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Measuring Stress Responses in Female Geoffroy's Spider Monkeys: Validation and the Influence of Reproductive State

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Abstract

Fecal glucocorticoid metabolites are increasingly used to investigate physiological stress. However, it is crucial for researchers to simultaneously investigate the effects of reproductive state because estradiol and placental hormones can affect circulating glucocorticoid concentrations. Reports on the relationships between glucocorticoids and reproductive state are inconsistent among females. Unlike several primate species that have heightened glucocorticoid activity during lactation, humans experience reduced glucocorticoid activity during lactation. Rather than a taxonomic difference, we hypothesize that this is a result of different environmental stressors, particularly the threat of infanticide. Here, we expand the number of wild primate species tested by validating a glucocorticoid assay for female Geoffroy's spider monkeys. We investigate the effects of reproductive state on their glucocorticoid concentrations. Utilizing a routine veterinary exam on a captive population, we determined that fecal glucocorticoid metabolites increase in response to a stressor (anesthesia), and this rise is detected approximately 24 hours later. Additionally, we found that extracted hormone patterns in a wild population reflected basic reproductive biology – estradiol concentrations were higher in cycling than lactating females, and in lactating females with older offspring who were presumably resuming their cycle. However, we found that estradiol and glucocorticoid concentrations were significantly correlated in lactating but not cycling females. Similarly, we found that reproductive state and estradiol concentration, but not stage of lactation, predicted glucocorticoid concentrations. Unlike patterns in several other primate species that face a relatively strong threat of infanticide, lactating spider monkeys experience reduced glucocorticoid activity, possibly due to attenuating effects of oxytocin and lower male-initiated aggression than directed at cycling females. More broadly, we conclude that future studies using fecal glucocorticoid metabolites to index stress should consider that reproductive state might confound glucocorticoid measurements.

Keywords

Ateles; estradiol; female reproductive state; glucocorticoids

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Introduction

Glucocorticoids (GCs) mobilize glucose in the bloodstream so that animals can respond quickly to severe threats, but chronic stress is detrimental to health and fertility [Sapolsky 1992]. Although fecal GC metabolites can indicate ecological and social stress, measurements in females may be confounded by reproductive state [von der Ohe and Servheen, 2002; Touma and Palme, 2005]. Life history theories incorporating allostasis predict GC concentrations will rise in response to pregnancy and lactation because these are predictable challenges crucial to reproductive success [Landys et al., 2006]. However, physiological mechanisms also influence GC concentrations within each reproductive state [Sapolsky, 1992; von der Ohe and Servheen, 2002]. Thus, it is necessary to disentangle the effects of physiology and life history before using non-invasive hormonal monitoring to investigate how external stressors affect GCs on an individual or group level.

Pregnancy is crucial to reproductive success but poses survival risks [Hoffman et al., 2008]. In anthropoid primates, the fetal adrenal cortex produces GCs and dehydroepiandrosterone sulfate (DHEAS), while the placenta releases corticotropin-releasing hormone (CRH) [Mesiano and Jaffe, 1997; Torres-Farfan et al., 2004; Power et al., 2010]. These hormones stimulate production of estrogens and GCs [Sapolsky, 1992; Rainey et al., 2004; Power et al., 2010]. Estradiol also promotes increases in circulating GC concentrations [Sapolsky, 1992; von der Ohe and Servheen, 2002; Touma and Palme, 2005]. As expected, the majority of studies on nonhuman primates report higher GC levels in latter stages of pregnancy [lemurs, *Lemur catta*: Cavigelli, 1999; callitrichids, *Saguinus oedipus*: Ziegler et al., 1995, *Callithrix kuhli*: Smith et al., 1997, *Leontopithecus rosalia*: Bales et al., 2005; capuchins, *Cebus* spp.: Ehmke, 2010; Carnegie et al., 2011; baboons, *Papio* spp.: Weingrill et al., 2004; Gesquiere et al., 2008; humans, *Homo sapiens*: Lockwood et al., 1996].

Cycling females may also experience elevated GCs due to estradiol. Resumption of cycling varies based on infant suckling frequency and duration, as well as maternal energetic condition [Ordög et al., 1998; Thompson et al., 2012]. Estradiol should increase as females near resumption of cycling [Altemus, 1995; Strier and Ziegler, 1997, 2005]. However, because estrogens peak in the periovulatory phase [Campbell et al., 2001; Hernández-López et al., 2010] we should expect fluctuations in both estradiol and GC concentrations.

