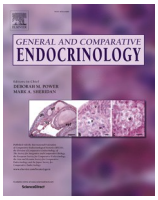
General and Comparative Endocrinology 334 (2023) 114212

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen



Assessing stress in wild black-and-white colobus monkeys non-invasively

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ARTICLE INFO

Keywords:
Biological validation
Cortisol metabolites
Circadian effect
Parturition
Reproductive state
African colobine

ABSTRACT

Analysis of glucocorticoid profiles serves as a valuable, multi-faceted tool for insight into the behavior and physiology of wild populations. Recently, the measurement of fecal glucocorticoid metabolites (FCMs) has exploded in popularity due to its compatibility with noninvasive techniques and remote environments A critical first step is to perform a biological validation to ensure that the assay accurately reflect changes in FCM levels. We use an enzyme immunoassay (EIA) to perform a biological validation on samples collected from two males and six females in a wild population of *Colobus vellerosus* in response to three naturally occurring potential stressors. We also describe the FCM response pattern in the week following parturition in three females and examine the influence of sex, reproductive state, and time of day on the concentrations of baseline samples collected daily from 13 adult individuals over a period of four months. We validated the assay: FCM levels increase in response to natural stressors with a two-day lag. In the two days surrounding parturition, FMC levels increased. Baseline concentrations were affected by collection time and female reproductive state, with lactating females having lower concentrations than pregnant or cycling females. Thus, we successfully carried out the first validation and characterization of FCMs in a wild African colobine. This will serve as an essential foundation for future studies of *C. vellerosus* and similar wild primates whose objective is to investigate the role glucocorticoids play in responses to social and ecological challenges.

1. Introduction

The hypothalamic–pituitaryadrenal (HPA) axis plays a central role in facilitating physiological responses to internal and environmental stimuli (Boonstra, 2013a). It operates on an ongoing basis, enabling individuals to cope with both unpredictable external disruptions ("stressors") as well as predictable changes (e.g., life history or seasonal transitions; Ricklefs and Wikelski, 2002). Accounting for the involvement of GCs in regulating myriad physiological systems such as metabolism, reproduction, and growth, the ability to measure glucocorticoids is key to understanding the ecology and evolution of vertebrates.

Whereas the collection of blood samples allows the measure of circulating GCs, a non-invasive alternative is the measurement of fecal cortisol metabolites (FCMs). GCs are metabolized first in the liver and secondarily by gut bacteria in the intestines prior to excretion via feces (and urine) in the form of FCMs, following a time delay ranging from hours to days, depending on taxa-specific transit time (Palme et al., 2005; Edwards et al., 2020). Thus, blood and fecal samples present two portraits of HPA activity, each with their own merits; blood samples offer a nuanced, but temporally narrow snapshot measure of GCs, where fecal samples provide a pooled, albeit indirect, estimate over an elongated period via the measure of FCMs (Sheriff et al., 2011). For those interested in addressing evolutionary or conservation-based questions, FCMs are a compelling choice, as they offer an integrative view of HPA activity and are subject to relatively less variation stemming from proximate causes, such as pulsatile secretion patterns and circadian rhythm (Möstl and Palme, 2002). Notably, however, the metabolized nature of these samples introduces a large degree of variation arising from metabolic and digestive differences that must be accounted for to appropriately quantify FCM levels (Palme, 2019).

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https://doi.org/10.1016/j.ygcen.2023.114212
Received 12 August 2022; Received in revised form 2 December 2022; Accepted 11 January 2023
Available online 13 January 2023
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A great variety of factors alter glucocorticoid regulation, metabolism, and excretion. For instance, captive and wild populations regularly differ in glucocorticoid metabolite ranges and excretion intervals, owing to their dissimilarities in activity patterns, diet, and sociality (Fanson et al., 2012; Van der Weyde et al., 2016; Young et al., 2017). Amongst wild populations, seasonal shifts in diet or gut microbiota may affect FCM levels (Dantzer et al., 2011; Palme, 2019). FCM excretion patterns may also be sex-specific, with males and females in some species varying in the proportions of glucocorticoid metabolites excreted via feces versus urine (Palme et al., 2005). Further, the methodologies used to assess FCMs introduce their own sources of random variation; differences in antibody selection (Fanson et al., 2017) or the protocols employed to collect, store, and extract FCMs are likely to drastically alter FCM preservation and/or sample concentrations (Palme et al., 2013; Palme, 2019; Sheriff et al., 2011). Thus, validations are essential for demonstrating methodological sensitivity to biologically meaningful changes in GC activity, as well as characterizing population and sex-specific ranges, which must be established to appropriately interpret FCM analyses (Palme, 2019; Touma and Palme, 2005).

The application of FCM methodologies for the study of wild mammalian populations has rapidly increased in frequency due to a recent expansion in the number of techniques suitable for remote field environments. Unfortunately, the use of validated FCM methodologies has not matched this same pace of proliferation; to some extent, this may be accounted for by enduring barriers to conducting physiological validations (in which circulating levels of GCs are induced pharmacologically) in many wild populations (Palme, 2019). Such constraints may include acquiring necessary permitting for administering injections to protected populations or working in environments with dense or inaccessible terrain, which may interfere with regular sample collection or repeated captures. Both concerns are troublesome for those studying primates, and likely contribute to a uniquely low percentage of FCM studies using validated methodologies in this order despite an otherwise high number of primate studies analyzing FCMs (Palme, 2019).

Ideally, physiological validations of wild populations are conducted in conjunction with a biological validation (in which increased GC circulation is induced by natural or human-instigated environmental disruptions) to demonstrate that biologically meaningful levels of variation can be detected in response to ecologically-relevant stressors (Sheriff et al., 2011). As such, biological validations are an important exercise for studies of all wild populations, irrespective of physiological validation feasibility. Further, biological validations are particularly valuable for those hoping to examine the adaptive role of GC regulation; natural stressors are likely to be representative of the intact ecological systems wild populations evolved within and may elicit more relevant responses to these ecological problems (Boonstra, 2013b). Traditionally, biological validations have relied on procedures such as capture, restraint, or transportation to induce a stress response, although naturally occurring events may also be leveraged for validation purposes as a less invasive option (Touma and Palme, 2005). Previous studies have performed biological validations via comparisons of glucocorticoid metabolites before and after a variety of natural potential stressors such as: agonistic social interactions or intergroup encounters (Eckardt et al., 2016; Goymann et al., 1999), rank reversal or male eviction (Braga Goncalves et al., 2016; Young et al., 2017), human-wildlife conflict (Nizeyi et al., 2011; Cremer-Schulte et al., 2017), and injury (Ganswindt et al., 2010).

