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Behavioural patterns associated with faecal cortisol levels in free-ranging female ring-tailed lemurs, *Lemur catta*

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The study of physiological stress and its context in free-ranging animals provides a means for understanding the challenges found in the natural habitat. Patterns of physiological stress in free-ranging animals have yet to be well characterized. Methodological difficulties in measuring physiological responses in the natural habitat have limited this area of research. In this research, physiological stress in free-ranging ring-tailed lemurs, *Lemur catta*, was estimated using a steroid-extraction method to measure cortisol levels from female faeces. Ten females were observed across two social groups in southwestern Madagascar during a 5-month period including portions of the annual wet and dry seasons. I used behavioural measures to estimate predation threat, food accessibility and individual dominance status, to determine whether these variables predict faecal cortisol levels. Faecal cortisol levels were relatively high during two distinct periods: one period coincided with late gestation and the other period corresponded with the end of the dry season, when high-intensity antipredatory behaviour and estimates of feeding effort were elevated. In addition, faecal cortisol measures were significantly correlated with dominance indices: high-index individuals had high cortisol values, and low-index individuals had low cortisol values. These results suggest that faecal cortisol measures can be used to assess seasonal and individual differences in adrenal activity in this lemurid primate, and that this measure could provide a means for quantifying physiological stress in free-ranging animals.

Physiological stress involves a cascade of neurological, hormonal and immunological responses that promote energy mobilization and facilitate effective behavioural responses to challenges in the environment. One physiological stress response involves increased catecholamine (e.g. epinephrine) release from the adrenal medulla, often followed by increased secretion of glucocorticoid hormone (e.g. cortisol) from the adrenal cortex into the blood stream (for review see Sapolsky 1992). Many studies have used elevated blood glucocorticoid levels as an index of physiological stress in animals. However, this measure can be difficult to collect from free-ranging animals and can be compromised because capture and handling can lead to rapid and significant increases in circulating glucocorticoid levels (Sapolsky 1982; Astheimer et al. 1994; Wingfield et al. 1994). Thus, studies conducted on the physiological stress response in free-ranging animals have been limited. Recent studies with felids have shown that traces of glucocorticoid hormones are detectable in faeces and can be used as a noninvasive measure of stress levels in free-ranging populations (e.g. Graham & Brown 1996). In the present study, I make use of this novel approach to track faecal cortisol levels in two groups of free-ranging lemurs during portions of the wet and dry seasons.

Past studies with both laboratory and free-ranging animals suggest several potential stressor stimuli for free-ranging animals. Laboratory studies with humans, tamarins and lagomorphs indicate that circulating glucocorticoid levels reflect reproductive state: late gestation is associated with a two- to three-fold increase in circulating cortisol levels, and postpartum is associated with a return to baseline cortisol levels (Mulay et al. 1973; Carr et al. 1981; Kriesten & Murawski 1988; Allolio et al. 1990; Ziegler et al. 1995; Lockwood et al. 1996). Field studies have led many researchers to propose that predation threat, decreased food accessibility and social aggression or competition provide some of the most significant challenges facing free-ranging gregarious animals (e.g. Hamilton 1971; Alexander 1974; Wrangham 1980; Sapolsky 1982; van Schaik 1989; Creel et al. 1996, 1997). However, laboratory studies (e.g. Weiss 1970; Hanson et al. 1976) and a recent report by Wingfield & Ramenofsky (1997) emphasize that the 'predictability' or 'controllability' of a stimulus are the most important characteristics

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that determine the degree of challenge it poses. In other words, noxious conditions that are predictable and/or controllable, including, for example, seasonal decreases in food accessibility or visually prominent predators, present less of a challenge to an organism than those that are not predictable or controllable.

Measures of glucocorticoid levels in free-ranging animals have been made primarily from blood samples. Several studies done with free-ranging avian, rodent and primate species have shown clear circannual and individual variability in glucocorticoid secretion. Circannual increases in adrenal activity are often associated with low food supplies (Sapolsky 1986; Astheimer et al. 1992; Boonstra & Singleton 1993) or unpredictable environmental conditions (Wingfield & Ramenofsky 1997). Studies that have investigated glucocorticoid levels relative to social environment report elevated levels during periods of social instability (Sapolsky 1983; Alberts et al. 1992) and, during stable periods, either low- or high-ranking individuals show the highest glucocorticoid levels within a group (Sapolsky 1982, 1983; Creel et al. 1996, 1997). At the same time, Wingfield et al. (1991) and Bercovitch & Clarke (1995) found no reliable differences in blood glucocorticoid levels relative to dominance status in white-browed sparrow weavers, *Plocepasser mahali*, and rhesus macaques, *Macaca mulatta*, respectively. In the only studies in which free-ranging animal faecal glucocorticoid measures have been reported, Creel et al. (1996, 1997) found elevated glucocorticoid levels in high-ranking female African wild dogs, *Lycaon pictus*. The results of field studies to date indicate a relationship between food accessibility and/or food predictability and glucocorticoid levels but they present a conflicting picture on the relation between dominance status and glucocorticoid hormones.

The aim of this study was: (1) to determine whether faecal cortisol levels fluctuate in synchrony with the common challenges of late gestation, predation threat, food accessibility and/or dominance status; and (2) to determine whether faecal cortisol levels relate to either the time of day samples are collected or to the weight of samples. Based on past research results, one can predict that faecal cortisol levels would be elevated during late gestation compared with postparturition, and during periods of increased antipredatory behaviour and/or decreased food accessibility. However, if decreased food accessibility is a predictable phenomenon, as it may be for the lemurs experiencing a yearly dry period of low food accessibility, it may be that lemurs can prepare physiologically for these periods, and thus, decreased food accessibility would not be a significant stressor.