Lactation poses challenges in terms of energetic costs, infant care and infant protection [Landys et al., 2006; Hoffman et al., 2008, 2010; Nguyen et al., 2008]. However, estradiol concentrations should remain low due to lactational amenorrhea [Lee, 1996; Strier et al., 2003], and studies in humans indicate reduced GC responsiveness to some stressors while breastfeeding [Wiesenfeld et al., 1985; Altemus, 1995; Uvnäs-Moberg, 1998; Mezzacappa et al., 2001]. Similarly, tufted capuchins (*Cebus apella*) experience the lowest stress levels while lactating [Ehmke, 2010]. This is supported by a large body of data in rodents indicating reduced GC responsiveness during lactation [reviewed in Tu et al., 2005]. However, other primate species show different patterns. For example, lactating rhesus macaques (*Macaca mulatta*) exhibit greater GC responsiveness [Maestriperi et al., 2008]. Similarly, in species that experience threats to infant safety, lactating females exhibit elevated GCs [*Papio hamadryas ursinus*: baboons: Weingrill et al., 2004; *M. mulatta*;

Hoffman et al., 2010] and the threat of male takeover can increase GC concentrations [baboons, *P. hamadryas ursinus*; Beehner et al., 2005; Engh et al., 2006a; howler monkeys, *A. pigra*; Cristóbal-Azkarate et al., 2007; capuchins, *C. capucinus*; Carnegie et al., 2011]. Individual differences such as parity [Nguyen et al., 2008] and dominance [Hoffman et al., 2010] also affect maternal stress. Thus, stress during lactation may be contextually dependent on risk to infant safety and individual differences in maternal status.

We predict that the different patterns seen in female mammal GC concentrations during lactation are related to differences in stress levels based on infant risk. To add to the body of empirical work on this topic, we chose to study variation in GCs based on estradiol concentrations, reproductive state, and stage of lactation among both captive and wild Geoffroy's spider monkeys (*Ateles geoffroyi*). Although predation [Matsuda and Izawa, 2008] and infanticide [Gibson et al., 2008; Alvarez et al., 2014] are potential risks for species in the *Ateles* genus, the frequency of these incidents are low. However due to potential risks, we may expect mothers of young infants to maintain higher levels of vigilance. Infants are not fully weaned until 24-36 months of age [van Roosmalen and Klein, 1988] and weaning age can vary based on maternal rank and offspring sex.

Campbell and colleagues [2000, 2001] demonstrated successful extraction of reproductive hormones from spider monkey feces, and determined that fecal metabolites lag behind urinary metabolites by 1-2 days. Subsequent studies have examined fecal or urinary GC concentrations. For example, in captivity, urinary GCs in both males and females rise in response to increased visitor presence [Davis et al., 2005]. Additionally, spider monkeys at zoos with diets high in carbohydrates and sugars, and low in protein and fibers, had the highest fecal GCs [Ange-van Heugten et al., 2009]. Rangel-Negrín and colleagues [2009] performed a limited biological validation, using one individual from each sex, and confirmed that GCs rise in response to a stress challenge. They then determined that spider monkeys in fragmented forest and captivity exhibited higher fecal GCs than those living in large tracts of conserved forest [Rangel-Negrín et al., 2009]. Additionally, in a conserved forest, GCs were highest in the dry season [Rangel-Negrín et al., 2009].

The goal of the current study is two-fold: 1) conduct the first validation of a fecal GC metabolite enzyme-immunoassay (EIA) for female Geoffroy's spider monkeys using anesthesia, which is a more reliable method than the stress challenges done previously [Wasser et al., 2000; Touma and Palme, 2005]; 2) determine the relationship between GCs and estradiol metabolites across reproductive states and lactation stages in this species. The validation was performed using captive cycling females, while the effect of reproductive state was examined in wild female spider monkeys. We predicted that fecal GC metabolite concentrations would increase following anesthesia, and that fecal GC metabolite concentrations would correlate with estradiol metabolite concentrations. We further predicted that pregnant females would have the highest GC concentrations, while cycling females were expected to have the lowest GC concentrations. Finally, we predicted that lactating females with young infants would have higher GC concentrations compared to females with older offspring.

