holding males for control of their social unit and showed an increase in T concentrations. Like the dominant females in our studies, harem-holding males presumably 'failed to rise to the challenge', exactly when they needed to most (Pappano and Beehner, 2014).

The mechanistic causes for our findings are still unknown, but one way to account for them is to consider the impact of contest-outcomes on androgens. Research across vertebrates suggests that changes in androgens during a social challenge depend on its outcome, with androgens often rising in winners relative to losers (reviewed in Hamilton et al., 2015; Oliveira 2009). In fact, long-term rank-related variation in contest outcomes and their subsequent effects on androgens are a common explanation for positive relationships between testosterone and social status in stable,

despotic hierarchies (reviewed in Oliveira, 2009; Hirshcenhauser and Oliverira 2006). It is possible therefore that changes in the strength and consistency of usually predictable rank-related contest outcomes could explain some of the variation seen across social contexts in studies such as ours. During times of social instability in despotic hierarchies, low-ranking animals may have more opportunities to engage in aggression and experience wins, both of which have been associated with elevations in testosterone (e.g. Williamson et al., 2017), whereas high-ranking animals may unexpectedly lose, potentially experiencing a drop in circulating testosterone.

In addition to social instability, our results suggest that that prior breeding experience is a significant modulator of the relationship between social status and testosterone in female hyenas. We found that on average, nulliparous females had lower fecal T concentrations than lactating and pregnant females; secondly, unlike lactating or pregnant females, they showed no apparent relationship between testosterone and social rank. This pattern appears to be largely driven by an increase in testosterone concentrations in high-ranking females at some point over the course of their first breeding experience. We suggest this point may be sometime late in or after first pregnancy, as we previously found no significant differences in the androgen profiles of primiparous high- and low-ranking females (see chapter1). Finally, the mechanistic causes and functional implications of this increase are also still unclear but represent potentially fruitful avenues for future investigation. This is especially true in light of testosterone's potentially pleiotropic effects on females and their offspring, and how these effects vary across reproductive states.

Overall, studies examining the relationship between androgens and rank across multiple reproductive states in female mammals are limited, and we know of no other study investigating the effect of prior breeding experience on this relationship. Our results therefore support recent calls for a focus on how testosterone in females varies across contexts relevant to female competition and physiology (Rosvall et al., 2019). They also emphasize that T secretion does not reflect the social environment in a uniform way across individual states or social contexts; further investigations into these patterns and their mechanistic causes can help us understand how hormones might flexibly link individual phenotypes to their environment.

APPENDIX

APPENDIX

“Assay_By”

Post-hoc comparisons (based on the “reproductive state*social rank” model) on the ‘assay_by’ variable showed there was no significant different between the two researchers (researchers 1 and 2) who used the same dilutions in the fecal T assays ($t = 0.862$, $p = 0.664$). As expected, they were both significantly different from the third researcher (researcher 3) ($p < 0.0001$), who used smaller solutions of fecal extract (a larger dilution) for performed assays. Researcher 1 assayed 145 samples, 2 assayed 368 samples, and 3 assayed 510 samples.

Results of Social Instability*Social Rank Model with 3 Instability Categories

The “social instability*social rank” model was run with social instability coded as a categorical variable with 3 levels (alpha death vs fission vs stable) instead of two (stable vs unstable, see Table 3.2). Results are as follows: the interaction between instability and rank was significant (LRT, chisq = 7.11, df = 2, $p = 0.03$). There was a significant positive relationship between testosterone and rank when the social hierarchy was stable (n = 67 samples, $t = 3.04$, $p = 0.003$), but not in the months following the death of an alpha female (n = 39 samples, $t = -0.396$, $p = 0.693$) or the months leading up to a fission event (n = 36 samples, $t = -0.341$, $p = 0.734$) (Figure A2.1). Interestingly, on average, animals during a fission appeared to have higher testosterone than animals during times of stability ($t = 2.61$, $p = 0.028$) or after the death of an alpha female ($t = 2.21$, $p = 0.09$). Further investigation is needed to examine how

this pattern depended upon rank, but it appears that the highest-ranking animals did not respond to fissions with an increase in fecal T concentrations (Figure A2.1).

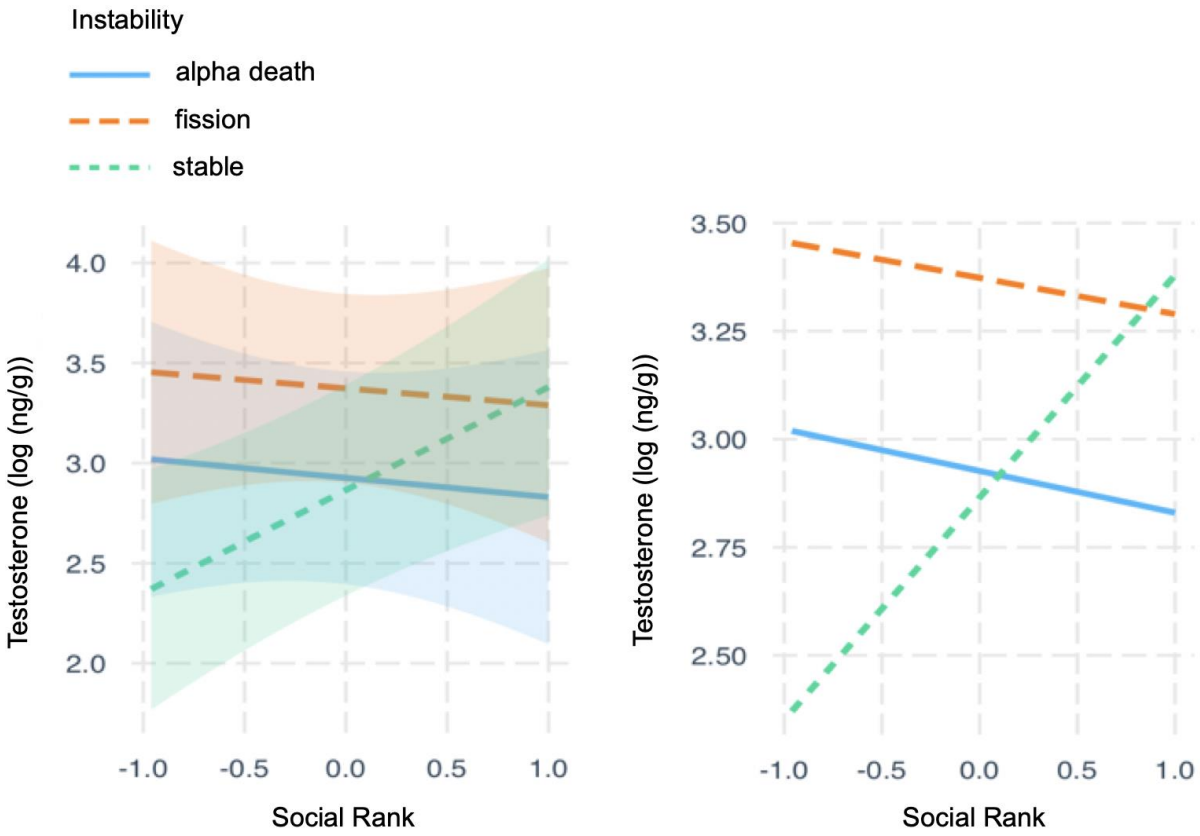

Figure A3.1. Plot of regression lines with (left) or without (right) ±95% confidence intervals relating logged fecal testosterone (T) concentrations (ng/g) to social rank in lactating females. Samples (n = 142) were collected from 51 individuals during periods after the death of an alpha female (solid blue n = 39), before a clan fission (orange dashed, n = 36), or of relative stability (green dashed, n = 67 samples). Social rank is standardized and centralized, with the highest-ranking hyena in the clan having a social rank of 1 and the lowest of -1. Regression coefficients and associated statistical estimates are obtained via simple slopes analysis, calculated from the results of a linear mixed effects model (see 'Statistical Analysis' section) using R package " jtools" (function interact_plot) (Long, 2019).

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CHAPTER 4

Serum Serotonin Concentrations in Spotted Hyenas: Physiological Predictors and Relationships with Social Dominance and Aggression in Males and Females.

Literature Review and Introduction

The serotonin (5-hydroxytryptamine or 5-HT) system is highly conserved across vertebrates, and, through its role as a neuromodulator, is involved in a wide array of behavioral and physiological functions, ranging from energy balance and digestion to sexual and social behavior (reviewed in Azmitia, 2010). This complex system is responsive to the social environment and plays a role in modulating social behaviors associated with both affiliation and competition, including parental attachment, parental care giving, play, cooperation, submissiveness, and most prominently, aggression (reviewed in Kiser et al., 2012; Olivier, 2015; Quadros et al., 2010; Reales et al., 2018). Some researchers suggest that serotonin's influence on these various social behaviors is driven primarily via its positive effect on impulse control and behavioral inhibition (Ferrari et al., 2005; Higley et al., 1996; Lesch and Merschdorf, 2000; Montoya et al., 2012). They suggest that, overall, serotonin facilitates behaviors that involve trading short term motivational gain for delayed gratification or rewards, including certain cooperative and affiliative behaviors, and inhibits behaviors associated with emotional reactivity and impulsive responses to the environment, including certain forms of aggression (Tops et al., 2009; Higley et al., 1996). Serotonergic-mediated behavioral inhibition may be especially potent in the face of potential aversive outcomes, with serotonin promoting behavioral suppression in response to cues predictive of punishment (e.g. Crockett et al., 2009). Particular attention has been paid to serotonin's role in inhibiting aggression, with studies across vertebrates finding that long-term exposure to 5-HT and its actions decreases the likelihood that an individual will behave aggressively toward conspecifics (see Olivier, 2015; Summers and Winberg, 2006 for reviews). Here we review serotonin's role in the interrelated concepts of behavioral

inhibition, aggression, and social dominance. We then discuss spotted hyenas as a potentially fruitful new model species with which to examine this role, and present results of an initial investigation into the relationship between serotonin and social dominance/aggression in spotted hyenas.

