Measuring salivary cortisol in wild carnivores

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ARTICLE INFO

ABSTRACT

Salivary hormone analyses provide a useful alternative to fecal and urinary hormone analyses in non-invasive studies of behavioral endocrinology. Here, we use saliva to assess cortisol levels in a wild population of spotted hyenas (*Crocuta crocuta*), a gregarious carnivore living in complex social groups. We first describe a novel, non-invasive method of collecting saliva from juvenile hyenas and validate a salivary cortisol assay for use in this species. We then analyze over 260 saliva samples collected from nearly 70 juveniles to investigate the relationships between cortisol and temporal and social variables in these animals. We obtain some evidence of a bimodal daily rhythm with salivary cortisol concentrations dropping around dawn and dusk, times at which cub activity levels are changing substantially. We also find that dominant littermates have lower cortisol than singleton juveniles, but that cortisol does not vary with age, sex, or maternal social rank. Finally, we examine how social behaviors such as aggression or play affect salivary cortisol concentrations. We find that inflicting aggression on others was associated with lower cortisol concentrations. We hope that the detailed description of our methods provides wildlife researchers with the tools to measure salivary cortisol in other wild carnivores.

1. Introduction

Endocrine processes are important mechanisms regulating behavior and physiology across diverse taxa, but directly measuring these processes is invasive and therefore not possible in many contexts. To circumvent this issue, non-invasive techniques, which don't require researchers to capture or manipulate animals, have allowed us to study endocrine function in natural populations and have helped address important questions in ecology, evolution, animal conservation, and animal welfare (Behringer and Deschner, 2017; Kersey and Dehnhard, 2014). The most common non-invasive endocrine approach is to collect and assay freely deposited feces and urine, which contain the metabolic products of many hormones. Concentrations of metabolites in the feces and urine are then measured to infer concentrations of circulating hormones within the body of the animal.

Although widely used, several inferential challenges are associated with measuring hormone concentrations in feces and urine. Because the metabolites in feces and urine reflect the products of hormones secreted

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throughout the body, it is not always possible to determine the source organ or the original plasma hormone of the excreted metabolites (Goymann, 2012; Touma and Palme, 2005). In addition, these methods cannot reveal real-time or short-term (within minutes) variation in endocrine physiology, such as circadian rhythms or effects of behavior on hormone concentrations, because excreted metabolites represent concentrations averaged over hours (urine) or days (feces) (Behringer and Deschner, 2017). Finally, because animals excrete on their own schedule and in their preferred locations, feces and urine can be difficult to collect. For instance, carnivores defecate infrequently compared to herbivores, and many animals defecate in latrine sites, where previously deposited feces might contaminate new samples (Buesching and Jordan, 2019). Urine can be especially difficult to collect as it quickly disperses or is absorbed by the substrate (Danish et al., 2015).

Salivary analyses overcome many of these shortcomings, making them a useful complement to fecal and urinary analyses in studies of behavioral endocrinology. First, salivary concentrations of steroid hormones accurately reflect the unbound plasma concentrations of hormones because the lipid-soluble steroids easily and rapidly diffuse from blood to saliva (Kirschbaum and Hellhammer, 1989; Wood, 2009). Second, salivary hormone sampling allows for short-term and frequent assessment of endocrine status, including daily patterns of hormone production (Cross and Rogers, 2004; Heintz et al., 2011) and short-term physiological responses to social interactions (Horváth et al., 2008; Wobber et al., 2010). Finally, saliva collection protocols typically involve offering the animal something to chew on, allowing researchers to sample at specific times instead of being bound by the excretion schedule of the study animal. Because of these advantages, the use of saliva collection for hormone measurement has recently been employed in captive animals and wild primates (Behringer and Deschner, 2017; Staley and Miller, 2020), but it is not yet broadly used for other free-living species. Because saliva collection from free-ranging animals requires that study subjects engage voluntarily with the collection device, saliva collection protocols must be somewhat tailored to the species under study.

Here, we develop a procedure for collecting saliva from wild carnivores, and we use it to collect saliva from members of a free-living population of spotted hyenas (*Crocuta crocuta*) in the Maasai Mara National Reserve, Kenya. Spotted hyenas are large, gregarious carnivores that live in matrilineal social groups called 'clans' (Kruuk, 1972). Much work has been done on the endocrine physiology of spotted hyenas using plasma (often from captive individuals, e.g., Glickman et al., 2006; Licht et al., 1992) and feces (usually from wild populations, e.g., Dloniak et al., 2006; Goymann et al., 2001).

However, many gaps remain in our understanding of spotted hyena endocrinology due to the limitations of fecal hormone sampling. For example, little is known about the short-term daily fluctuations (circadian rhythms) in hormones in hyenas or other carnivore species exhibiting crepuscular activity patterns, i.e. most active around dawn and dusk (Holekamp and Dloniak, 2010). Particularly little is known about the endocrinology of juveniles, because juveniles defecate infrequently and their feces are difficult to collect (Benhaiem et al., 2013). Finally, as with most wild populations (but see Hohmann et al., 2009; Leeds et al., 2018), it is still unclear how circulating hormone concentrations are immediately affected by social interactions among hyenas.

We use our non-invasive salivary hormone collection procedure to fill these knowledge gaps, focusing on salivary glucocorticoids (i.e., cortisol and corticosterone), a class of steroid hormones conserved across vertebrates. Glucocorticoids are known to regulate metabolism, energy, and activity levels by modulating the availability of glucose (Sapolsky et al., 2000), and they thus exhibit clear circadian variation in the saliva and plasma of many diurnal and nocturnal animals (Kumar Jha et al., 2015). Glucocorticoids are also secreted in response to a variety of internal or external challenges, including predation events, food scarcity, inclement weather, and social interactions (Wingfield et al., 1998). Glucocorticoids can be accurately measured using saliva: salivary

cortisol is highly correlated with plasma cortisol concentrations in many species (Sheriff et al., 2011). Following stress onset, cortisol levels in saliva and in plasma rise in parallel, with salivary cortisol lagging only minutes behind plasma cortisol (Beerda et al., 1998; Riek et al., 2019).