One can also predict a relationship between dominance rank and cortisol measures, although the direction of this relationship might not be clear. In addition, evidence of circadian rhythms in primate blood, salivary and urinary glucocorticoid levels (e.g. Wiebe et al. 1984; Allolio et al. 1990; Czekala et al. 1994; Coe & Levine 1995) indicate that time of day could affect faecal cortisol measures. Finally, faecal weight may affect cortisol measures in that increased weight may dilute faecal cortisol concentrations, which would primarily reflect diet bulk and not changes in circulating cortisol levels.

In this study, I used behavioural observations to assess challenging stimuli (gestation, predation threat, food accessibility and dominance status), and I used a method of extracting faecal steroid hormones that allowed me to monitor daily cortisol levels in free-ranging female ring-tailed lemurs in southwestern Madagascar. Females of this species provide ideal subjects for an exploratory study on stress in free-ranging primates: they live in harsh habitats and it has been argued that they undergo significant nutritional stress during gestation and lactation, coincident with the annual dry season in their natural habitat (Jolly 1984; Richard & Dewar 1991; cf. Kappeler 1996). Moreover, ring-tailed lemurs are diurnal, terrestrial, and live in large groups, which facilitates behavioural observations and faecal sampling.

METHODS

Prior to conducting fieldwork, I tested whether a steroid-extraction method, originally designed to assess reproductive hormones in anthropoid primates (Wasser et al. 1994), could be used to assess faecal cortisol levels in ring-tailed lemurs. I then applied the faecal cortisol measure in the field to document fluctuations in cortisol excretion and to examine environmental conditions associated with periods of elevated cortisol production in free-ranging ring-tailed lemurs. The field study involved monitoring behaviour and cortisol excretion in two groups living in the Beza Mahafaly Special Reserve during 4.5 months that included portions of the wet and dry seasons. I monitored the following variables: (1) reproductive state (gestating versus lactating); (2) frequency of antipredatory responses; (3) relative activity associated with food acquisition; (4) frequency of intragroup agonistic interactions; (5) faecal weight; and (6) time of daily faecal production.

Comparison of Faecal Cortisol Levels to Serum Cortisol Levels

To assess the applicability of Wasser et al.'s (1994) steroid-extraction procedure to measure cortisol-like substances in ring-tailed lemur faeces, I compared individual faecal cortisol measures to serum cortisol levels. I collected blood samples from ring-tailed lemurs at the Duke University Primate Center, analysed them for cortisol concentration (using the RIA procedure described below), and compared them to corresponding faecal cortisol measures. For this procedure, semifree-ranging lemurs were corralled into a holding cage within their normal forest enclosure, and caught and bled within 40 min of being corralled. The corralling procedure has been used repeatedly with these lemur groups (on a monthly basis), and is always coupled with a food reward, and occasionally, with release from the cages without further manipulations. Lemurs detained in these holding cages increase their activity at the sight of an animal technician holding a white capture net. Based on this response and the fact that white nets signal further manipulations, the arrival of technicians with nets was used as the onset of the capture procedure. I recorded the time delay between the arrival of technicians and the bleeding of the lemurs to control for the onset of capture stress on cortisol levels. A veterinarian collected 2–3 ml of blood from the femoral vein of 30 individuals and I collected faecal samples during the capture procedure. The veterinarian collected blood into Vacutainer SST tubes (Becton Dickinson, Franklin Lakes, New Jersey), allowed it to clot for approximately 30 min, then centrifuged the blood samples at 1200 *g* for 20 min. I decanted and stored the resulting serum supernatant at −80°C until extracted. I collected faecal samples at the time of blood collection and individually stored the samples in Whirl-Pak bags (Nasco, Fort Atkinson, Wisconsin) at −20°C until assayed.

I compared the serum cortisol values for each lemur to the cortisol levels found in the extract of their faeces collected during the blood collection procedure. Because serum cortisol levels can increase within minutes of an encounter with a stressor such as capture (Sapolsky 1992), I conducted an analysis to determine the point at which circulating cortisol levels increased significantly after capture and then eliminated serum samples collected after this time prior to comparison with faecal cortisol levels. Serum cortisol levels increased significantly after 16 min from the onset of animal capture (Wilcoxon–Mann–Whitney test: $W_x=158$, $N=20$, $P<0.01$), a delay comparable to baboons (Sapolsky 1982). Based on the 12 serum samples collected within 16 min, serum and faecal cortisol levels were significantly correlated ($r=0.56$, $N=13$, $P<0.05$). When I repeated this analysis with only those serum samples collected within 9 min of capture onset and the corresponding faecal samples, as would be suggested from data on baboons (Sapolsky 1982), the correlation coefficient increased to 0.99 ($N=4$, $P<0.01$). These correlation coefficients were taken as good support for the application of the extraction method of faecal steroids for estimating circulating cortisol levels in ring-tailed lemurs.