Serotonergic Mediation of Aggression

Evidence for serotonin's role in modulating aggression has been found across vertebrate species, in studies focusing on different components of the serotonin system. Results from both correlational and experimental studies lend support to the hypothesis that chronic activation of the central serotonin system, also referred to as "high serotonergic function," inhibits aggression. These studies use a wide range of methods because, due to the complexity of the system, serotonergic function can be manipulated and measured in a variety of ways. Drug or dietary manipulations that increase or decrease central serotonergic activity have been found to reduce or escalate aggressive behavior, respectively, in lizards, fish, and various mammals (reviewed in Ferrari et al., 2005; Olivier, 2015; Summers and Winberg, 2006; Umukoro et al., 2013). These manipulations include selective serotonin reuptake inhibitors (SSRIs), 5-HT 1A/1B receptor agonists or antagonists, dietary tryptophan (trp) supplementation or depletion, and direct administration of 5-HT. A number of correlational studies have also linked aggressive behavior to variation in serotonin system function, assessed using a variety of measures; post mortem concentrations of brain 5-HT and its major metabolite (5-hydrxyindoleacetic acid or 5-HIAA) are often used in laboratory animals, including fish and rodents, but studies on non-rodent mammals most often use less direct biological markers of central serotonergic function. These include concentrations of 5-HT and 5-

HIAA in blood and cerebrospinal fluid (CSF), respectively; 5-HIAA is usually measured in CSF and 5-HT in blood due to differences in relative concentrations and detectability. Concentrations of 5-HT in blood have been shown to correlate with CSF concentrations (e.g. Audhya et al., 2012; Bianchi et al., 2002; Nakatani et al., 2008), and CSF 5-HIAA reflects the sum of 5-HT metabolism in the central nervous system (Higley and Linnoila, 1997; Stanley et al., 1985). Indirect biological markers of central serotonergic function also include physiological responses to pharmacological challenge, used to assess net brain serotonergic responsivity (e.g. Shively et al., 1995). A common test is prolactin response to challenge by fenfluramine, a 5-HT receptor agonist. Overall, central serotonergic function can be indicated by several different measures. For example, low serotonergic function can be indicated by low concentrations of blood 5-HT (e.g. Audya et al., 2012), low CSF concentrations of 5-HIAA (e.g. Higley and Linnoila, 1997), and diminished prolactin response to fenfluramine challenge (e.g. Shiveley et al., 1995) (but see Shively et al., 2014 for caveats).

An array of studies have linked natural variation in serotonergic function with individual differences in aggressive traits. For instance, studies on various rodent and fish species, have found a relationship between 5-HT and individual coping styles in the face of stress. Individuals who tend to react more ‘proactively, responding to stressors with aggression and/or escape behavior, tend to have lower brain 5-HT levels than those who do not (e.g. Koolhas et al., 1999, 2007; . Øverli, et al., 2002, 2003; Butler et al., 2018 for review). A number of studies in humans and other primates (most on monkey species such as rhesus macaques) have found that interindividual differences in brain concentrations of 5-HIAA are relatively stable over time and across situations

(e.g. Higley and Linnoila, 1997; Lesch et al., 2000, Fontenot 2009 ; Shively et al. 1998); furthermore, that they seem to vary with individual predisposition towards aggression. Generally, these studies, report that individuals who have relatively low serotonergic function are more likely to engage in high-intensity, unprovoked, and, in the human literature, 'impulsive' aggression (defined in Montoya et al., 2012 as aggression that is unplanned and driven by effect) specifically, as opposed to more species-typical aggression, (e.g. Shively et al., 1995, Raleigh et al., 1986; Montoya et al., 2012; Umukoro et al., 2013; Westergaard et al., 2003)).

The fact that low serotonin function is often specifically related to high-intensity or impulsive aggression supports the hypothesis that serotonin's influence on aggression is due to a more general influence on behavioral inhibition, with low central serotonergic function leading to a decrease in impulse control and, in turn, to an increase in related forms of aggression (Ferrari et al., 2005; Lesch and Merschdorf, 2000; Montoya et al., 2012). This idea is supported by studies on several monkey species showing a connection between low serotonin function and an individual's likelihood of experiencing injury or early mortality (e.g. Mehlman et al., 1994; Higley et al., 1996; Higley and Linnoila, 1997), as well as their tendency to engage in risky behaviors, including early dispersal (e.g. Fairbanks et al., 1999) , taking long leaps through the tree canopy (Higley and Linnoila, 1997; Mehlman et al., 1994) and short latencies to approach (and more assertive interactions with) a social stranger (e.g. Fairbanks et al., 2001, Kinally et al., 2006). Studies on other species have also indicated that the role of serotonin in modulating aggression depends on the type of aggression under consideration. Though studies on nonprimate animal models do not often attempt to differentiate impulsive and

other types aggressive behavior, a meta-analysis focusing on a range of different animal models (Carrillo et al., 2009) found that serotonergic mechanisms are implicated in the mediation of offensive and predatory aggression, as opposed to defensive aggression. Interestingly, a study on golden hamsters (Cervantes and Delville, 2007) found that animals engaging in higher levels of aggression were also more impulsive in a delay-discounting paradigm, suggesting that findings in rodents may resemble those in primates.

Although overall, most studies have found an inhibitory effect of 5-HT on aggression (Carrillo et al., 2009), it is important to note that this result is far from universal, with some studies finding no relationship between 5-HT and aggression, and some even finding a positive relationship between 5-HT and aggression (see Ferrari et al., 2005; (Kiser et al., 2012)). For instance, Carrillo et al.'s (2009) meta-analysis concluded that while 5-HT had an overall significant inhibitory effect on aggression, this effect was dependent on the species studied (and in the case of laboratory rodents, the strain), the type of aggression studied, and the type of drug treatment used to modulate serotonergic function.

The mechanistic relationship between serotonin and aggression is far from straightforward and our understanding of it is complicated by the fact that the serotonin system has many modes of action. The effect of 5-HT on behavior varies with duration of exposure (e.g. Balázsfi et al., 2018; reviewed in Summers, 2002; Winberg and Thörnqvist, 2016) , brain regions involved (e.g. Vindas et al., 2014; Balázsfi et al., 2018), and receptor subtype (e.g. Hassanain et al., 2003; reviewed Umokoro et al., 2013). 5-HT can even have opposite effects on aggressive behavior, depending on the

level (usually duration) of exposure and the area of the brain or receptor type targeted. For example, one study on female rats (De Almeida and Lucion, 1997)) found that stimulation of 5-HT1A receptors in the median raphe nucleus, dorsal periaqueductal gray, and corticomedial amygdala nuclei decrease maternal aggression in rats, but that certain levels of stimulation in the medial septal area can increase it. This functional variation in the brain likely underlies some of the variation in findings on the relationship between serotonin and aggression and can contribute to difficulty in interpreting correlational studies linking general measures of 5-HT function to behavior.

Serotonin measures commonly used in studies of non-rodent mammals, including blood 5-HT, CSF 5-HIAA, and responses to pharmacological challenge, have been shown to be useful biological markers of central serotonergic function. However, results from these studies may be difficult to interpret. For instance, low levels of CSF 5-HIAA, the most commonly used measurement, is thought to reflect the sum of 5-HT metabolism in the central nervous system and may be particularly indicative of prefrontal cortex activity. However, low levels may be due to a number of factors, including low 5-HT concentrations, which is the usual interpretation, or because 5-HT metabolism is low (i.e. the amount that is transmitted across the synapse and re-synthesized to replace the amount of serotonin used), which could actually reflect *higher* brain 5-HT availability (Shively et al., 2014). Secondly, blood concentrations of 5-HT, while often correlating with brain 5-HT concentrations (e.g. Audya et al., 2012) are confounded by the various peripheral functions of 5-HT, including its role in digestion and metabolism. Nevertheless, despite some mixed results and the difficulty inherent in assessing the functioning of a complex system, it is compelling that across a multitude

of study systems using a host of methods, serotonergic function has been implicated in the modulation of behavioral inhibition and in the setting of individual thresholds for aggression.

Serotonin and Social Dominance

Given the serotonin system's prominent role in modulating social behaviors such as aggression, it follows that serotonergic function often varies with social status in animals living in social hierarchies. The relationship between serotonin and social dominance appears to be bidirectional - serotonergic activity affects a range of behaviors, including aggression, that can influence an individual's ability to attain or maintain social dominance; on the other hand social interactions (e.g., repeated social defeat and other forms of social stress) can have lasting influences on the serotonin system (Bacqué-Cazenave et al., 2017; Kiser et al., 2012b; Summers, 2002; Summers and Winberg, 2006; Veenema, 2009) . For instance, social defeat has been found to have numerous effects on serotonin functioning, including both stimulatory effects (e.g., increases in the activation of serotonergic neurons, serotonin transporter activity, and 5-HT2a receptor binding) and depressive effects (e.g., decrease in 5-HT1a receptor numbers/responsivity) (reviewed in Mooney et al., 2014).

Unsurprisingly, given 5-HT's role in inhibiting aggressive behavior, a negative relationship between serotonergic activity and social dominance has been demonstrated in many studies of rodent (e.g. Blanchard et al., 2001), fish (e.g. Loveland et al., 2014) and reptile species (e.g. Larson and Sommers, 2001). In these study systems, dominance is obtained and maintained largely through aggression, and manipulations of the serotonin system often result in changes to aggressive behavior

and thus social status (Ferrari 2005). For instance, in several species of social rodents, manipulations of the serotonin system have been shown to reverse dominance relationships, with artificial elevations in serotonergic activity resulting in more subordinate status and vice versa (e.g. Blanchard et al., 2001; Bonson and Winter, 1992; Hilakivi et al., 1989). Similarly, in *Anolis carolinensis* lizards, SSRI treatment of dominant lizards resulted in chronic brain 5-HT elevation, reduced aggression, and lowered social status (Larson and Summers, 2001). On the other hand, social status, in particular low social status, can impact serotonergic function. For instance, in Anolis lizards (Summers, 2002) and several species of social fish, subordinate status is associated with chronic activation of the brain serotonergic system (e.g. zebrafish (Dahlbom et al., 2012), African cichlid fish (Loveland et al., 2014) and rainbow trout (Øverli et al., 1999; Winberg et al, 1993). This pattern is largely thought to be due to chronic elevation of serotonergic activity in response to social stress, such as social defeat or harassment, commonly experienced by subordinate individuals (e.g. Higuchi et al., 2019, Summers 2002; reviewed in Backström and Winberg, 2017; Winberg and Thörnqvist, 2016).

