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Fecal glucocorticoids reflect socio-ecological and anthropogenic stressors in the lives of wild spotted hyenas

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Abstract

Our aim was to identify natural and anthropogenic influences on the stress physiology of large African carnivores, using wild spotted hyenas (*Crocuta crocuta*) as model animals. With both longitudinal data from a single social group, and cross-sectional data from multiple groups, we used fecal glucocorticoids (fGC) to examine potential stressors among spotted hyenas. Longitudinal data from adult members of a group living on the edge of the Masai Mara National Reserve, Kenya, revealed that fGC concentrations were elevated during two periods of social upheaval among adults, especially among younger females; however, prey availability, rainfall, and presence of lions did not influence fGC concentrations among hyenas. Our results suggested that anthropogenic disturbance in the form of pastoralist activity, but not tourism, influenced fGC concentrations among adult male hyenas; rising concentrations of fGC among males over 12 years were significantly correlated with increasing human population density along the edge of the group's home range. As hyenas from this social group were frequently exposed to anthropogenic disturbance, we compared fGC concentrations among these hyenas with those obtained concurrently from hyenas living in three other groups undisturbed by pastoralist activity. We found that fGC concentrations from the undisturbed groups were significantly lower than those in the disturbed group, and we were able to rule out tourism and ecological stressors as sources of variation in fGC among the populations. Thus it appears that both social instability and anthropogenic disturbance, but not the ecological variables examined, elevate fGC concentrations and represent stressors for wild spotted hyenas. Further work will be necessary to determine whether interpopulation variation in stress physiology predicts population decline in groups exposed to intensive anthropogenic disturbance.

Keywords

Crocuta; Glucocorticoids; Stress; Anthropogenic disturbance

Introduction

Whereas endocrine studies have traditionally been used in conservation to monitor reproduction in rare or threatened species, hormones are now being used to monitor stress physiology as an indicator of how animals cope with social and ecological challenges in their daily lives (Millspaugh and Washburn, 2004; Walker et al., 2005a; Wingfield et al., 1997). In this context, fluctuating glucocorticoid (GC) concentrations, released from the adrenal glands

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in response to stressful stimuli, are often measured as an expression of how animals perceive and respond to both predictable (e.g. seasonal changes in food availability) and unpredictable (e.g. encounters with predators) changes in their environment (Walker et al., 2005a; Wingfield et al., 1997). In the short term, circulating GC concentrations released in response to an acute stressor help mobilize energy reserves, and suppress non-essential processes like growth and digestion. However, chronic elevation of GC concentrations can lead to pathology, including compromise of cardiovascular, immune, neural, and reproductive function (Sapolsky, 2002).

In recent years, our understanding of responses by free-living animals to potential stressors has been greatly enhanced by the development of methods for non-invasive hormone sampling (Keay et al., 2006; Millspaugh and Washburn, 2004; Walker et al., 2005a; Wasser et al., 2000), mainly because more traditional methods of blood sampling can themselves be stressful (e.g. Sapolsky, 1985). Fecal steroid analysis, in particular, offers an economical alternative to drawing blood for frequent collection of samples without disrupting the normal behavior of individuals or groups. Many species excrete multiple steroid metabolites, which can be extracted and measured with immunoassays like those used to measure circulating steroid hormones (Millspaugh and Washburn, 2004; Wasser et al., 2000).

