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Validation of a field technique and characterization of fecal glucocorticoid metabolite analysis in wild chimpanzees (*Pan troglodytes*)

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Abstract

Monitoring adrenocortical activity in wild primate populations is critical, given the well-documented relationship between stress, health and reproduction. Although many primate studies have quantified fecal glucocorticoid metabolite (FGM) concentrations, it is imperative that researchers validate their method for each species. Here, we describe and validate a technique for field extraction and storage of FGMs in wild chimpanzees (*Pan troglodytes*). Our method circumvents many of the logistical challenges associated with field studies while yielding similar results to a commonly-used laboratory method. We further validate that our method accurately reflects stress physiology using an ACTH challenge in a captive chimpanzee and a FGM peak at parturition in a wild subject. Finally, we quantify circadian patterns for FGMs for the first time in this species. Understanding these patterns may allow researchers to directly link specific events with the stress response.

Keywords

chimpanzee; fecal glucocorticoid metabolites; field methods; ACTH; parturition; circadian rhythm

INTRODUCTION

In response to a perceived stressor, an individual has a physiological reaction, which involves the release of glucocorticoids by the adrenal glands via the hypothalamic-pituitary-adrenal (HPA) axis. Glucocorticoids increase readily-available energy by hepatic gluconeogenesis, inhibiting the uptake and storage of glucose from the bloodstream, and suppressing non-essential functions [Sapolsky, 2002]. These effects are adaptive and can ensure survival during an acute or short-term stressor. However, chronic exposure to elevated glucocorticoid concentrations can have deleterious consequences. Chronic stress can result in immunosuppression, muscle wasting, and reduced reproduction [Sapolsky,

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2002]. For example, chronic social stress in long-tailed macaques increased visceral fat depositions and incidences of coronary artery atherosclerosis [Shively et al., 2009]. Glucocorticoids were positively related to parasite richness [male chimpanzees: Muehlenbein & Watts, 2010; male mandrills: Setchell et al., 2010].

Given the relationship among stress, health, and reproduction, numerous studies have therefore investigated the environmental, social, and demographic correlates of primate glucocorticoid production. A full review is beyond the scope of this paper, but we summarize some of the variables that have previously been linked to glucocorticoids. Nutritional stress is often reflected in seasonal variation in glucocorticoid levels [e.g. chimpanzees: Muller & Wrangham 2004; capuchins: Carnegie et al., 2011]. It is interesting to note that environmental predictability may also play an important role. For example, Rosenblum & Andrews [1994] found that a variable foraging regime was more disruptive than either high or low food availability in captive bonnet macaques. Other ecological variables also correlate to HPA activity, including day length [chacma baboons: Weingrill et al., 2004], temperature, and altitude [gelada baboons: Beehner & McCann, 2008]. In short, these studies demonstrate that ‘challenging’ ecological conditions correlate to higher glucocorticoid levels.

Studies have further investigated the relationship between glucocorticoids and social and demographic variables. The influence of dominance rank on glucocorticoids has a long history in particular [chimpanzees: Muller & Wrangham, 2004; Verreaux’s sifakas: Fichtel et al., 2007; savanna baboons: Gesquiere et al., 2011] but the relationship may depend upon dominance hierarchy stability and social support [reviewed in Abbott et al., 2003; chacma baboons: Bergmann et al., 2005; baboons: Engh et al., 2006; blue monkeys: Foerster et al., 2011]. For example, Crockford et al. [2008] found that a focused grooming network reduced the impact of an unstable male hierarchy in female chacma baboons. Glucocorticoid levels relate to a range of other social and demographic factors, including age, reproductive state, and immigration status [chacma baboons: Weingrill et al., 2004; chimpanzees: Khahlenberg et al., 2008; Seraphin et al., 2008; savanna baboons: Altmann et al., 2010].

The interest and importance of quantifying glucocorticoids in wild populations is clear. Free-ranging populations likely face much higher ecological stress than captive populations. Understanding how environmental and social perturbations affect animals allows us to examine individual survival and reproductive success, as well as population level questions and conservation efforts across many taxa [starlings: Cyr & Romero, 2007; snowshoe hares: Sheriff et al., 2009]. Despite the significance of wild studies, they have lagged behind captive work due to the practical and logistical difficulties of field conditions. In the last decade, endocrine studies in wild populations have increased due to resolution of these issues [spotted hyenas: Goymann et al., 2001; tufted capuchins: Lynch et al., 2002; ground squirrels: Mateo & Cavigelli, 2005; elephants: Freeman et al., 2010].