Methods

All research was conducted in accordance with The Ohio State University's Institutional Animal Care and Use Committee protocol #2008A0098. Field research was conducted in adherence to the regulations of Costa Rica's Ministerio de Ambient, Energía, y Mares. This research adheres to the American Society of Primatologists' principles for the Ethical Treatment of Non-Human primates.

Captive animals

Subjects in the captive study were five female Geoffroy's spider monkeys at the Brookfield Zoo, in Brookfield, Illinois. These females reside in a group that also includes two vasectomized male Geoffroy's spider monkeys and one female black spider monkey (*A. paniscus*). The group is housed in a public enclosure measuring 30.48×30.48 m (approximately 863,097.48 cubic meters of space) during the day, and four connected enclosures (each $3.66 \times 4.27 \times 2.44$ m) at night. In their public enclosure, the spider monkeys share the space with capuchin monkeys (*C. apella*), a great anteater (*Myrmecophaga tridactyla*) and a tapir (*Tapirus bairdii*). The subjects have periodic interactions with the capuchins and have visual contact with cotton-top tamarins (*Saguinus oedipus*), Goeldi's monkeys (*Callimico goeldii*), golden lion tamarins (*Leontopithecus rosalia*) and a two-toed sloth (*Choloepus hoffmanni*).

The spider monkeys are fed canned Zupreem® Primate Diet, Mazuri® New World monkey chow, fruit, vegetables, and sweet potatoes every day. They also receive hard-boiled eggs three times a week. They receive the canned Zupreem® (435 g), leafy greens (250 g), and other vegetables (450 g) in the morning prior to going on exhibit around 10 am, and additional leafy greens in their public enclosure. Upon their return to their night enclosure around 5 pm, they are fed monkey chow (500 g), fruit (765 g), sweet potatoes (123 g), leafy greens (1500 g), and hard-boiled eggs.

Anesthesia stimulates adrenocortical activity, and thus is a viable alternative to using an ACTH challenge for physiological validation [Wasser et al., 2000; Touma and Palme, 2005]. A routine veterinary exam was performed on all subjects on June 11, 2008, and each examination took between 45-75 minutes. Animals were anesthetized using a combination of metomidine and ketamine by zoo veterinarians. Under anesthesia, each animal underwent a full physical exam, tuberculosis test, blood draws, and radiographs. Additionally, some animals underwent rectal cultures, pinworm checks, and tongue scrapes.

Twenty-two fecal samples were collected from four subjects from June 7-14, 2008 (before and after the veterinary exam), although we were not able to sample all females equivalently after the exam. A fifth female (CH) was excluded from analysis because we were unable to sample her at all after the exam. All females were presumed to be cycling because they are housed with vasectomized males. Identified samples were collected by keepers from the night enclosure floor, frozen at -20°C , transported to the Ohio State University, and extracted following the same protocol used in the field.

Wild animals

Data were collected on wild Geoffroy's spider monkeys at El Zota Biological Field Station in Costa Rica. El Zota is a 1000 ha private reserve situated in the northeastern region of the country at 10°57.6 N, 83°75.9' W approximately 20 km from Tortuguero National Park and Barro del Colorado Reserve [Pruetz and LaDuke, 2001; Lindshield, 2006]. This area receives approximately 4000-5000 mm of rainfall annually and exhibits very mild seasonality [Sanford et al., 1994; Wolfe and Ralph, 2009]. The reserve consists of anthropogenically modified habitat, and includes primary forest, secondary forest, fragmented banana plantation, former pastureland, and harvestable monoculture [Lindshield, 2006].

Two spider monkey communities are present at El Zota: the well-habituated Pilón community, which ranges throughout the southern secondary forest and on neighboring properties to northwest and southeast of the reserve, and another community that ranges in the northern primary forest [Pruetz and LaDuke, 2001; Lindshield, 2006; Rodrigues, 2007]. Approximately 39-41 individuals were in the Pilón community, including 17 adult and subadult females, 15 infant and juvenile offspring, and 7-9 adult and subadult males. All adult and subadult females were study subjects. Individuals were identified based on pelage, facial, and genital characteristics.