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

We inquired about likely sources of stress experienced by spotted hyenas. First, seasonal variation in ecological factors, including local prey abundance and rainfall, might affect their stress physiology. Second, competition and intra-guild predation occur among carnivores occupying similar ecological niches (Caro and Stoner, 2003). In most African ecosystems, lions (*Panthera leo*) are the most important interspecific competitors for spotted hyenas; lions not only compete with hyenas for access to live prey and carcasses, but they also represent a significant source of hyena mortality (Honer et al., 2002; Kruuk, 1972; Watts and Holekamp, 2009). Thus, lions may represent important stressors for hyenas. Third, many gregarious carnivores, including spotted hyenas, also experience intra-specific social stress (Creel, 2005; Goymann et al., 2001; Young et al., 2006). Unlike other gregarious carnivores, which are predominantly cooperative breeders, all adult spotted hyenas have the opportunity to breed despite status-related reproductive skew occurring in both sexes (Holekamp et al., 1996; Engh et al., 2002). Spotted hyena society is structured by strict hierarchical rank relationships like those found in troops of cercopithecine primates, and it appears that, as in these primate societies (Abbott et al., 2003; Creel, 2001), there is no relationship between rank and fecal GC concentrations among male hyenas (Dloniak, 2004; Goymann et al., 2003). Although one study demonstrated a weak relationship between rank and fecal GC concentrations among female hyenas that were neither pregnant nor lactating (Goymann et al., 2001), these results were not confirmed in our study population (Dloniak, 2004). Rare events such as the overthrow of the alpha matriline have been found to increase GC concentrations among wild baboons (Engh et al., 2006; Sapolsky, 1983). Such events, and the ensuing periods of social instability, could potentially affect animals in any hierarchical society, so we asked whether social instability might affect stress hormone concentrations among spotted hyenas.

Finally, burgeoning human populations near many African wildlife reserves have put increasing pressure on protected lands and resources in recent decades (Wittemyer et al., 2008). Humans now represent an important mortality source for hyenas and other carnivores living inside protected areas (Kolowski and Holekamp, 2006; Watts and Holekamp, 2009; Woodroffe, 2000; Woodroffe and Ginsberg, 1998). In addition, recent work has suggested that anthropogenic activity, particularly that occurring in the form of pastoralists and grazing livestock within the boundaries of protected areas, alters the behavior of spotted hyenas and forces them to make energetic compromises not observed where pastoralists are absent (Boydston et al., 2003b; Kolowski and Holekamp, in press). Measures of tourist activity have also been shown to influence stress physiology in vertebrates (e.g. Creel et al., 2002; Pereira et al., 2006; Walker et al., 2005b). Therefore, pastoralist activity, and perhaps also seasonal variation in visitation by tourists, might be expected to affect hyena stress physiology.

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

Materials and methods

Study populations and subjects

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

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

Female spotted hyenas are promiscuous and breed throughout the year (Holekamp et al., 1999; Kruuk, 1972). Reproductive histories were known here for female members of all study clans. Ages of all individuals born into each clan during the study period were known, and mother–offspring relationships were established on the basis of nursing associations. Birth dates (±7 days) were assigned based on appearance and behavior of cubs when they were first seen (Holekamp et al., 1996). Female spotted hyenas reach reproductive maturity at around 2 years of age (Glickman et al., 1992; Holekamp et al., 1996; Van Meter et al., 2008), and were considered here to be adults at 24 months. Duration of gestation in spotted hyenas is 110 days (Kruuk, 1972), so, counting backward from parturition dates, we could determine conception dates. We classified adult females as "nulliparous" from 24 months until their first known conception; first parturition could be detected by the extensive tearing of the phallus that occurs then, even when the cubs do not survive (Frank and Glickman, 1994).

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

Longitudinal study of the Talek clan

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

Socio-ecological variables

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

To examine the effect of lions on spotted hyena fGC concentrations, the presence of lions was recorded when one or more lions were present within 200 m of any Talek hyena. Fecal samples reflect a general pattern of circulating hormone concentrations depending on the internal clearance time of the hormone (Brown et al., 1994; Wasser et al., 2000). Captive spotted hyenas administered an adrenocorticotrophic hormone (ACTH) challenge, which increases circulating GC, exhibited elevated fGC concentrations from 1 to 8 days after the challenge (Dloniak, 2004; Goymann et al., 1999). Acute stressors, such as caged transport, also produced measurable elevations in fGC concentrations for several days after the incident (Goymann et al., 1999). Therefore, we identified fecal samples from individuals present with lions up to one week prior to sample collection. We coded lion presence as a binary predictor variable such that each fecal sample was or was not associated with the presence of a lion during the preceding week.