Free-ranging Behaviour and Cortisol Levels

Subjects

From August 1995 to January 1996, I observed 10 females from two social groups of ring-tailed lemurs living in a gallery forest reserve (Beza Mahafaly Special Reserve) in southwestern Madagascar. The Beza Mahafaly area undergoes a marked annual dry season accompanied by a period of decreased food variability during October and November (Sauther 1992). Prior to the study, females had been individually marked with a unique collar and tag combination (by R. Sussman and M. Sauther). Visibility throughout the study was good. Underbrush was minimal and the lemurs spent over 70% of their active hours within 10 m of the forest floor. Each social group was followed for seven 6-consecutive-day follows, and follows were alternated between the two groups during the study. During this time, all but one of these seasonal reproducing females went from late gestation to mid-lactation in the reproductive process. The one exceptional female, in group 2, gave birth to a seemingly healthy offspring in synchrony with the other females, then lost the infant after it suffered a severe wound to the head between the eighth and 10th weeks of observation. In addition, females in group 1 showed qualities of a fission–fusion social organization. Two females, along with three or four males, would occasionally travel, feed and sleep separately from the rest of the group. Group 2 similarly showed signs of group flux shortly after the completion of the study when two low-ranking females were outside of their original group (L. Gould, personal communication).

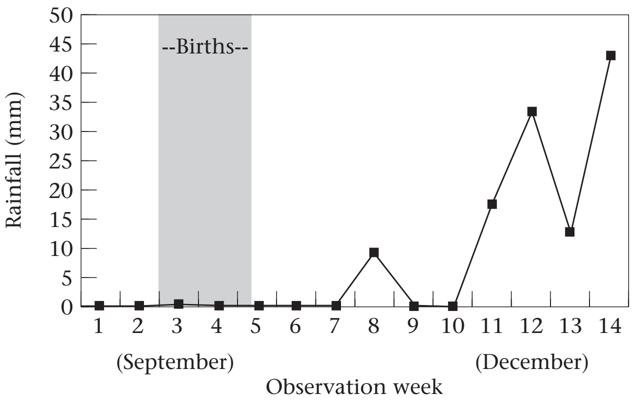

Figure 1. Time line of study identifying rainfall levels during each observation week (1–14), the period of births for all females, and the months when the study began and ended. Group 1 was observed during observation weeks 1, 3, 5, 7, 9, 11 and 13. Group 2 was observed during weeks 2, 4, 6, 8, 10, 12 and 14.

Throughout this paper, behavioural and faecal cortisol means are reported for each observation week (i.e. 6-consecutive-day follows). I collected 14 weeks of data, with seven alternating weeks from each group. Thus, means from ‘week 1’ refer to the first week of data collected from group 1, and means from ‘week 2’ refer to the next week of data collected, which were from group 2. Landmark events during these weeks are outlined below and in Fig. 1. The 14 observation weeks occurred over a period of 4.5 months (late August to early January). Births occurred between weeks 2 and 5 (September). Less than 10 mm of rain fell during observation weeks 1–10 (late August to mid-November) and 114 mm of rain fell during observation weeks 11–14 (mid-November to early January). A rush of new vegetation appeared in the reserve after week 12. The end of the dry season provided an ideal opportunity to assess faecal cortisol patterns during an annual period of decreased food accessibility.

Behavioural observations

I collected behavioural data to estimate the following factors: degree of predation threat, relative food accessibility and dominance indices. I conducted observations from approximately 0530 to 1200 hours and from 1500 to 1800 hours each day. The period from 1200 to 1500 hours included an extended sleeping period for the lemurs.

Daily observations were made for a mean (±SD) of 8.3±2.1 h/day (*N*=85). During the 5-month period, I observed group 1 for 378 h, and group 2 for 330 h.

Predation threat was estimated from all-occurrence records of 'high-intensity' antipredatory responses. I identified 'high-intensity' responses as the highly conspicuous 'yap' and 'shriek' vocalizations defined by Jolly (1966) and Macedonia (1990). These vocalizations, which are displayed by the group as a whole, are extremely loud and are associated with a rapid cessation of ongoing activity. Thus, it is unlikely these vocalizations would be overlooked during observation sessions. I collected focal animal samples (Altmann 1974) daily to calculate feeding effort and dominance indices based on social agonistic behaviour. Two 9-min focal samples were collected from each female daily: one sample in the morning and one sample in the afternoon. The time and order of focal samples were pseudo-randomized so that each female was observed for equal amounts of time during each 1-h block within observation sessions. Behavioural states recorded during focal samples included sleeping, resting, travelling and feeding, as defined by Jolly (1966) and Pereira & Kappeler (1997). Aggressive and submissive acts, as defined by Pereira & Kappeler (1997), were recorded during focal samples. I collected faecal samples opportunistically.

Weekly behavioural means were calculated to estimate changes in environmental conditions. I estimated weekly predation threat as the mean frequency of high-intensity antipredatory calling bouts per hour of observation. Feeding effort for each female was estimated as a ratio of time spent travelling and 'actively foraging' divided by time spent sleeping, resting and 'passively foraging'. I defined 'active foraging' as foraging from a standing position, and 'passive foraging' as foraging from a sitting position. Lemurs used active foraging most often when food items were small or were located at the ends of branches, and passive foraging primarily when feeding on grasses, leaves or large fruit. 'Foraging' included chewing and drinking. I calculated dominance indices for each female as the number of times a female approached another adult female and elicited a submissive signal without overt signs of aggression, divided by the total number of times a female was involved in this kind of interaction in either direction. I used this method of estimating relative dominance in addition to the more traditional method of developing a hierarchy based on interaction matrices.

Faecal sample storage and extraction

Faecal samples collected during behavioural observation sessions were stored in 40-ml screw-top polypropylene vials (Curtis Matheson Scientific, Inc., Norcross, Georgia) containing 15 ml of a 0.3% sodium azide in 100% ethanol solution. For each female, I analysed a mean (±SD) of 1.5±0.4 samples (*N*=10) from each observation day, with a total of 742 samples from all females.

