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Correlations between Social Context and Fecal Glucocorticoid Metabolite Concentrations in Free-ranging Female Gray-cheeked Mangabeys (*Lophocebus albigena*) in Kibale National Park, Uganda*

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An elevated concentration of glucocorticoids is an indicator of stress, and chronically high glucocorticoid levels are often associated with poor health and reduced fertility. We explored conditions that might be stressors in the lives of adult female gray-cheeked mangabeys (*Lophocebus albigena*) by measuring fecal glucocorticoid metabolites (fGCM) concentrations. During a six-month study we collected 109 fecal samples from 28 adult females from five groups in Kibale National Park, Uganda. We examined fecal fGCM levels of individual females relative to their own reproductive status (cycling or not cycling) and that of other females and to the presence of newcomer (immigrant) males. We found elevated fGCM concentrations in females when other females in their groups were at the peak of sexual swelling, and when immigrant males joined the group.

Key words: Estrus, fecal glucocorticoid, immigrant males, sexual swelling, stress.

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When humans and other animals perceive a stressful situation, they elevate glucocorticoid concentrations that help mobilize resources for a response while down-regulating other vital processes. While promoting survival in the short run, prolonged periods of elevated glucocorticoid concentrations can jeopardize health and interfere with reproduction (SAPOLSKY 2005). Therefore, measurement of glucocorticoid concentrations in animals in different contexts as well as longitudinally within individuals as their context changes, can throw important light on the socio-ecology of animals. Firstly, psychological stressors can be identified and their relative importance assessed. Secondly, correlations between stress and fitness (e.g., reproduction and health) can be investigated.

Such questions need to be addressed under natural conditions. By measuring glucocorticoid metabolite concentrations in fecal samples (fGCM) it is possible to identify correlates in wild animals without causing handling stress. Another advantage of using fecal samples is that fGCM levels reflect the episodic fluctuations of hormones over a certain period of time (hours to days), so that data are less affected by episodic fluctuations or the pulsatility of hormone secretion than glucocorticoid concentrations in blood.

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Several stressors of wild animals have been identified by measuring fGCM. For male mammals, such stressors include immediate but short-lived events, e.g., attacks by predators (gray-cheeked mangabeys, *Lophocebus albigena*: ARLET & ISBELL 2009), and longer-term, more chronic situations, e.g., competition over estrous females (Japanese macaques, *Macaca fuscata*: BARRETT *et al.* 2002; tufted capuchins, *Cebus apella nigritus*: LYNCH *et al.* 2002; gray-cheeked mangabeys: ARLET *et al.* 2009), instability in the dominance hierarchy (olive baboons, *Papio anubis*: SAPOLSKY 2005), and effort required to maintain high rank (African wild dogs, *Lycaon pictus*: CREEL *et al.* 1996; olive baboons, *Papio anubis*: SAPOLSKY 1992; Japanese macaques: BARRETT *et al.* 2002).

Environmental correlates of fGCM in females have been less studied, partly because reproductive condition affects glucocorticoid levels (ALTMANN *et al.* 2004; BALES *et al.* 2005; CHENEY & SEYFARTH 2009). For instance, pregnant females often have higher levels of glucocorticoids than non-pregnant females, and levels increase throughout pregnancy (CAVIGELLI 1999; WEINGRILL *et al*. 2004). Although lack of awareness of the relationship between cortisol and reproductive condition can potentially confound our interpretation of environmental stressors, it need not prevent investigation of stress in females, and much can be gained. For example, understanding potential stressors on females can help to clarify differences in reproductive success among individuals. In their review, CHENEY and SEYFARTH (2009) noted that glucocorticoid levels in female primates can be elevated 1) by ecological conditions (e.g., reduced food availability or extreme temperatures), 2) in high-ranking females when there is high reproductive skew, but not necessarily when there is low reproductive skew, 3) in high-ranking females who are actively involved in aggressive intergroup encounters, and 4) when females face social instability.