Whereas the social hierarchy of the Talek clan was generally stable and unchanging over time (Frank, 1986; Holekamp et al., 1993), during the course of our longitudinal study two events occurred that resulted in brief periods of social instability within the clan. First, in May of 1999, the highest-ranking (alpha) female died after having occupied that rank since the mid 1980s

(Holekamp et al., 1993). For some months after her death, the daughters of the alpha female fought vigorously for position, and observers noted an increase in serious wounding among them. Here we considered the six months following the alpha's death to be a period of social instability. Second, in 2000, the Talek clan split into two smaller clans; by 2001, contact between the two groups resulting from this fission event was rare, and restricted to only a single individual (J.E. Smith, unpublished data). Therefore we considered the last six months of 2000 to represent a second period of social instability. For the purpose of analysis we coded social stability as a binary predictor variable indicating stable or unstable monthly periods.

Anthropogenic disturbance

Since the mid-1990s, increasing anthropogenic activity in the Talek area has led to altered use of dens and territory, altered temporal patterning of activity, and frequent human–hyena conflict (Boydston et al., 2003b; Kolowski and Holekamp, 2006, in press; Kolowski et al., 2007). Although illegal, the once rare presence of livestock and herders inside the reserve was a daily occurrence by 2005 (Kolowski and Holekamp, 2006Kolowski and Holekamp, in press). The Talek region now supports a high density of pastoralist settlements along the northern border of the MMNR (Reid et al., 2003), and thus along the northern edge of the Talek clan territory. Kenyan census data indicated that the human population in the Talek area has approximately doubled every 15 years since 1950 (Lamprey and Reid, 2004). To investigate the effect of this burgeoning human population along the border of the home range of the Talek clan, we asked whether or not fGC concentrations increased over the 13-year duration of our study, measured in samples collected during twenty-six 6-month intervals.

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

Cross-sectional comparison among clans

To compliment our longitudinal data, we investigated variation in fGC concentrations among several clans in a cross-sectional study. Starting in September 2002, a second clan within the MMNR was monitored, the Mara River clan. The territory of the Mara River clan was located deep within the Reserve (Fig. 1), and too far from pastoralist settlements for livestock grazing. However, the closest border of the territory of the Mara River clan was only 6 km from the Talek clan's territory. Also, from October 2003 through July 2005, two hyena clans were monitored that occupied adjacent territories in ANP, the Ol Tukai clan and the Airstrip clan (Fig. 1). In our cross-sectional study we restricted the study period in Talek to correspond with the onset and duration of the studies conducted on the other three clans. Therefore, Talek data in this analysis were limited to 2002 through 2005. In all clans we used identical methods for continuous collection of demographic, behavioral, and ecological data.

The population densities of hyenas living in the MMNR and ANP during the period of the cross-sectional study were similar (Kolowski et al., 2007; Watts and Holekamp, 2008) (Table 1); however, other ecological differences between ANP and the MMNR might potentially affect the stress physiology of resident hyenas. First, abundance of prey did not differ significantly between the territories of Talek and Mara River clans (Kolowski et al., 2007), but prey density in the MMNR was over twice that in ANP (Table 1). Second, the MMNR typically received more rain on average than ANP, and had slightly lower average daily temperatures during the study period than did ANP (Table 1). Finally, the lion density in ANP was less than half that in the MMNR (Watts and Holekamp, 2008).

The four clans also varied in their exposure to anthropogenic disturbance. Similarly large numbers of tourists visit both ANP (141581±1331 tourists) and the MMNR (139127±7864 tourists) each year (Okello et al., 2005), although the smaller size of ANP generates a much higher tourist density than that in the MMNR (Table 1). Within the MMNR, researchers encountered tour vehicles 4.8 times more frequently while observing hyenas in Talek than in the Mara River territory (Kolowski et al. 2007). Although comparable data were not available from the ANP clans, the territories of both of these clans were located close to tourist lodges, and tourist visitation to both ANP territories more closely approximated that in Talek than that in the Mara River territory.