Below, we present methods for collecting and analyzing saliva from wild carnivores, and we implement these methods to reveal new insights into the behavioral endocrinology of spotted hyenas. First, we present our saliva collection and analysis protocol, and we confirm that our cortisol assay of hyena saliva samples meets analytical and biological validation criteria using adrenocorticotropic hormone (ACTH) stimulation tests. Second, we examine daily patterns of variation in cortisol concentrations among wild juvenile hyenas. We predict that juveniles exhibit crepuscular cortisol rhythms based on their activity patterns (Holekamp and Dloniak, 2010; Kolowski et al., 2007). Third, we determine how cortisol values vary with respect to biologically meaningful ecological and demographic characteristics of the juveniles tested. We predict that age, sex, and litter status affect cortisol concentrations in juveniles based on studies of fecal glucocorticoid concentrations (Benhaiem et al., 2013, 2012a; Greenberg, 2017). Finally, we investigate how short-lived social interactions such as aggression or play affect cortisol concentrations. We predict that receiving aggression increases cortisol concentrations, as demonstrated in lab animals and in wild primates (Hsu et al., 2006; Wittig et al., 2015). We also predict that social play decreases cortisol concentrations, as theorized in the 'socialization' hypothesis for the adaptive function of play behavior and as demonstrated in domestic dogs (Horváth et al., 2008; Pellis et al., 2015).

2. Methods

We studied three clans of wild spotted hyenas in the western part of the Maasai Mara National Reserve in Kenya; these clans have been monitored continuously since 2008. We monitored clans daily during two observation periods, in the morning from 0530 h to 0930 h and in the evening from 1700 h to 2100 h. When we encountered a subgroup of one or more hyenas, we initiated an observation session and recorded the identities of all hyenas present, using their unique spot patterns and ear damage to recognize individuals. Sessions lasted from five minutes to several hours and ended when behavioral interactions ceased and/or observers left that individual or group. In all sessions, we recorded common agonistic behaviors using all-occurrence sampling (Altmann, 1974), including threat behaviors ranging from intention movements (e. g., head-waves) to aggressive contact (Kruuk, 1972). We also recorded social play behavior, which we defined as two or more individuals engaged together in chasing, wrestling, jumping, or chewing on one another (Tanner et al., 2007). We recorded social play behavior using two-minute scan sampling, where sampling was initiated when social play was observed, repeated at two-minute intervals as long as any social play behavior was occurring, and terminated when two scan samples had passed with no social play behavior. If hyenas began to play later in the session, the two-minute sampling protocol was re-started. All sampling was carried out following the guidelines of ASAB Ethics Committee and the ABS Animal Care Committee (Animal Behavior, 2020) and was approved by the Michigan State University IACUC Committee (#05/14-087-00).

We monitored several environmental variables throughout the study. Daily temperatures (°C) were measured with an outdoor min/max thermometer. Daily rainfall (mm) was measured using a standard plastic rain gauge. Both gauges were located in our field camp near the borders of all three clan territories. We monitored prey availability during biweekly surveys by counting all wild herbivores within 100 m of 2–3 line transects (1.5–5.4 km long) in each clan's territory (Holekamp et al., 1999). For each clan, monthly mean prey density was calculated as prey counted per square kilometer based on the number of animals sighted during line transect surveys that month.

2.1. Saliva sample collection from juveniles

Between 2015 and 2018, we collected 262 saliva samples from 33 female and 35 male ($n = 69$) wild juvenile spotted hyenas (Table 1). We were not able to collect saliva samples from all juveniles in our clans; thus, our data are likely skewed toward the bolder and/or better habituated individuals in the population. Saliva samples were collected using a hand-held pole apparatus (Fig. 1) modified after Lutz et al. (2000). Our device consisted of a 1-meter PVC pipe (diameter 2.5 cm) with a bolt crossing the pipe near the bottom and secured with a nut. A 9-inch piece of solid braid polyester rope (3/8-inch diameter, Quality Nylon Rope) was knotted at the top and coated with ~1 tsp. of vegetable fat (Kimbo, Bidco Africa; Fig. 1A). Polyester rope was chosen for its limited effect on steroid hormone concentrations (Gröschl et al., 2008; Hansen et al., 2008), and vegetable fat was chosen as bait due to its wide availability in Kenya and low phytoestrogen content (Verleyen et al., 2002). The baited rope was inserted into the PVC pipe and the bolt secured under the knot to hold the rope in place (Fig. 1B). A single known juvenile hyena was allowed to chew on the rope for 1–5 min (Kobelt et al., 2003), after which the apparatus was withdrawn into the vehicle (Fig. 1C,D). To prevent contamination from other food sources, juveniles that had fed or nursed within the preceding 10 min were not permitted to chew the saliva collection device. After saliva collection, the white rope was inspected for traces of blood, because blood contamination may artificially elevate measured salivary cortisol levels (Kivlighan et al., 2004). If no traces of blood were found, the saturated portion of the rope was cut away with clean scissors, placed in a Salivette (Sarstedt) tube with a perforated inlay, and stored at ambient temperature for the remainder of the observation period.

Upon returning to camp, samples were centrifuged at relative centrifugal force (RCF) 1318 g at ambient temperature for 10 min, and the saliva collected at the bottom of the tube was again inspected for possible blood contamination: a pink coloration appears at a blood concentration of 0.1–0.2% (Wood, 2009). If the saliva appeared clear or white, it was transferred to a cryotube with a disposable pipette and then frozen in liquid nitrogen. In association with each saliva sample, we recorded the identity of the hyena, the date, the start and stop time of chewing, and the time at which each sample was frozen in liquid nitrogen. Saliva volume was not recorded because cortisol levels are unaffected by salivary flow rate across species (Kirschbaum and Hellhammer, 1989; Sheriff et al., 2011).

2.2. Biological validation: ACTH stimulation tests

To compare cortisol concentrations measured in blood and in saliva, we performed adrenocorticotropic hormone (ACTH) stimulation tests (Goymann et al., 1999) on three wild adult spotted hyenas (2 males, 1

Table 1
Summary of saliva samples ($n = 262$) collected from wild juvenile spotted hyenas ($n = 69$) and used in this manuscript.