We use three potential stressors to validate the assay. First, we use receiving a bloodied bite wound during agonistic interactions, as such injuries are well-established acute stressors necessitating energetic mobilization (Barton, 1985). Second, we include the injury or sudden death of a group member, since these events alter social networks of individuals within groups, leading to social consequences in primates such as increased vulnerability to future agonisms or intragroup encounters (Campbell et al., 2016; Dittus and Ratnayeke, 1989; Gonçalves and Carvalho, 2019). Third, we assess whether acute stress associated with parturition is reliably detectable in FCMs. Although the physiological stress of birth is widely accepted, parturition events have not previously been used as natural stressors in biological validations.

The black-and-white colobus (*Colobus vellerosus*) is an arboreal monkey endemic to western Africa. Like other colobines, they consume a folivorous diet that primarily relies on foregut fermentation for digestion. Adults have moderate sexual dimorphism with males and females weighing approximately 8.5 kg and 7 kg, respectively (Oates et al., 1994). They reside in bisexual groups consisting of one or several adult males (Wong and Sicotte, 2006). Both males and females can disperse in this species; while males always disperse at sexual maturity, females disperse facultatively in response to infanticide risks associated with male takeover and group size (Sicotte et al., 2017; Teichroeb et al., 2009, 2011). When new males immigrate, sometimes ousting former resident males, immigrant males may attack and/or kill young infants (infanticide) to accelerate the availability of new mating opportunities with females in the group (Teichroeb and Sicotte, 2008). Births are not seasonally constrained in this species; females typically give birth to a new infant approximately every 11–16 months (Vayro et al., 2021). Analyses of FCMs in *C. vellerosus* will give insight into the physiological mechanisms that underpin the high degree of behavioral flexibility in this species.

There are no EIA validations or detailed FCM characterizations available for any African colobine. Our objectives were to: (a) validate a non-invasive method for collecting, analyzing, and quantifying fecal glucocorticoid metabolites in a wild population of black-and-white colobus (using naturally-occurring potential stressors; (b) determine the time course of FCM excretion; (c) examine variation in baseline FCM concentrations as it relates to three factors (time of day, sex, female reproductive state); and (d) describe the FCM change pattern immediately around parturition.

2. Materials and methods

2.1. Study site and animals

We collected behavioral and fecal samples from a wild population of black-and-white colobus monkeys (*Colobus vellerosus*) from August through November 2019 during the rainy and 'pre-dry' seasons in the Boabeng-Fiema Monkey Sanctuary (BFMS), Ghana. BFMS is a 1.9 km^2 forest fragment that has been protected as part of a community-managed conservation program since the 1980 s. The upper canopy height is approximately 30–50 m (Teichroeb et al., 2012). These monkeys travel down to the understory (5 m) or forest floor for a few hours a day, spending the bulk of their time at heights greater than 20 m (Schubert, 2011). As such, observation of our study population typically occurs from a distance of 10–30 m, limiting our team's ability to reliably collect other biological sample types beyond feces, such as hair or blood.

This population has been under observation by our research team since 2000. The identity of all individuals in our study groups are known and can be individually recognized. An estimated 19 groups reside in BFMS (Kankam and Sicotte, 2013); all are protected by national law and local taboo (Saj et al., 2006, 2005). Currently, there are no predators of *C. vellerosus* there. During our study our groups ranged in size from 13 to 17 individuals (see Table 1 for full composition). Our sample consisted of four adult males and ten adult females from three groups (SP, WT, WW). All adult males were included in the sample; however, one male emigrated and was excluded from analyses due to a low sample count. All adult females were included in our samples. We opportunistically selected specific individuals based on current reproductive status and relative ease of consistently collecting fecal samples.

To assess hormone profiles of females before and after birth events we included females who we predicted to be pregnant based on daily observations and birthdates of females' most recent infant. Even for highly trained observers, pregnancies are difficult to visually identify due to the shape of the abdomen and long hair obscuring the stomach area. In our study, all pregnant females sampled were in the late stage of

Table 1
Composition of study groups and number of samples collected from each group by sex.

Group	Full group composition	Days observed	Study sample			
			Adult females		Adult males	
			n individuals	*n* samples	*n* individuals	*n* samples
SP	1 AM, 6AF, 2 SM, 1SF, 1JM, 1JF, 2IM, 2IF	37	2	61	1	29
WT	1 AM, 5AF, 1SM, 2SF, 1JF, 1IM, 2IF	46	4	202	1	65
WW	2 AM, 7AF, 2JM, 1JF, 2IM, 3IF	80	4	229	2*	78

Not all adult group members were included in the study.
* Only one male was reliably present in group throughout the study. As a result of this, only five samples could be collected from the second male. Samples from the second male were excluded from analyses based on the limited sample size. (M = male, F = Female).

pregnancy. We were able to collect samples from one multiparous female, XY, both before and after birth. For one multiparous female, IS, and one primiparous female, L1, we did not intentionally collect samples prior to birth but began collecting daily samples immediately following parturition. All three females gave birth to healthy, singleton infants.

Data collection protocols were approved by the Boabeng-Fiema Monkey Sanctuary's management committee, Ghana Wildlife Division, and the University of Calgary Animal Care Committee (BI 2006–28, 2009–25). Protocols also conformed to all laws and guidelines of appropriate governing bodies.

2.2. Feces collection and storage

We collected samples five days each week from 06:00 to 15:00 and two days each week from 06:00 to 10:00. Where terrain permitted, we collected samples from all observed defecation events from study individuals. When multiple samples were collected from a single individual in one day, samples were collected, stored, and extracted separately, except when defecation events were separated by<20 min. Given their daily activity patterns, samples were most frequently collected during early and mid-morning hours, as they primarily spend afternoons resting with reduced activity.

To increase the number of study subjects, not all groups were followed for the full length of the study. The number of sampling days for each individual ranged between 37 and 80 days. We collected fecal samples opportunistically, with priority given to minimizing the number of days between sample collections for each study subject. Samples were collected fresh (within 10 min of defecation), homogenized by hand, and stored in plastic zip-top bags. Samples were labeled with date and time of collection, subject ID, location, and completeness of sample (whether whole or partial sample was collected). Whenever possible, we collected samples in their entirety, but removed large herbaceous materials and avoided collecting portions of samples with adhered soil particles to minimize contamination. Samples at risk of contamination (in contact with or located too closely to urine or another individual's feces) were not collected. Following collection, samples were kept on ice (no more than eight hours) until returning to our field laboratory where they were stored at −20° C. To minimize sample degradation, samples were extracted within 80 days of collection (mean storage time = 24 days, range = 1–77 days; pre-extraction storage time was not associated with sample concentration r^2 = -0.096).