In contrast to the Talek clan, none of the other three study clans experienced any significant exposure to pastoralist activity within their territories (Table 1). In ANP, livestock and herders were seen only once during this study, within the Airstrip clan’s territory (herd size=75 head), and were never seen within the Ol Tukai territory (*n*=22 months). Likewise, livestock were never seen in the territory of the Mara River clan (*n*=25 months) (Kolowski et al., 2007). In the Talek territory, we monitored the presence of livestock accompanied by herders and conducted detailed head counts of livestock. The mean number of livestock counted in the Talek territory was 1386±181 head (Kolowski and Holekamp, in press). Thus the Talek clan had a much greater exposure to pastoralist activity than did either Mara River or ANP hyenas.

The similarities and differences among our four study clans permitted us to develop the four sets of predictions shown in Table 2. If ecological variables, such as prey availability or rainfall, act as stressors (H1), then we expected to see significant differences in fGC concentrations between the two parks, with higher fGC concentrations among hyenas living in the harsher ANP conditions. However, if local lion density affected the stress physiology of spotted hyenas (H2), we predicted fGC concentrations would be higher in both clans in the MMNR than those in ANP, because MMNR has a higher lion density. If hyenas find tourist visitation stressful (H3), then fGC concentrations among hyenas in the Mara River clan should be markedly lower than in any of the other three clans. Finally, if anthropogenic disturbance in the form of pastoralist activity represents an important stressor to wild hyenas (H4), then we expected Talek hyenas to have higher fGC concentrations than hyenas in any of the other three study clans, regardless of the park in which they were located.

Fecal sample collection, extraction, and immunoassay

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

Frozen fecal samples were lyophilized and extracted in ethanol as described previously (Dloniak et al., 2003; Van Meter et al., 2008). Extracts were further diluted 1:20 in steroid diluent, and assayed with a corticosterone radioimmunoassay kit (ImmuChem Double Antibody Corticosterone ^{125}I RIA Kit, MP Biomedicals (formerly ICN), 07-120102). The corticosterone antibody displays a high affinity to the major glucocorticoid metabolites present in the feces of a wide variety of vertebrates (Wasser et al., 2000), including spotted hyenas

(Goymann et al., 1999). The minimum detection limit for this assay was 10 ng of glucocorticoid per gram hyena feces. Precision of the assay was monitored by assaying serial dilutions of pooled hyena fecal extracts. The intra-assay coefficient of variation was 4.79±2.76%, and the interassay coefficient of variation was 10.12% (n=17 assays). Serial dilutions of pooled samples produced displacement curves parallel to that produced by the corticosterone standards from the kit.

Statistical analysis

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

Longitudinal analysis of the Talek clan

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

Based on the results of our methodological models, we further separated models into groups based on sex and reproductive condition (immigrant males, nulliparous females, non-pregnant females, and pregnant females) to test our variables of interest along with group-specific covariates previously shown to influence fGC concentrations. We controlled for lactation state among non-pregnant females (lactating or non-lactating) (Dloniak, 2004; Goymann et al., 2001), and day of gestation for pregnant females (Dloniak et al., 2006b). We examined the main-effects of each parameter as well as interactions with covariates.

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

Cross-sectional comparison among clans

For the cross-sectional comparison, a nested ANOVA was used to test for significant differences among clans in fGC concentrations. Before performing the nested ANOVA we investigated the procedural variables that were found to have significant effects in the longitudinal dataset. Males and females, and morning and evening samples were equally represented in each study group. However, we had an unequal distribution of pregnant females among clans, so samples from pregnant females were excluded from the nested analysis. For the nested ANOVA, clan (Talek, Mara River, Ol Tukai, or Airstrip) was nested within park (MMNR or ANP) to assess the main effects of park and clan. A post hoc analysis was performed using Fisher's LSD test; means and standard errors are presented.