Number of samples		
Sex	Male	149
	Female	112
Litter status	Singleton	85
	Dominant twin	92
	Subordinate twin	85
Weaning status	Nursing	205
	Weaned	49
Distribution of samples		
Age (months)	Mean	7.3
	Median	6.9
	Range	2.2 to 23.4
Maternal social rank (standardized)	Mean	0.06
	Median	0.11
	Range	-1 (low) to 1 (high)

female) in June–July 2015. ACTH is released by the anterior pituitary gland, and it stimulates the adrenal cortex to release glucocorticoid hormones such as cortisol. Hyenas were anesthetized with 6.5 mg/kg tiletamine-zolazepam (Telazol, Zoetis) diluted in 3 mL distilled water, administered intramuscularly in a pressurized dart fired from a CO_2-powered rifle (Holekamp and Sisk, 2003). Immobilizations took place between 0700 h and 0900 h. Within 10–15 min of darting, we drew our first blood sample from the jugular vein of each individual into a heparinized vacutainer tube. Approximately 20 min after the first blood draw, we injected 0.25 mg of synthetic ACTH (Cortrosyn, Amphastar Pharmaceutical) diluted in 1 mL distilled water into the thigh muscle of a back leg. Serial blood samples were drawn at 10-minute intervals from the first blood draw until 90 min after ACTH injection. Serial saliva samples were taken at the same 10-minute intervals as the blood samples by carefully pipetting saliva out of the mouth and into a cryotube using a disposable pipette. To keep the hyena hydrated over the 2-hour test period, we injected a 20 mL bolus of saline solution under the loose skin of the hyena's neck or legs every 15–20 min. Supplementary doses of Telazol were administered as necessary throughout this procedure to maintain deep anesthesia.

After placing the anesthetized hyena in a safe and shaded place to recover from anesthesia, we returned to camp where saliva samples were inspected for possible blood contamination (Wood, 2009) and then frozen in liquid nitrogen. Blood samples were centrifuged at RCF = 1000 g at ambient temperature for 10 min; plasma was then drawn off, aliquoted, and stored in liquid nitrogen until it was shipped on dry ice to the United States, where it was stored at −80 °C until assay. Plasma samples were assayed in duplicate using a corticosterone radioimmunoassay kit (MP Biomedicals CortiCote RIA kit, #06B256440); further details of this plasma assay are published in Holekamp and Smale (1998).

2.3. Salivary cortisol assay

Within one year of collection, all saliva samples were transported on dry ice to a -20 °C freezer in the United States. Prior to analysis, samples were thawed, briefly vortexed, and centrifuged at RCF = 1811 g at 12 °C for 15 min. Centrifuged samples often had a coating of vegetable fat on top; clear saliva was pipetted out from below the vegetable fat and stored in a fresh microcentrifuge tube for future analysis. Samples were then either re-frozen or assayed immediately.

To measure concentrations of salivary cortisol, samples were assayed in duplicate using a cortisol enzyme immunoassay kit previously validated for use in humans and some animals (Salimetrics Cortisol Enzyme Immunoassay Kit, #1-3002). Cross-reactivity of the antibody with steroids was as follows: cortisol: 100%; dexamethasone: 19.2%; prednisolone: 0.568%; corticosterone: 0.214%; 11-deoxycortisol: 0.156%; cortisone: 0.130%; triamcinolone: 0.086%; 21-deoxycortisol: 0.041%; progesterone: 0.015%; testosterone: 0.006%. All other steroids tested: < 0.004%. Analytical sensitivity for cortisol was 0.007 µg/dL. We performed analytical validations of parallelism, accuracy, and precision based on Brown et al. (2004).

2.4. Temporal effects

Most mammals exhibit a daily circadian rhythm in their cortisol concentrations (Kumar Jha et al., 2015). To determine whether juvenile spotted hyenas' cortisol concentrations vary predictably across the day, we explored the distribution of log-transformed cortisol concentrations relative to the time of day at which that sample was collected (Fig. S1). Juvenile hyenas dwelling in the communal den are most active around dawn and dusk, when mothers visit the den to socialize and to nurse their cubs. Thus, we investigated the relationship between time and juvenile cortisol concentrations in two ways: (1) by comparing samples collected in the morning vs. evening, and (2) by examining the time at which those samples were collected relative to the time of sunrise

Fig. 1. Methods for collecting saliva from juvenile spotted hyenas. **A.** Pieces of polyester rope knotted at the top and coated with vegetable fat. **B.** Baited rope inserted into the PVC pipe, with a bolt secured under the knot. **C.** Research assistant safely collecting saliva from a hyena. **D.** Juvenile hyena depositing saliva on the collection rope. Photos by Erin Person and Jadelys Tonos.

(morning) or sunset (evening). To investigate activity at the communal den, we also visually examined patterns of maternal den attendance using data collected in the same clans in the years prior to this study.

2.5. Demographic characteristics of sampled hyenas

For each saliva sample, we recorded the age, sex, social rank, litter status, and weaning status of the juvenile spotted hyena at the time of sampling. We calculated the age in months of each hyena; we estimated juvenile birthdates (to ±7 days) from their appearance when first seen above ground (Holekamp et al., 1996). All hyenas were sexed based on the morphology of the erect phallus (Frank et al., 1990). Because hyenas exhibit maternal rank inheritance (Engh et al., 2000), we assigned juveniles the same social rank as that of their mother at the time of sampling. The social rank of each adult female hyena was determined based on the occurrence of submissive behavior during dyadic agonistic interactions (Strauss and Holekamp, 2019); for each clan, standardized social ranks ranging from −1 (lowest-ranked) to +1 (highest-ranked) were calculated annually for adult females using the MatReorder method in R package *DynaRankR* (Strauss, 2020). We assigned litter status for each juvenile to one of three categories based on whether we ever observed that juvenile with a littermate. If a sibling was never observed, a juvenile was considered a 'singleton.' If we did observe a littermate at any point in time, we determined the 'dominant' and the 'subordinate' juvenile based on the outcome of aggressive interactions between the littermates during early life (Smale et al., 1995). We assigned weaning status for each juvenile (whether or not they had been weaned) based on observations of nursing conflicts and observations of cubs subsequently seen with their mother when no nursing occurred (Holekamp et al., 1996).