The studies mentioned above all quantified fecal glucocorticoid metabolite (FGM) concentrations. Fecal hormone analyses are particularly useful for studies where it is impossible to collect either blood or saliva from subjects. Although captive work with chimpanzees (*Pan troglodytes*) has reported fecal methods [Whitten et al., 1998; Heistermann et al., 2006], other studies in wild populations have focused exclusively on urine [Muller & Wrangham, 2004; Muller et al., 2007; Kahlenberg et al., 2008; Emery Thompson et al., 2010] even though fecal samples are much more readily collected. Despite the advantages of fecal steroid analysis, there are several difficulties that warrant consideration and emphasize the need to validate a particular method. Studies have demonstrated that there is little to no native cortisol or corticosterone in feces [Palme et al., 2005]; researchers must therefore identify which metabolic steroid hormone by-product can

be detected in their species. This is most readily demonstrated through an adrenocorticotrophic hormone (ACTH) challenge on a captive subject. Exogenous administration of ACTH is known to stimulate the adrenal glands to release glucocorticoids, and cause a rise and fall of these hormones which can be tracked in feces.

Fecal hormone concentrations can also be affected by the storage method [Khan et al., 2002; Terio et al., 2002] and bacterial degradation. Naturally-occurring bacteria decompose FGM within hours if the sample is not properly stored [Wasser et al., 1998; Moestl et al., 1999]. Wasser et al [1998] demonstrated that storage in ethanol prevents hormone degradation but shipping ethanol internationally is problematic [Ziegler & Wittwer, 2005]. Given the challenges inherent in fecal hormone analysis and inter-species differences in hormone production and excretion, it is imperative that researchers validate their method of choice for a given species and extraction technique [Touma & Palme, 2005]. Such validations are becoming standard in the field of primate behavioral endocrinology [e.g. gelada baboons: Beehner & McCann, 2008] and set the stage for future research in the species of interest.

Here, we report a new technique for monitoring fecal glucocorticoids and characterize diurnal FGM patterns for the first time in wild chimpanzees. We first validated our extraction and storage method by comparing our method to a commonly-used laboratory method [Wasser et al., 1991; 1993; Brown et al., 1994]. We then conducted biological validations of our methods using an ACTH challenge on a captive chimpanzee and by demonstrating the stress response surrounding parturition in a wild chimpanzee at Gombe National Park, Tanzania. The latter represents a unique opportunity to demonstrate the biological validity and relevance of our method in a wild setting given the well-documented spike in circulating glucocorticoids at parturition in other mammals [heifers and goats: Hydbring et al., 1999; dogs: Olsson et al., 2003]. Studies in primates have demonstrated a general increase over the course of pregnancy [gorillas and chimpanzees: Smith et al., 1999] and a group-level and mother-specific increase after parturition [bonobos: Behringer et al., 2009] but we are not aware of any wild great ape studies that have captured the stress response to the birth event.

Finally, we have quantified and reported FGM circadian patterns from samples collected during a 6-month field season. Diurnal patterns have been reported in a broad range of mammals and birds [reviewed in Touma & Palme, 2005]. These patterns have been mirrored in chimpanzee rhythms with higher cortisol concentrations during the morning [saliva: Heintz et al., 2011; urine: Muller & Lipson, 2003], but this is the first study to demonstrate how FGM varies diurnally in wild chimpanzees. Fecal samples represent pooled hormone concentrations over several hours and previous research has produced mixed results on diurnal patterns with FGM. Marmosets have higher FGM in afternoon samples [Raminelli et al., 2001; Sousa & Ziegler, 1998] compared with morning samples while gorillas do not appear to have a discernible FGM rhythm [Peel et al., 2005]. Understanding diurnal FGM patterns are necessary for linking social events with subsequent fecal measurements in future studies.