To assess the role of stress responses in the evolution of primate social systems, comparative data are needed. Gray-cheeked mangabeys are useful to compare with well-studied baboons because the two genera are phylogenetically closely related (HARRIS & DISOTELL 1998; BURRELL *et al.* 2009) yet differ markedly in habitat, gray-cheeked mangabeys being arboreal. The social system of gray-cheeked mangabeys is similar to that of baboons in that they live in multi-male, multi-female social groups, females typically remain in their natal groups and have linear dominance hierarchies, males disperse from their natal groups around sexual maturity, and dispersing males may join other groups (OLUPOT 1999; OLUPOT & WASER 2001, 2005; CHANCELLOR & ISBELL 2009a, b). As in baboons, male gray-cheeked mangabeys can be highly aggressive toward females, particularly when females are in estrus, and they can also be aggressive toward immatures (ARLET *et al.* 2008). However, direct evidence for infanticide is still lacking. In contrast to baboons, gray-cheeked mangabeys live in much smaller groups: while baboons live in groups of up to around 85 individuals (CHENEY & SEYFARTH 2009), gray-cheeked mangabeys seldom live in groups larger than about 20 individuals.

We investigated possible stressors in the lives of wild female gray-cheeked mangabeys as revealed by fGCM. From our previous studies we know that females receive aggression from males when they are in estrus, that particular immigrant males are aggressive towards infants, and that the presence of estrous females is associated with aggression among males and elevated fGCM in males (ARLET *et al.* 2008, 2009). Therefore, we tested the hypotheses that for females the following contexts are associated with elevated fGCM concentrations: 1) being in estrus, 2) the presence of estrous females in the group, and 3) having an infant while there is an immigrant in the group. In addition, we made preliminary observations on the effects of group size, rank, and births and deaths of infants on fGCM. The time of day was investigated as a co-factor, because glucocorticoids are known to display diurnal patterns (e.g., SOUSA & ZIEGLER 1999).

Material and Methods

Study site and subjects

The study complies with the current laws of Uganda. This study is based on fecal samples and demographic data collected from February to August 2007. The site was in Kibale National Park, Uganda (0°13′-0°41′ N and 30°19′-30°32′ E), near the Makerere University Biological Field Station at Kanyawara (CHAPMAN & LAMBERT 2000). The study period included a wet (April-June) season and two dry seasons. All adult females in four of five study groups (Mikana (MK), CC, Butanzi (BT1), and Lower Camp 1 (LC1) have been individually identified since 2003 (Table 1). We were able to indentify confidently only one of five females in LC2. However, all five groups were well habituated to human presence as a result of Olupot's studies (e.g., OLUPOT 1999; JANMAAT *et al.* 2009), and we were able to walk right under them without apparent response from the monkeys.

Data collection

The five groups were studied for one week in succession so that each group was followed once every five weeks. Data were recorded on group

Table 1

Group compositions of five study groups of gray-cheeked mangabeys in Kibale National Park, Uganda. Abbreviations: AM- adult male, AF-adult female, SAM-sub-adult male, SAF-sub-adult female, JM-juvenile male, JF-juvenile female, INF-infant

Group	AM	AF	SAM	SAF	JM	JF	INF	Total
BT1	3	6	1	0	2	3	3	18
CC	4	5	0	0	6	2	2	19
LC1	4-5	7	2	1	2	1	2-3	19-20
LC2	1	5	0	0	1	0	3	10
MK	1	5	1	0	2-3	2	0	12-13

composition and size, including number of adult, non-immigrant, resident males in the group. Males can stay in groups for a variable amount of time. In this study, a male who immigrated into a group during the six months was considered as a new male during the whole study period, because immigrant males typically have fGCM concentration lasting about six months (ARLET *et al.* 2009). All other males in this study could be assigned resident status with confidence, because the individual males present in all groups have been recorded following the above schedule since December 2005.