2.6. Statistical analyses

All analyses were conducted using R Version 4.0.5 and R Studio Version 1.4.1106. Prior to any analysis, we explored our data by investigating outliers, distribution, and collinearity (Zuur et al., 2010).

All cortisol concentrations were log-transformed to achieve normality. Forest plots were created using R package *sjPlot* (Lüdecke et al., 2021b), and all other plots were created using R package *ggplot2* (Wickham et al., 2020). For the biological validation, we performed a Pearson's product-moment correlation on log-transformed values of cortisol concentrations in 25 paired saliva and plasma samples taken from 3 hyenas during ACTH stimulation tests.

2.7. Modeling cortisol concentrations in juvenile spotted hyenas

We built a global linear mixed model of methodological, ecological, and demographic variables with the potential to influence measured cortisol concentrations (Table S1). Because this is the first time that saliva has been collected from wild spotted hyenas for hormone measurement, we approached this as an exploratory analysis, examining a number of methodological variables with the potential to influence cortisol concentrations: (1) hyena chew time on the saliva collection device (minutes), (2) time between collection and freezing of saliva sample (hours), (3) time between collection and assay of saliva sample (months), and (4) number of freeze-thaw cycles undergone by each sample.

We also included a number of other factors known to affect salivary cortisol levels in other taxa such as: (5) collection time of day (AM/PM) and (6) collection time relative to sunrise/sunset (minutes) to determine if there were any temporal effects. We included an interaction between time of day and time relative to sunrise/sunset in case the cortisol concentrations changed at different rates in the morning versus evening. We included daily temperature (°C; both (7) minimum and (8) maximum) and (9) daily precipitation (mm) to account for potential thermal stress or dehydration (de Bruijn and Romero, 2018). We included an interaction between minimum and maximum temperature in case thermal challenges were due to the change in temperature rather than the minimum or maximum. We included (10) monthly clan prey density to account for potential juvenile or maternal nutritional stress (Benhaiem et al., 2013; Dloniak, 2004).

Lastly, we included the demographic covariates of (11) hyena age

(months), (12) sex, (13) maternal social rank, (14) litter status, and (15) weaning status (Behringer and Deschner, 2017; Creel et al., 2012). We included interactions between age and sex, and between age and rank, to account for the possibility of different developmental trajectories based on sex or rank. We also included an interaction between rank and prey density to account for the effect of rank on access to resources among adult females (Holekamp et al., 1996).

Prior to creating our global model, we tested model predictors for multicollinearity using both correlation coefficients and variance inflation factors (VIFs), and we removed collinear predictors until none were collinear, with all correlation coefficients ≤0.7 and all VIFs ≤3 (Harrison et al., 2018). Numeric model predictors were z-score standardized immediately before modeling using the scale function in R to allow comparison of coefficients (Harrison et al., 2018). The global model was built using R package *lme4* (Bates et al., 2020) and included the above fixed effects and interactions, as well as hyena identity as a random effect (Table S1). We performed model selection on the global model using AIC criteria and the dredge function in R package *MuMIn* (Bartoń, 2020). The top model was visually inspected to confirm assumptions regarding multicollinearity, normality of residuals, normality of random effects, heteroscedasticity, and homogeneity of variance using R package *performance* (Lüdecke et al., 2021c). We also inspected groups and observations for disproportionate influence on the model. Between-group comparisons of litter status were conducted using a Tukey post-hoc test for multiple comparisons of means in R package *multcomp* (Hothorn et al., 2021). Predicted cortisol values for plotting were obtained using the ggpredict function in R package *ggeffects* (Lüdecke et al., 2021a).

We performed two sensitivity analyses for this model. First, we added the term for weaning status to our top model to ensure that the change in energy balance caused by weaning did not affect our results. Second, we ran the top model on a restricted dataset of only twin littermates to ensure that our results concerning dominant and subordinate littermates were robust.

2.8. Investigating behavioral correlates

Earlier studies have demonstrated a relatively predictable relationship between mild stressors and cortisol excretion in saliva: salivary cortisol concentrations peak 20–30 min post-event in humans and 16–20 min post-event in dogs (Beerda et al., 1998; Kirschbaum and Hellhammer, 1989). Based on these studies and our ACTH stimulation tests, we chose a behavioral sampling window of 15–45 min post-event (Fig. S2). Using our field notes, we categorized saliva samples based on whether the sampled hyena had either emitted aggression, received aggression, engaged in social play, or none of the above in the 15–45 min before saliva sample collection. Samples were only included in the analyses if the individual was active and had exhibited a single type of behavior during the time window to avoid conflicting behavioral signals (e.g., individuals who both emitted and received aggression during the sampling window were excluded from the analysis). We then used residuals from the top model explaining salivary cortisol concentrations to investigate the effect of social behavior on cortisol; by using residuals, we controlled for known methodological, ecological, and demographic predictors (e.g., social rank) to reveal the remaining variation (Pierce and Schafer, 1986; Whitehead and James, 2015). We built a linear model, where residuals were modeled as a function of whether the individual had emitted aggression ('aggressor'), received aggression ('recipient'), engaged in social play ('play'), or none of the above ('other'). In primates, aggressive behavior, both giving and receiving, may increase glucocorticoids (Wittig et al., 2015), and social play behavior may reduce stress and lower glucocorticoids (Norscia and Palagi, 2011; Wooddell et al., 2017).

3. Results

3.1. Validation of salivary cortisol measurement in spotted hyenas

3.1.1. Analytical validation

Our assay passed all three analytical validation tests. For parallelism, the slopes of the kit standard curve and the serial dilution of the hyena saliva pool were not significantly different ($t = -0.024$, $p = 0.981$), indicating that these lines were parallel. Mean cortisol recovery was $99.8 \pm 9.2\%$, indicating the accuracy of our salivary cortisol measurements across different concentrations. Our intra-assay CV was 7.4% ($n = 6$ replicates), while our inter-assay CVs for all salivary assays were 10.2% (low concentration control; $n = 15$ assays), 4.7% (high concentration control; n = 15 assays), and 13.2% (hyena saliva pool, 45% binding; $n = 7$ assays), indicating the precision of our salivary cortisol measurements.