During fieldwork, 134 fecal samples were collected from 17 individually recognized females from July 2010-August 2011 (Table I). Reproductive condition of subjects was determined retroactively as “cycling”, “lactating,” or “pregnant.” Females were considered pregnant by noting estimated date of birth and calculating a typical gestation length of 7.5 months [Eisenberg, 1973; Chapman and Chapman, 1990] to determine approximate time of conception. Two females, JI and ST, gave birth during the study period; however, we were only able to sample JI during pregnancy. Females were considered lactating if they had offspring <24 months of age and were subdivided into three stages of lactation depending on the age of their offspring (early: 0-6 months; middle: 6-12 months; late: 12-24 months). Offspring ages were estimated based on size, nursing, predominant travel method, and bridging following van Roosmalen and Klein (1988). Females without offspring and females with offspring greater than 24 months were considered cycling and were further divided into adult and subadult categories. Although this categorization was imprecise, 24 months was used as the cutoff between the “lactating” and “cycling” categories because 24 months is approximately the earliest age at which offspring could be fully weaned [van Roosmalen and Klein, 1988].

Samples were collected opportunistically, with a target goal of obtaining one sample per female every two weeks. However, due to the difficult nature of finding individuals and collecting fecal samples, this goal was only met for three females (AS, LE, and JL) and sample collection was less frequent for other females. Samples were collected by MAR or a field assistant using either a Ziploc bag or plastic spatula and test tube. They were then stored in a thermos with a cold pack and returned to the field station within an hour for immediate processing.

Fecal sample processing

Samples were processed and stored using Solid Phase Extraction (SPE) following the protocol outlined by Ziegler and Wittwer [2005], with modifications suggested by Erin Ehmke (pers. comm.). Fecal material (0.1 g) was mixed with 2.5 ml distilled water and 2.5 ml ethanol. The mixture was shaken by hand for five minutes and spun for 10 minutes. The mixture was allowed to settle for at least 30 minutes. Approximately 3 ml of the supernatant was removed and transferred to a clean test tube. Then, 2 ml of the filtered supernatant was removed and passed through a Prevail C18 Maxi-Clean SPE Cartridge (Alltech ©, Lexington, KY). To prevent continued extraction, cartridges were washed with 2ml of distilled water following suggestions by Ziegler and Wittwer [2005]. To ensure preservation in the field, cartridges were stored in Ziploc bags with silica gel, and then placed in a cooler with additional silica gel. Samples remained in storage within the SPE cartridges for 72-460 days.

Hormone analysis

Samples were analyzed at the Wisconsin National Primate Center Core Assay facility by DW. GCs were assayed using Enzyme-Immunoassay (EIA) technique, whereas estradiol was assayed using Radio-Immunoassay (RIA) technique. SPE cartridges were washed with 1 ml of a 95:5 water: methanol solution. Next, 2 ml of methanol was added to the SPE cartridge and collected. Methanol was dried and re-suspended in ethanol and stored in the refrigerator until assayed.

Cortisol is the primary GC in most primates [Heistermann et al., 2006]. Therefore, we used an in-house cortisol EIA using a polyclonal antibody (R4866, raised in rabbits) and conjugate purchased from Coralie Munro (UC-Davis) and standards H4001 from Sigma-Aldrich (St. Louis, MO). This assay cross-reacts with other steroids, particularly cortisone and other GCs. First, 200 µl of sample were evaporated, re-suspended in 300 µl of F:HRP at a concentration of 1:150,000, and plated. Plates were then incubated for 2 hours, unbound material was washed off the plate, and substrate was added. Stop solution was added after sufficient color developed. Assays were calculated using log-logit regression. The cortisol assay was validated for accuracy where the percentage of observed values from expected were 93.49% (± 1.09 , N=6). To determine if the assay was measuring the GCs in the samples as in the standards, we tested serial dilutions of a pooled sample from the spider monkeys for parallelism to our standards. The slope of the line was determined to not be different from the standard curve serial dilutions ($t=1.14$, $df=22$, $N=7$, $P>0.05$). Using internal controls in order to determine precision, we found inter- and intra-assay coefficients of variation of two pools were acceptable (15.8/9 and 13.1/6).