A range of studies on old world monkeys, and others on humans, have demonstrated that a relationship between serotonin and social dominance can also be found in complex mammalian social hierarchies. The majority of primate studies (summarized in Table 1) asked whether social dominance correlated with 5-HT functioning (as indicated by biological markers such as blood 5-HT and CSF 5-HIAA) in captive populations of various monkey species. Social groups were often formed of unrelated animals and were either established before, during, or after physiological

sampling. As in most fish, reptile, and rodent study systems, studies on several monkey species, including talapoin monkeys (Yodyingyuad et al., 1985) , long-tailed (cynomolgus) macaques (Kaplan et al., 2002; Riddick et al., 2009; Shively et al., 1995) and rhesus macaques (Howell, et al., 2007; Asher et al., 2013) found that socially dominant animals appear to have lower serotonergic function than subordinate individuals. . However, many studies on primates found either no relationship (e.g. Bowden et al., 1989; Kaplan et al., 2002) or the reverse relationship, with social dominance being associated with *higher* central serotonergic function. These include studies on female pigtailed macaques (Westergaard et al., 1999), male common squirrel monkeys (Steklis et al., 1986), the majority of several studies on male vervet monkeys (Mcquire et al., 1983; Raleigh et al., 1991; but see Fairbanks et al., 2004) and several on humans (Tse et al., 2014; reviewed in Qu et al., 2017). Perplexingly, a positive relationship has also been found in several studies of female rhesus macaques (Higley et al., 1996; Westergaard et al., 1999) and long-tailed macaques (Shively et al., 2003), groups for which other studies found an inverse association (Asher et al., 2015, Shively et al., 1995; Riddick et al., 2009) (see Table 1).

Table 4.1. The relationship between social dominance and measures of serotonergic function in various monkey species. Information about the study subjects (species, sex, age group) is presented, including the sex composition of their social groups. As most studies involved captive, and often unrelated animals, social hierarchies were not always established at the time of the study. We list whether the measurement of 5-HT function occurred before, after, and/or during the establishment of the focal individual's social status ("Timing of measurement"). Studies varied in how they assessed (or manipulated) 5-HT function and their methods are indicated under "5-HT measure" (measurements include CSF 5-HIAA, whole blood (WB) 5-HT, fenfluramine challenge, and others). The direction of the relationship between the 5-HT measure and social dominance is listed in "Relationship to dominance" (positive if dominant individuals had higher activity, negative if subordinate individuals had higher activity, and none if no significant relationship was found). General comments or additional information related to other columns (indicated by *) is in the "comments" column.

Table 4.1 (con't)

A) Vervet (C. aethiops)

Sex	Age	Sex Composition	Timing of Measurement (before, during, or after rank establishment)	5-HT Measure	Relationship to Dominance	Study (1st author, year)	Comments
M	Adult	Multi M/F	Before, During, After	WB 5-HT	Positive	Mcquire et al., 1983;	
				CSF 5-HIAA	Positive	Mcquire et al., 1983	
				Manipulation*	Positive	Raleigh et al., 1991	* Drugs and dietary modifications (trp) used to increase or decrease 5-HT activity
	Young adult	Multi M/F	Before *	CSF 5-HIAA	Negative	Fairbanks et al., 2004	* CSF 5-HIAA taken before a simulated dispersal correlated with status obtained in new group.

Table 4.1 (con't)

B) Long Tailed Macaque (M . fascicularis)

Sex	Age	Sex Composition	Timing of Measurement (before, during, or after rank establishment)	5-HT Measure	Relationship to Dominance	Study (1st author, year)	Comments
F	Adult	Multi F	Before, After*	CSF 5-HIAA	Negative	Riddick et al., 2009	* CSF 5-HIAA measured before social group formation, was predictive of social status attained, and did not change afterwards ** serotonin transporter
			After	CSF 5-HIAA	None	Kaplan et al., 2002	
				Trp concentration in lower dorsal raphe	Positive	Shively et al., 2003	Females were ovariectomized.
				Fenfluramine challenge	Positive	Shively et al., 1998	Females ovariectomized.
				Fenfluramine challenge	Negative	Shiveley et al., 1995	
M	Adult	Multi M	After	CSF 5-HIAA	Negative*	Kaplan et al., 2002	* Significant after correcting for CSF HVA

Table 4.1 (con't)

C) Talpoin (M.talapoin)

Sex	Age	Sex Composition	Timing of Measurement (before, during, or after rank establishment)	5-HT Measure	Relationship to Dominance	Study (1st author, year)	Comments
F	Adult	Multi F	Before, During, After*	CSF 5-HIAA	Negative*	Yodyingyuad et al., 1985	*Significant relationship only between dominance and measurement taken after rank established
M	Adult	Multi M	Before, During, After	CSF 5-HIAA	Negative	Yodyingyuad et al., 1985	*Significant relationship only between dominance and measurement taken after rank established

Table 4.1 (con't)

D) Rhesus macaque (M. mulatta)

Sex	Age	Sex Composition	Timing of Measurement (before, during, or after rank establishment)	5-HT Measure	Relationship to Dominance	Study (1st author, year)	Comments
F	Range*	Multi F	Before	CSF 5-HIAA	Positive	Westergaard et al., 1999	* Mean age 23 months
	Adult	Multi F, 1-2 M	Before, During, After*	CSF 5-HIAA	Positive	Higley et al. 1996	*Measure was predictive of rank attained and did not change afterwards
			After	CSF 5-HIAA	None*	Michopolous et al., 2012	*Positive trend
				CSF 5-HIAA	Negative	Asher et al., 2013	Females were ovariectomized and some treated with estrogen (E2). Result not dependent on E2 replacement.
M	Young adult	Multi M/F	Before (adult), After (natal)*	CSF 5-HIAA	Negative	Howell et al., 2007	*Juvenile CSF correlates with natal rank and predicts rank after dispersal. This is free-ranging population, but they are food-supplemented and without predators

Table 4.1 (con't)

E) Pig-tailed macaque (M. nemestrina)

Sex	Age	Sex Composition	Timing of Measurement (before, during, or after rank establishment)	5-HT Measure	Relationship to Dominance	Study (1st author, year)	Comments
F	Adult	Multi M/F	After	WB 5-HT	None	Bowden et al., 1989	
	Range*	Multi F	Before	CSF 5-HIAA	Positive	Westergaard et al., 1999	*Average age was 29 months
M	Adult	Multi M/F	After	WB 5-HT	None	Bowden et al., 1989	

Table 4.1 (con't)

F) Common Squirrel (S. sciureus)

Sex	Age	Sex Composition	Timing of Measurement (before, during, or after rank establishment)	5-HT Measure	Relationship to Dominance	Study (1st author, year)	Comments
M	Adult	Multi M	After	WB 5-HT	Positive	Steklis et al., 1985	

The most common explanation of a positive relationship between dominance and serotonin in primates is due to its proposed role in promoting 'social competence'. That is, serotonergic function in primates is often positively associated with certain affiliative behaviors that can be helpful in attaining/maintaining dominance and negatively associated with emotional reactivity and escalated/impulsive aggression, traits often seen in subordinate monkeys (Ferrari et al., 2005; Higley et al., 1996; Raleigh et al., 1991). This idea is supported by early studies on established groups of captive vervet monkeys, which showed that enhancement or suppression of serotonin signaling via drug manipulation could induce social dominance or subordinance, respectively, in treated monkeys (e.g. Raleigh et al., 199l; see Westergaard et al., 1999 for review). This causal relationship seemed largely driven by changes in behaviors towards females. Males who were or became socially dominant showed the most affiliation and the least aggression towards females, whose support in aggressive coalitions allowed them to attain and maintain dominance against other males. (Raleigh et al., 1991)

Variation in the behavioral methods used to attain and maintain social dominance may help account for variation in the relationship between social dominance and serotonin among species and studies. Several researchers have suggested that a negative relationship between serotonergic function and social dominance is more likely when individuals assert dominance status primarily via aggression (often physical), as is common in many rodent, fish, reptile, and some despotic primate species studied (Ferrari et al., 2005, Tops et al., 2009). This is consistent with findings of a negative relationship between serotonergic function and dominance in despotic long-tailed and rhesus macaques, and a positive relationship in studies on less despotic groups, such

as male vervet monkeys and female pig-tailed macaques. It could also explain some variation in findings on the same species living under different captive conditions. Variation in group sex composition, relatedness of group members, and physical enclosure (e.g. escape opportunities) could all potentially influence dominance-related social behavior. For instance, the only study finding a negative relationship between 5-HT function and social dominance in captive male vervet monkeys found that juvenile CSF 5-HIAA was predictive of adult dominance status after a simulated dispersal. Contrary to the studies on established groups, which found that affiliative interactions with females were important in the establishment of dominance (Raleigh et al., 1991), this study found that high body weight and social impulsivity were correlated with adult dominance (Fairbanks et al., 2004).

Overall, it does appear that differences in social structure and conditions can create situations where diverse behavior patterns, and thus activity in associated neuroendocrine systems, is connected to social dominance. However, this idea requires further testing and does not account for opposite findings in studies of the same species living in similar captive conditions, including those on female long-tailed and rhesus macaques (e.g. Shively et al., 1995 vs Shively et al., 1998). In particular, it doesn't explain findings of a positive relationship between social dominance and serotonergic function in these species, which are considered highly despotic. Differences in reproductive state of the study animals appear to underlie at least some of the conflicting findings. For instance, interactions between the serotonin system and estrogens likely account for opposite findings in intact versus ovariectomized adult female long-tailed macaques (Shiveley et al., 2003). This idea is supported by a later

study by Asher et al. (2013) with female rhesus monkeys, that found that E2 replacement in ovariectomized females decreased CSF 5HIAA levels, but only in dominant individuals; however, they found a negative relationship between dominance and CSF 5HIAA regardless of hormone treatment. Clearly, the inter-relationship between serotonin and social dominance is complex, varies with sex, and species, and includes interactions with other neuroendocrine systems. This, in combination with variation in methods (e.g. how serotonin function was measured and whether it was measured before, during, or after hierarchy establishment), makes it difficult to come to a unifying theory explaining the relationship between serotonin function and social dominance in primates.

The Spotted Hyena as a Model System

Although our knowledge of serotonin's role in the mediation of aggression and social dominance is rapidly expanding, the vast majority of mammalian studies involve captive animals from only a handful of primate and rodent species. Here, we seek to add comparative data to the literature by examining the connection between serotonin, social dominance, and aggressive behavior in a wild population of spotted hyenas. Spotted hyenas live in social groups called clans, each of which is organized by a despotic, linear hierarchy characterized by maternal rank inheritance and youngest ascendency, as also occurs in many cercopithecine primates including rhesus macaques, baboons and vervet monkeys. However, patterns of sex-specific competitive behavior in hyenas differ from those in most mammals, in which males are usually socially dominant to females, and engage in more intense and frequent combat than do

females. In contrast, spotted hyena females emit higher rates and intensities of aggression than do males, and adult females are socially dominant to adult males (Szykman et al., 2003; Van Meter Dissertation, 2009).