2.3. Steroid extraction and analyses

We processed and extracted fecal samples at our Ghanaian field site. Samples were dehydrated using a small oven at 60° C for approximately 6–8 h until completely dehydrated. Following this, we removed any remaining undigested herbaceous material from the feces, pulverized samples using a mortar and pestle, and sieved through a fine stainless steel mesh sifter. Clean, pulverized samples were added to a 2.0 mL microtubule and weighed to a mass of 0.048 – 0.052 g. We added 0.5 mL of 80 % methanol to each sample and then vortexed for 30 min (IKA VXR Basic Vibrax, 1450 r.p.m.) and centrifuged for 15 min (2500 g). Supernatants were transferred into 1.5 mL microtubules and placed in a warm oven (40° C) for 1–2 days until fully desiccated. Microtubules were then sealed and returned to −20° C storage until transportation to the University of Toronto Scarborough (UTSC) for enzyme immunoassay (EIA) analyses. Once at UTSC, fecal extracts were redissolved and diluted in assay buffer at a 1:100 dilution and run in duplicate. Extracts were assessed using an 11-oxoetiocholanolone enzyme immunoassay (originally described by Palme and Möstl, 1997); we selected this antibody based on its successful application with other similar-sized primates (Martínez-Mota et al., 2008; Scheun et al., 2020). The average intra-assay coefficients of variation (CV) of high- and low-value pools were 6.5 and 16.4 %, respectively. The average inter-assay CVs of high- and low-value pools were 11.8 and 13.8 % (n = 22).

2.4. Biological validation

Given the movement patterns and protected status of our study population, we were unable to conduct an investigator-induced physiological validation experiment on this population. There are no captive *C. vellerosus* populations at present. Thus, to validate our methods, we compared fecal samples of all group members before and after three potential stressors: two incidences of group member injury acquired during agonistic interactions (for which we cumulatively collected 49 samples from 5 individuals) and one unexpected group member death (for which we collected 11 samples from 3 individuals).

2.4.1. Description of injury event #1

O1, the adult male of WT group was observed with a fresh and bloodied open wound approximately 2.5 X 5 cm in size located halfway down his tail when the group was located at 7:25 on 22 October 2019. While the cause of injury was not observed, the injury was not present when the group was left late in the previous afternoon; loud calls were recorded early the morning of the injury (vocalizations that are regularly associated with intergroup encounters; Delgado, 2006). Bite wounds of this severity are typically the product of agonistic interactions during intergroup encounters or with extra-group males (Cheney, 1987). We did not observe any visiting males near WT group in the week prior to and following the injury. Based on our focal and *ad libitum* observations, we estimated that O1 cumulatively spent at least one hour grooming his wound each day in the weeks following the injury. By 4 November 2019 we recorded that his wound had tripled in size, but by late December it was fully healed.

2.4.2. Description of injury event #2

We observed IS, a lactating female with a young infant in WT group, participating in an intergroup encounter with WW group on the morning of 30 October 2019. After chasing an adult female from WW, we observed C7, the adult male of WW group, grab and bite IS at 9:15, who vocalized in response. Following the intergroup encounter, we observed that IS's tail was bloodied with a visible open wound approximately 2.5 X 2.5 cm on her tail. In the days following the intergroup, we observed IS frequently inspecting her tail. When group members inspected and attempted to groom her tail wound, we observed IS swatting their hands

away. IS's injury was fully healed within approximately-two weeks.

2.4.3. Description of group member death

On 23 October 2019, we located the body of BY3, a juvenile male belonging to WW group, on the forest floor. The juvenile male's death appeared to have been the result of falling from a tree. BY3 had been observed in group every day the week leading up to the death, including the day prior. Because the event occurred overnight, we estimated the event time as 00:00 (midnight) and calculated group members' responses based on this estimated timeline.

2.5. Data analysis

It was not possible to monitor our study population at all times. As a result, our team missed recording some potential stressors, resulting in FCM peaks that we are not able to explain based solely on observations. To account for this, we used an iterative process to classify samples as either "baseline" or "peak" for each individual. Baseline samples were identified for each subject using an iterative process with the R package "hormLong" (Fanson and Fanson, 2015) in which samples with a concentration exceeding two standard deviations of the mean were eliminated, with the process repeated until all remaining sample concentrations fell inside two standard deviations of the mean (Brown et al., 1994). Based on the three "potential stressor" events used in our biological validation, we calculated a time-to-peak interval for each individual – the amount of time between the event onset and maximum FCM concentration in our fecal record within the five days following an event. Other primates of similar body sizes vary in their time-to-peak interval, but one day is considered average (*Alouatta caraya*, 1–2 days: Cantarelli et al., 2017; *Alouatta seniculus*, 1 day: Rimbach et al., 2013; *Macaca fascicularis*, 1 day: Bahr et al., 2000; *Trachypithecus geei*, 2 days: Sarmah et al., 2017). However, some folivorous mammals have an extended excretion lag time to compensate for the longer digestion necessary for a highly fibrous diet, as reflected by longer time-to-peak intervals (*Giraffa camelopardalis*, 2–6 days: Bashaw et al., 2016; *Gorilla beringei beringei*, 1–6 days: Eckardt et al., 2016; *Lasiorhinus latifrons*, 3 days: Hogan et al., 2011). Thus, we selected a window of five days to account for a potentially extended excretion lag time owing to the specialized diet of *C. vellerosus* and to ensure that no peaks would be missed. For the maximum FCM calculation, we included all samples, regardless of whether they were considered within the baseline range by the iterative process (Fanson et al., 2017). Notably, due to individual differences in sampling frequency, we did not calculate a time-to-onset interval (i.e., excretion lag time) for this population, which is the time between event onset and initial detectable rise in FCMs in the fecal record.

To test whether we could detect a rise in FCMs above the baseline in the five days immediately following physical injury events, we constructed a generalized linear mixed effects model (GLMM) using the "glmer.nb()" function in the "lme4" R package (Bates et al., 2015). To accommodate overdispersion in the model, we assessed the model using a negative binomial distribution. We narrowed our analyses to include only samples from individuals in groups experiencing the potential stressor events and excluded peak samples outside the time window of interest (0–5 days post-stressor). Our model included sample type (injury response, group member response to potential stressor, pre-/post-stressor baseline) as a fixed effect and collection period (morning/afternoon) and subject ID as random effects.

To test which intrinsic factors were associated with elevated baseline FCMs, we constructed GLMMs with peak samples excluded from analyses ("glmer" function, "lme4" package; Bates et al., 2015) and tested model residuals for normality with Shapiro-Wilks testing. We assessed whether sex or circadian patterns influenced baseline FCMs by including sex, sample collection period ("early morning" 06:00–10:30, "late morning - afternoon" 10:30–15:00), and an interaction between sex and collection period as fixed effects. Subject ID and sample collection month were included as random effects. To test the influence of female reproductive cycle state on baseline FCMs, we constructed a GLMM with reproductive state ("cycling", "lactating", "late pregnancy") as a fixed effect with subject ID, age in years (scaled), and sample collection period included as random effects.