Faecal samples were dried in a centrifugal evaporator (Savant, Farmingdale, New York) and 0.2 g of dry dust-like faecal matter was extracted. Seeds and fibrous material were avoided. I extracted cortisol according to the 'short method' of faecal sex steroid extraction described by Wasser et al. (1994). For the boiling step of the procedure, I used 95% ethanol.

Faecal cortisol measure

I analysed faecal-extract cortisol concentrations using a radioimmunoassay (RIA) protocol developed in the Laboratory of Comparative Human Biology at Emory University (J. Stallings, personal communication). The protocol involved modifications to a commercially available RIA kit designed for human serum cortisol analyses (Pantex CORTISOL (CORT) ^{125}I, Santa Monica, California). The assay had a sensitivity of 0.1 μg/dl and the cortisol antiserum was known to cross-react 35% with corticosterone, 30% with 21-desoxycortisol, 17.5% with 11-desoxycortisol, 2.9% with progesterone, and less than 0.01% with androstenedione, androsterone, cholesterol, cortisone, DHEA, dihydrotestosterone, alpha and beta-oestradiol, oestriol, oestrone and testosterone. Prior to assay analysis, faecal extracts were dried and reconstituted in a buffer solution made of 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (pH 7.4), which was also used to dilute kit standards, antibodies, controls and serum samples. For the RIA, I added 20 μl of ^{125}I cortisol tracer and 100 μl of diluted (1:5) antiserum to 20 μl of each of the following: diluted (1:10) kit standards, diluted Bio-Rad Lypocheck control solutions (control I, 1:10; control II, 1:10; control III, 1:20) (Bio-Rad Clinical, Anaheim, California), diluted (1:5) serum samples, and reconstituted faecal samples. After vortexing and subsequent overnight incubation at room temperature, I added 500 μl of diluted (1:5) PEG second antibody, vortexed the solutions, then incubated them for 1 h at room temperature. I then centrifuged (1800 *g*) the solutions at room temperature for 1 h, decanted supernatants and measured radioactivity of the remaining precipitates using 10-min counts in an Apex automatic gamma counter (ICN Biomedicals, Inc., Horsham, Pennsylvania) equipped with AGC plus software (v. 2.06). Standards, controls, serum samples and faecal samples were run in duplicate. Cortisol concentrations are expressed as ng cortisol/g of dry faeces.

To assess the accuracy of the RIA, I divided four dry faecal samples into four 0.2-g portions and added an increasing amount (0, 16, 32, 64 ng) of cortisol to each portion. Spiked portions were then extracted and assayed, and the resulting slope of their curve was 0.99, and the mean (±SD) recovery of the cortisol doses was 112.4±16.9% (*N*=12). I determined the degree of parallelism for the RIA by serially diluting three faecal extracts each four times (1:2–1:16) and then running each dilution through the assay procedure. The slope calculated from each set of dilutions was compared to the slope of the standard curve using Student's *t* tests with the statistical threshold set at 0.05. The dilution slopes did not differ significantly from the standard curve slope. I calculated interassay coefficients of variation from repeated measures of three commercial control solutions (Lypocheck controls I, II, III) diluted with buffer. Interassay coefficients of variation were 6.53% (*N*=7) for the low-cortisol standard, 5.73% (N=5) for the mid-cortisol standard, and 13.84% (N=5) for the high-cortisol standard. Intra-assay coefficients of variation were calculated from four repeated within-assay measures of these control solutions. The mean (±SD) intra-assay coefficient of variation was 6.05±5.18% (N=9). I estimated steroid recovery during extraction by adding a known amount (1200 cpm) of radiolabelled cortisol to each sample prior to the extraction procedure. I assessed recovery of radiolabelled cortisol for 72% of the samples and the mean (±SD) recovery was 88.9±7.5% (N=532).

Statistical Analyses

Mean cortisol levels and behavioural measures were calculated for each female for each week. I used Bartletts' test of homogeneity of variance (Sokal & Rohlf 1995) to determine whether data across weeks/conditions were homoscedastic. Feeding effort estimates were heteroscedastic and transformed using a square-root transformation. Faecal cortisol data were skewed to the right and were transformed using a logarithmic scale. Females in group 2 gave birth to their offspring over a period of approximately 3 weeks, whereas females in group 1 all gave birth within a period of 8 days, during which time no observations were made on them. Because the births in group 2 were spread out over a 3-week period, confounding synchronous environmental conditions were decreased. I compared pre- and postgestation cortisol levels for females in group 2 only. Paired t tests were used to compare mean cortisol levels in group 2 females just prior to and just following parturition. Single factor ANOVAs were used to determine whether behavioural frequencies (antipredatory behaviour, feeding effort) differed across study weeks. A nested ANOVA was used to compare faecal cortisol levels, time of sample collection, and mean weight of samples across weeks. When an ANOVA indicated a significant difference among weeks, I used a Tukey–Kramer multiple comparisons or a Welsch step-up procedure to determine weeks that were significantly different from others (Sokal & Rohlf 1995). Statistical threshold for the Tukey–Kramer and Welsch tests was set at $P<0.05$. A multiple linear regression analysis, including a backward stepwise elimination procedure, was used to assess which of the following variables best accounted for cortisol variability: (1) reproductive status, (2) antipredatory behaviour, (3) feeding effort, (4) faecal sample weights, (5) individual dominance indices and (6) days into the study. I categorized reproductive status simply as gestation ('1') or postparturition ('0').