For both male and female hyenas, the nature of their dominance-interactions vary throughout ontogeny. Female hyenas often give birth to twins, and intra-litter dominance is determined early in life via physical aggression. In mixed-sex litters, the female twin is dominant approximately 80% of the time (Smale et al., 1995). Within the clan at large, both males and females acquire ranks just below that of their mothers via a learning process called “maternal rank inheritance”. By six months of age, juveniles of both sexes begin to challenge lower-ranking adult females, and by 18 months, juvenile females and males dominate 76% and 50% of lower-ranking adult females, respectively. Juvenile females appear to be better at enforcing their rank, partially due to their persistence in attempts to dominate lower ranking adults, as they are more likely to counterattack when threatened by them than are young males (Smale et al., 1993). At 2-5 years of age, males disperse from their natal clan, and subsequently become socially subordinate to all natal animals in their new social group. For the most part, they appear to passively accept subordinate status, never failing to appease even the youngest natal animals (Smale, 1995). Immigrant males ‘queue’ for status, and the males who have been in that clan the longest are dominant to newer arrivals. Although rates of intra-sexual aggression are similar for adult males and females (Curren, 2012), females engage in more intense aggression than do males, and the majority of this aggression is directed down the social hierarchy (Van Meter, 2009). Furthermore, studies on females have also shown that unprovoked aggression directed against

others closely below them in social status may be important for the maintenance of their social rank (Van Meter dissertation, 2009).

Here we use blood serum serotonin concentrations as a biomarker for central 5-HT levels; peripheral concentrations of 5-HT and 5-HIAA have been shown to correlate with CSF concentrations, and can serve as biomarkers of central serotonergic function (Audhya et al., 2012; Bianchi et al., 2002; Nakatani et al., 2008); several studies on other mammals have found blood 5-HT levels to correlate with aggression and impulsivity (e.g. Kinnally, et al., 2006; Rosado et al., 2010). Because ours is the first study to quantify levels of 5-HT in spotted hyenas, one of our objectives is to provide descriptive data on the relationship between blood 5-HT and a number of physiological and demographic factors for which we have data available. These include age, sex, female reproductive status, and two measures of energetic status: an index of body condition and blood glucose concentrations.

Concentrations of 5-HT and 5-HIAA in both the brain and blood have been found to vary with age in several mammals, including rodents (e.g. Morgan, 1987), dogs (e.g. Reisner et al., 1996), humans (e.g. Eklundh et al., 1996) and nonhuman primates (e.g. Fairbanks 1999; Fontenot et al., 2009; Higley 1991); in mammalian females they can also vary with reproductive state (e.g. Jury et al., 2015; Sekiyama et al., 2013). Concentrations of 5-HT and its metabolites in the peripheral system in particular may vary with energetic status. The peripheral serotonin system plays a key role in energy balance and metabolism, and seems particularly important for the regulation of glucose and lipid homeostasis (El-Merahbi et al., 2015; Namkung et al., 2015; Yabut et al., 2019). Accordingly, peripheral levels of 5-HT and 5-HIAA can fluctuate with both long-

and short-term changes in diet or food intake. For instance, recent food ingestion has been found to significantly increase blood 5-HT in horses (Alberghina et al., 2011) (Alberghina et al., 2011), humans (Blum et al., 1992), and other mammals (Namkung et al., 2015). Secondly, blood 5-HT correlates with various indices of body condition in mammals, including humans (e.g. Hodge et al., 2012), rodents (e.g. Crane et al., 2015) and dogs (e.g. Park et al., 2014).

Here, we made use of two different indicators of energetic status available to us. First, we examined the possible relationship between 5-HT and blood glucose concentration, a measure of energy immediately available to an animal. Second, we utilized a mass-based ratio index of body condition, calculated by dividing body mass by an indicator of overall body size. Such measures are meant to reflect individual differences in energy reserves after accounting for differences in body size, with high values considered indicative of higher energy reserves, usually in terms of total or percent body fat. For inclusion in our analyses, we considered two commonly utilized indices: mass/length and, a measure equivalent to human BMI (commonly used in studies of blood serotonin's relationship to metabolism), mass/length^2 (Hodge et al., 2012; see Labocha et al., 2014; Peig et al., 2017 for discussions on body mass indices)

The main aim of our study was to test the hypothesis that interindividual and intersexual variation in aggressive behavior among spotted hyenas is mediated, in part, by serotonergic function. We therefore expected to see lower blood 5-HT concentrations in animals more able to express aggression (socially dominant animals and females) relative to those who more often inhibit it (socially subordinate animals and males). We expected to find an inverse relationship between social dominance and 5-HT in females

specifically. Males are less likely to aggressively assert their social status even at young ages, and adult males must inhibit aggression in the presence of all natal clan members, regardless of their status within the immigrant male hierarchy. Additionally, and in light of literature showing that social defeat/harassment early in life can have long term impacts on serotonergic function and related behavior (e.g. Maloney et al., 2018; Veenema, 2009; Veenema et al., 2006), we included an investigation of the effect of early life social rank on 5-HT concentrations in adult females. Finally, we also expected interindividual variation in blood 5-HT to be relatively stable, and we expected individuals with relatively low 5-HT concentrations to be more likely to engage in aggressive behavior, particularly high-intensity aggression, given the opportunity to do so. Due to limitations in sample size, these last questions were addressed only in females.

Materials and Methods

Study Populations and Demographic Information

Our subjects were part of a long-term field study of free-living spotted hyenas within the Masai Mara National Reserve (MMNR), located in southwestern Kenya. Hyena clans are territorial, fission-fusion societies consisting of multiple matrilines of adult females, their offspring, and several immigrant males that join the clan as adults (reviewed in Smith et al., 2017). Samples were collected between August 1993 and June 2012 from members of 5 clans: Emarti Hill (n = 11 samples), Limping Lion (n = 24 samples), Mara River (n = 44 samples), Fig Tree (n = 28 samples), and Talek (n = 80 samples). Members of each clan defended a stable group territory within the north-

central region of the MMNR. Individuals included here were recognized by their distinct spot patterns and ear damage. Certain demographic (sex and age estimates) and physiological data (body condition and blood glucose) were available from individuals of all clans, but data on maternity, agonistic interactions, and social status was only available for individuals from the more intensively monitored clans - Mara River, Talek, and Fig Tree. Observation sessions were conducted in the morning from 0600 to 1000 and in the evening from 1600 to 2000 h.

Animals in this study ranged from 8 to 271 months old (mean = 47 months) at the time of blood sample collection. Animals were sexed based on the sexually dimorphic glans of the erect phallus (Frank et al., 1990) or, for some individuals in the less intensively monitored clans, during immobilization by inspection of the scrotum for evidence of testes. Ages for natal animals from well-monitored clans were determined (+/- 7 days) based on their appearance when first seen, as described previously (Holekamp et al., 1996). For immigrant males and animals from less well-known clans, ages were assigned (plus or minus 6 months) with an age-estimation model based on tooth measurements taken at darting (Van Horn et al., 2003). Maternity was established based on genotyping and observations of cubs nursing (Holekamp and Smale, 1993).

Reproductive Status

Female spotted hyenas are promiscuous and breed year-round, generally giving birth to litters containing only 1-2 cubs (Holekamp et al., 1999; Kruuk, 1972). They give birth through a pseudopenis, which tears at first parturition, leaving a vertical band of pink scar tissue. Thus, the date of a female's first conception can be estimated relatively

accurately, regardless of litter survival, from recording the appearance of fresh pseudopenis scarring. (Frank and Glickman, 1994). Reproductively mature females (> 24 months) were considered nulliparous until the date of their first conception. Females were categorized as pregnant for the 110 days, the length of the gestation period in this species, prior to the known birth of a litter (Kruuk, 1972). Females were considered to be lactating from the day they gave birth to a litter until the first of three milestones: (1) the date of conception of the next litter, (2) the date of weaning of the last litter, or (3) the disappearance of the last litter (Holekamp et al., 1996). Weaning dates were assigned as described by Holekamp et al. (1996).

Non-pregnant parous females were classified as non-lactating during the gap between the weaning/disappearance of one litter and the conception of 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.

Reliable information on reproductive status was only available for females in continuously monitored clans during periods of time with good den visibility. Therefore, our analysis of reproductive status was restricted to a subset of lactating, nulliparous, and pregnant adult females. Our sample size for females known to be in the 'non-lactating' (n =4) reproductive state was considered too small (n = 4 non-lactating females) to include in the analysis of reproductive status.

Social Rank

Due to differences between natal animals (adult females and their juvenile offspring) and immigrant adult males in how social rank is attained and maintained, we

ranked them within two separate social hierarchies. Ranks within the two hierarchies ("immigrant" and "natal") were assigned independently of one another, so that if an adult female was the lowest ranking natal animal she would be assigned the lowest possible rank, even though she was still socially dominant to all immigrant males in the clan. To account for temporal variations in clan sizes, we calculated each hyena's standardized relative rank by dividing it's absolute numerical rank by the total number of ranked animals in its respective social hierarchy (natal or immigrant) at the time. The highest-ranking individual in the hierarchy was assigned a rank of 1 and the lowest a rank of -1.

The social ranks of adult females were assigned using a dominance matrix based on observations of dyadic agonistic interactions (Holekamp & Smale 1993; Smale, Frank & Holekamp 1993). Subsequently, rank was updated annually to accommodate demographic changes in each clan. Juvenile animals were assigned the rank of their mother, in accordance with maternal rank inheritance and youngest ascendancy, until they reached reproductive maturity at 24 months of age (Glickman et al., 1992; Holekamp et al., 1997). At sexual maturity, young females were assigned their own ranks in the adult female hierarchy.

Social ranks of immigrant males were determined strictly by their tenure in the clan (East and Hofer 2001; Smale et al. 1997), with the earliest arrival holding the highest rank and the most recent arrival holding the lowest. We therefore deduced an immigrant male's rank based on his date of arrival in the clan (as in Holekamp and Smale 1998), and confirmed this assignment based on outcomes of dyadic agonistic interactions (Holekamp and Smale 1993; Smale et al. 1993). Occasionally, adult natal males were included in calculations of the relative ranks of immigrant male because

there have been a few who never dispersed and instead became sexually active and sired offspring within their natal clan. These animals eventually fell in social rank from their natal rank to the top of the immigrant male hierarchy, and accordingly were given a rank of first in the immigrant male queue once they reached 36 months of age (after East and Hofer, 2001). Though they were included in calculations of immigrant male standardized relative ranks, adult natal males were not included in the analysis examining social rank as a predictor of 5-HT concentrations. This was to account for differences in social behavior between immigrant males and adult natal males before they dispersed.