Results

Longitudinal analysis of the Talek clan

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

Methodological variables

Table 3 shows the results of the methodological analysis. For immigrant males, only sample collection time explained a significant portion of the variation among fGC concentrations, indicating that samples collected in the morning were higher than those collected in the evening. The same was true in the model for adult females; here reproductive state also significantly affected fGC concentrations. We controlled for these variables in all subsequent models.

Immigrant males

Terms included in the model for immigrant males were sample collection time, study duration time, tourism, and social instability (AICc=230). The further removal of study duration time (AICc=227), tourism (AICc=227), or social instability (AICc=227) generated equally parsimonious models, so we present the parameter estimates for the model containing all terms together (Table 4). Whereas only sample collection time and study duration were significant variables in this model, the model estimates also indicated that fGC concentrations increased among immigrant males during months of heavy tourism, increased over the course of our study, and were relatively high during periods of social instability.

Nulliparous females

Only sample collection time and social instability remained in the model for nulliparous adult females (Table 4). Here, although the parameter estimate for sample collection time indicated that morning samples contained higher fGC concentrations than evening samples, the estimate was not significant in the model. Fecal samples collected during periods of social instability contained significantly higher fGC concentrations than those collected during stable periods.

Non-pregnant females

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

Pregnant females

The model including sample collection time and day of gestation (AICc=86) was equally parsimonious with the model that included these two terms plus social instability (AICc=87; Table 4). Significant terms in the model included sample collection time and day of gestation, which indicated that fGC concentrations increased during the course of gestation. Samples collected during periods of social instability had higher fGC concentrations than those collected during stable periods, although this term was not significant in the model.

Cross-sectional comparison among clans

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

Discussion

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

Socioecological variables

In our longitudinal study, natural ecological variation was not found to be a significant stressor in the lives of spotted hyenas. Seasonal variation in prey availability and monthly rainfall did not explain variation in fGC concentrations among hyenas. Nor did our data support the notion that the immediate presence of lions elevates fGC concentrations among hyenas. Even large carnivores like hyenas experience competition from intra-guild predators (Caro and Stoner, 2003); lions and hyenas compete for access to both carcasses and live prey (Cooper, 1991; Honer et al., 2002; Kruuk, 1972). Watts and Holekamp (2009) found that lions cause the majority of known deaths among adult hyenas in the Talek clan, but that varying levels of competition with lions could not explain patterns of mortality among adult hyenas. Here we were unable to detect a physiological stress response, measured as elevated fGC concentrations, among individuals that had encountered lions 1 to 7 days before fecal sample collection. Although lion–hyena encounters were common, violent interactions between lions and hyenas were rare, and it may be only the latter type of interaction that elicits a stress response measurable in hyena fecal samples. However, like our longitudinal analysis, our cross-sectional data comparing fGC concentrations among clans exposed to dramatically different lion densities (H2; Table 2) also failed to suggest that lion presence represents a chronic stressor to hyenas. Although the lion density in the MMNR was twice that in ANP, there was no indication that park influenced hyena fGC concentrations, and there were no significant differences in fGC concentrations between the ANP clans and the Mara River clan.

In carnivore societies structured by linear dominance hierarchies, patterns of social stress vary among dominant and subordinate members of a group (Creel, 2005), but this does not seem to be true among hyenas living in the MMNR (Dloniak, 2004; Dloniak et al., 2006b). However, here we examined instability in the social hierarchy as a predictor of social stress; such instability could potentially affect members of any hierarchical society. Our data indicate that nulliparous females experienced elevated fGC concentrations in response to social instability, and, although not significant, we observed trends in the same direction among pregnant and non-pregnant females, and among immigrant males. Social instability was the only predictor, other than sample collection time, to explain any variation in fGC concentrations among nulliparous females. Juvenile spotted hyenas gain their rank by “inheriting” their mother’s position in the dominance hierarchy, but it is not until their second year of life that their ranks finally become established relative to those of older clan members (Engh et al., 2000; Smale et al., 1993). The recency of their rank acquisition may account for the heightened response to social instability apparent among nulliparous females.