METHODS

Methodological validation

Subjects—To compare the efficacy of our field method to laboratory extraction, we collected samples from two adult females that were group-housed at Lincoln Park Zoo (Chicago, IL) from June 6, 2006 – June 21, 2006. Female 1 was born in captivity and was 16 years old at the time of data collection. She was group-housed with three juvenile (2:1) and three adult chimpanzees (2:1). Female 2 was wild-caught with an estimated age of 41 years old at the time of collection. She was group-housed with four adult chimpanzees (3:1). Both

subjects had access to water *ad lib* and were fed a mixture of vegetables, fruits, Leaf Eater chow, and Hi-Pro chow under the direction of the Lincoln Park Zoo Animal Care staff.

We collected fecal samples ($n = 24$ samples, 11 from Female 1 and 13 from Female 2) and froze them at -20°C within 2 hours until they were processed. Each sample was extracted by two methods: a laboratory method commonly-used in other studies [Loeding et al., 2011] and our field extraction method which allows samples to be extracted in the field and then stored in a dry form. The research protocol for this study was approved by the Research Committee at The Lincoln Park Zoo (Chicago, IL) and adhered to the American Society of Primatologists principles for the ethical treatment of primates.

Laboratory Processing Method—We weighed out 0.50 g (± 0.02 g) wet feces and added 5.0 ml of 90% ethanol:distilled water. The tubes were then capped, shaken for 30 minutes (Glas-col Mixer, Terre Haute, IN), and centrifuged at 1500 rpm for 20 minutes. We poured off the supernatant into clean, labeled 16 $\times$ 125 mm tubes and added an additional 5 ml of 90% ethanol:distilled water to the pellet that remained in the original extraction vial. Those tubes were re-vortexed for 30 seconds and centrifuged as above for 15 minutes to extract additional hormones from the pellet. The two supernatants (from the first and second extractions) were combined, and we dried down 1 mL aliquots in a warm bath (60°C) using air until completely desiccated. The dried samples were reconstituted in 1 mL of phosphate buffered saline (PBS; 0.2 M NaH_2PO_4 , 0.2 M Na_2HPO_4 , NaCl), sonicated for 20 min and stored in the freezer until analyzed using an enzyme immunoassay.

Field Processing Method—We weighed out 0.50 g (± 0.02 g) wet feces and added 5.0 ml of 90% ethanol:distilled water into 16 $\times$ 100mm tubes, which has been shown in previous studies to give a high recovery [Santymire et al., 2012]. The tubes were then capped and hand-shaken for 30 seconds. The tubes were rotated on a low-energy rotator (Barnstead International, Model 400110, DuBuque, IA) for 2 hours, and centrifuged for 20 minutes at 1500 rpm on an electric centrifuge. We poured the resultant supernatant into another set of labeled 16 $\times$ 100mm tubes. For the field processing method and ACTH validation, 1 ml aliquots were removed and dried in a warm bath (60°C) using air. When dry, the samples were capped and stored at room temperature until reconstitution. Samples collected in the field (stress response to parturition and characterization of diurnal FGM patterns) were processed using identical methods as above except for drying. Field samples were placed in a pelican case with reusable desiccant (Eva-dry, Westchase, FL) to dry; this drying method requires no electricity (and is hence, field-friendly). Samples were capped after they were completely desiccated (approximately 2 weeks).

Biological Validation

ACTH Challenge

Subject: To validate that our assay is accurately measured FGM associated with the stress response, we conducted an ACTH challenge on a captive, pair-housed male chimpanzee at Yerkes National Primate Research Center (Atlanta, GA). The subject was 21 years old at the time of the challenge (August 2008) and had been raised in captivity from birth. He was well-trained to receive injections so we could be confident that a peak in FGM would correspond to the ACTH injection, not the handling or injection procedure. Water was available *ad lib*, and he was fed fresh fruits and vegetables, and monkey pellets twice per day. We used a green food coloring agent to distinguish the subject's fecal matter from that of his housemate.