3. Results

3.1. Baseline FCM concentrations

We collected a total of 657 samples from 13 individuals, with a mean of 51 samples per monkey (range = 10–94). The mean sampling intensity was 0.83 samples/day (range = 0.60–1.20); thus, we collected one sample from each individual every 1.26 days (range = 0.83–1.67). We identified mean overall, baseline, and peak FCM concentrations for all individuals (Supplementary Table) and observed considerable variation both between and within individuals (Fig. 1). Sample concentrations ranged from 120 to 3309 ng/g dried feces, with mean baseline and peak concentrations of 558 ± 9 and 1347 ± 48 ng/g dried feces, respectively.

3.2. Biological validation

The mean time at which maximum FCM concentration was reached post-injury was 2.01 days (range = 0.91–4.06 days; Fig. 2). Maximum FCM concentrations represented an increase of 434 % above baseline in the male and 152 % above baseline in the female following injury events. For individuals reacting to the death or injury or a group member, maximum FCM concentration increased by a mean of 254 % (range = 123–486 %) above baseline. Samples collected in the five days following potential stressors (personal injury, group member injury or death) had higher concentrations than baseline samples collected from the same individuals outside this window regardless of whether the response was to one's own injury or the injury or death of a group member (injury response - baseline: $Z = 2.80$, $p = 0.01$; group response - baseline: $Z = 3.94$, $p = 0.002$; LS means: baseline = 6.36 ± 0.06, injury response = 6.68 ± 0.13, group response = 6.60 ± 0.08; Fig. 3). FCM concentrations did not differ when comparing individuals' responses to personal injury with responses to group member injury or to death ($Z = -0.71$, $p = 0.76$).

3.3. FCM concentrations following parturition events

We detected at least one peak FCM sample in the two days immediately preceding or proceeding parturition events for all three females who gave birth during the study (Fig. 4). Maximum FCM concentrations represented a mean increase of 227 % (range = 211–259 %) above baseline concentrations. For IS and L1, these peaks were the second highest concentration samples collected from these individuals during the entire study period; for XY, this peak represented her sample with the third highest concentration over the course of the study. Likewise, for all three females, we also detected an abrupt decline in FCM concentrations following these peaks, with FCM concentrations maintaining relatively lower FCM concentrations in the week following parturition. Contrary to the similar response pattern of the three females following their births, group members did not have consistent responses to parturition events; one birth elicited peaks from nearly all group members whereas the other two birth events resulted in no change in group members' sample concentrations.

3.4. Baseline FCM concentrations in relation to time of day, sex, female reproductive state

Baseline FCMs showed noticeable circadian variation, with samples collected during collected during morning being higher than those collected in the afternoon ($t = -2.55$, $p = 0.01$; Fig. 5). Baseline FCMs did

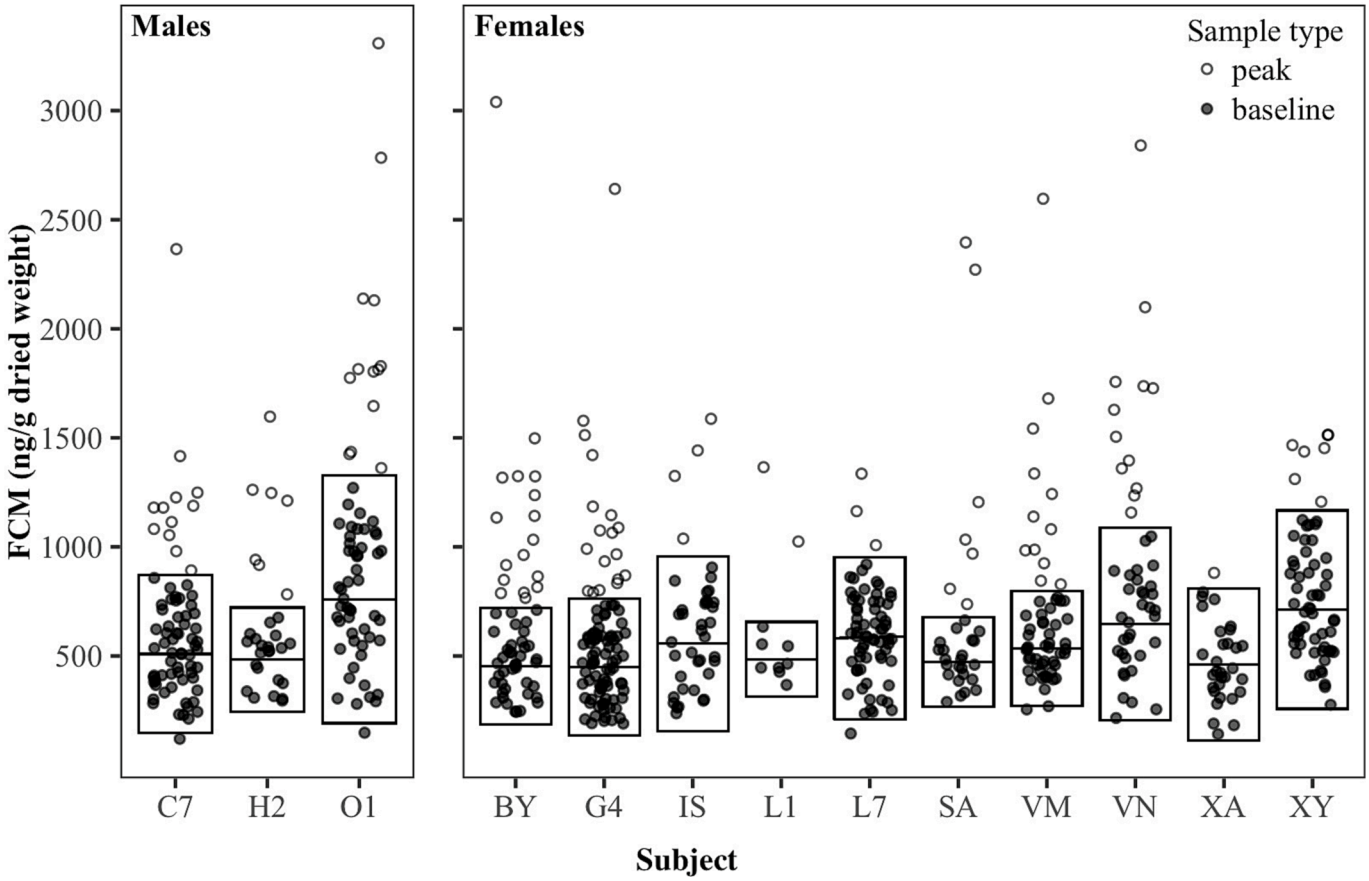

Fig. 1. Fecal glucocorticoid metabolite (FCM) concentrations (ng/g dried weight) by subject for all samples collected (n = 657). Open and closed points represent peak and baseline samples, respectively. Baseline and peak samples were identified using an iterative process. Black boxes represent individual baseline mean ± 2 SD.

not differ significantly between males and females (t = -0.57, p = 0.58; Fig. 5; Supplementary Table), nor was there an interaction between sex and collection period (t = 0.54, p = 0.60). Female reproductive state was associated with differences in baseline FCMs (LS means: cycling = 8.54 ± 0.26, late pregnancy = 9.06 ± 0.27, lactating = 7.90 ± 0.15; Fig. 5); lactating females had lower baseline FCMs than both pregnant (t = -6.06, p < 0.001) and cycling females (t = 2.72, p = 0.05). There was no difference between the baseline FCM concentrations of cycling females and those of pregnant females (t = -2.07, p = 0.12).