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

Anthropogenic variables

Tourists have been present in large numbers in both the MMNR and ANP for more than 3 decades (Okello et al., 2005). Within the MMNR, the Talek and Mara River territories differed very little except with respect to the intense daily exposure of Talek hyenas to the presence of tour vehicles and livestock (Kolowski et al., 2007). The Mara River territory was visited by tourists at lower rates than were the territories of any of our other study clans. Therefore, had tourist visitation been generally stressful to hyenas, we expected to observe lower fGC concentrations among Mara River hyenas than among hyenas in any of the other three study clans (H3; Table 2). Tourists in vehicles pose no direct threat to hyenas, and in both parks, hyenas are well habituated to vehicle presence and do little to avoid them. However, data from our longitudinal analysis suggest that immigrant males may not be as well habituated to tour vehicles as are the natal members of the Talek clan.

In contrast to tourists, pastoralist herdsmen represent a direct threat to hyenas; they are an important source of mortality for the Talek population, second only to lions (Watts and Holekamp, 2009). Additionally, hyenas appear to perceive herdsmen as threats because hyenas often flee from guarded cattle herds whereas cattle left unattended by herders are not avoided (Kolowski and Holekamp, in press). Our longitudinal data indicate that fGC concentrations among immigrant male hyenas have increased since the early 1990s; this increase has been correlated with the increasing size of the human population living along the border of the Talek clan's home range (Lamprey and Reid, 2004). Although our study did not directly test proximate mechanisms linking human population growth with hyena stress physiology, a recent study comparing the behavior of Talek and Mara River hyenas indicated that it was the presence of pastoralists and their livestock, not tourism, that accounted for altered behavior among Talek hyenas relative to that observed in the undisturbed Mara River clan (Kolowski et al., 2007).

Variation in pastoralist activity, but not tourism or ecological variables, was also consistent with the patterns of variation we observed among clans with respect to fGC concentrations. A study of Serengeti hyenas living inside and outside the Ngorongoro Crater found lower fGC concentrations among females (Goymann et al., 2001) but not males (Goymann et al., 2003) living within the Crater. The authors attributed the lower fGC concentrations to ecological differences experienced by the two populations, and the lower energetic demands enjoyed by females inside the Crater. In our own cross-sectional study, we observed no effect of park on fGC concentrations in our samples, suggesting that the differences in ecology experienced by hyenas living in ANP and the MMNR did not influence hyena stress physiology (H1; Table 2). Furthermore, differences in pastoralist activity among monitored clans (H4; Table 2) better predicted elevated fGC concentrations than did any natural ecological variables. Growing human populations adjacent to protected areas, as has been described here for the Talek region, are common in many African and Latin American countries (Wittemyer et al., 2008). Whereas this form of anthropogenic disturbance has clear ties to reduction in resources available to protected carnivores, here our data suggest that growing human populations along borders of protected areas may also have direct physiological consequences for the animals living within these areas. Together the results of our longitudinal and cross-sectional studies suggest that increasing activity of local pastoralists in the Talek region represents a source of stress to Talek hyenas.