Estradiol was analyzed using an in-house RIA technique. The polyclonal antibody (raised in rabbits) was purchased from Holly Hill Biologicals Inc. (Hillsboro, OR), the tritiated estradiol from Perkin Elmer (Waltham, MA), and standards (E8875) from Sigma-Aldrich. First, 50 µl of the sample was evaporated, antibody and trace added, and refrigerated overnight. After overnight incubation, 1ml of charcoal solution was added. After another 15 minutes incubation, the solution was centrifuged to remove charcoal. Supernatant was poured into a scintillation vial and the radioactivity was counted in a beta counter. Assays

were calculated using log-logit regression. The estradiol assay was validated for accuracy (98.84 ± 1.53 , $n=8$) and parallelism ($t=-1.83$, $df=22$ $p>0.05$, $n=5$) using internal controls as described above. The inter-assay and intra-assay coefficients of variation were the two pools were acceptable (8.4/2 and 7.4/1.8).

Data analysis

Because sample sizes were small in the captive validation study, nonparametric tests (Spearman's rank, Wilcoxon signed rank and Mann Whitney tests) were used. For the wild study, sample sizes were large enough to use a Pearson's correlation to test for the relationship between the two hormones and generalized linear mixed models (GLMM) to test the effect of multiple predictors simultaneously (including several potentially confounding effects) on both GCs and estradiol. This GLMM is particularly suited to this study because it accounts for repeated measurements from the same individuals [reviewed in: Bolker et al., 2009] and therefore minimizes risk of pseudoreplication because individual identity can be included as a random effect. Because GC and estradiol data were not normally distributed, we used a gamma probability distribution and a log link function.

The GLMM examining GC concentrations as the dependent variable included individual as a random factor, and estradiol concentration, stage of lactation (early, middle or late), reproductive state (cycling or lactating), parity (nulliparous or parous), age (subadult or adult), time collected (AM or PM), and days in storage as fixed factors. The GLMM examining estradiol concentration as the dependent variable included individual as a random factor, and GC concentration, reproductive state, stage of lactation, parity, age, time collected and days in storage as fixed factors. Because only one pregnant female was sampled, we eliminated samples collected during gestation from the analysis. However, results remain essentially the same if we included her in the models.

All analyses were run in SPSS (IBM corp. Armonk, NY). Alpha was set at $\alpha=0.05$. Because the captive validation was based on a directional hypothesis, a one-tailed P-value is reported for this test. Two-tailed P-values are reported for all other tests.

Results

Captive Validation Study

Females had significantly higher GC concentrations post-exam (Wilcoxon signed ranks test: $W=1.826$, $N=4$, $P=0.034$). Because fecal samples were not recovered from two individuals (EV and ME) on days one and two after the veterinary exam, their peak GC values were likely missed. However, complete cortisol profiles of the two most thoroughly sampled females suggest that peak GC concentrations are reached one day post-exam, with GC concentrations returning to approximately baseline by day two (e.g., Figure 1). The estradiol and GC concentrations of these cycling females were not correlated (Spearman correlation: $r_s = 0.174$, $N=22$, $P=0.437$).

Wild Study

GC and estradiol concentrations were positively correlated in samples from lactating females (Pearson correlation: $r=0.367$, $N=67$ samples from nine females, $P<0.01$; Figure 2). However, the two hormones were not correlated in samples from cycling females ($r=0.097$, $N=64$ samples from 11 individuals, $P=0.445$).

Estradiol (GLMM: $F_{1,121} = 5.111$, $P = 0.026$) had an effect on GC concentrations and the effect of reproductive state on GCs approached significance ($F_{1,121} = 3.453$, $P = 0.066$), with cycling females having higher GC levels (Figure 3). There was no effect of lactation stage ($F_{3,121} = 1.042$, $P = 0.377$; Figure 4) or of three potentially confounding variables (age: $F_{1,121}=0.108$, $P=0.295$; parity: $F_{1,121} = 1.518$, $P = 0.220$; time collected: $F_{1,121} = 1.909$, $P = 0.170$) on GC concentrations. However, there was a nonsignificant trend for days in storage ($F_{1,121} = 2.967$, $P = 0.088$) to affect GC concentrations (see below).