4. Discussion

4.1. Biological validation using natural stressors

The 11-oxoetiocholanolone EIA used in our study detected significant increases in FCM concentrations in both males and females following three natural stressors – physical injury, injury of a group member, and death of a group member. Peak FCM concentrations were 2.5 times baseline values, providing strong support that our methods reliably detect significant rises in FCMs in this population. Peak FCM concentrations occurred between one and four days following stressors, with a mean lag time being two days. This lag is somewhat longer than other primates of similar body sizes (*Alouatta seniculus, Ateles hybridus*: Rimbach et al., 2013; *Macaca fascicularis*: Bahr et al., 2000), but parallels individual variation and lag times observed in other folivorous primates, such as gorillas (Eckardt et al., 2016; Nizeyi et al., 2011; Shutt et al., 2012) and golden langurs (Sarmah et al., 2017). In several individuals, peaks were brief, with samples collected hours later having concentrations near baseline levels, highlighting the need for intensive sampling practices to capture responses to stressors in this population. Although our sample size of thirteen individuals is relatively small, the sampling intensity was high for a study of wild primates. Ideally, when possible, larger sample sizes should be sought to further increase the likelihood of capturing a full breadth of responses to natural stressor events in wild populations.

There was a high degree of individual variation in FCM concentrations in response to a group member's injury or death, demonstrating that similar events can incur disproportionate stress for different individuals. All adult females experienced more substantial elevations in FCM concentration following the injury of the group's adult male than they did in response to the injury of another female, though both injuries were acquired during agonistic intergroup interactions. However, in response to both events, we observed that lactating females experienced more dramatic increases to FCM concentrations than did pregnant or cycling group members. Intergroup encounters can represent competition for valuable food or mate resources and serve as opportunities for assessing males' strength and potential resistance to future male takeover events (Cheney, 1987; Steenbeek, 1999; van Schaik et al., 1992); both functions have been documented previously in African colobines (*Colobus guereza:* Fashing, 2001, Harris, 2010; *C. vellerosus:* Sicotte and Macintosh, 2004). Either scenario should in theory impact lactating females more heavily than pregnant or cycling females, as they shoulder the highest energetic demands (Gittleman and Thompson, 1988; Hanwell and Peaker, 1977) and are most at risk of male attacks to themselves or their infants (infanticide) following male takeover events (Teichroeb and Sicotte, 2008). Of these two potential risks (reduced food patch access, male takeover), infanticide attacks would confer the highest and most direct cost to females with young infants (van Schaik, 2000). Physical injury that results in an adult male's diminished strength may signal increased vulnerability to male takeover to females in the group.

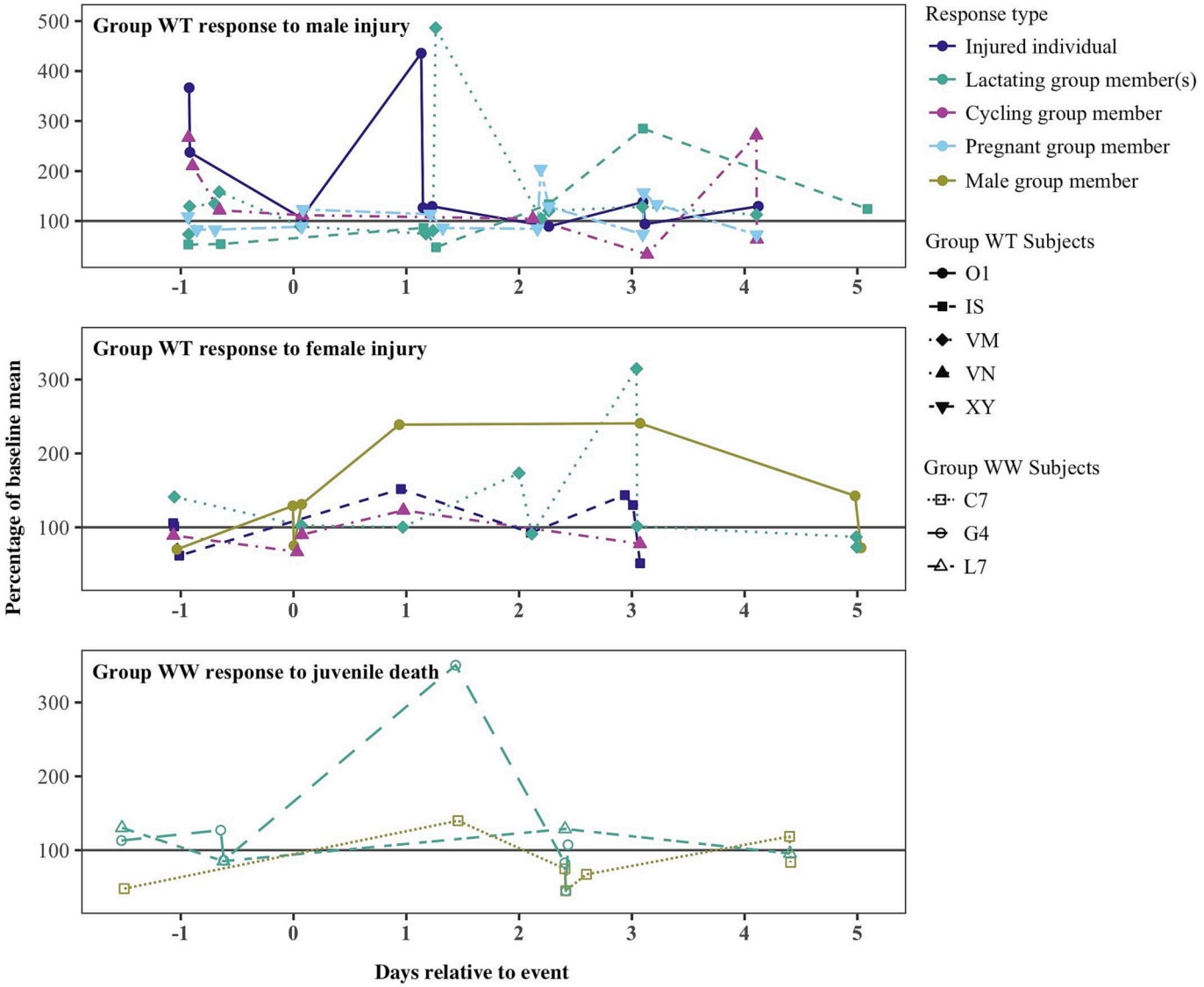

Fig. 2. Fecal glucocorticoid metabolite (FCM) responses of all group individuals to adult male and female physical injury events as well as group member death. FCM concentrations are expressed as a percentage of the individual's mean baseline FCM concentration. Each plotted line denotes the FCM response of one individual in the day before and five days after a stressor event.