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

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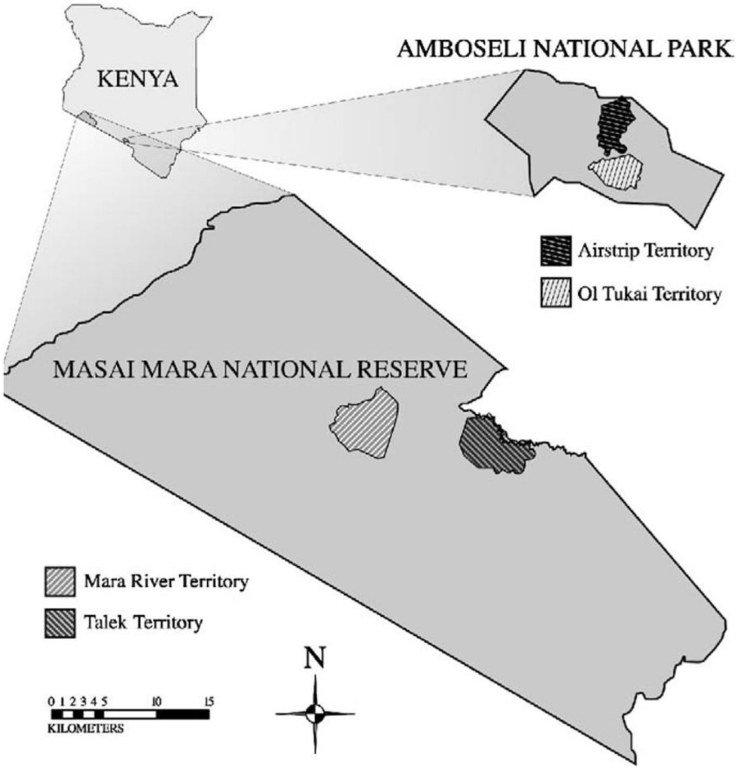

Fig. 1.
Map of parks and clan territories.

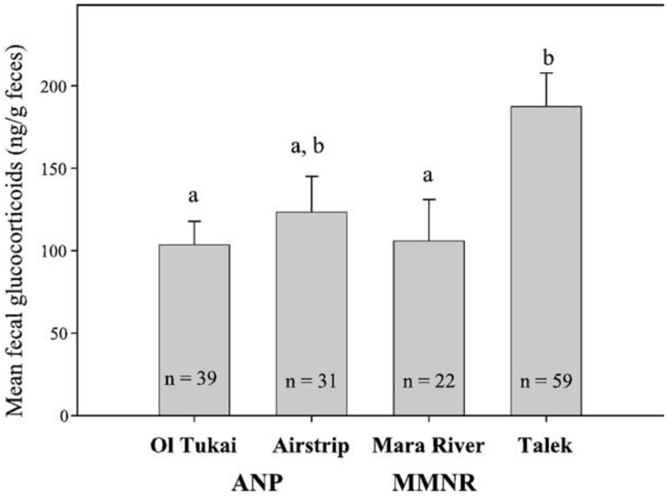

Fig. 2.

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

Table 1

Ecological features of parks and clans

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

[a] Watts and Holekamp (2008).

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

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

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

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

[f] Okello et al. (2005).

[g] Kolowski and Holekamp (in press).

Table 2

Summary table showing relative ecological and anthropogenic differences among parks and clans, and the respective sets of predictions generated for our nested cross-sectional analysis

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

The first prediction was based on differences in the ecological conditions experienced by hyenas in each clan and park (H1), the second was based on variation in lion density among clan territories (H2), the third was based upon variation in tourism (H3), and the fourth was based on variation in pastoralist activity (H4). Only the last of these predictions was confirmed, as shown in Fig. 2.

Table 3

Models (GLMM) investigating methodological sources of variation in fGC concentrations

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

Males and females were tested in separate models; all variables included in the models are shown. Individual identity was entered as a random variable.

Table 4

Parameter estimates from the selected models (GLMM) explaining fGC concentrations among each group

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

Individual identity was entered as a random factor in all models.

[a] The negative estimate for collection time indicates that morning samples had higher fGC concentrations than evening samples.

[b] The positive estimate for duration of study indicates fGC concentrations increased as our study progressed.

[c] The positive estimate for tourism indicates that fGC concentrations were higher during months of peak tourism.

[d] The negative estimate for instability indicates that samples from periods of instability had higher fGC concentrations than those from stable periods.

[e] The negative estimate for lactation status indicates that samples from non-lactating females had higher fGC concentrations than those from lactating females.

[f] The positive estimate for day of gestation indicates that fGC concentrations increased during gestation.