GCs (GLMM: $F_{1,121} = 9.010$, $P = 0.003$), reproductive state ($F_{1,121} = 20,911.876$, $P < 0.001$) and lactation stage ($F_{3,121} = 8837.816$, $P < 0.001$) all had effects on estradiol concentrations. Overall, cycling females had higher estradiol levels than lactating females (Figure 3) and females in late lactation had higher estradiol levels than early or middle lactation stages (Figure 4). Although there was no significant effect of three potentially confounding variables (age: $F_{1,121} = 0.154$, $P = 0.696$; parity: $F_{1,121} = 2.047$, $P = 0.155$; time collected: $F_{1,121} = 0.040$, $P = 0.842$), there was a nonsignificant trend for days in storage ($F_{1,121} = 3.220$, $P = 0.075$) to affect estradiol.

Because days in storage emerged as a trend in both models, we examined these relationships. GC concentrations had a very weak negative relationship with days in storage (Pearson's correlation: $r=-0.129$, $N=131$), whereas estradiol concentrations had a very weak positive relationship with days in storage ($r=0.103$, $N=131$). For both hormones, these weak relationships appear to be due to outlying values (GCs: 142.54 ng/g, 280.65 ng/g; estradiol: 377.35 ng/g).

Because we only obtained samples from one pregnant female, we considered her separately as a case study. Pregnant female JI gave birth on or slightly before 11/15/2010, and exhibited a rise in estradiol 8-9 weeks prior to birth (during the third trimester; Figure 5). However, because some estradiol may be converted and excreted as estrone [Ziegler et al., 1989], it is possible that estradiol rose earlier but fecal estradiol metabolites did not measurably increase until the third trimester. GCs increased slightly during the period in which estradiol exhibit this rise. After birth, JI's estradiol concentrations plummeted, and remained low for the remainder of the study.

Discussion

GC response to a stressor

Results from our captive validation suggest that fecal GC metabolites in female Geoffroy's spider monkeys are associated with physiological stress induced by anesthesia. Data from all four female subjects that were successfully sampled indicate elevated GC concentrations after the veterinary exam. Although small sample sizes limit conclusions, data from the two

best-sampled individual females in the captive study indicate that fecal GCs peaked approximately one day after anesthesia. This is congruent with the 20-25 hour peak concentration reported previously for a female spider monkey [Rangel-Negrín et al., 2009]. The time lag between experience of a stressor and a rise in fecal metabolites should depend on steroid metabolism and gut passage rate [Touma and Palme 2005; Ziegler and Wittwer 2005]. Although Milton [1981] determined that gut passage rate for spider monkeys is generally 4-8 hours, she found that some markers were still excreted over 24 hours after ingestion, which is congruent with our findings.

Reproductive state

Lactating female spider monkeys exhibited lower GC concentrations than cycling females. This finding is similar to studies showing reduced GC response during lactation in humans [Wiesenfeld et al., 1985; Mezzacappa et al., 2000, 2001; Groer et al., 2002] and tufted capuchins [Ehmke, 2010]. In humans, the pattern of low GC responsiveness is attributed to the effects of oxytocin and prolactin, as well as reduced CRF activity during lactation [Altemus, 1995; Uvnäs-Moberg, 1998; Tu et al., 2005]. In particular, oxytocin is released during nursing, and is implicated in both maternal bonding and reducing stress response and fear.

However, our results contrast the findings in other primate species such as baboons [Weingrill et al., 2004; Beehner et al., 2005; Engh et al., 2006] and macaques [Maestripieri et al., 2008; Hoffman et al., 2010]. These patterns may reflect differences in the social context that affects lactating females. Lactating macaques, particularly low ranking females, face the threat of kidnapping [Silk, 1980], whereas lactating baboons face high risks of infanticide [Kitchen et al., 2004; Engh et al., 2006]. In comparison, the risk of infanticide in spider monkeys is comparatively low. In a single population of chacma baboons, 40% of infant mortality could be attributed to infanticide [Palmobit et al., 2000], whereas only eight infanticides have been reported across the *Ateles* genus [Gibson et al., 2008; Alvarez et al., 2014].