Aggressive Behavior

All aggressive acts emitted by the hyenas during all observation sessions were recorded using all-occurrence sampling (Altmann, 1974). Observation sessions were initiated whenever we saw one or more hyenas separated from others by at least 200 meters and are described in detail by Yoshida (2012). However, here we restricted our analysis of aggressive behavior to female hyenas from the Talek clan. The intensive, longitudinal study of this clan resulted in the most comprehensive data set with which to assess variation in aggressive behavior in relation to 5-HT values. Furthermore, because female hyenas are philopatric and male disperse, sometimes changing clans multiple times during adulthood, females were observed for a much greater portion of their lives than were males. Following Yoshida (2012) and Van Meter (2009), two measures of aggression were calculated for the females included in this analysis: *average lifetime aggression rate* and *average lifetime intensity of aggression*. Only dyadic aggressions emitted during adulthood were included in these calculations.

Females included in this analysis were observed in between 286 and 1687 observation sessions for a total of 53-1,050 hours.

To calculate the *average lifetime aggression rate* for each Talek female, an hourly adjusted aggression rate was calculated for each observation session during which the female was present as an adult. Following Yoshida (2012), only sessions in which the hyena was observed for at least ten minutes were included in this calculation. The rate was calculated as follows:

$$\frac{\text{Number of aggressive acts} \div \text{number of potential targets}}{\text{Duration of the observation session (hours)}}$$

The number of aggressive acts was a count of aggressions emitted by the focal individual towards 'potential targets' during the session. Potential targets were defined as all lower-ranking hyenas present in the session, including adult females, juveniles, and immigrant males. Because aggressive acts directed up the hierarchy are rare, this measure controlled for variation in the number of opportunities each hyena had to aggress during the session. To calculate a lifetime rate for each hyena, we averaged its adjusted aggression rates in all its sessions after reaching reproductive maturity (24 months).

To calculate *average lifetime aggression intensity,* aggression intensity, or the severity of each aggressive act, was measured on a scale of 1 (low) to 3 (high). Aggressive acts with intensities of 1 were intention movements to bite. Aggressive acts with intensities of 2 were intermediate threats, involving snapping or lunging, but no actual contact. Aggressive acts with intensities of 3 were high-intensity aggressions involving a biting attack. The intensities of all aggressive acts emitted by each female

during all her sessions during adulthood were averaged to attain a lifetime average aggression intensity.

Immobilization Procedure and Physiological Variables

Anesthetization and Blood Collection

As part of the ongoing study, hyenas are routinely anaesthetized with tiletamine–zolazepam (6·5 mg kg^{-1} Telazol; Fort Dodge Animal Health, Fort Dodge, IA, USA) administered in a plastic dart fired from a CO_2-powered rifle (Telinject Inc., Saugus, CA, USA) (Holekamp & Sisk 2003). All immobilizations were conducted during the day, most early in the morning, and all were carried out according to guidelines specified by the American Society of Mammologists and approved by the Institutional Animal Care and Use Committee at Michigan State University (MSU; AUF #05/14-087-00). Following Telazol administration, hyenas were weighed (kg) and morphological measurements (cm) taken (see Figure1, Swanson et al., 2011). Morphological measurements included skull length and body length (as depicted in Figure 1 Swanson et al., 2011), which were summed to use as a measure of total body length. This measure was used in our calculations of body condition (mass/length and mass/length^2) (see following 'Statistical analysis' section).

Whole blood was collected from the jugular vein of anesthetized hyenas using both heparinized and non-heparanized vacutainer tubes. Glucose measurements (mg/dL) were taken from whole blood in heparinized tubes immediately after blood draw using a glucose meter (OneTouch Verio, LifeScan Inc, 2014, Lug, Switzerland) Blood in non-heparanized tubes was allowed to clot at ambient temperature and

centrifuged. Sera were then drawn off, aliquoted, and promptly frozen in liquid nitrogen (−196°C) until shipped on dry ice to MSU, where samples were stored at −80 °C. Samples remained frozen until prepped for high performance liquid chromatography (HPLC) analysis.

Quantification of Serum 5-HT Concentrations with High Performance Liquid Chromatography

Between August and October, 2012, we measured serum 5-HT concentrations using high performance liquid chromatography (HPLC) with electrochemical detection, a well-validated method for the analysis of 5-HT concentrations in tissue and biological fluids (e.g. Kema et al., 2000; Peaston and Weinkove, 2004). Sample preparation and HPLC detection protocols were based on protocols in Diaz et al., 2008, briefly summarized as follows. Tubes of blood serum were thawed and a portion (550 ul) was aliquoted into a separate tube for sample preparation. 0.5M Trichloroacetic acid (TCA) was added in a 1:10 dilution to deproteinate samples. Samples were vortexed and then sat on ice for 10 minutes. Tubes were then centrifuged at 4500 x g for 20 minutes at 4 ^{o}C. The supernatant was transferred to a second tube, where it underwent this procedure for a second time (beginning with the addition of TCA). The resulting supernatant was then ultra-centrifuged at 280,000 x g for 2 hours, and the top layer of each sample (20 ul) was placed into a loading tray for auto-injection into a C18 reverse-phase analytical column (KinetexTM 2.6 µm C18 100 Å LC Column, 100 x 4.6mm, Phenomenex, Torrance, CA). 5-HT concentrations were measured using electrochemical detection HPLC (HPLC/ESA Coulchem system) at 0.2 V and 0.6 ml/min flow rate. The HPLC mobile phase solution consisted of 90mM sodium phosphate

dibasic heptahydrate (NaH2PO4), 50mM citrate, 50mM EDTA, and 1.7mM SOS (sodium octyl sulfate). Sample 5-HT concentrations were determined by comparing peak areas in samples with those obtained from standards. 5-HT standards (10 standards ranging from 1.875 to 1500 pg/ul serotonin creatine sulfate monohydrate) were always run prior to experimental samples, to create a standard curve against which sample concentrations could be calculated.

Statistical Analysis

The overall data set, including animals from all clans, consisted of 222 serum samples collected from 213 individual hyenas. Unless otherwise noted, one sample was randomly selected from individuals who were sampled more than once to avoid pseudo-replication. The data set as a whole was evaluated for outliers using an analysis of absolute deviation around the median (R package Routliers; Delacre and Klein, 2019), as suggested by Pollet and Meij (2017). Five points were considered low potential outliers (high and negative deviation from the median). The three lowest were removed from subsequent analyses presented here; when they were included in analyses, they caused violations to model assumptions (e.g. normally distributed residuals).

All statistical analyses were performed in R version 3.1.3. 3 (R Core Team, 2015). For models in which 5-HT serum concentration (ng/ml) was the response variable, values were logged to normalize their distribution. Unless otherwise noted, males and females were analyzed in separate models because the serotonin system is known to interact with sex steroids, and sex differences in serotonergic function are commonly found among vertebrates (e.g. Terranova et al., 2016).Effects of predictor

variables were assessed via linear regression (package lme4; Bates et al., 2015) using the restricted maximum likelihood method of estimation. All continuous predictor variables were scaled and centered for ease of analysis and interpretation. The significance of categorical variables involving more than 3 categories was assessed via likelihood ratio tests. Post-hoc pairwise comparisons with Tukey-adjusted p-values were conducted using package emmeans (Lenth, 2018). When implemented, model comparison was performed using Akaike's information criterion, adjusted for small sample sizes (AICc), with R package AICcmodavg (Mazerolle, 2016). A lower AICc value indicated a better fit, and all models within two AICc points of the best-fitting model (dAICc $\leq$ 2) were considered to be equally good fits to the data (Burnham and Anderson, 2002). The data met assumptions for tests used, and alpha was set at 0.05.. Models were evaluated for multicollinearity using variation inflation factors (VIF). Further model diagnostics were conducted via an examination of diagnostic plots associated with the base lme4 package as well as the ols_diag package. Data points having a Cook's Distance (CD) of > 4/n were considered to be potential outliers (Van der Meer et al., 2010) and further investigated via other model diagnostics. When relevant, the main results of models are reported with and without removal of potential outliers. In terms of reporting our main results, we included all data points in the analysis, unless we specifically described our rationale for excluding certain points.

Our models varied in sample size depending on which variables were included so we report the sample size used in each analysis below. Data on social and certain demographic variables were only available for a subset of individuals. Data collected during immobilization (body condition and glucose) were sometimes not available due to

factors specific to individual dartings (for example, if the animal was waking up too quickly for data collection to be completed). When sample size allowed for it, all predictor variables were retained in models.

Preliminary Analyses

Our samples were collected over the course of 19 years, so we first tested for an effect of storage time (days between serum collection/freezing and HPLC analysis) on 5-HT concentrations. A bivariate linear regression showed no significant effect ($n = 213$, $t = 0.763$, $p = 0.446$), in agreement with previous work showing that 5-HT remains stable for years in frozen blood (Korpi, 1984). Furthermore, including this variable in subsequent analyses did not change model results, so we excluded it from the analyses presented here.

Because our analysis included individuals from several different clans, we tested for an effect of clan on serum 5-HT concentrations with a one-way ANOVA. We found a significant effect ($n = 213$, $df = 4$, $chisq = 4.54$, $p = 0.0001$), driven by one clan in particular. The Mara River (MR) clan had significantly lower serum 5-HT values than Emarti Hill ($t = 2.780$, $p = 0.0463$), Limping Lion ($t = 3.55$, $p = 0.004$), or Talek ($t = 4.102$, $p = 0.0006$). Therefore, for analyses presented here we either a) excluded individuals from Mara River clan, if they did not contribute significantly to the sample size of the relevant model or b) included a binomial categorical variable indicating whether an animal was a member of Mara River (MR) or another ("Other") clan.

Finally, we conducted a preliminary analysis to determine which of two body condition indices to include in final analyses: mass/length or mass/length^2 (Quételet index). We assessed these indices separately in the two sexes, as suggested by

Labocha et al., 2014. The mass/length index was potentially problematic because it was highly collinear with age in juvenile animals. Additionally, it was highly correlated with body length (pearson's correlation, $r = 0.79$, $p < 0.0001$.). One common concern with ratio indices such as these is that if they are correlated with body size overall, they are of little value in comparing individuals of different sizes (Labocha et al., 2014). We therefore used the mass/length^2 index as our measure of body condition.

Statistical Models of 5HT Concentrations

i) Demographic and Physiological Predictors of Serum 5-HT

We utilized our larger data set, including individuals with unknown social ranks, to assess the effects of physiological and demographic variables (later controlled for in our social rank models) (n = 82 males, n = 97 females). We included the predictor variables of age (days), clan (Mara River vs Other), blood glucose (mg/dL), and body condition (kg/cm^2). In the male model, which included both immigrant and natal adult males, residence status (immigrant versus natal male) was also included.