Procedure: The subject received a 0.45 IU/kg [Heistermann et al., 2006] dose ACTH (Cortrosyn; Amphastar Pharmaceuticals, Inc. Rancho Cucamonga, CA) via intramuscular

injection at 11:00 AM on August 11, 2008. We collected every feces voided by the subject over 11 days, 4 prior to and 6 after the injection ($n = 27$ fecal samples). We focused on samples collected on the day of the injection and for 2 days thereafter ($n = 12$ fecal samples) to determine the ACTH profile. Samples were stored at -80°C , shipped on dry ice, and then stored at -20°C until processing via the lab method described above. ACTH administration and fecal collection was approved by Yerkes IACUC (#151-2008Y).

Stress Response to Parturition: During a 6-month field study at Gombe National Park, Tanzania (May–October 2010), one of our female subjects was encountered in the morning after giving birth overnight. The birth window was extrapolated from prior observations of the subject and since the umbilical cord was still attached to the infant. Such a well-known stressor represents a unique opportunity for biological validation in field settings. We therefore followed the mother intensively for 4 days in order to collect fecal samples ($N = 9$ sample) and test for a spike in her FGMs. The mother was 29 years old at the time of parturition and the infant was her fifth. Samples were stored frozen (-20°C) until extraction via our field method. Fecal collection was approved by Tanzanian Wildlife Research Institute.

Characterization of Diurnal FGM Patterns

Subjects: We collected fecal samples from mothers ($N = 61$ fecal samples from 9 individuals) during a 6-month field season at Gombe National Park, Tanzania, in order to quantify diurnal patterns. The focus on mothers was a logistical decision since this study was rolled into another project on mother and infant behavior. We confined our analyses to days on which multiple samples were collected from the same target (range 2–7 samples). Fecal sample collection occurred between 6:00–1900 hours. Samples were frozen at -20°C at Gombe until processed via our field method. Fecal collection was approved by Tanzanian Wildlife Research Institute.

Enzyme-immunoassay: For all analyses, our dried samples were reconstituted in 1 ml PBS immediately before analysis. To ensure that all the dried hormone extract was in solution, we added 3–4 glass beads and sonicated each tube for 20 minutes. We then shook the tubes on a mixer for an additional 30 minutes (Glas-col Mixer, Terre Haute, IN).

We quantified FGM via cortisol enzyme immunoassay (EIA) with a method previously described [Wasser et al., 2000; Young et al., 2004]. C. Munro (University of California-Davis, CA) provided horseradish peroxidase (HRP) ligands and polyclonal antiserum (R4866). Cortisol antiserum and HRP were used at dilutions of 1:8500 and 1:20000, respectively [Loeding et al. 2011]. Cross-reactivities of cortisol R4866 antibody are reported as: cortisol 100%, prednisone 6.3%, corticosterone 0.7%, 21- deoxycorticosterone 0.5%, progesterone 0.2%, pregnenolone 0.1%, androstenedione 0.1%, dehydroisoandrosterone-3-sulfate 0.1%, estradiol-17 β 0.1%, estriol 0.1%, cholesterol 0.1%, prednisolone 9.9%, cortisone 5.0%, deoxycorticosterone 0.3%, 11- desoxycortisol 0.2%, 17 α -hydroxyprogesterone 0.2%, 17 α -hydroxypregnenolone 0.1%, testosterone 0.1%, dehydroepiandrosterone 0.1%, aldosterone 0.1%, estrone 0.1%, and spironolactone 0.1% [Young et al., 2004]. The EIA was biochemically validated for chimpanzees by demonstrating 1) parallelism between the binding inhibition curves of fecal extract dilutions and the cortisol standard ($R^2 = 0.969$), and 2) significant recovery ($> 90\%$) of exogenous cortisol added to fecal extracts ($y = 0.82x + 3.54$, $R^2 = 0.998$).

Statistical Analyses: To compare the efficacy of our method to the laboratory method, we used a simple paired t-test.

To quantify FGM elevation during the ACTH challenge, we calculated baseline and elevated FGM concentrations using an iterative process [Brown et al., 1994; Moreira et al., 2001]. In brief, the mean value is first calculated from all samples. Any sample greater than the mean + 1.5 standard deviation (SD) is then removed from subsequent calculations. The process is repeated on all samples that were not removed until all remaining samples fall within 1.5 SD of the new mean value (the 'baseline'). Samples were considered 'elevated' when they were above the baseline value + 1.5 SD.