Additionally, increased GCs may be contingent on present risks. For example, infanticide and male takeover is well-documented for capuchins [Fedigan, 2003; Ramírez-Llorens et al., 2008]. However, lactating tufted capuchins had lower GC concentrations than females in other reproductive stages [Ehmke, 2010] and lactating vs. cycling/anovulatory white-faced capuchins exhibited no difference in GC concentrations [Carnegie et al., 2011]. However, when the white-faced capuchins experienced an acute threat of male takeover, females of every reproductive state exhibited elevated GCs [Carnegie et al., 2011].

Although we did not have the sample sizes necessary to examine individual differences, factors such as rank, parity, and individual differences in maternal behavior can affect maternal GCs [Nguyen et al., 2008; Hoffman et al., 2010]. Additionally, male infant spider monkeys may face greater infanticide risk [Alvarez et al., 2014] and the effects of offspring sex remains to be tested. Thus, it is possible that patterns within a particular group may reflect current demographic composition, rather than species-wide patterns.

In many primate species, females are the targets of male aggression [Smuts and Smuts, 1993]. Female-directed aggression initiated by males is well-documented among spider monkeys [Fedigan & Baxter 1984; Campbell 2000; Campbell 2003; Slater et al. 2008]. Although this aggression occurs across reproductive states, male pursuits resulting in prolonged chases are more likely when directed toward cycling females [Campbell 2003; Slater et al. 2008]. Although the same aggressive targeting of cycling females by males occurs in other species [e.g., baboons, Kitchen et al., 2009] these females retain lower GC concentrations than pregnant and lactating females [Beehner et al., 2005]. The pattern of relatively high GC concentration in cycling female Geoffroy's spider monkeys may therefore indicate that the high frequency of male aggression directed at females outweighs the relatively rare threat of infanticide in this species.

Although we removed our only pregnant female's samples from analyses, her patterns supports this conclusion as her GC concentrations remained low for approximately three months post-partum, during lactation (Figure 5). We suggest that further study is warranted to examine the GC profiles of females throughout gestation, and how GCs vary with estradiol before and after birth.

Stages of Lactation

Although lactating females should have lower concentrations of estradiol than cycling females [Altemus, 1995], the process of weaning should be associated with increases in estradiol concentrations as female begin to resume cycling [Strier and Ziegler, 2005]. The high variability in weaning age within spider monkeys should result in similar variability in resumption of cycling [McFarland Symington, 1987; van Roosmalen and Klein, 1988]. As predicted, we found that estradiol peaked during late lactation, and females in this stage exhibited the highest variation in estradiol concentrations (Figure 4). We further predicted that females in early or middle lactation, with offspring who were most vulnerable to infanticide [Crockett and Janson, 2000] would have higher GC concentrations than females in late lactation. However, this prediction was unsupported, likely due to the low threat of infanticide.

Collection Time and Days in Storage

Although estradiol and GC concentrations were slightly higher in morning than afternoon samples, this relationship was not significant. Our pattern is different from those reported for other New World monkeys [conflicting results in tufted capuchins: Lynch et al., 2002; Ehmke, 2010; Wheeler et al., 2013; higher in afternoon in common marmosets, *Callithrix jacchus*: Sousa and Ziegler, 1998]. A number of studies indicate that GC metabolites from fecal samples lack the diurnal variation of GC expression observed in urine and serum [*Lemur catta*: Cavigelli, 1999; *Alouatta pigra*: Martínez-Mota et al., 2008; *Macaca sylvanus* and *Pan troglodytes*: Heistermann et al., 2006; *Papio ursinus*: Beehner and Whitten, 2004; *Gorilla gorilla* Shutt et al., 2012]. Any difference between fecal and other analyses may be due to a combination of liver metabolism and gut passage rates consolidating hormone metabolites [Touma and Palme, 2005].

Although we found a trend toward storage time affecting both GC and estradiol concentration in the GLMMs, this trend was weak even in single-variate analyses, and may be due to outliers. Previous studies exhibit conflicting results regarding storage time. While some studies exhibit fluctuations with GC concentrations over time in storage, the direction and timing of patterns are inconsistent [Beehner and Whitten, 2004; Pappano et al., 2010; Shutt et al., 2012; Kalbitzer and Heistermann, 2013; Wheeler et al., 2013]. Because of inconsistencies over storage conditions, these fluctuations are attributed by to inter-assay reliability rather than true changes in hormone concentrations during storage [Shutt et al., 2012; Kalbitzer and Heistermann, 2013; Wheeler et al., 2013]. Although Pappano and colleagues [2010] suggest preserving samples with sodium azide for RIA analyses, this preservative interferes with the EIA analyses used in our study [Kalbitzer and Heistermann, 2013].