In a separate model, the effect of reproductive status was assessed for adult females whose reproductive status at the time of sampling was known (n = 13 lactating, 8 nulliparous, and 7 pregnant females*)*. For this model, we kept only predictor variables that were significant in the model described above in order to not further limit our already small data set.

ii) Sex Differences in Serum 5-HT Concentrations

We tested for sex differences in serum 5-HT in juvenile (n = 34 males, n = 41 females) and adult (n = 57 males, n = 68 females) hyenas using a linear regression

model that included an interaction between sex and age. Besides clan (MR vs Other), this model included no other predictor variables, as the results of the above model showed that their effects on 5-HT concentrations varied by sex.

iii) Relationship between Social Rank and 5-HT

For our analysis of the relationship between serum 5-HT concentrations and social rank, juvenile males (n=27), juvenile females (n=33), adult females (n= 37), and immigrant males (n=18) were analyzed in separate models. Models included the following predictors: blood glucose (mg/dL), body condition (kg/cm^2), age (days), and an individual's social rank at the time it was sampled. Clan (MR vs Other) was also included for the immigrant male and adult female models, as these models included individuals from the Mara River clan.

In our analysis on immigrant males, we report the main results (Table 4.2A) after the removal of one outlying data point. Along with being associated with the largest Cook's distance score in the model, well above our cut-off (CD = 0.45, cut off = 0.22), its removal resulted in a much-improved model fit (the associated adjusted R^2 went from - 0.054, indicating extremely poor model fit, to 0.17). Finally, notes associated with processing of that sample specify that the color of the serum was pink as opposed to the usual light yellow, which could indicate the occurrence of hemolysis (rupture of the protective membrane around red blood cells). Hemolysis is a common preanalytical error in the analysis of blood samples and can contribute to measurement error for a number of blood metabolites (Kamlage et al., 2014).

We next investigated whether an individual's social rank at birth affected 5-HT concentrations later in life using a subset of adult females included in the above analysis, all from Talek clan (n= 22). We often did not know ranks at birth for the females from less intensively monitored clans, and most immigrants were born in unmonitored clans. Juveniles were not included in this analysis because their ranks at

sampling were virtually identical to their ranks at birth. We ran two models to compare the predictive power of social rank at birth and social rank at sampling on 5-HT concentrations in the same subset of females; one model was identical to the models above and the other model used *social rank at birth* instead of *social rank at the time of sample collection*.

iv) Inter-Individual Stability in Serotonin Concentrations

A linear mixed model was run on a subset of 7 Talek females (n = 15 samples) for whom we had repeat samples (either 2 or 3) over the course of their lifetime (between 8.3 and 134.0 months old). Individual ID was included as a random effect. Due to our limited sample size, only fixed effects that had been significant predictors of female 5-HT in our other analyses (age and social rank at birth) were included.

The stability of inter-individual differences in serum 5-HT was quantified using the intra-class correlation coefficient (ICC), calculated as the between-subjects variance divided by the sum of the between – and within- subjects variances (Tucker et al., 2007). Parametric bootstrapping was utilized (10,000 simulations using bootMer function in lme4 package; Bates et al., 2015) to obtain an associated 95% confidence interval.

v) Serum 5-HT as a Predictor of Female Aggressive Behavior

As an initial investigation into the relationship between blood 5-HT and aggression, we inquired whether individuals with the highest and lowest average lifetime rates or intensities of aggression also had the highest and lowest 5-HT concentrations, respectively. Our overall dataset included 23 females (n = 27 samples) who were sampled in adulthood and for whom we had enough behavioral data over their lifetime

to include in the analysis. We averaged the 5-HT concentrations of the seven females with the lowest 5-HT serotonin concentrations into a “low serotonin” group, and the seven females with the highest concentrations into a “high serotonin” group. If a female was sampled more than once during adulthood, an average of those sample values was used in this calculation. Two models were run, one with *average lifetime aggression rate* and one with *average lifetime aggression intensity* as the response variable, to test whether individuals in the high serotonin group differed behaviorally from those in the low group. Non-parametric tests (Kruskal-Wallis) were used to account for the non-normal distribution of the two response variables.

Results

Demographic and Physiological Predictors of Serum 5-HT

Serum 5-HT was negatively correlated with body condition in male ($t = -2.88$, $p = 0.005$) (Figure 4.1) but not female hyenas ($t = -1.04$, $p = 0.302$) (Table 4.2). Although blood glucose did not have a significant effect on 5-HT concentrations in females ($t = -1.04$, $p = 0.77$), there was a near-significant trend in males ($t = -1.88$, $p = 0.06$) (Table 4.2). Serum 5-HT increased with age in females ($t = 2.50$, $p = 0.014$) but had no significant relationship with age in males ($t = -0.198$, $p = 0.843$) (Table 4.2). There was also no overall difference between natal and immigrant males in terms 5-HT concentrations ($t = -0.725$, $p = 0.47$) (Table 4.2). (Note that this is also true if the model is limited to only adult males ($t = -0.999$, $p = 0.32$), which vary less in overall size than males of all age groups).

In adult females, we found no evidence that serum 5-HT varied with reproductive state; we found no significant differences in 5-HT concentrations between nulliparous (n = 8), lactating (n = 13), and pregnant (n = 7) females (n = 28, LRT, $F = 0.058$, $p = 0.79$).

Table 4.2. Results of linear regression models testing demographic and physiological predictors of logged serum 5-HT concentrations (ng/ml) in male (A) (n = 82 samples) and female (B) (n = 97 samples) spotted hyenas. Model parameter estimates and associated values (standard errors (SE), t-statistics (t), and p-values) are presented. Predictors include body condition (kg/cm^2), blood glucose (mg/dL), age (days), clan (Mara River (MR) vs Other, with Other as the reference level), and, for males (A) resident (res.) status (immigrant vs natal, with immigrant as the reference level). Continuous predictor variables are centered and standardized. Statistically significant effects are shown in bolded font.

A) Males

Fixed effect	**Estimate**	**SE**	**t**	***p*-value**
Intercept	6.44	0.108	59.76	**< 2e-16**
Body Condition	-0.178	0.062	-2.88	**0.005**
Glucose	-0.110	0.059	-1.88	0.064
Age	-0.018	0.091	-0.198	0.843
Clan (MR)	-0.229	0.117	-1.96	0.053
Res. (Natal)	-0.115	0.158	-0.725	0.471

B) Females

Fixed effect	**Estimate**	**SE**	**t**	***p*-value**
Intercept	6.40	0.047	134.74	**< 2e-16**
Body Condition	-0.051	0.049	-1.04	0.302
Glucose	-0.012	0.041	-0.287	0.775
Age	0.147	0.059	2.50	**0.014**
Clan (MR)	-0.393	0.121	-3.22	**0.002**

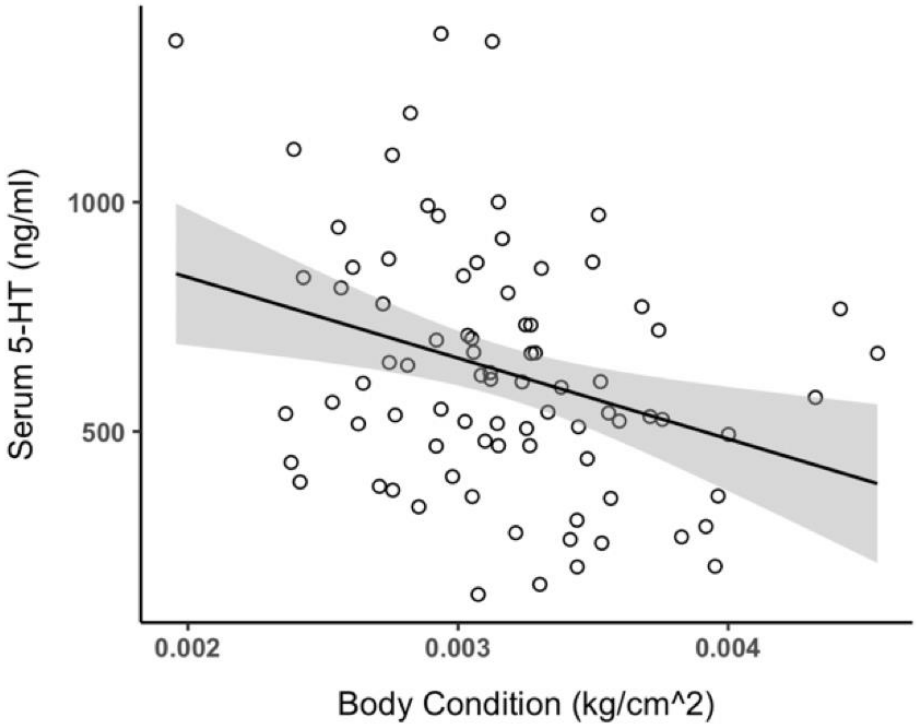

Figure 4.1 Correlation (±95% interval) between body condition (kg/cm^2) and serum serotonin (5-HT) concentrations (ng/ml) in male spotted hyenas. There is a significant, negative effect of body condition on 5-HT concentrations, according to the linear regression model on logged 5-HT concentrations (n = 82, t = -2.88, p = 0.005, Table 4.2A).

Sex differences in serum 5-HT across ontogeny

There was no evidence of a sex difference in average 5-HT concentrations (t = -0.936, p = 0.35). Post-hoc analysis of the trending age by sex interaction (t = 1.89, p = 0.06), further revealed that this was the case for both juveniles (t = - 1.65, p = 0.10, n = 34 males, 41 females) and adults (t = 0.936, p = 0.35, n = 57 males, 68 females).

Relationship between Social Rank and 5-HT

Social Rank at Time of Sample Collection

Social rank at sampling was not a significant predictor of serum 5-HT concentrations in either immigrant or juvenile natal males, (t = -0.511, p = 0.6192 and t

= -1.17, p = 0.25, respectively) (Table 4.3A and B). For juvenile females (*t = -2.65, p = 0.01*) (Table 4.3D, Figure 4.2), but not adult females (*t = -1.44, p = 0.163*) (Table 4.3C), social rank had a significant negative effect on serum 5-HT concentrations, with more socially dominant animals having lower 5-HT than more subordinate ones. The effects of social rank in these models did not change with the removal of potential outliers; however, body condition for juvenile males went from non-significant (Table 4.3B) to significant (t = -2.022, p = 0.0561), and age for adult females went from trending (Table 4.3C) to significant (t = 2.861, p = 0.008) after the removal of one high outlying point for each model.