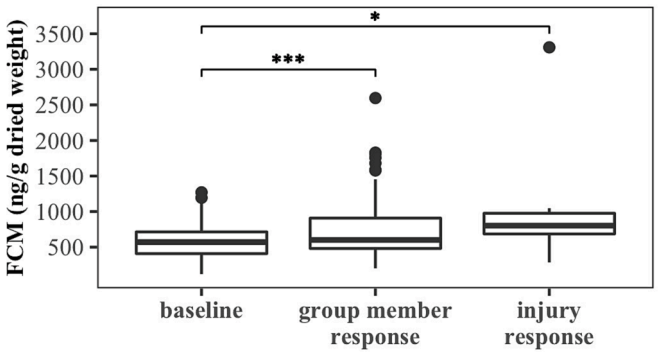

Fig. 3. Comparing fecal glucocorticoid metabolite (FCM) concentrations (ng/g dried weight) of baseline samples collected pre- and post-potential stressors to all samples collected from the same individuals in the five days following a potential stressor event (receiving bite wound, injury of a group member, sudden death of group member). Plotted values display the raw mean, with the lower and upper hinges representing the 25 % and 75 % quartiles. Significance codes: $p \leq 0.05$ *; $p < 0.001$ ***.

Notably, in comparison to both group member injury events, responses of group members to the unexpected death of a juvenile resulted in relatively lower FCM peaks. Unexpected death inevitably disrupts social relationships in-group at least temporarily (Adam-Troian et al., 2021), but the death of a juvenile is less likely to pose direct energetic or reproductive costs to nonmaternal group members. Though the current study presents a small sample of hormonal responses to injuries resulting from intergroup events, our dataset suggests future analyses of females' hormonal responses to social environment or agonistic interactions may add valuable insight regarding energetic costs of group-living in this species.

4.2. FCM responses to parturition events

Given the physically stressful nature of parturition, we examined whether mothers responded to birthing with a consistent FCM pattern in this population. In the two days surrounding parturition events, our subjects showed high FCM concentrations followed by an abrupt decline. Dense longitudinal FCM sampling of females immediately post-parturition in the wild has been performed only in one other primate,

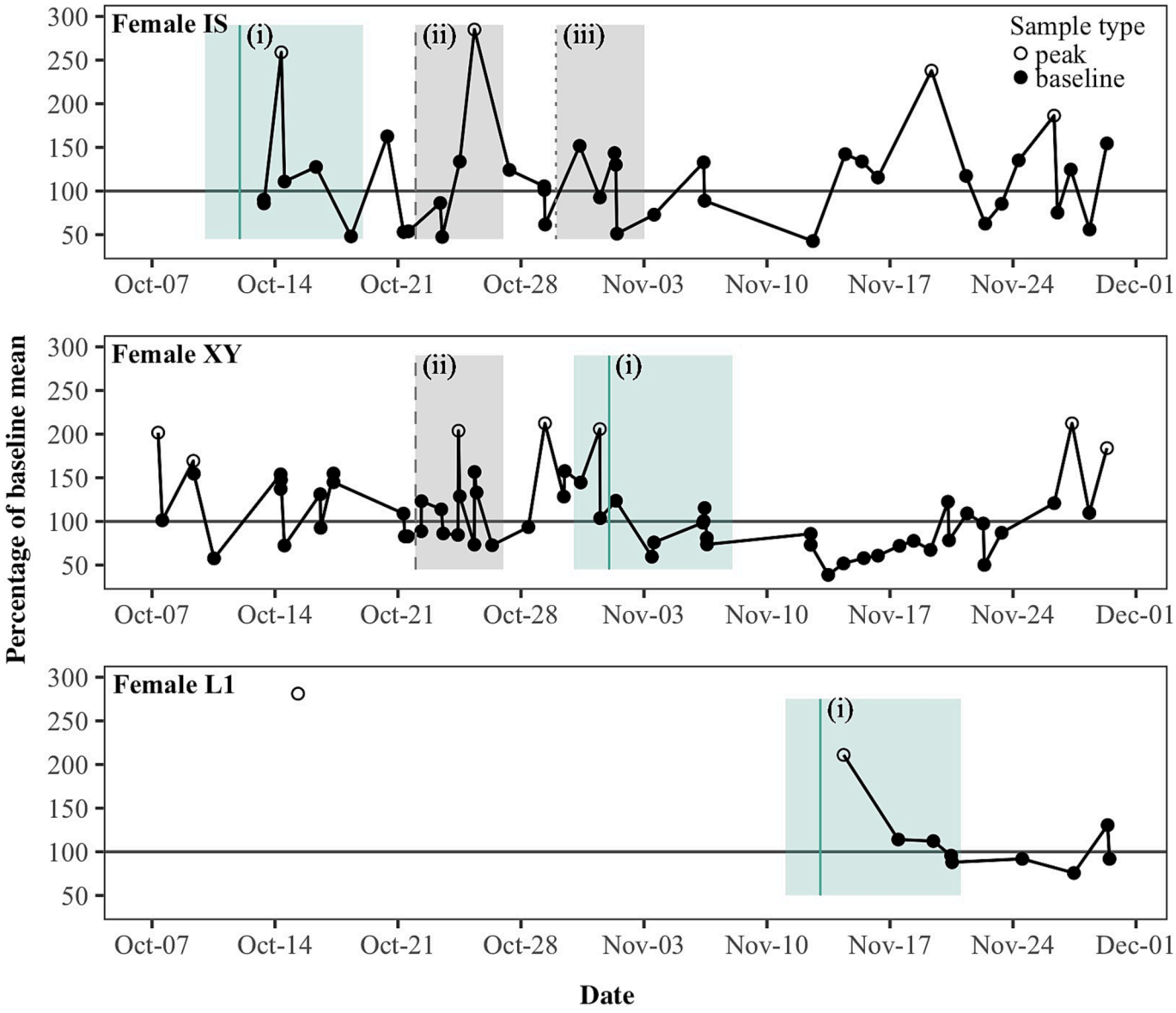

Fig. 4. Complete longitudinal FCM profiles for three females who gave birth during the study: IS, XY, L1. Closed and open points represent samples identified as "baseline" and "peak" using the iterative process. (i) Solid lines indicate date of parturition events, (ii) dashed lines indicate date of adult male's injury in group and (iii) dotted lines indicate date of physical injury. For parturition events, the green shaded boxes highlight the two days prior to and seven days following birth. For other natural stressors (injury, response to group member injury), the grey shaded boxes highlight the five days following an event. We collected only one fecal sample from female L1 opportunistically during October in the period before she was selected as a study subject. After the birth of her first infant in November, we formally added her to our sampling regime and her fecal samples were collected on a routine basis until the end of the study.