Like baboons, female gray-cheeked mangabeys have exaggerated sexual swellings. It is reasonable to assume that female gray-cheeked mangabeys ovulate while at peak swelling because that is the case for baboons and for less closely related chimpanzees (EMERY & WHITTEN 2003; GESQUIERE *et al.* 2007). Female reproductive stage was assessed visually as: inflating – with a small, pink sexual swelling, that increases over a period of 4-14 days; peak – with a large and highly visible, red swelling (2-4 days); deflating – with a wrinkled purple swelling that decreases in size over 7-14 days, and non-cycling – all others. Reproductive assessments and male presence in groups were linked temporally to fecal samples collected one to three days afterwards. This takes into consideration the mean digestive transit time and mean retention time (22.7-38 hrs) reported for gray-cheeked mangabeys (LAMBERT 1998), and the experience-to-gland-to-blood-to-feces process. Female ranks were based on the outcome of antagonistic interactions organized in dominance matrices (CHANCELLOR & ARLET, unpublished data) with the exception of group LC2.

The order of fecal sample collection in each group was opportunistically determined by first sighting of females that had not yet been sampled during a day. We attempted to collect one sample per female per week. The samples were collected between 0700-1700 hrs. The samples were collected from known individuals only, except LC2 group, and no further precautions were needed. Fecal sample collection, treatment, and analysis were carried out following the protocol described in ARLET *et al.* (2009). When a focal female defecated, the time was noted and the sample was immediately collected, placed in a sterile scintillation vial, and stored on ice in a cooler. At the end of the day, all samples were placed in a -20^{o}C freezer until hormone suspension (STRIER & ZIEGLER 1997, 2005; WHITTEN *et al.* 1999). Hormones were suspended at the field station using the protocol developed for red colobus (CHAPMAN *et al.* 2006). A fecal sample was removed from the freezer, thawed, and homogenized using a spatula. Then, 0.50 g was solubilized using a 5.0 pH citrate buffer/95% ethanol solution (10 ml, 1:1) that was mixed for 21-27 hrs. After mixing, samples were spun in a centrifuge for 30 min at 3200 rpm to separate the supernatant containing the hormones from the fecal pellet, and then 2 ml of the supernatant was passed through a solid phase extraction cartridge (Alltech maxi-clean filter Standard C18) for storage and transport to the U.S.A.

The samples were then sent to the Wisconsin National Primate Research Center's (WNRPC) Assay Services, where the method for glucocorticoid measurement was validated and samples analyzed. At the WNPRC, glucocorticoid metabolites were extracted from the filters. The cartridges were washed with 1 ml of 20% methanol and the columns were eluted with 2 ml methanol. This methanol was dried, resuspended in 1 ml ethanol, and 50 μl was taken for the enzyme immunoassay (EIA). The WNPRC lab used the antibody R4866, which was developed by MUNRO & STABENFELDT (1984) and is well characterized. These analyses provided data on the metabolites of cortisol found in the supernatant, tested the EIA for parallelism, and produced a measure of the amount of glucocorticoid metabolites in each sample in nanograms per gram of dry feces. Since fresh (not dried) feces were used for hormone extraction, the dry weight of each sample was calculated using the percent water of a sample from the same homogenized fecal sample. Water content was determined by weighing these samples before and after drying to constant weight in the field (CHAPMAN *et al.* 2006). Six samples were spiked with a cortisol solution to help validate the methods and results from these samples were corrected for this addition. Glucocorticoid metabolites were extracted twice from the same homogenized sample for nine different samples to get insight into the repeatability of the extraction and method of

analysis: the CV% varied between 1.2 and 8.1 (average 4.0), which is well within the acceptable range of 0-10%.