Social Rank at Birth

Unlike social rank at sampling, social rank at birth was a significant predictor of adult female serum 5-HT, with serum 5-HT decreasing as social rank increased (t = -1.55, p = 0.14, Table 4.4) (Figure 4.3). When a model on the same subset of data was run with social rank at sampling instead of social rank at birth, the effect of rank was non-significant (t = -1.549, p = 0.14) and the model fit was reduced (dAIC = 2.8). Thus there is strong evidence that social rank at birth is a better predictor of adult serum 5-HT than their current social rank.

Social rank's effect in this model was robust to outlier removal, but the removal of the same outlying point mentioned above again resulted in age becoming significant (t = 3.24, p = 0.005).

Table 4.3. Results of linear regression models testing the effect of social rank at sampling on logged serum 5-HT concentrations (ng/ml) in (A) male adult (immigrant) (n = 18 samples), (B) male juvenile (n = 27 samples), (C) female adult (n = 37 samples) and (D) female juvenile (n = 33 samples) spotted hyenas . Model parameter estimates and associated values (standard errors (SE), t-statistics (t), and p-values) are presented. Predictors include body condition (kg/cm^2), blood glucose (mg/dL), age (days), social rank. For adult male (A) and adult female models (C) clan (Mara River (MR) vs Other, with Other as the reference level) is also included as a predictor. Continuous predictor variables are centered and standardized. Statistically significant effects are shown in bolded font.

A) Adult (Immigrant) Males

Parameter	**Estimate**	**SE**	**t**	***p*-value**
Intercept	6.15	0.250	24.59	**5.8e-11**
Body Condition	-0.416	0.184	-2.26	**0.045**
Glucose	-0.053	0.140	-0.375	0.715
Age	0.098	0.209	0.470	0.647
Clan (MR)	0.160	0.259	0.618	0.548
Social Rank	-0.181	0.355	-0.511	0.619

B) Juvenile (natal) Males

Parameter	**Estimate**	**SE**	**t**	***p*-value**
Intercept	6.96	0.783	8.89	**9.8e-09**
Body Condition	-0.186	0.113	-1.65	0.114
Glucose	-0.002	0.077	-0.021	0.983
Age	0.625	1.01	0.619	0.542
Social Rank	-0.142	0.123	-1.17	0.254

Table 4.3 (con't)

C) Adult Females

Parameter	Estimate	SE	t	*p*-value
Intercept	6.46	0.085	76.00	**2e-16**
Body Condition	-0.095	0.071	-1.34	0.193
Glucose	-0.034	0.084	-0.401	0.692
Age	0.179	0.094	1.90	0.068
Clan (MR)	-0.366	0.195	-1.88	0.071
Social Rank	-0.148	0.103	-1.44	0.163

D) Juvenile females

Parameter	Estimate	SE	t	*p*-value
Intercept	7.69	0.731	10.51	**3.19e-11**
Body Condition	-0.092	0.131	-0.701	0.489
Glucose	-0.048	0.050	-0.964	0.343
Age	1.61	0.919	1.75	0.091
Social Rank	-0.360	0.136	-2.65	**0.013**

Table 4.4. Results of a linear regression model testing the effect of social rank at birth on logged serum 5-HT concentrations (ng/ml) in adult female (n = 22 samples) spotted hyenas. Model parameter estimates and associated values (standard errors (SE), t-statistic (t) are presented. Predictors include body condition (kg/cm^2), blood glucose (mg/dL), age (days), and social rank. Continuous predictor variables are centered and standardized. Statistically significant effects are shown in bolded font.

Parameter	**Estimate**	**SE**	**t**	***p*-value**
Intercept	6.46	0.101	64.95	**<2e-16**
Body Condition	-0.111	0.089	-1.25	0.227
Glucose	-0.061	0.090	-0.676	0.508
Age	0.169	0.154	1.10	0.286
Rank at birth	-0.270	0.121	-2.22	**0.039**

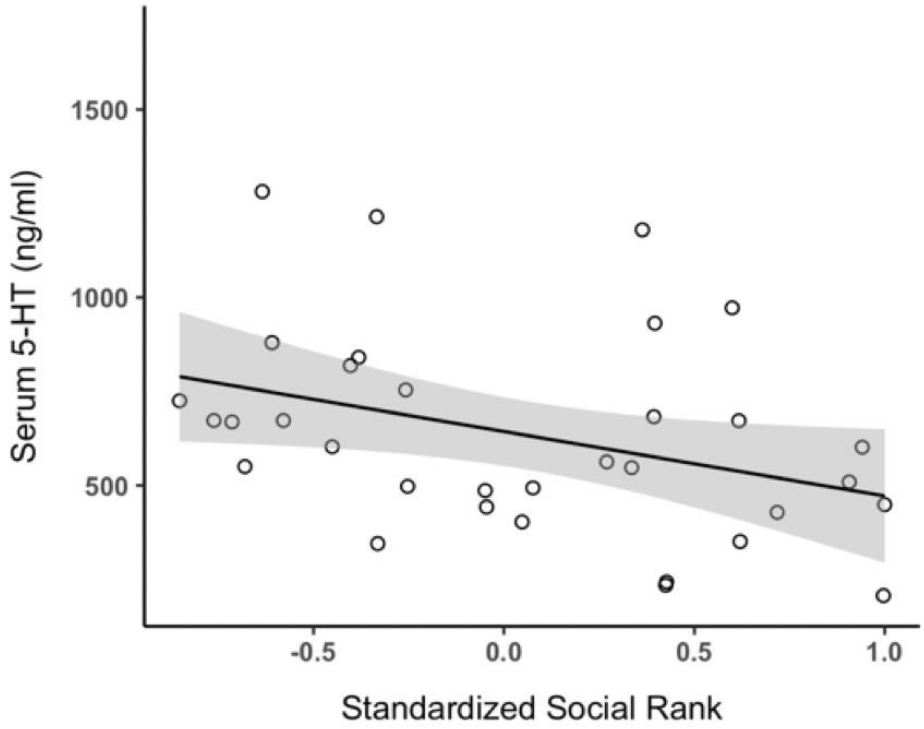

Figure 4.2. Relationship between social rank at sampling and serum serotonin (5-HT) concentrations (ng/ml) in female juvenile spotted hyenas. Social rank is standardized and centered, with the highest-ranking hyena in the clan having a social rank of 1 and the lowest of -1. Socially dominant juvenile females have significantly lower concentrations of serum 5-HT (ng/ml) than subordinate ones (n = 33, t = -2.65, p = 0.01, Table 4.3D).

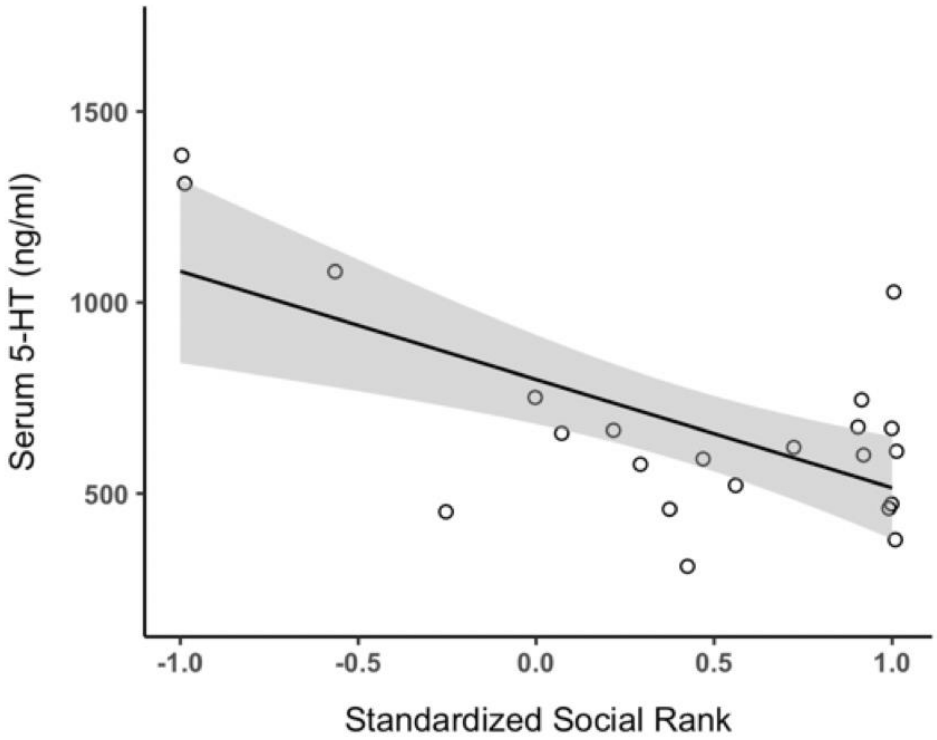

Figure 4.3. Relationship between social rank at birth and serum serotonin (5-HT) concentrations (ng/ml) in adult female spotted hyenas. Social rank is standardized and centered, with the highest-ranking hyena in the clan having a social rank of 1 and the lowest of -1. Females with higher social rank at birth (born to socially dominant mothers) have significantly lower concentrations of serum 5-HT (ng/ml) than those born at lower social ranks (n = 22, t = -2.24, p = 0.04, Table 4.4).

Inter-Individual Stability in Serotonin

Our analysis of inter-individual stability in serum 5-HT samples showed a high correlation (ICC = 0.90 95% CI:[0.51, 0.98]) between serum 5-HT in samples taken from the same individual females (n = 15 samples from 7 females). According to published benchmark ranges (Landis and Koch, 1977), which designate ranges for slight (0.0- 0.2), fair (0.2 – 0.4), moderate (0.4—0.6), substantial (0.6-0.8) and almost perfect agreement (0.8-1) between data collected from the same individual at different time points, even the ICC estimate at the lowest range of our calculated 95% interval

(ICC = 0.51) indicates moderate inter-individual stability in the blood. Our parameter estimate (0.90) at the high end of our 95% CI (0.98) indicates extremely high stability.

Serum 5-HT as a Predictor of Female Aggressive Behavior

Females with low serum serotonin concentrations in adulthood did not differ from those with high concentrations in either average rates (chi-squared = 0.037, df = 1, p = 0.85) or intensities (chi-squared = 0.690, df = 1, p = 0.41) of aggression.

Discussion

Here we present results of the first study in spotted hyenas examining behavioral and physiological correlates of a commonly used biomarker of central 5-HT function, serum 5-HT concentrations. We aimed to provide descriptive information on a number of physiological and demographic correlates of serum 5-HT in males and females across ontogeny, and to test the hypothesis that interindividual and intersexual variation in agonistic behavior among spotted hyenas is mediated by variation in serotonergic function. Our prediction that we would see lower blood 5-HT concentrations in animals more likely to aggress relative to those who more often inhibit aggression was only partially supported, but our results suggest interesting avenues for future research on the link between early life social status and 5-HT function.