RESULTS

Gestation and Faecal Cortisol Levels

Cortisol levels in group 2 females just prior to birth were significantly higher than levels just following parturition (paired t test: t_4=2.97, $P<0.05$). Cortisol measures during late gestation were twice as great as levels just after parturition (Fig. 2).

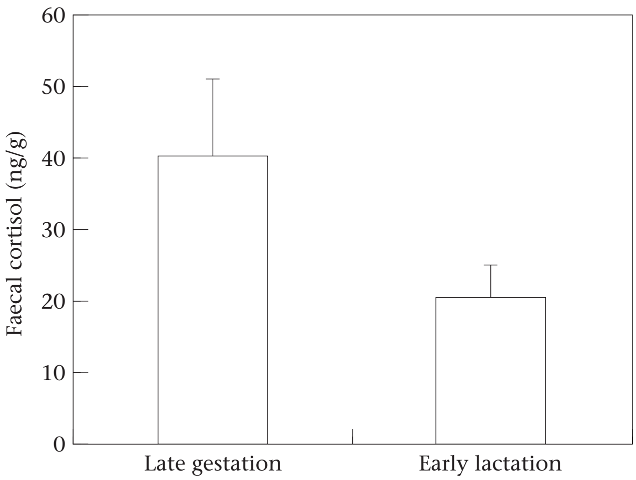

Figure 2. Mean (±SE) faecal cortisol levels for females from group 2 during late gestation and early lactation.

Behavioural Measures

During weeks 5–14, antipredatory behaviour was most frequent between weeks 7–11 (Fig. 3a). The frequency of antipredatory behaviour across weeks differed significantly for group 1 (ANOVA: $F_{4,25}$=2.90, $P<0.05$) but not for group 2 (ANOVA: $F_{4,25}$=0.491, NS). For group 1, antipredatory behaviour was significantly more frequent during weeks 7–11 than during weeks 5 or 13 (Tukey–Kramer multiple comparisons).

Mean feeding effort values calculated for each female for each week indicated a significant change in feeding effort across weeks 5–14 (group 1: ANOVA: $F_{4,20}$=17.21, $P<0.001$; group 2: ANOVA: $F_{4,17}$=14.32, $P<0.001$). For group 1, feeding effort values were greatest during week 11, for group 2, feeding effort was greatest during weeks 8, 10 and 12 (Tukey–Kramer multiple comparison, Fig. 3b). Weeks 9–12 coincide with the end of the dry season, when food availability has been shown to decrease (Sauther 1992).

Agonistic interactions were too infrequent to calculate meaningful weekly dominance indices. Based on all-occurrence observations, individual female dominance status was stable during the study period, and dominance indices were comparable to normally calculated discrete dominance ranks.

Faecal Cortisol Levels

A nested ANOVA indicated that cortisol measures differed significantly across females in one group, and that for both groups, within-female cortisol measures differed significantly across weeks 5–14 (group 1: between-females ANOVA: $F_{4,19}$=15.47, $P<0.001$; within-females, between-weeks ANOVA: $F_{19,191}$=4.47, $P<0.001$; group 2: between-females ANOVA: $F_{4,20}$=2.22, NS; within-females, between-weeks ANOVA: $F_{20,260}$=4.98, $P<0.001$). In general, females had relatively low cortisol levels during weeks 5–8 and elevated levels during weeks 9–12 (Fig. 3c).

Sample collection time did not differ across weeks for any of the females (single factor ANOVAs calculated