3.1.2. Biological validation

Our results indicated that cortisol measured in saliva closely reflects cortisol measured in plasma in spotted hyenas (Fig. 2). The correlation between cortisol measured in saliva versus plasma was $r = 0.77$ ($p < 0.001$).

3.1.3. Lag-time in salivary cortisol

When we compared the initial increase in cortisol in plasma to the initial increase in saliva, we detected a time lag of approximately 20 min (Fig. S2). Plasma cortisol peaked at approximately 55 min post-injection; however, we were unable to determine the salivary peak in cortisol because our ACTH stimulation tests did not capture the salivary decline. At present, all we can say is that the salivary peak was at least 20–25 min later than the plasma peak.

3.2. Temporal effects

Juvenile spotted hyenas exhibited two daily phases of decline in their salivary cortisol levels, once around sunrise and the other around sunset (Fig. 3A). This means that there are likely at least two daily peaks in salivary cortisol concentrations, although we cannot determine exactly when those peaks, or the troughs that must follow them, occur, nor can we rule out the possibility that there are more than two peaks. The
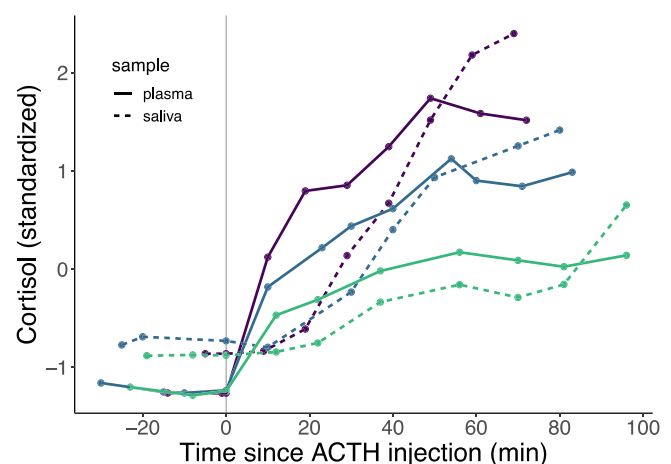
Fig. 2. Biological validation of saliva as a measure of plasma cortisol in three adult wild spotted hyenas. Each individual is represented by a different color. Dots represent sampling points, and lines are drawn between each sampling point for visualization. The vertical gray line represents the time of ACTH injection. Raw cortisol concentrations are z-score standardized within sample type for visualization. Cortisol concentrations from samples collected prior to the ACTH injection were averaged and plotted again at time 0 for visualization.
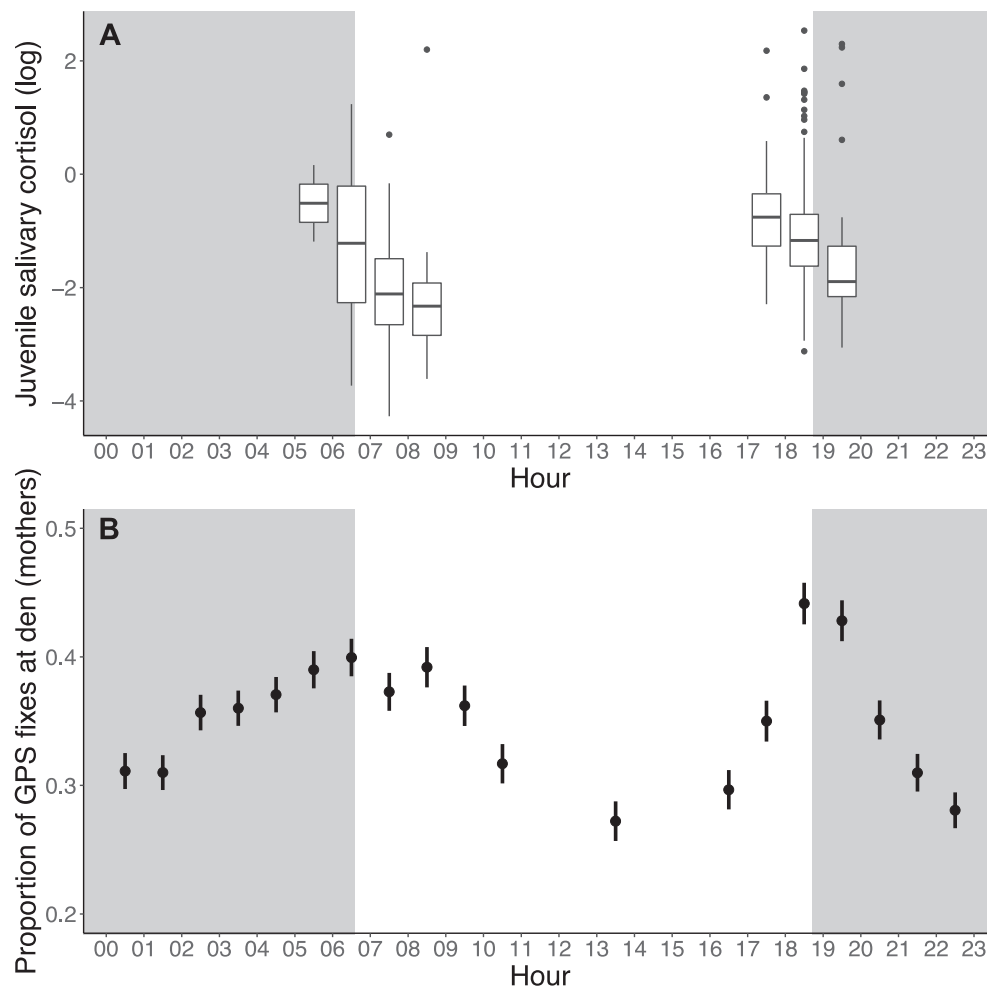
Fig. 3. Temporal effects on salivary cortisol concentrations. Gray shaded areas indicate night, and white areas indicate daylight. **A.** Cortisol concentrations in saliva samples collected from juvenile spotted hyenas. Samples were collected from three clans between 2015 and 2018, primarily at the communal den. Each bin represents the average log-transformed cortisol concentrations (µg/dL) from all juvenile samples collected within that hour. **B.** Proportion of time spent at the communal den by GPS-collared adult female hyenas (*n* = 10) who were nursing juveniles in two of the same three clans between 2012 and 2014. Collars were programmed to record GPS locations hourly from 1600 h to 1000 h, and also once at 1300 h, for a total of 20 location fixes per 24-hour period. Maternal presence at dens was determined by calculating the proportion of each female's total fixes that occurred within 100 m of the clan's active communal den. Fig. 3B modified from Greenberg (2017).

declining phases that we document here (and the peaks that must precede them) seem to align with maternal activity at the communal den (Fig. 3B), although the saliva samples and the activity data were not collected concurrently.