To test for a diurnal pattern in FGM, we log transformed the wild chimpanzee FGM diurnal rhythm data to normalize the dataset. All values exceeding two SDs from the mean of log transformed values were removed. We ran a mixed model that accounted for repeated measures on the same female and that included time (AM/PM) and month to account for temporal variation in food availability that may affect glucocorticoid values as seen in seasonal results at other sites [Muller & Wrangham, 2004]. Values are presented as mean $\pm$ SE. For all analyses, $p < 0.05$ was considered significant.

RESULTS

Methodological Validation

There was no difference between the laboratory and field methods (paired t-test: $t(24) = -0.41$, $p = 0.68$). Mean FGM concentration for samples processed with the lab method was 17.85 ± 1.21 ng/g wet feces compared to a mean FGM concentration of 18.57 ± 1.33 ng/g wet feces) for samples processed with our field method.

Biological Validation

ACTH Challenge—The subject exhibited a pronounced increase in FGM concentration following the ACTH injection (Fig. 1). We averaged samples that were voided overnight since we could not ascribe a precise time of defecation. The peak FGM concentration occurred approximately 29 hours post-injection. Mean FGM concentration for all samples collected over the 11-day period was 11.90 ± 0.68 ng/g wet with a baseline of 9.94 ng/g wet feces and elevation above 11.76 ng/g wet feces were considered elevated.

Response to Parturition—On the day after parturition, the mother showed a marked increase in FGM. Figure 2 plots all samples collected within 1 week of the parturition event as well as the monthly mean. As illustrated, the FGM concentration for the mother peaked at 158 ng/g wet feces on the day after parturition; this is approximately six times higher than her average FGM concentration before pregnancy ($20.01 \pm \text{SE } 1.67$ ng/g wet feces, $N = 22$ samples) and approximately three times higher than average during her the birth month ($51.06 \pm \text{SE } 12.52$ ng/g wet feces, $N = 12$ samples).

Diurnal FGM Patterns—We found that both time of day and month were significantly correlated to FGM values (time of day: $F_{1,49} = 5.13$, $p = 0.03$, month: $F_{4,49} = 3.37$, $p = 0.02$). The mean morning FGM was 19.83 ± 1.42 ng/g wet feces ($N = 33$ samples) while the mean afternoon FGM was $22.71 \pm$ (N = 28 samples) (Figure 3).

DISCUSSION

Understanding the socio-ecological correlates of the stress response is critical given the impact of stress on health and reproduction. In the last decade, numerous studies have therefore reported relationships between adrenocortical activity and a suite of stressors, including nutrition, dominance rank, dominance hierarchy stability, and immigration [e.g. blue monkeys: Foerster et al., 2011; savanna baboons: Gesquiere et al., 2011]. As studies

become increasingly common, it is imperative that researchers validate their method for a particular species [Touma & Palme, 2005]. This study therefore validates a method that will be the basis future research on wild chimpanzee stress physiology. The method may further be useful to primate behavioral endocrinologists working in other species.

Previous work investigating glucocorticoid concentrations in wild chimpanzees have relied on urine analysis [Muller & Wrangham, 2004; Muller et al., 2007; Kahlenberg et al., 2008; Emery Thompson et al., 2010]. For example, Muller and Wrangham [2004] demonstrated a positive correlation between male dominance rank and urinary cortisol, which may reflect the increased energetic costs of maintaining rank through physical aggression. They also reported nutritional and circadian results that matched expectations based on work in other species. Urinary cortisol is a useful method. Like feces, it can be collected non-invasively and does not have the episodic patterns apparent in blood. However, it is more difficult to collect than feces since urine absorbs quickly into a forest substrate. Our method is therefore a powerful alternative to urine analyses in this species and may allow researchers to increase sample size in future chimpanzee research efforts.