Pan troglodytes (Murray et al., 2013). The post-parturition FCM pattern described in our study is consistent with the pattern found by Murray et al. (2013) and, more broadly, with FCM and serum GC patterns observed in other mammalian studies, including those using less dense sampling procedures (captive *Bos taurus*: Smith et al., 1973; captive *Lama glama*: Leon et al., 1990; captive *Orcina orca*: Robeck et al., 2017; captive *Oryctolagus cuniculus*: Mulay et al., 1973; captive *Pan paniscus*: Behringer et al., 2009; wild *Theropithecus gelada*: Carrera et al., 2020) in which GCs spiked just before or after parturition before returning to baseline or lower levels. In our study, the timing of peak FCM concentrations varied amongst females, with one female's peak sample collected two days prior to birth of her infant, while the other two females' peak samples were collected within two days post-birth. Such differences likely reflect individual differences in intestinal transit time or may be the result of a lack of available samples from two of the females preceding birth events, for whom higher pre-birth peaks could have occurred but were not recorded.

Previous studies on human and nonhuman primates suggests that maternal GCs are not directly involved in the induction of parturition, though they may indirectly facilitate gene expression changes in the placenta in preparation for labor (Liggins, 1979; Zannas and Chrousos, 2015). The surge in FCMs observed in our study around the time of parturition likely represents a physiological response to inherent physical stresses associated with late pregnancy and labor in the form of inhibited mobility and onset of contractions, rather than a triggering of labor by GCs. The high FCM values observed around the time of parturition mirror or exceed those recorded in the same females in response to the natural stressors used for biological validation in our study, suggesting birth events could reliably operate as natural stressor events for use in future biological validations of FCM methods in other wild populations. Many long-term studies already closely monitor demographic events non-invasively. Parturition events, which occur

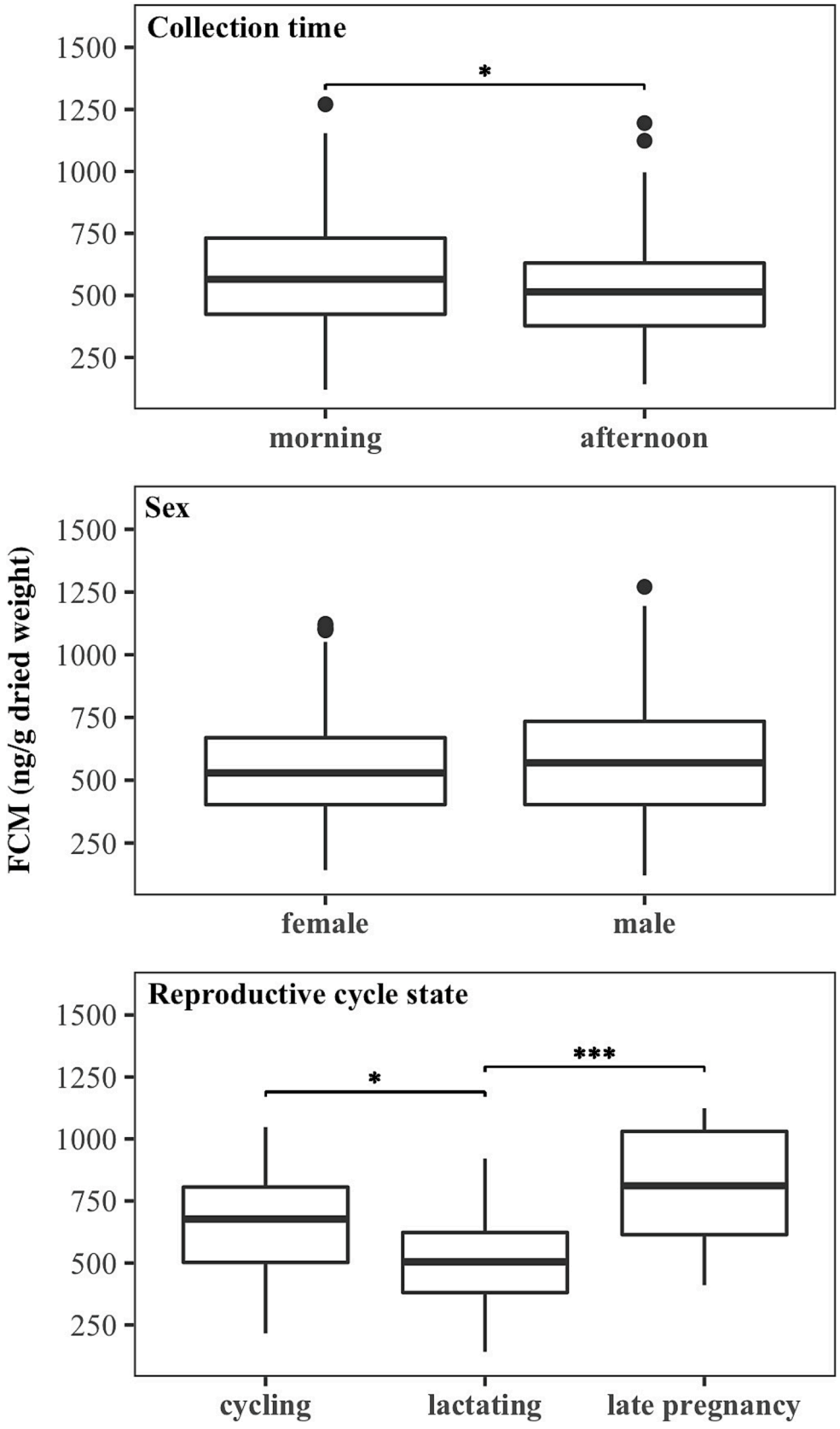

Fig. 5. Effects of collection time, sex, and female reproductive cycle state on baseline FCM concentrations (ng/g dried weight). Plotted values display the raw mean, with the lower and upper hinges representing the 25 % and 75 % quartiles. Significance codes: $p \leq 0.05$ *; $p < 0.001$ ***.

regularly and with some predictability, can be captured in the fecal record more readily than many other sporadic natural stressors. Given the obstacles associated with conducting validations in wild populations, consideration of parturition may be helpful in closing the current research gap between studies analyzing FCMs with and without validated methodologies, an especially serious problem in primates.