3.3. Juvenile cortisol concentrations

Our top model (*n* = 256; Fig. 4) predicting salivary cortisol concentrations in juvenile hyenas included the time between collection and assay (months), time of day (am/pm), time relative to sunrise/sunset (minutes), maximum temperature (°C), and litter status (dominant, subordinate, singleton) (Fig. 4A). Cortisol concentrations were higher in samples collected closer to their assay date ($\beta = -0.44$, $p < 0.001$; Fig. 4B), indicating that cortisol concentrations decreased as storage time increased. Samples collected in the evening had higher cortisol than samples collected in the morning ($\beta = 0.49$, $p < 0.001$; Fig. 4B), and samples collected before sunrise or sunset contained more cortisol than samples collected later that same morning or evening, respectively ($\beta = -0.20$, $p = 0.001$; Fig. 4B). Samples collected on hotter days had higher cortisol than samples collected on cooler days ($\beta = 0.26$, $p < 0.001$; Fig. 4B). Samples from dominant littermates had lower cortisol than samples from singleton juveniles, although other comparisons did not differ significantly (Tukey post-hoc test: [dominant - singleton]: $\beta = -0.67$, $p = 0.007$; [dominant - subordinate]: $\beta = -0.47$, $p = 0.102$; [subordinate - singleton]: $\beta = -0.20$, $p = 0.693$; Fig. 4C). Chew time (mean = 3.5 min, range = 1–8), time between collection and freezing (mean = 2.1 h, range = 0.6–5), number of freeze-thaw cycles (mean = 2.1, range = 1–4), minimum temperature, precipitation, prey density,

age, and maternal social rank were not included in the top model or any model within 5 AIC of the top model, nor were any interaction terms. Sex was included in a model within 2 AIC of the top model but not in the top model.

In a sensitivity analysis where we included weaning status as a covariate in this model, the magnitude, direction and precision of all estimates were comparable to the above model in which weaning status was not included (Fig. S3). In a sensitivity analysis where we restricted our dataset to only cubs from twin litters, the magnitude, direction and precision of all estimates were comparable to the above model in which our dataset included singleton juveniles (Fig. S4).

3.4. Behavioral correlates of cortisol concentrations

Our model (*n* = 87; Fig. 5A) investigating the effect of social behavior on salivary cortisol concentrations compared cortisol concentrations in samples from individuals that had emitted aggression, received aggression, or engaged in social play with samples from individuals that had not engaged in any of these behaviors. Samples from aggressors had marginally lower cortisol concentrations than samples from recipients, although no other behavior types differed significantly (Tukey post-hoc test: [aggressor-recipient]: $\beta = -0.67$, $p = 0.061$; [all other comparisons]: $p > 0.1$; Fig. 5B).

4. Discussion

We successfully measured cortisol in saliva collected non-invasively from wild juvenile spotted hyenas (Table 1, Fig. 1, Fig. 2), although our
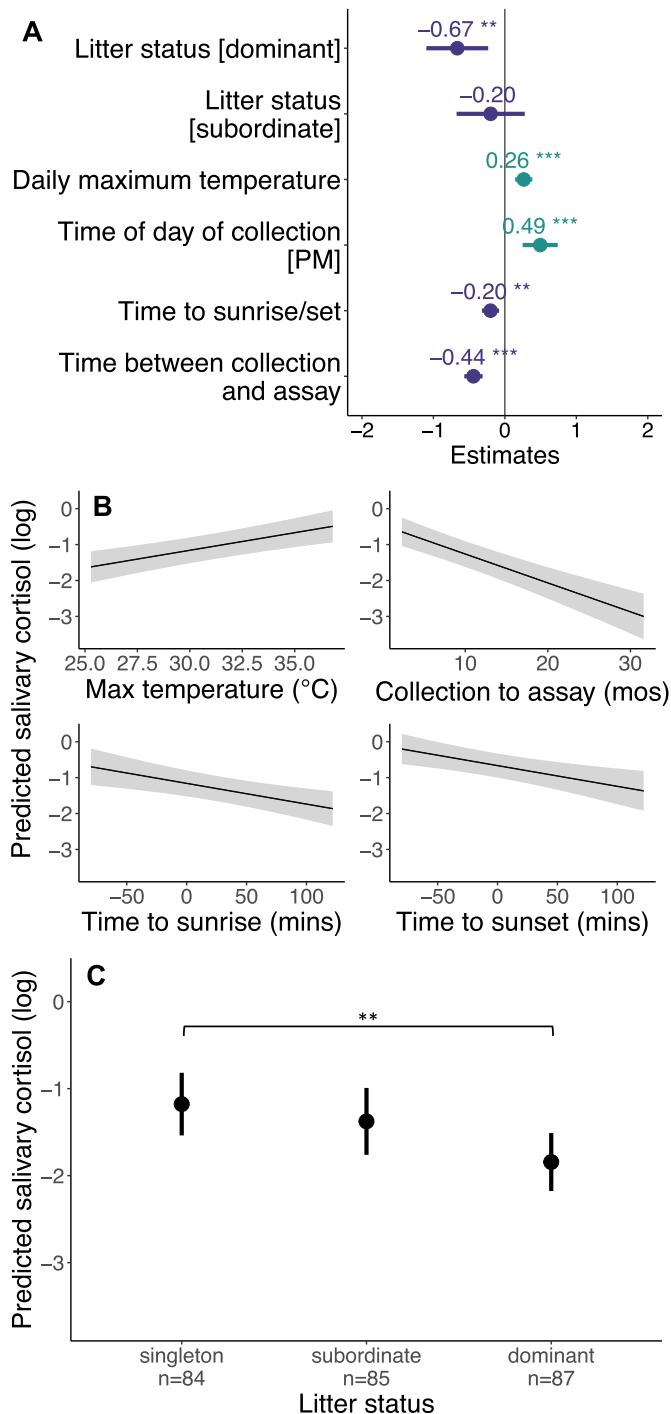
Fig. 4. Top model of predicted salivary cortisol concentrations (n-samples = 256, n-hyenas = 69). Asterisks depict significance at the following *p*-values: * = 0.05; ** = 0.01; *** = 0.001. **A.** Dots depict coefficient estimates, lines depict 95% confidence intervals. **B.** Lines depict estimated marginal means and shaded areas depict 95% confidence intervals. **C.** Dots depict estimated marginal means and vertical lines depict 95% confidence intervals. Asterisks depict significance in a Tukey post-hoc test.