Fecal hormone analyses have become much more common in recent years for the reasons outlined above. In the past, some studies have desiccated wet feces or stored samples in a preservative until they could be shipped for analyses [ethanol: Terio et al., 2002; formalin: Millspaugh et al., 2003]. However, desiccation can itself alter hormone metabolite concentrations [Terio et al., 2002] and storage in preservatives also has disadvantages. It can be difficult to transport preservatives due to shipping regulations and potential spillage, and fecal hormone extraction can occur inadvertently [Wielebnowski & Watters, 2007]. Field extraction methods are therefore increasingly popular, with adaptations specific to certain species and study sites [e.g. Chacma baboons Beehner & Whitten, 2004; African wild dog: Santymire & Armstrong, 2010]. Following in the vein of those studies, we developed and presented a method that works well for chimpanzees. It allows researchers to extract in the field into small vials that are easily stored and shipped without special consideration or the need for prolonged freezer storage.

Our method was biologically validated through an ACTH challenge. Despite low concentrations of native cortisol in feces compared to other substrates, FGM peaked in the typical pattern with a delay of approximately 29 hours post-injection which is concordant with gut times reported in other ACTH challenges in captive populations using different methods [Bahr et al., 2000; Heistermann et al., 2006; Whitten et al., 1998]. Our findings also indicate there is a higher affinity for the cortisol EIA compared with corticosterone [Santymire et al., 2012] for chimpanzees feces. While ACTH challenges are reliable validations, our field study afforded us an interesting and insightful opportunity to determine how a well-known stressor presents itself in FGM in wild chimpanzees. Numerous captive studies have shown a marked spike in glucocorticoids around parturition [cow & goat Hydbring et al., 1999; dog: Olsson et al., 2003], including one study from captive bonobos [Behringer et al., 2009]. However, this is the first study to demonstrate the expected peak in a wild great ape. The mother in our study is assumed to have given birth in the early morning prior to the peak (based upon a fresh and attached umbilical cord). Her FGM values peaked around 4 PM and (even with a wide birthing window of 12:00 – 7:00 AM) suggest the gut delay in wild chimpanzees is substantially shorter than in captive species.

The reduced gut time is echoed in our circadian data. Our data show that wild chimpanzee FGM is higher in the afternoon which has the same pattern as FGM in marmosets [Sousa & Ziegler, 1998; Raminelli et al., 2001]. The time delay in feces is much larger than saliva and urine and produces a different diurnal pattern compared with salivary cortisol in captive chimpanzees [Heintz et al., 2011] and in urinary cortisol metabolites for both captive and

wild chimpanzees, gorillas, and humans [Czekala et al., 1994; Muller & Lipson, 2003; Anestis & Bribiescas, 2004]. No previous studies have reported FGM patterns for wild chimpanzees but our results (parturition and circadian rhythm) suggests that raised glucocorticoid metabolites manifest in feces approximately 12 hours after a stressor. Coupled with our parturition data, the circadian rhythm data presented here suggest a fecal delay on the order of 12 hours and much less than estimates for the peak in the ACTH challenge. It is important to point out that wild individuals have much higher fiber content in their diet, which has been shown to influence hormone metabolite excretion [Dantzer et al., 2011; Wasser et al., 1993]. Future studies investigating glucocorticoid response to a stressor should account for diet and time delay in each specific environment rather than relying on captive data.

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FGM Concentration During ACTH Challenge

Figure 1. Fecal glucocorticoid metabolite (FGM) concentrations following adrenocorticotrophic (ACTH) injection

The injection in a captive-housed male chimpanzee occurred at Time 0 and every feces was collected thereafter. Open data markers indicate that overnight samples were averaged and assigned a time of 8.00 AM. We calculated baseline and elevated values from samples collected over 10 days, 3 of which were prior to the ACTH challenge. The solid line represents the baseline with the dashed line representing the elevated values.

Figure 2. Peak in fecal glucocorticoid metabolite concentrations at parturition

Parturition time is inferred from first observation observing the new infant early on the morning of 16-September-2011 with the umbilical cord still attached. Maternal fecal glucocorticoid metabolites (FGM) peaked that afternoon and were over three times the monthly average for the mother (indicated by a dashed line).

Figure 3. Diurnal patterns in fecal glucocorticoid metabolite concentrations

Mean ($\pm$ SEM) fecal glucocorticoid metabolites (FGM) for samples excreted in the morning (6.00 – 11.59 AM) and afternoon (12.00 – 7.00 PM) are provided. When we controlled for month, time was significantly different with higher mean afternoon FGM.