4.3. Other sources of variation in baseline FCM concentrations in C. Vellerosus

As part of our characterization of FCM profiles in this population, we examined three potential sources of variation – collection time, sex, and female reproductive state – which may inform future analyses of FCMs in this population. We detected a circadian effect in baseline concentrations, with samples collected in the morning having higher concentrations than those collected in the afternoon. Peak circulating GCs typically coincide with the onset of the circadian active period (Spencer and Deak, 2017), as such the circadian effect in *C. vellerosus* reflects a lag time between blood GC and excreted FCM levels. Typically, diurnal FCM variation is associated with relatively short gut passage times or a greater number of daily defecation events, since these characteristics limit the excretion of glucocorticoid metabolites in each bowel movement to only those circulating during a shorter temporal range (Touma and Palme, 2005). Like *C. vellerosus*, primates exhibiting diurnal variation generally defecate multiple times daily, (Mendonça-Furtado et al., 2017; Murray et al., 2013; Rimbach et al., 2013). On the other hand, folivory is often associated with the absence of a circadian effect, since highly fibrous diets necessitate longer intestinal transit times for sufficient nutrient absorption. Interestingly, many other folivorous primates with similar or shorter lag times than *C. vellerosus* do not have a detectable circadian effect in the FCM record (*Alouatta pigra*: Martínez-Mota et al., 2008, van Belle et al., 2009; *Alouatta seniculus*: Rimbach et al., 2013; *Theropithecus gelada*: Beehner and McCann, 2008). This pattern suggests that other physiological adaptations beyond intestinal transit time may contribute to the absence of diurnal variation in folivores. For instance, disparities in body size, digestive pathways, or levels of reliance on gut microbiota communities for digestion could all alter metabolite pooling or the extent to which metabolites are degraded prior to elimination (Goymann, 2012). It is possible that the use of foregut fermentation in colobines explains the presence of diurnal variation observed in our population compared to other folivorous primates. We recommend that future studies of African colobines consider including time of day in their analyses to offset a potential circadian effect.

We found no difference between male and female baseline FCM concentrations. There are no consistent FCM patterns across mammals in relation to sex (Palme, 2019). In addition to our population, the absence of a sex-mediated difference in FCMs has been reported in a number of other primates (*Ateles hybridus*, *Ateles seniculus*: Rimbach et al., 2013; *Alouatta pigra*: Behie et al., 2010; *Macaca arctoides*: Pineda-Galindo et al., 2017) and mammals (Braga Goncalves et al., 2016; Harris et al., 2012; Spaan et al., 2017).

Female reproductive state was associated with differences in basal FCM concentrations with lactating females having significantly lower concentrations than pregnant or cycling females. Our findings are consistent with a broad mammalian pattern in which circulating GCs increase during pregnancy, reaching their highest levels during late gestation (Edwards and Boonstra, 2018). Further, similar results have been reported in studies of both seasonally and non-seasonally breeding primates (Cavigelli, 1999; Charpentier et al., 2018; Gesquiere et al., 2008; Rudolph et al., 2020).

Maternal investment in mammals is high (Bronson, 1985), and thus, reproductive state can vastly alter energetic priorities of and demands on females. For instance, the relatively high levels of FCMs observed in our study during late pregnancy is likely attributable to proximate causes of GC increases, such as estrogen-mediated upregulation of GC activity or internal recalibrations for energy mobilization and storage necessary for pregnancy (Brann and Mahesh, 1991). GCs play a preparative role for life after birth for both fetus and mother. A late gestation peak in maternal GCs has been credited with facilitating fetal organ maturation and the development of lung surfactant prior to birth across mammals (Ballard and Ballard, 1995; Fowden et al., 1998; Liggins, 1994). GCs are involved in structural development of mammary tissue and assist in milk production (Stead et al., 2021). Following this, elevated GCs in *C. vellerosus* appear to play a functional role in preparing mother and fetus for life after parturition.

It is widely accepted that, compared to other reproductive states, lactation is the most energetically demanding (Hanwell and Peaker, 1977). Traditionally, it has been suggested that GCs should rise during lactation to facilitate sufficient glucose mobilization during this energetically intense period (Kenagy and Place, 2000), but studies of wild mammalian populations have demonstrated that this is not always the case (Edwards and Boonstra, 2016; Romero, 2002). Low basal FCMs in our study suggests that lactating females primarily rely on other forms of phenotypic flexibility to manage the energetic requirements of lactation besides increasing GC circulation. For instance, in both human and nonhuman primates, mild to moderate reductions in food availability during lactation are often compensated for with adjustments to the timing of future births (Emery Thompson, 2013). Flexible reproductive timing has been observed in *C. vellerosus*; females may shorten interbirth intervals by overlapping investment in consecutive offspring ("direct stacked investment") in response to increased stability in male residence patterns (Vayro et al., 2021). Beyond demonstrating an existing adaptive responsiveness to social environment in this species, this pattern suggests that during socially stable periods, the day-to-day energetic demands of nursing an older infant are flexible enough to facilitate investment in an additional offspring.

5. Conclusion

We found significant increases in FCM concentrations in a wild population of *C. vellerosus* following three naturally occurring stressors (injury, group member injury, sudden death of a group member) using field-friendly extraction protocols and an 11-oxoetiocholanolone EIA. We observed marked increases in FCM concentrations in three adult females in the two days surrounding parturition in this population, suggesting that birth events may be a viable natural stressor in future biological validations of other wild primate populations. Further investigations by other researchers focusing on parturition events will help to contextualize the generality of our observations for primates. Our study identified the excretion lag time of FCMs (2 days) and contributed comparative data not yet available for African colobines, such as FCM ranges by sex and reproductive state. Together, our validation demonstrates that our methods are appropriate for evaluating the impact of acute stressors on fecal glucocorticoid metabolites in this population. Building on this, future studies of this and other wild primates should investigate with larger sample sizes to what extent individuals cope with variation in social structure, social stability, and reproductive state by altering glucocorticoid circulation.

Funding

This study was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grants (#2016–05540; #2017–03768) and a Canadian Association for Biological Anthropology (CABA) Shelley R. Saunders Thesis Research Grant.

CRediT authorship contribution statement

Allyson G. King: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition. **Phoebe D. Edwards:**

Methodology, Investigation, Writing – review & editing, Project administration. **Susanne Cote:** Writing – review & editing, Supervision. **Rupert Palme:** Methodology, Resources, Writing – review & editing. **Rudy Boonstra:** Methodology, Resources, Writing – review & editing, Supervision, Funding acquisition. **Pascale Sicotte:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

We thank the Boabeng-Fiema Monkey Sanctuary management committee and Ghana Wildlife Division for granting permission to conduct this research. We acknowledge Robert Koranteng, Charles Kodom, Bismark Owusu Anfwi, and Lori Sande for their contributions to field data collection and Sampson Amponsah for electrician support at our field site laboratory. We also thank Diana Christie for general field site support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2023.114212.

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