technique admittedly requires well-habituated individuals and samples only individuals bold enough to interact with the apparatus. We hope that this description of our methods provides other researchers with a relatively simple way to measure salivary cortisol in other carnivores, both captive and free-living. To our knowledge, these data represent the first systematic attempt to collect and measure salivary hormones from wild carnivores without darting or capturing them. Although we only
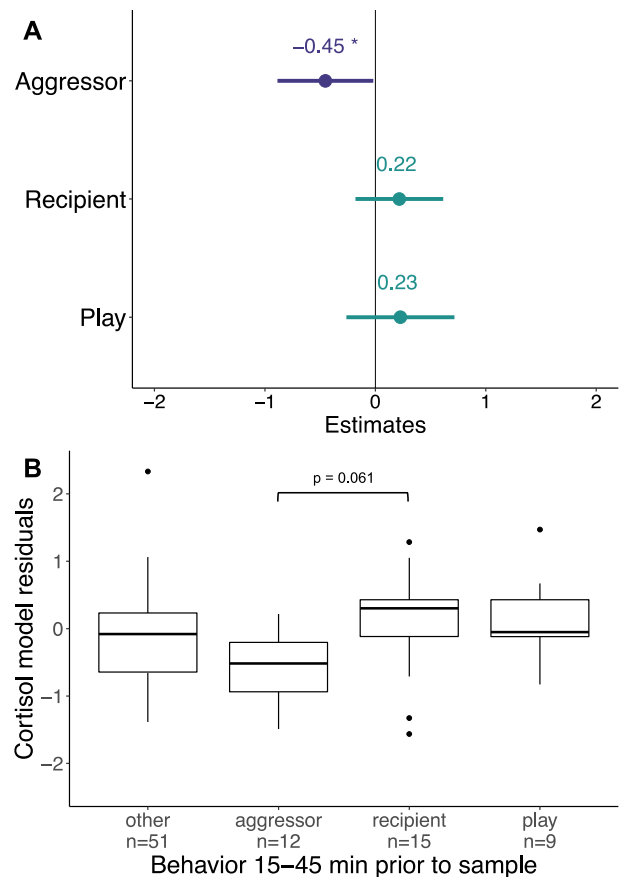

Fig. 5. Effect of social behavior on juvenile salivary cortisol concentrations. Samples categorized based on individual behavior in the 15–45 min before sampling. Residuals are taken from the model shown in Fig. 4. **A.** Asterisks depict significance at the following p-values: * = 0.05; ** = 0.01; *** = 0.001. **B.** *P*-value indicates significance in a Tukey post-hoc test.

sampled saliva from our subjects during morning and evening observation periods, we documented at least two clear phases of decline in salivary cortisol concentrations, at dawn and dusk. We found that cortisol covaried with daily temperature and was visually correlated with maternal den attendance but not with other socio-ecological covariates. We also found that cortisol varied among juvenile hyenas depending on their litter composition, but did not vary with cub age, sex, or maternal social rank. Finally, we were able to observe the effects of short-term aggression on the salivary cortisol concentrations of young hyenas. These data provide important insights into the behavioral ecology of social carnivores.

4.1. Methodological considerations for other researchers

The literature is inconsistent regarding most of the methodological covariates we tested, including the effect of storage at different temperatures (Garde and Hansen, 2005; Gröschl et al., 2001; Toone et al., 2013). We found that spotted hyena saliva can be stored up to 5 h at ambient temperature prior to freezing with no effect on measured cortisol concentrations, but that concentrations decrease across months and years during storage at −20 °C. Our results also indicate that the number of freeze-thaw cycles (up to 4) does not affect cortisol concentrations (Garde and Hansen, 2005), nor does the amount of time that a hyena spent chewing on the collection device (Kobelt et al., 2003). Overall, the difference in results between hyenas and other species suggests that researchers should assess these covariates for their specific study species and sampling procedures.

4.2. Daily cortisol rhythm

We found some evidence of a daily rhythm in spotted hyena salivary cortisol concentrations that has two distinct periods of decline, one around sunrise and one around sunset (Fig. 3A). This pattern suggests that there are two peaks and two troughs in what may be a bimodal daily rhythm of salivary cortisol (although the possibility that there are more than two peaks cannot be ruled out). This observed daily cortisol rhythm might be generated either internally (i.e., by a circadian clock), by responses to daily changes in the environment (e.g., the arrival of mothers at the den), or by an interaction between the two (Boulos and Terman, 1980).

We suspect that the proposed daily rhythm in cortisol concentrations is associated with the bimodal distribution of activity of juvenile spotted hyenas. Adult hyenas exhibit peaks in activity just before dawn and dusk (Kolowski et al., 2007), a pattern that is even more evident in juveniles living at the communal den. Mothers of young hyenas in our study population typically visit the communal den in the morning and again in the evening to nurse their cubs (Fig. 3B); this leads to elevated activity in juveniles twice per day, as cubs are usually resting in or near the den when adults are not present (Kruuk, 1972; White, 2007). To our knowledge, a bimodal daily cortisol rhythm has been documented in only two other mammals, the domestic pig (*Sus scrofa domesticus*; Hillmann et al., 2008; Ruis et al., 1997) and the Sudanian grass rat (*Arvicanthis ansorgei*; Verhagen et al., 2004). Given that cortisol secretion is tightly coupled with awakening in both diurnal and nocturnal species (Kumar Jha et al., 2015), it may be that a 12-hour rhythm evolved in crepuscular species, including hyenas, as a consequence of selection favoring maintenance of a tight link between cortisol and activity. The proximate mechanism mediating the rise of cortisol around dawn and dusk might involve anticipation of food (i.e., milk) that the mother often (but not always) provides when she arrives at the den. This may then be followed by a decline in both activity and cortisol, whether or not there has actually been a bout of nursing.