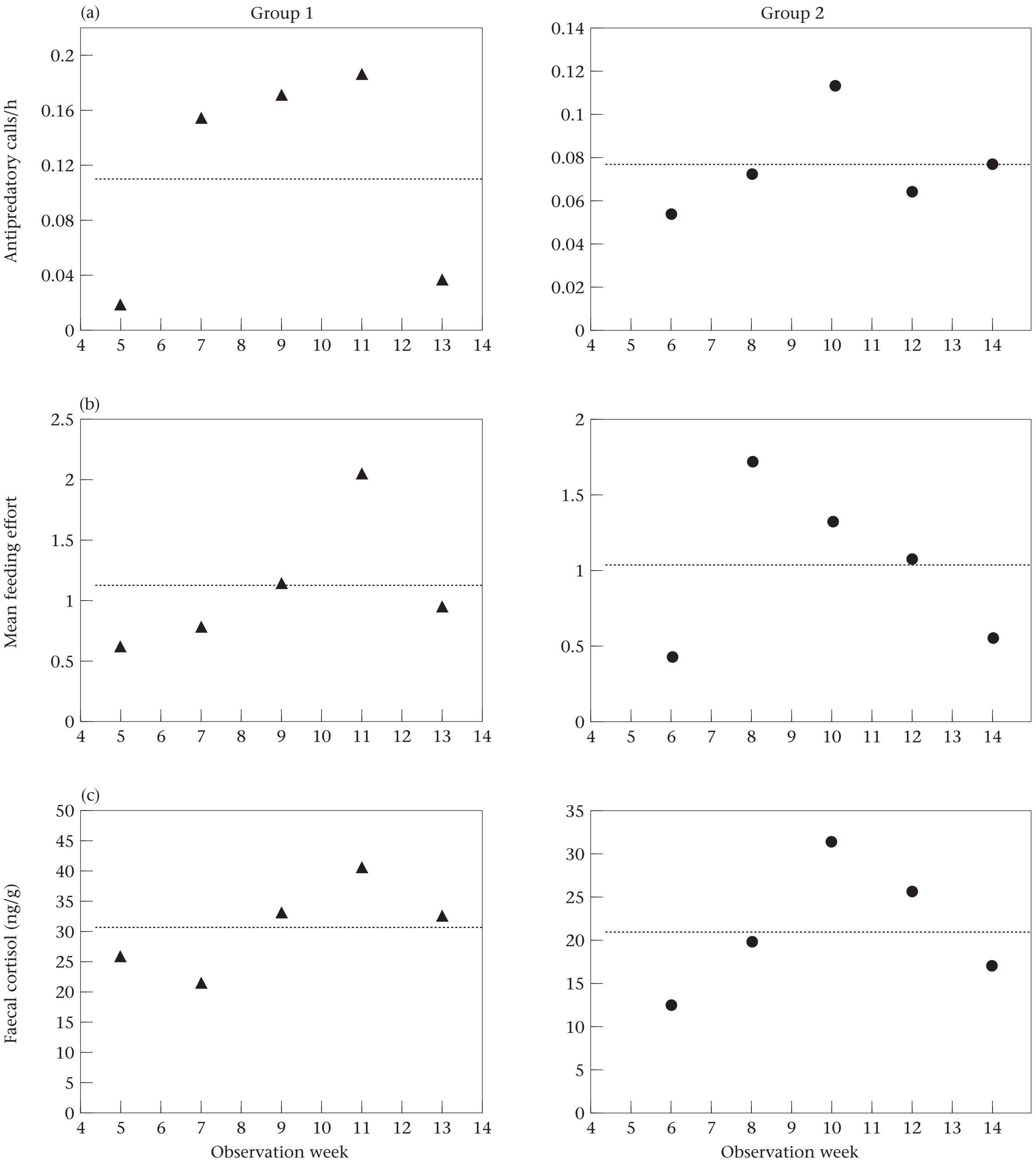

Figure 3. (a) Mean frequency of high-intensity antipredatory vocalizations, (b) mean feeding effort measure, and (c) mean faecal cortisol levels for two groups of female ring-tailed lemurs during nongestational weeks (weeks 5–14). The dashed horizontal lines represent across-week means for each measure.

for each female produced a mean *P* value of 0.355 with no female's data producing a *P* value less than 0.05). In addition, no diurnal cortisol patterns were evident when multiple daily samples from the same female were compared, and there were no significant ($P<0.05$) correlations between cortisol values and time of day for any of the individual female weeks.

Faecal weights differed significantly for eight of 10 females across the 10 weeks (ANOVA: $P<0.05$), with the lowest weights occurring during weeks 9–12. Comparisons of faecal

Table 1. Results of multiple linear regression analyses for each social group

Predictor	Coefficient	*t*	*P*
Group 1			
Reproductive	1.004	4.14	0.0003
Predators	0.839	0.80	0.430
Effort	0.221	1.31	0.204
Faecal weight	0.077	1.71	0.100
Dominance	0.758	4.34	0.0002
Day	0.003	1.02	0.317
Adjusted R^2=0.568, $F_{6,25}$=7.81, P=0.00084			
Group 2			
Reproductive	0.846	3.60	0.001
Predators	0.223	0.13	0.901
Effort	−0.077	−0.27	0.792
Faecal weight	−0.094	−2.66	0.012
Dominance	0.661	3.90	0.001
Day	0.004	1.30	0.204
Adjusted R^2=0.542, $F_{6,30}$=8.11, P=0.00003			

Logarithmically transformed faecal cortisol was the dependent measure, and reproductive status (pregnant, ‘1’, versus postparturition, ‘0’), antipredatory behaviour, effort measures, faecal weight, dominance indices and days into study were the predictor variables.

cortisol values and faecal weights within each female week indicated that eight of 66 female weeks showed a significant (P<0.05) negative correlation between cortisol values and faecal weight. To evaluate further the relative impact of faecal weight on cortisol measures, weight was included as a predictor variable in the multiple regression analysis described below.

In summary, cortisol levels were elevated prior to parturition and again, to a lesser degree, during weeks 9–12. The second peak occurred at the end of the dry period, when antipredatory behaviour and feeding effort measures were elevated (Fig. 3) and faecal weights were low. This weekly cortisol pattern was apparent in all females, including the one female that lost her offspring between weeks 8 and 10.

Faecal Cortisol Levels Related to Behavioural Measures

A multiple linear regression analysis was used to assess the relative relationships between logarithmically transformed faecal cortisol measures and the following variables: reproductive status (pregnant versus postparturition), antipredatory behaviour, feeding effort, faecal weight, dominance indices and days into study. For group 1, 56.8% of cortisol variability could be accounted for when all six predictors were included in the multiple linear regression analysis (ANOVA: $F_{6,25}$=7.81, P=0.0008; see Table 1). A stepwise, backward elimination of predictors with P values greater than 0.10 retained the reproductive status, feeding effort, faecal weight and dominance indices variables, which accounted for 57.6% of the cortisol variability (ANOVA: $F_{4,27}$=11.54, P=0.00001). For group 2, 54.2% of the cortisol variability was accounted for by the six predictor variables (ANOVA: $F_{6,30}$=8.11, P=0.00003; see Table 1). A stepwise, backward elimination procedure retained three of the predictors: reproductive status, dominance indices and faecal weight. Together, these four variables accounted for 55.7% of the cortisol variability (ANOVA: $F_{3,33}$=16.09, P=0.000001). I conducted further regression analyses without the faecal weight variable, because this variable relates to measures of feeding effort and may deflate the predictive value of feeding effort. Significance of variables in the overall or backward stepwise regression analyses for either group did not change substantially when the faecal weight variable was excluded from the regression analysis.