Demographic and Physiological Predictors

We tested a number of demographic and physiological predictors of serum 5-HT; however, we were particularly focused on accounting for the effects of energetic status because peripheral serotonin plays a key role in energy balance and metabolism.

Because blood 5-HT has been shown to spike after recent food ingestion in some species (e.g. Alberghina et al., 2011; Blum et al., 1992), we might have expected a positive relationship between serum 5-HT concentrations and blood glucose, a measure of energy immediately available to the individual that can be indicative of whether they have fed recently. However, we found no significant relationship between blood glucose and serum 5-HT and, if anything, there was slight evidence for a negative correlation between glucose and 5-HT in males ($t = -1.88$, $p = 0.064$). This could be due to the particulars of digestion in carnivores, whose blood glucose concentrations are more stable, and do not fluctuate as strongly after meals, than those of omnivores (Hewson-Hughes et al., 2011; Schermerhorn, 2013; Zoran, 2002). Therefore, blood glucose might not serve as a good indicator of whether a mammalian carnivore has recently fed. Furthermore, peripheral 5-HT has a complex role in glucose metabolism, so correlations between overall blood 5-HT and glucose concentrations may be difficult to predict at any given time (El-Merahbi et al., 2015).

We found that 5-HT concentrations were negatively related to our measure of body condition in male hyenas, but not in females. A negative correlation between 5-HTconcentrations and body fat has been found in humans and other animals, including animal models of obesity (e.g. Park et al., 2014, Hodge et al, 2015). Furthermore, the relationship between 5-HT concentrations and nutritional status has been found to be

sex-dependent in other studies. For instance, Hodge et al (2015) found that, while body mass index (equivalent to our measure of body condition here) was inversely correlated with blood serotonin in both women and men, other measures, such as total percent body fat were only negatively related to 5-HT in men. However, we caution that further biological validation of our measure of body condition is required to interpret these results (Labocha et al., 2014). Specifically, it is still unclear exactly what our measure of body condition indicates about energetic status in male and female hyenas and whether it indicates something different about energetic status in the two sexes.

Our study also shows that in spotted hyenas, 5-HT concentrations increase with age in females but not in males. Central concentrations of 5-HT and 5-HIAA have been found to increase (e.g. Fairbanks 1999), decrease (e.g. Eklundh 1997, Reisner 1996) in some studies, but to remain unchanged (e.g. Higley, 1996) with age in studies of other mammals. The relationship between age and 5-HT may depend on several factors, including the age range included in the study, sex , and early life environment (e.g. Higley 1991, 1996).

In adult female hyenas, we found no evidence that blood 5-HT concentrations varied among lactating, pregnant, and nulliparous females, though it has been found to vary with reproductive state in other adult female mammals (Sekiyama et al 2013; Jury et al 2014). However, these studies utilized more precise information about reproductive state to detect differences between stages of the ovarian cycle or across pregnancy (Kaplan, et al., 2002). Little is known about cycling in spotted hyenas, and the effect of reproductive status and associated changes in sex steroids on 5-HT in female hyenas warrants further investigation.

Sex Differences in 5-HT and Implications for Female Dominance

We found no support for our prediction that sex differences in 5-HT concentrations relate to sex differences in social dominance and aggression, as there were no significant differences in serum 5-HT concentrations between male and female spotted hyenas either in early life or in adulthood. Although there is some evidence for higher serotonergic function in females of species showing more mammalian-typical sex differences in aggression (e.g. Schwandt et al, 2010; Higley et al., 1991), overall results are mixed, and there is no clear evidence demonstrating that sex differences in baseline 5-HT concentrations are related to sex differences in aggressive behavior (de Almeida et al., 2015). It is possible that (a) serotonin is unrelated to dominance relationships between sexes in spotted hyenas or (b) serotonin helps drive sex differences in aggression and social dominance via its interaction with sex steroids and sex-dependent effects on other brain systems (de Almeida et al., 2015)

Relationship between 5-HT and Social Dominance in Males and Females

Because of their more frequent opportunities to engage in aggression, we expected socially dominant individuals to have lower serum 5-HT concentrations than subordinates. In particular, we expected this to be true for females, who, throughout ontogeny, assert their social status more aggressively than do males (Smale et al., 1995; Smale et al., 1993; Van Meter, 2009). This prediction was supported in that we found a significant inverse relationship between social rank and 5-HT concentrations in female, but not male, hyenas. Our findings thus contribute support for the hypothesis that a negative relationship between 5-HT and rank may be more likely in despotic

social hierarchies (in which rank is attained/maintained primarily via aggression) than in less despotic ones.

Nevertheless, it is possible that sex differences in the relationship between serotonin and social status are driven by factors other than sexually dimorphic competitive behavior. This is especially true in light of serotonin's involvement in a wide range of potentially rank-related physiological processes (e.g. immune function) and behaviors (e.g. affiliative and cooperative behavior) (reviewed in Azmitia, 2010; Howell et al., 2014).

Relationship between 5-HT and Early Life Social Rank versus Social Rank at Sampling

We obtained several results suggesting that early life social rank in particular affects female 5-HT concentrations. Firstly, social rank at sampling is a significant predictor of 5-HT for juvenile but not adult females. Despite the stability of the hyena social hierarchy, social ranks for older females may differ quite a bit from their ranks earlier in life due to demographic changes within the clan and the births of younger siblings, who invariably rank above them. Secondly, although social rank at sampling was not significantly related to adult female 5-HT concentrations, there *was* a significant inverse correlation between adult female 5-HT concentrations and their social rank at birth. This finding, together with our finding that blood 5-HT levels are consistent within individuals over ontogeny, suggests that early life social rank plays a role in determining individual 5-HT concentrations.

Further study would be needed to determine exactly when during early life these possible effects of social rank on serotonergic function might be taking place, as well as what is driving them. One possibility is that an individual's early social interactions have

long term impacts on their serotonin system. A range of studies in young rodents, humans, primates (Veenema, 2009) and fish (e.g. Abbey-Lee et al., 2018), show that a variety of social stressors can have long term effects on serotonergic function, often increasing it. These social stressors include social harassment and repeated social defeat, which are often experienced by low-ranking animals in despotic hierarchies.

Another possibility is that the relationship between early social rank and 5-HT is driven by maternal effects, specifically rank-related variation in maternal neuroendocrine functioning during pregnancy (e.g. Maloney et al., 2018). Previous research in hyenas has found evidence for rank-related variation in exposure to maternal androgens, and androgen exposure early in life has been found to have organizational effects on both the serotonin system and aggression. For example, testosterone treatment in neonatal female rats reduces extracellular levels of serotonin in the amygdala compared to controls, suggesting that organizational effects of testosterone on aggression could be partly driven by a reduction in brain serotonin (Sunblad & Eriksson 1997). Also, experimentally increasing androgen concentrations at or shortly after birth causes a significant decrease in serotonergic activity in the frontal cortex and dorsal raphe nucleus of rats (Dominquez et. al 2002), two brain areas involved in the serotonergic control of aggressive behavior. These studies suggest a plausible mechanistic explanation for our finding that females born to high-ranking mothers have lower serotonin concentrations than those born to relatively low-ranking mothers - perhaps higher exposure to maternal androgens in the offspring of socially dominant females (as shown by Dloniak et al., 2006) causes a long-term reduction in serotonergic function, reflected in lower serum 5-HT concentrations.

Our findings also have interesting implications for the role of 5-HT as a modulator of behavioral plasticity. If early life social rank could help establish lifelong serotonin function, it may provide female hyenas a means to match their behavior to their lifelong social environment. Further investigations into the effects of serotonin on dominance-related behaviors are required to see if there is any merit to this idea; however recent research in rodents does provide evidence that changes to the serotonergic system early in life can have long term consequences on dominance-related behaviors. Firstly, it is well established that 5-HT plays a significant role in neurodevelopment (Sodhi and Sanders-Bush,2004; Whitaker-Azmitia, 20; Gaspar et al., 2003). Alternations to the serotonin system either prenatally, via exogenous maternal or endogenous fetal sources, or postnatally, can have long term impacts on social circuitry in the brain, and thus play a role in establishing life-long behavior patterns. For instance, a series of rodent studies have shown that early life exposure to fluoxetine, an SSRI, can have long term and sometimes irreversible impacts on offspring aggressive and dominance behaviors (Lisboa et al.; 2007; Kiryanova et al., 2016; Svirsky et al., 2016: Maloney 2018;) Furthermore, there is a large body of evidence showing that early life social stress can cause both long term alternations in 5-HT function and a change in serotonergic-mediated social behaviors, including aggression. The mechanistic causes are still unclear, but recent work provides evidence that stress-induced changes in the hypothalamic 5-HT system is one component underlying these effects.(Veenema, 2009; Veenema et al., 2006).

Serum 5-HT as a Predictor of Female Aggressive Behavior

Finally, we found no evidence for a direct relationship between serum 5-HT and aggressive behavior emitted by females, specifically their average lifetime rates and intensities of aggression. Previous work found that average aggression rates in female hyenas were heavily influenced by situational variables, as opposed to average intensities of aggression, which were found to be more specific to the individual (trait-like) (Yoshida, 2012). Because individual differences in serotonergic function have been most often linked to trait-like differences in intense forms of aggression, it is perhaps unsurprising that we found no relationship between 5-HT and average lifetime rates of aggression. However, we also failed to find a relationship between 5-HT concentrations and average lifetime intensity of aggression. This could be due to limitations of our study. For instance, due to sample size, we did not distinguish among different contexts of aggression; it is possible that serotonin is more directly related to rates of certain types of (e.g. unprovoked) aggression than others. One potentially fruitful avenue for future research would be to look at the synergistic effects of the serotonin system with cortisol and sex steroids on aggression. Particular attention has been paid to the interaction between testosterone and 5-HT in the mediation of aggressive behavior (e.g. Ambar and Chiavegatto 2009; Bonson et al., 1994). Specifically, several researchers have suggested that the facilitative effect of low 5-HT on escalated or 'impulsive' aggression may only be apparent in individuals with high levels of testosterone (Montoya et al., 2012). This could be a particularly interesting avenue of research in female spotted hyenas, which are heavily androgenized compared to females of most other mammals (Glickman, 1987). High-ranking females have higher androgen

concentrations than low-ranking females (Dloniak et al., 2006) and, in light of these findings, potentially lower serotonergic function than subordinate females. This physiological profile in particular might enhance aggressive behavior in dominant females relative to their subordinates.

LITERATURE CITED

LITERATURE CITED

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