4.3. Cortisol covaries with temperature and litter status

4.3.1. Ecological covariates

Maximum daily temperature was positively correlated with salivary cortisol concentrations (Fig. 4). Although this trend could reflect changes in saliva due to dehydration, we do not see the same trend in salivary testosterone analyzed in the same samples (unpublished data), suggesting that dehydration cannot explain our results. Instead, our results might reflect a seasonal trend of thermal stress in either juveniles or mothers, leading to higher cortisol during the hottest parts of the year (Hollanders et al., 2017; Whittington-Jones et al., 2011). In contrast to maximum temperature, we found no effect of minimum temperature or precipitation on cortisol concentrations; juvenile hyenas may be able to easily regulate cold stress via huddling together, as is often observed on cooler mornings (de Bruijn and Romero, 2018; Gilbert et al., 2010).

Finally, we found no effect of local prey abundance on salivary cortisol in juvenile hyenas. In the Serengeti ecosystem, adult hyenas sometimes commute long distances on foraging expeditions that may last up to six days (Hofer and East, 1993), during which time juveniles experience substantial nutritional stress and have elevated fecal glucocorticoid concentrations (Benhaiem et al., 2013). However, this commuting behavior is rare in our study area, in which prey is more consistently available. Juvenile fecal glucocorticoid concentrations are not correlated with prey density (Greenberg, 2017), and consumption of their mother's milk likely buffers juveniles from any nutritional stress due to low prey abundance (Hofer et al., 2016; Holekamp et al., 1996).

4.3.2. Demographic covariates

Litter status was correlated with salivary cortisol concentrations: singletons had the highest cortisol, subordinate juveniles from twin litters had the second highest, and dominant juveniles from twin litters

had the lowest cortisol concentrations, although only the difference between singletons and dominant littermates was statistically significant (Fig. 4C). Singletons may have higher cortisol concentrations because they lack social support (i.e., from a sibling) at the communal den; social support and close partnerships are known to reduce stress in other species (Wittig et al., 2016). We also observed a non-significant trend for subordinate twin littermates to have higher cortisol than dominant twins, which is similar to patterns documented in hyena feces (Benhaiem et al., 2013). Subordinate littermates may have higher cortisol concentrations due to either higher rates of received aggression or lower food intake; dominant littermates frequently aggress on subordinates to prevent subordinates from nursing (Benhaiem et al., 2012b; Wahaj and Holekamp, 2006).

Although fecal glucocorticoids are influenced by age and sex (Benhaiem et al., 2013, 2012a; Greenberg, 2017), we found no effect of either variable on salivary cortisol concentrations. There are at least two possible explanations for the discrepancies. First, sex differences in gut composition might lead to sex differences in fecal glucocorticoid metabolites without affecting salivary cortisol (Goymann, 2012; Rojas et al., 2020). Second, juveniles sampled via saliva tended to be younger than juveniles sampled via feces in earlier studies (Greenberg, 2017), perhaps affecting the trends we found. Maternal rank did not explain glucocorticoid concentrations in either saliva or feces (Greenberg, 2017), nor does social rank influence fecal glucocorticoid metabolites in adults (Dloniak, 2004; Goymann et al., 2001).

4.4. Behavior affects short-term cortisol concentrations

Although a correlation between glucocorticoid concentrations and aggressive behavior has been established in a wide range of taxa, most studies of wild animals are unable to evaluate the immediate effects of emitting or receiving aggression (but see Wittig et al., 2015). Saliva sampling, however, allows for precisely this short-term assessment of glucocorticoids. We found that emitting aggression was correlated with decreased salivary cortisol (Fig. 5A), especially when compared to recipients of aggression (Fig. 5B). Across species, the effect of emitting aggression, or winning, is more variable, with some species exhibiting no change and others exhibiting increases in glucocorticoids similar to that of losers (Hsu et al., 2006). In hyenas, where the outcomes of aggressive interactions are highly predictable, emitting aggression may be a form of stress relief in much the same way as redirected aggression, or scapegoating (Kazem and Aureli, 2005). Given that hyenas are highly attuned to dominance relationships within the group (Engh et al., 2005), reinforcement of dominance over subordinates through ritualized aggression might actually buffer individuals against socially-induced stress.

The socialization hypothesis suggests that social play provides both immediate and delayed social benefits, including reduced stress or tension (Graham and Burghardt, 2010; Pellis et al., 2015). Social play has been shown to reduce stress-related behaviors and cortisol levels in dogs and primates (Horváth et al., 2008; Norscia and Palagi, 2011). While we did not observe any difference in cortisol between individuals that had recently engaged in social play and those that had not, this could be due to our small sample size ($n = 9$) or due to differences between species.

5. Conclusion

Our work confirms that salivary analyses offer a useful alternative to fecal and urinary analyses in naturalistic studies of hormone-behavior relationships in wild carnivores. Our results also demonstrate that salivary hormone measurements are particularly useful for assessing short-term effects of specific behavioral interactions, while also accounting for other ecological or demographic variables likely to affect hormone concentrations. Finally, our work raises many new questions and opens pathways for further research exploring phenomena such as daily

rhythms in hormone concentrations and variation in glucocorticoid concentrations due to litter composition.

CRediT authorship contribution statement

Declaration of competing interest

None.

Acknowledgements

Funding

Data availability

All data and analysis code are available through public GitHub pages (https://github.com/tracymont/hyena_saliva_cortisol).

Appendix A. Supplementary materials

Supplementary figures and tables to this article can be found online at https://doi.org/10.1016/j.yhbeh.2021.105082.

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