Data analysis

We analyzed the data in complementary ways. First we used Generalized Estimating Equations (GEE) to estimate the effects on fGCM of Morning or Afternoon, Number of Other Females with Sexual Swellings, Peak or Non-Peak Swelling, Having an Infant, Presence or Absence of Immigrant Males, and possible interactions between these factors. The GEE method is most appropriate when there are one or a few samples from relatively many individuals, and utilizes both variation among and within individuals (HARDIN & HILBE 2003). We could not use mixed models because these require at least five observations per individual. While useful for accounting for correlations within individuals, the GEE method has the disadvantage that model selection is based on p-values rather than AIC. Moreover, while mixed models provide informative standard errors of random effects, the GEE method generates a correlation structure (working correlation matrix) which is not expressed as a single number that can be reliably used for comparisons of within individual correlations. Finally, we did not include any temporal correlation between samples of the same individual in the model because virtually all samples from the same individuals were taken more than a week apart and in males any correlation between samples breaks down over this time period (ARLET *et al*. 2009). In LC2 we could identify only one adult female individually and so we treated fGCM measures from non-recognizable individuals as if each was from a different individual. Parameter selection was done based on p-values with p-critical 0.1 (this p-value was chosen *a priori* because of the exploratory nature of the study and limited sample size). We first added parameters singly and then added other parameters and then interactions. A full model is provided to show non-significant factors.

Because results of the GEE analyses could potentially be confounded with e.g., group size or female rank, we investigated whether the main results from the GEE analyses were consistent with within-individual patterns of fGCM. For each female for which we had the needed data, we compared average fGCM concentrations of samples collected when 1) no immigrant males vs one or more immigrant male were in her group, 2) when she had no swelling, but when all other females in her group had no swellings vs when at least one had peak swelling, and 3) the female herself had no sexual swelling vs. when she had a peak swelling. These contrasts were considered irrespective of any potential confounding factors, and tested using *t*-tests paired within individuals.

Other potentially important factors could not be included in these analyses due to limited sample size or lack of variation in time, and were explored separately. These included the factors group and group size, female rank, and births and deaths of infants. We did not include predator presence (i.e., crowned eagles, *Stephanoaetus coronatus*) because predator attacks are relatively rare. Moreover, males that do not chase eagles show no increase in fGCM levels when besieged by eagles (ARLET & ISBELL 2009), and females do not chase eagles. We did not collect behavioral data from females during this study.

Results

We collected 109 fecal samples: 97 from 26 individually recognized females in four social groups and 12 from at least three females in different reproductive conditions that were not individually recognizable in LC2 (six from females with sexual swellings, four from adult females with infants, and two from adult females without sexual swellings or infants). We analyzed the data both with and without samples from LC2 and found that the direction of the results did not change, so LC2 was included in results presented here. For individually recognized females, sample sizes ranged from 1 (3 females) to 7 (1 female), all at least one week apart.

The most consistent results were that the presence of immigrant males was associated with elevated fGCM levels and that the presence of females with peak swellings was associated with elevated fGCM levels for other females in the group. These results were found both with the GEE method (Table 2, Fig. 1) and within individuals over time (Fig. 2).

Figure 1 shows that fGCM concentrations in females are higher when estrous females are present in the group and when an immigrant male is in the group, and these effects appear to be additive, thus illustrating our GEE model results presented in Table 2. All eight females for whom we had the necessary data had higher fGCM levels when an immigrant male was present in her group than when such males were absent (paired *t*-test: $t=-6.48$, $p<0.001$; Fig. 2a). The mean fGCM level of these females was only 30.1 ± 0.2 SE ng/g when no immigrant males were in the group, and 53.7 ± 0.6 SE ng/g when at least one immigrant male was present (Table 2; Fig. 2a).

Similarly, mean fGCM levels were significantly elevated for other females (55.2 ± 1.05 SE ng/g) when at least one female in the group had a peak

Table 2

Model estimates from Generalized Estimating Equations analysis with exchangeable working correlation matrix on the natural log of fGCM concentrations (ln ng/g) in female gray cheeked mangabeys in Uganda. Both the number of females at the peak of sexual swelling and the number of immigrant males had significant effects (p.001, treated as continuous variables), and time of day was also significant with our p-critical of 0.1 that reflects the exploratory nature of this study

