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FECAL CORTISOL LEVELS RELATIVE TO BEHAVIOR IN A FREE-RANGING PRIMATE: ADAPTIVE ASPECTS OF A GLUCOCORTICOID HORMONE

by

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Date: 30 November 1998

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Psychology in the Graduate School of Duke University

1998

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ABSTRACT

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ABSTRACT

Release of glucocorticoid hormones into the bloodstream is one of the many responses subsumed within the classic description of physiological stress. Glucocorticoids facilitate mobilization of stored energy into useable energy (glucose, fatty acids, and amino acids). By monitoring these hormones in animals in their natural habitat, we can gain insight into the conditions under which their secretion may have evolved to better understand their adaptive function. Until present, such research has been difficult to conduct because the manipulation required for a traditional blood measure itself affects glucocorticoid secretion. The research described here employed a non-invasive method to measure a specific glucocorticoid, cortisol, in the feces of free-ranging social primates.

The subjects chosen for this work -- female ringtailed lemurs (Lemur catta) -- are thought to experience significant nutritional stress in their dry forest habitat. To identify conditions under which elevated cortisol levels occur in the natural habitat, fecal cortisol levels were monitored and related to three behavioral measures (feeding effort, anti-predatory behavior, and dominance status) in adult females living in southwestern Madagascar (Beza Mahafaly Special Reserve). Cortisol levels were elevated during the end of the dry season when anti-predatory behavior and feeding efforts were highest. The most reliable finding was that dominant females exhibited significantly higher mean cortisol levels than did subordinate females.

To determine how decreased food accessibility may affect cortisol excretion in these females, manipulations of food accessibility were conducted with forest-dwelling ringtailed lemurs at the Duke University Primate Center in Durham. North Carolina. In these trials, as food became less accessible and females put more effort into feeding, fecal cortisol levels decreased. When these experimental results were analyzed with the Madagascar results the relationship between feeding effort and fecal cortisol levels appeared non-linear suggesting that cortisol secretion may be most beneficial under certain conditions relating to present and possible future energy sources available to an organism.

The results of these studies suggest that glucocorticoid hormones may be secreted in free-ranging animals as a strategy to survive periods of low environmental resources and that individual differences in glucocorticoid excretion may reflect alternative strategies for living in a dynamic habitat.

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beyond this door. The sense of accomplishment derived from identifying and interpreting a novel phenomenon in nature becomes enormous within this context. In addition, thank you Carl for helping me improve my writing by enduring and critiquing many written products.

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The Duke University Primate Center, where I have conducted the majority of my research, is a unique facility, not only in its intriguing collection of prosimian primates, but more importantly in its outstanding collection of top-notch animal caretakers. The primate center rivals all other centers in their maintenance of semi-free-ranging lemur populations for scientific research. These forest reserves provided stomping grounds for me to hone my sensory capabilities to observe fine details of animal behavior and to develop observational protocols that would carry me through some trying field work. I cannot thank David Brewer enough for his countless hours of assistance and for his interest in research and for curious conversations about the lemurs in the natural habitat enclosures. Bill Hess provided more help than he had the time to commit. Although results of the work we conducted together do not show up in this dissertation, I want to thank Bill for all the help with the unpleasant task of lemur leg-holding and "swabbing". And thanks to both David and Bill for restraining so many lemurid, teeth-baring heads for crucial sample collection protocols. May your immune systems function all the better for these trying experiences.

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CHAPTER 1

GLUCOCORTICOIDS IN THE NATURAL HABITAT

Animals in their natural habitat must accommodate their behavior to dynamic stimuli in their environment such as changing food availability, changing degrees of vulnerability to predation, and changing social relationships. The underlying physiological mechanisms that allow for such behavioral flexibility in the natural habitat are not well understood. The physiological stress response, as originally described by Selye (1936), seems to have evolved as a complex set of mechanisms that prepare an organism to respond to challenging or changing environmental conditions. One of the many physiological responses included under the guise of the stress response is secretion of glucocorticoid hormones which facilitate energy mobilization. By understanding glucocorticoid secretion patterns in free-ranging organisms we can gain insight about the conditions under which catabolic processes are stimulated in the natural habitat and how this may enable organisms to accomodate to dynamic environmental conditions.

Stress physiology

The concept of "stress", whether qualified as "psychological" or "physiological", is often cast in a negative light (e.g. Uno *et al.* 1994, Chan *et al.* 1996, Landfield *et al.* 1996). In the present work, I will focus on the potentially adaptive aspects of one part of the physiological stress response: glucocorticoid secretion. The classic description of the physiological stress response involves neurological, hormonal and immunological responses that, in general, promote energy mobilization and facilitate effective behavioral responding to challenges. Some of the benefits of the complex of responses include heightened attention and memory (McEwen & Sapolsky 1995), elevated blood sugar levels (Sapolsky 1992), and a redistribution of leukocytes to peripheral areas of the body (Dhabhar 1998). Negative aspects of the stress response (e.g. cell death) are seen primarily when the response becomes chronic or long-lasting.