Conclusions

Results of this study confirm that elevated fecal GC metabolites indicate physiological stress, and thus can be used to measure stress in female spider monkeys. Cycling females had higher concentrations of both hormones than lactating females, and estradiol began rising during late lactation. This finding is consistent with studies of human and tufted capuchin females, but differs from the patterns exhibited by macaques and baboons. We suggest that primate GC patterns vary in conjunction with typical social challenges during each reproductive state. In species with a relatively low threat of infanticide, oxytocin may have an attenuating effect on GC concentrations. Additionally, male chases directed toward cycling females may be a greater stressor relative to the low risks of infanticide in this species. Although conclusions are limited by the opportunistic nature of fecal sampling, we suggest that future studies on GCs in female primates incorporate estradiol concentration or reproductive state to account for potential confounding effects.

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Figure 1. Representative example of glucocorticoid (GC) profile for adult female spider monkey RI at Brookfield Zoo, Illinois, before and after veterinary exam

Figure 2.

Scatterplot of estradiol and glucocorticoid (GC) concentrations in wild lactating females. Estradiol and GCs were significantly correlated. To improve the view of the graph, three outlying values (GC: 280 ng/gram, E: 29.34 ng/gram; GC: 142.52 ng/gram, E: 4.94 ng/gram; GC: 27.71 ng/gram, E: 377.35 ng/gram) are not shown.

Figure 3.
The effects of reproductive state on glucocorticoid (GC) and estradiol (E) concentrations. Reproductive state had an effect on GC concentration that approached significance, and a significant effect on estradiol concentration.

Figure 4.

The effect of lactation stage on glucocorticoid (GC) and estradiol (E) concentrations. Early lactation is when offspring are 0-6 months, middle includes offspring 6-12 months, and late includes offspring 12-24 months. Cycling (not lactating) females are included for comparison. Lactation stage had no effect on GC concentration, but had a significant effect on estradiol concentration.

Figure 5. Glucocorticoid (GC) and estradiol (E) profile of pregnant female JI: arrow reflects that birth occurred between 11/4/2010 and 11/15/2010

Table 1
Individually recognized focal subjects in the Pilón community at El Zota Biological Field Station, their offspring, and number of fecal samples collected

<u>Individual</u>	<u>Age</u>	<u>Parity</u>	<u>Reproductive State</u>	<u>Offspring Age</u>	<u>Samples</u>
AG	Adult	parous	Middle Lactation	Dorsal infant	5
AS	Subadult	nulliparous	Cycling	None	17
AR	Adult	parous	Middle Lactation	Dorsal infant	13
BU	Subadult	nulliparous	Cycling	None	9
DA	Adult	parous	Cycling	Juvenile-2	2
EV	Adult	parous	Late Lactation	Juvenile-1	1
FA	Adult	parous	Middle Lactation	Dorsal infant	1
HO	Subadult	nulliparous	Cycling	None	9
IS	Adult	parous	Cycling	Juvenile-2	5
JI	Adult	parous	Pregnant/early lactation	Juvenile-3/Ventral infant	8
JL	Adult	parous	Middle Lactation	Dorsal infant	17
LE	Adult	parous	Late lactation/cycling	Juvenile-1	18
MC	Adult	nulliparous	Cycling	None	11
MI	Adult	parous	Cycling	Juvenile-2	2
RU	Adult	parous	Late lactation/cycling	Juvenile-1	12
ST	Adult	parous	Cycling/pregnant/early lactation	Ventral infant	2
ZE	Adult	parous	Late lactation	Juvenile-3/Juvenile-1	2
N = 17 females (10 cycling, 10 lactating, 2 pregnant)					Total
					134

Ventral infants are approximately 0-6 months old, Dorsal infants 6-12 months old, Juvenile-1 12-24 months old, Juvenile-2 24-36 months old, and Juvenile-3 36-50 months old (following van Roosmalen and Klein 1988).