Parameter	95% Wald CI				Hypothesis test		
	Effect size B	SE	Lower	Upper	Wald Chi^2	Dif.	P-value
Final model:							
intercept	3.2	0.07	3.07	3.33	2172.8	1	<0.001
1 immigrant male compared to 0	0.55	0.1	0.34	0.75	27	1	<0.001
2 peak females compared to 0	0.51	0.05	0.4	0.61	90.1	1	<0.001
1 peak female compared to 0	0.12	0.08	-0.04	0.28	2.2	1	0.14
Afternoon compared to morning	0.14	0.08	-0.01	0.29	3.5	1	0.06
Full model:							
intercept	3.3	0.11	3.08	3.49	960.1	1	<0.001
1 immigrant male	0.39	0.35	-0.30	1.07	1.23	1	0.27
1 peak female compared to 0	0.15	0.06	0.02	0.28	5.3	1	0.21
Afternoon vs morning	0.15	0.08	-0.13	0.305	3.3	1	0.07
Individual is peak	-0.10	0.14	-0.38	0.17	0.55	1	0.48
Infant*immigrant male	0.052	0.23	-0.39	0.49	0.05	1	0.82
Peak*immigrant male	0.12	0.30	-0.47	0.71	0.16	1	0.69

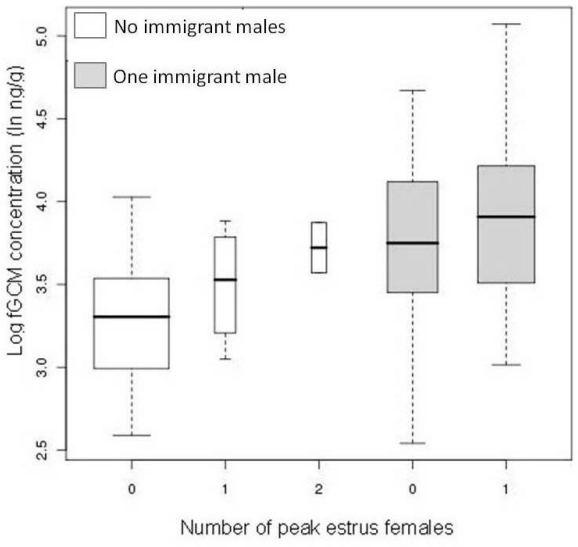

Fig. 1. Boxplots of log-transformed fGCM concentrations, by number of peak estrous females and number of immigrant males present in a group at the time of fecal sample collection. The width of each box is proportional to the square-root of the number of samples represented in the box. Statistical results are given in Table 2.

sexual swelling, compared to when no females had peak swellings (35.2 ± 0.25 SE ng/g; Fig. 1). Among the 15 females for which we had such data, 11 (73%) showed elevated fGCM levels when other females in the group developed sexual swellings (paired *t*-test: t=-2.11, p=0.05; Fig. 2b).

Neither the GEE procedure nor the within-individuals analyses recognized the estrous stage of the individuals as a predictor of fGCM, and averages over individuals were inconsistent with the trend suggested by the sparse within-individual data. The averages suggest that estrous females are less stressed than the other females in their group at that time: fecal samples from females that had peak sexual swellings had mean fGCM levels that were lower (37.5± 1.6 SE ng/g) than samples from females that had no peak swelling themselves but were in the company of another female with peak swellings (54.5± 0.96 SE ng/g; Table 3). In contrast, examination within females shows that in four of the five females (80%) for which we had such data, mean fGCM levels were elevated when they themselves were at peak sexual swelling (paired *t*-test: t=-1.75, p=0.15; Fig. 2c).

According to the GEE analysis, fGCM concentrations in samples taken in the afternoon were higher than those taken in the morning based on our criterion for statistical significance (Table 2). Other factors or interactions did not attain significance, even with our relaxed p-critical value of 0.1.