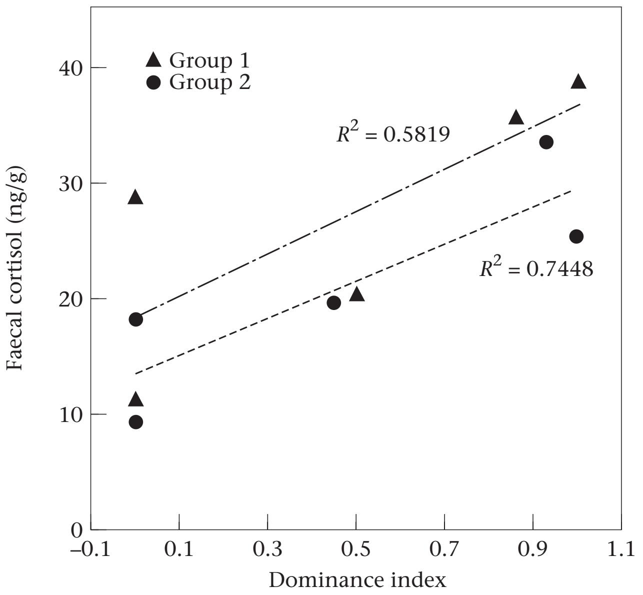

Figure 4. Mean individual faecal cortisol measures related to dominance indices for all females. Females with higher dominance indices had higher cortisol values than females with low dominance indices.

For both groups, reproductive status and dominance indices provided the most reliable predictor of individual weekly faecal cortisol measures, with late gestation (versus postparturition) and increasing dominance indices predicting increased cortisol measures.

Dominance and Cortisol Measures

Ranking females according to their dominance indices yielded the same hierarchy as did a more traditional matrix estimation of dominance ranks. The female with the highest index elicited submissive signals from all other females in her group, the female with the second highest index elicited submissive signals from all but the highest-ranking female, and so forth. Individual dominance indices were significantly correlated with mean faecal cortisol measures (r=0.76, P<0.05; Fig. 4). Females with the highest dominance indices had the highest faecal cortisol measures.

Dominance indices were also significantly correlated with rates of initiating aggression (r=0.86, P<0.01) and feeding efforts (r=0.65, P<0.05) among females. The correlation coefficients between rates of initiating aggression and faecal cortisol measures (r=0.60, P<0.10) and feeding effort estimates and cortisol measures (r=0.62, P<0.10) were moderately high but not statistically significant. These relationships may begin to explain why high-index females had high faecal cortisol values; high-index females appear to expend more effort on initiating aggressive interactions and on feeding, and this increased energy expenditure may explain their elevated cortisol levels (e.g. Borer et al. 1992).

DISCUSSION

Faecal cortisol levels measured in free-ranging female ring-tailed lemurs during this 14-week study coincide with current beliefs that predation threat, decreased food accessibility and social status can present significant challenges to free-ranging animals. During the latter half of 1995, free-ranging female ring-tailed lemurs at Beza Mahafaly Special Reserve experienced two periods of elevated faecal cortisol levels: one period at the end of gestation and a second period during the end of the dry season, when behavioural repertoires indicated decreased food accessibility and increased predation risk. In addition, dominant females had greater mean faecal cortisol levels than their subordinate group mates. These results support the initial predictions of the study to varying degrees. The relationship between predation threat and cortisol was very weak. There was a weak relationship between food accessibility and cortisol. Finally, the relationship between individual dominance status and cortisol levels was very strong.

The prediction that cortisol levels would be elevated during periods of increased antipredatory behaviour was not well supported. Cortisol levels were elevated during weeks when antipredatory behaviour was elevated, but rates of antipredatory behaviour did not provide a strong predictor of cortisol variability. Lack of predictive power could suggest that predators do not present a significant challenge to lemurs or that threats of predation are predictable and/or some how controllable and therefore not stressful for female ring-tailed lemurs. Alternatively, the lack of correspondence may suggest that antipredatory behaviour either does not reflect predation risk or is not a linear predictor of risk. In other words, when predation risk is either very high or very low, antipredatory behaviour may be minimal, and only when risk is moderately high (e.g. predators are common but distant), would antipredatory behaviour be highest as a means of warding off predators. If this is the case, then rates of antipredatory behaviour may not be the best behavioural estimate of predation risk, and therefore would not provide a reliable, linear predictor of cortisol levels. Another possibility is that predation risk is a predictable and relatively controllable threat and therefore does not predict a stress response.

The prediction that cortisol would be elevated when food accessibility was low was not supported. At the end of the annual dry season, when active foraging behaviour increased, there was a measurable rise in faecal cortisol levels, but the feeding effort variable provided a reliable predictor of cortisol variability for only one of the two study groups. This weak relationship between feeding effort and cortisol may indicate that decreased food accessibility at the end of the dry season did not pose a significant challenge. Perhaps the annual decrease in food accessibility is predictable, and therefore lemurs can prepare for the decrease and thereby minimize the challenge. The fact that rainfall levels in 1995 at Beza Mahafaly were similar to mean rainfall levels reported by Sauther (1992) in 1987 and 1988 at Beza provide some support for this notion (comparison of 1995 and 1987/1988: 9 versus 12 mm in October, 41 versus 50 mm in November, and 73 versus 85 mm in December).