Other factors may not have contributed statistically to elevated cortisol levels, but there are indications that they may be important or confounded with other factors. Females in groups with only one resident male (LC2, MK) had mean cortisol

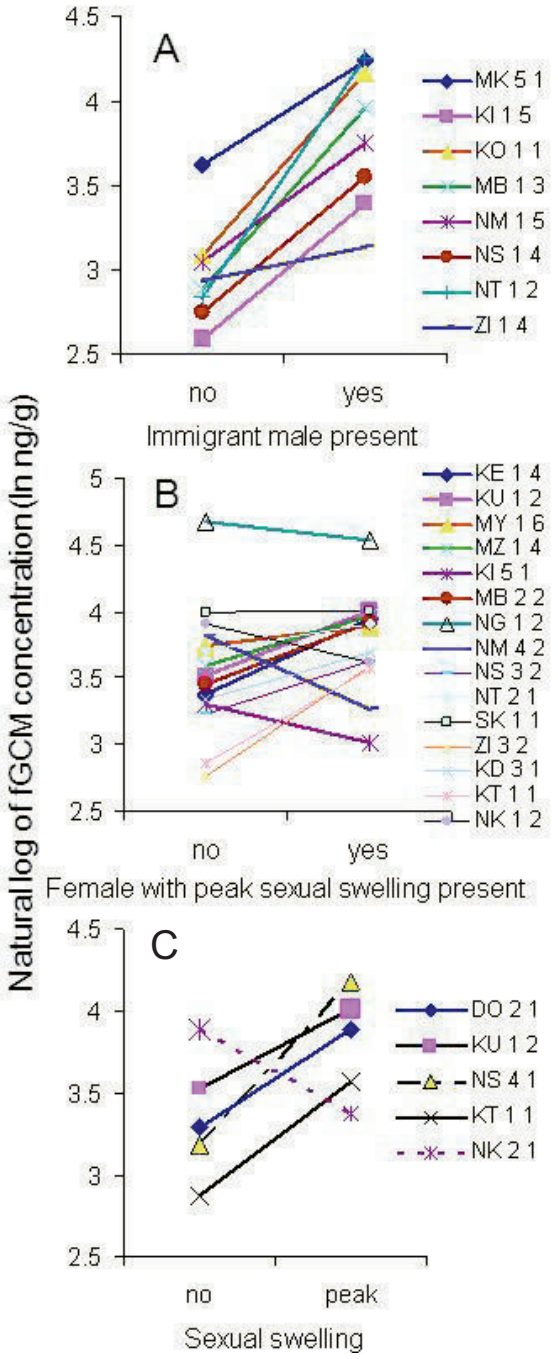

Fig. 2. Within-individual patterns of fGCM with respect to: A) whether a new male was present in her group, B) the presence of (one or two) females with peak sexual swelling, C) if the sample came from a female with or without peak sexual swelling. The legends on the right hand side represent individual females with the respective sample size for each situation. Values are averages if more than one value was available and any confounding factors are not corrected for. fGCM was elevated in all eight cases when there was an immigrant male in her group, in 11 out of 15 cases when a female with peak swelling was present, and in 4 out of 5 when she was at peak swelling.

levels of 32.1 ng/g ± 0.3 whereas females in groups with three (BT1) or more resident males (CC, LC) had mean cortisol levels of 45.4 ng/g ± 0.4. Within two days after females lost their infants (n = 2), their fecal cortisol concentrations increased from 36 to 68 ng/g and 36.2 to 80.6 ng/g. After one female gave birth (the only birth during our study), her cortisol level increased from 49.8 to 98.6 ng/g. Finally, the highest-ranking females in each of the two largest groups (LC and CC) had the highest cortisol levels (>100 ng/g) of all females.

Table 3

Sample sizes and fGCM concentrations (ng/g) of female mangabeys of different reproductive states (non-cycling, estrous inflating, estrous peak and estrous deflating), when estrous peak females were present in the group, when immigrant males were present in the group

FCGM for females	N samples	Mean	SE	Range
non-cycling	73	41.9	0.39	12.7-159.2
estrous inflating	6	40.7	2.23	25.0-60.9
estrous peak	13	37.5	1.59	15.5-91
estrous deflating	16	41.7	1.58	17.8-122.0
all when estrous peak female(s) present	35	54.5	0.96	20.4-159.2
all when immigrant male(s) present	52	53.7	0.6	12.7-159.2

Discussion

Our analyses suggest that the occurrence of both immigrant males and estrous females in groups is associated with increased fGCM concentrations in female gray-cheeked mangabeys. The presence of such group members can thus be interpreted as generally stressful for females. Moreover, these factors are more common in larger groups (ARLET *et al.* 2011) and suggests a disadvantage of living in larger groups because greater numbers of estrous females appear to attract more males (OLUPOT & WASER 2001; ARLET *et al.* 2008). Although the analysis shows that the average female's corticosteroid levels are higher under such circumstances, we note that there is variation among females, and future research should examine causes of individual variation further than our small sample size allowed.

Immigrant males may increase stress for females in at least three ways. First, they may increase aggression in the group as they compete with resident males for dominance or access to estrous females. Second, previous studies showed that particular immigrant gray-cheeked mangabey males are aggressive towards infants but not females (ARLET *et*

al. 2008), and this may stress mothers. Among chacma baboons, immigrant males most often commit infanticide and the presence of such males elevates fGCM levels significantly in lactating females (ENGH *et al.* 2006). Also, BEEHNER (2005) reports that in chacma baboons male social instability itself does not necessarily elicit a stress response from females. Rather, it is the specific male that rises to the alpha position that prompts a stress response, and only from the females at risk of infanticide. If immigrant males stress females through their threat to infants, an interaction between having an infant and presence of immigrant males would be expected. That we did not find such interaction may be due to limited sample size or constitute a difference with baboons. Because direct observation of infanticide is lacking after many years of study, it is probably at least less common in gray-cheeked mangabeys than in baboons. That, on average, all individuals show elevated fGCM when immigrant males are in the group (and thus aggressive interactions are more common; ARLET *et al.* 2011) may be related to the arboreal habitat. Because the number of possible paths of escape is limited by the branches, and animals can fall from the trees, all individuals are at risk when some individuals are chasing each other. Finally, previous research with gray-cheeked mangabeys has shown that when immigrant males enter a group, resident males become more aggressive toward females (ARLET *et al.* 2007, 2008). It is possible that immigrant males are merely catalysts for increased stress on females because they increase aggression of resident males toward females.

The presence of at least one female at peak swelling in the group may increase stress for all females because of the attractiveness of estrous females to males and the resulting competition among males as they compete aggressively. This again contrasts with baboons where only individuals directly targeted by aggressors tend to show elevated fGCM, and this may be a result of the arboreal habitat that promotes collateral damage of conflicts. We have no indication that females in estrus are aggressive towards other females (ARLET *et al.* 2007).

The uncertainty of whether females at peak sexual swelling themselves had elevated fGCM levels (as has been found in other species) may be due to small sample size or confounding factors, such as group size and rank. For instance, we had only 13 fecal samples from females at peak swelling, compared to 73 from non-cycling females, and only five individuals with at least one sample at peak swelling and one at another stage. Moreover, any effect such as suggested in Fig. 2c may be stress-related or part of the reproductive cycle.

Our results suggest a difference in fGCM found in samples collected before and after noon, which may point at diurnal variation in glucocorticoid concentrations in this species. Even though diurnal variation in blood glucocorticoid concentrations is common, such effect is, however, unusual in this type of study and should be investigated further before any conclusions are drawn.

In conclusion, our results suggest that tension resulting from competition among males over access to receptive females elevates stress, on average, in female gray-cheeked mangabeys, particularly in larger groups. This contrasts with findings in their terrestrial cousins where only targeted individuals (receiving aggression/threat of infanticide) tend to be stressed and may be related to risks associated with living in the canopy on one hand, and different male sexual strategies on the other. Future studies might take advantage of this as a starting point to clarify which particular behaviors cause elevated fecal glucocorticoid levels and, by inference, stress in non-estrous as well as estrous females, and to investigate how other factors such as variation in food availability, group size, social stability, and female rank affect stress.

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