The fact that feeding effort was a more significant predictor of cortisol variability in the stepwise regression for group 1 than for group 2, may indicate that the two study groups were differentially sensitive to the stimuli in their environment. Group 1, for which the feeding effort was a significant predictor, lived in the western, drier section of the Beza Mahafaly reserve and spent more time in feeding-related activities relative to group 2, which lived closer to the river bed in a more lush environment. Food access may be a more important challenge to the females in group 1 situated in a drier forest area.

Apart from food scarcity, another possible explanation for the elevation in cortisol levels at the end of the dry season may be changes in female physiological state associated with lactation or rearing of offspring. Because all females gave birth within a 3-week period, changing lactational demands were experienced synchronously, as were changing environmental conditions. Furthermore, because all females in this study gave birth to and lactated for one offspring, the influence of postpartum reproductive demands on cortisol levels are difficult to assess here. However, one female that lost her offspring after observation week 8 showed elevated levels of cortisol during weeks 10 and 12, similar to that of continually lactating females. This elevation occurred 2–4 weeks after the female was last seen feeding her offspring. These results are difficult to interpret because there may be a confound from stressors associated with the loss of an infant.

Reproductive status and dominance indices were significant predictors of cortisol variability in the female ring-tails. Cortisol levels during late gestation were two to three times higher than those during early gestation. The magnitude of this difference is comparable to that seen in other species (e.g. Kriesten & Murawski 1988; Allolio et al. 1990; Lockwood et al. 1996), and this replication supports the applicability of Wasser et al.'s (1994) faecal-cortisol extraction method for assessing cortisol levels in ring-tailed lemur females and possibly other free-ranging mammalian species.

The prediction that cortisol levels would be related to dominance status was strongly supported. Dominance indices were a reliable predictor of cortisol variability. High-index females had high mean faecal cortisol levels and low-index females had low levels. These findings coincide with Creel et al.'s (1996, 1997) findings with female African wild dogs, although the social structure of ring-tailed lemurs and African wild dogs present some clear differences (e.g. male versus female dispersal, individual versus cooperative breeding, etc.). The results from the present study differ from those reported for free-ranging male baboons, where high-ranking individuals tend to have the lowest serum cortisol levels of a troop (Sapolsky 1982). These differences may mirror differences in social organization between these species or may suggest that dominance presents two different sets of circumstances for male and female social animals. Alternatively, high rates of aggression, associated with high rank in African wild dog and ring-tailed lemur females, may account for elevated cortisol levels in high rankers (Creel et al. 1996). High-ranking male baboons are not necessarily the most aggressive males in a group (Sapolsky 1983), which may explain the discrepancy between female lemurs and male baboons. Another complicating factor in the relationship between dominance and cortisol levels is that the females in this study may have been experiencing a period of social instability, which has been identified as a period when high rankers may show higher cortisol titres (e.g. Sapolsky 1983). Recently analysed data from three stable social groups at Berenty Private Reserve in southeastern Madagascar indicate that results similar to those of this study from Beza Mahafaly also exist in the stable social groups at Berenty (S. Cavigelli, W. Levash, T. Dubovick & A. Jolly, unpublished data).

Finally, dominance ranks and indices correlate with a number of behavioural categories, which may best explain the relationship between cortisol and dominance. Glucocorticoid levels, and other measures of physiological stress, may relate best to factors other than, but associated with, dominance measures; for example, age, rate of being attacked or attacking, quality of diet, differential costs of reproduction, and so forth (e.g. Bernstein 1981; Creel et al. 1996; de Villiers et al. 1997). More information is needed on the relations among dominance status, social behaviour profiles and glucocorticoid levels, as well as other measures of physiological stress, in both male and female free-ranging animals.

The results of the multiple regression analyses indicate that dry faecal weight should be taken into account when interpreting faecal cortisol measures. However, the relationship between weight and cortisol concentrations is not clear from the data presented here. For one group, the relationship was negative (i.e. as faecal weight increased, cortisol measures decreased), and for the second group, the relationship was positive. Future resolution of this relationship will strengthen the faecal glucocorticoid measure. Results from this study also suggest that time of day does not significantly affect faecal cortisol measures even though the circadian pattern in glucocorticoid secretion has been clearly documented in other primate species (Wiebe et al. 1984; Allolio et al. 1990; Czekala et al. 1994; Coe & Levine 1995). A lack of diurnal variation may result from the fact that faeces accumulate hormones over several hours and therefore mute hormonal peaks and troughs. Again, such issues should be addressed in further faecal steroid analyses.

This study presents a preliminary application of a faecal steroid measure to assess cortisol levels in free-ranging primates and to determine the context for elevated glucocorticoid excretion. The results of the faecal cortisol analyses are consistent with ecological theories suggesting that predation threat and food accessibility pose significant challenges to animals in the natural habitat. However, these two variables were less reliable predictors of cortisol variability in this study than were reproductive state and dominance indices. The lack of predictive power in the predation threat and feeding effort measures may reflect the fact that these variables fluctuated in a predictable fashion and were therefore a negligible challenge relative to other unpredictable fluctuations. Most organisms encounter regular (hourly, seasonal, etc.) fluctuations in environmental conditions, and some may very well be able to predict such fluctuations and prepare for change. It is the unpredictable and/or uncontrollable situations that must present the greatest challenge to free-ranging animals. Measures of challenging stimuli in the natural habitat must include some aspect of stimulus predictability and/or controllability. The findings of this study, in concert with findings from other research studies, encourage further exploration of circannual and individual differences in faecal glucocorticoid patterns in free-ranging animals. Successful application of this method could open a new arena of research that can provide insight into inter- and intraspecific differences in glucocorticoid responses to naturalistic environmental conditions.

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