Hormonal aspects of the stress response, which involve the hypothalamic-pituitary-adrenal (HPA) axis, includes two major classes of hormones: catecholamines (e.g. epinephrine) and glucocorticoids (e.g. cortisol). At the onset of a stress response, the sympathetic branch of the autonomic nervous system is activated, stimulating release of epinephrine from the adrenal medulla. Epinephrine stimulates responses such as increased heart beat, constriction of blood vessels, and increased breathing rate. The hypothalamus is also responsible for stimulating adrenocorticotropic hormone (ACTH) release from the pituitary gland, which then stimulates release of glucocorticoid hormones from the adrenal cortex into the blood stream within minutes of catecholamine release. Glucocorticoids inhibit energy storage and stimulate breakdown of complex energy substances (triglycerides, proteins and glycogen) into more simple, useable energy substances (fatty acids, amino acids and glucose) (review in Sapolsky 1992). Catecholamine release is relatively rapid and short-lived (seconds to minutes); glucocorticoid release is slower and longer-lasting (minutes to hours).

Glucocorticoids

Glucocorticoid hormones are steroid hormones and include specific hormones such as cortisol and corticosterone. The major function of glucocorticoids is to stimulate mobilization of complex energy units, particularly glycogen stored in muscle and liver, into simple energy forms, like glucose, for use throughout the body. Other responses that occur during a physiological stress response complement this catabolic glucocorticoid function. For example, insulin release, which promotes energy storage, is inhibited when glucocorticoid secretion is stimulated. The complement of increased glucocorticoids and decreased insulin in circulation promotes an increase in catabolic activity. The amount of glucocorticoids in the blood stream provides an index of the energetic state of an animal, e.g. catabolic versus anabolic. This information provides insight into how animals respond to changing environmental conditions.

Blood samples for circulating glucocorticoid measures are difficult to collect from free-ranging animals and, in addition, the measure can be compromised by blood sampling 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). For these reasons, studies conducted on glucocorticoid secretion in free-ranging animals are limited. New methods being developed to measure steroid hormones in feces have provided a new means to assess this response in the natural habitat. Studies with bighorn sheep, cats, and chimpanzees have shown that glucocorticoid hormones are detectable in feces and that the method could be used with free-ranging populations (Miller *et al.* 1991, Graham & Brown 1996, Whitten *et al.* 1998). This thesis made use of these new techniques to study glucocorticoid excretion in a free-ranging primate that lives in a highly seasonal, often thought to be rather challenging, habitat in Madagascar. The fecal cortisol measure provided the following two advantages over blood measures: (1) fecal measures could be collected without disturbing either the animals or their environment, and (2) the measure could be easily collected from multiple animals within a study group at the same time of day and on a daily basis.

Study species

I chose to study cortisol excretion in female ringtailed lemurs (Lemur catta) for several reasons. The most important reason is that ringtailed lemurs live in dry forest habitats and the females are thought to experience significant nutritional stress because they lactate at the end of the dry season when food availability is limited (Jolly 1984, Richard & Dewar 1991, c.f. Kappeler 1996). These females provide an ideal subject for some of the first investigations of fecal cortisol levels in free-ranging animals. In addition, ringtailed lemurs, being an early evolved primate with large social groups and a high degree of olfactory communication, resemble in some ways, both primates and rodents, two of the more well-studied species in physiological stress research. Also, ringtails are a model study species in that their behavior facilitates ample and reliable data collection: (1) the lemurs are rather terrestrial so visibility is good for behavioral observations, (2) the lemurs are social animals that live in large groups and engage in frequent social interactions, (3) these interactions and the lemurs' behavior have been well-described by many researchers, and (4) the lemurs defecate close to the ground so that entire fecal samples can be collected. In addition, the lemurs' behavior and ecology have been well-studied in two reserves in Madagascar (Jolly 1966, Budnitz & Dainis 1975, Jolly *et al.* 1982, Sauther 1989, 1991, Jolly *et al.* 1993, Sauther & Sussman 1993) so that much longitudinal background data exist for this species in the natural habitat. And finally, several well-established, well-studied ringtail social groups have been living in naturalistic enclosures at the Duke University Primate Center (DUPC) for the past 12 years (Macedonia 1988, Pereira & Izard 1989, Kappeler 1993, Pereira & Kappeler 1997) which provides an arena for experimental hypothesis testing within a naturalistic setting.

Specific goals

The specific goals of this research were to determine under what conditions cortisol levels are elevated in social primates in naturalistic conditions. Observational and experimental studies were conducted to determine conditions associated with increased cortisol excretion. Because cortisol secretion promotes catabolic processes, we might presume that cortisol levels should increase in such a way as to activate catabolic processes when resources are scarce but that some moderation of this response is required if organisms are to survive long-term periods of decreased resource availability. Glucocorticoid secretion strategies provide a window into adaptive strategies used by organisms to survive in changing environments.

In Chapter 2, I review how a fecal steroid extraction method, originally developed by Wasser and colleagues (1994), was tested for use with ringtailed lemurs. Chapter 3 involves the application of this tested method with free-ranging adult female ringtailed lemurs in southwestern Madagascar during a five-month period. Results of this field work indicate that female ringtailed lemurs had significantly elevated cortisol levels at the end of the dry season, when food accessibility decreased and when the lemurs' increased their frequency of anti-predatory behavior. The other pattern to emerge from this field study was that mean cortisol levels were significantly higher in the higher-ranking females than in the lower-ranking females.

To determine whether decreased food accessibility and the accompanying increase in feeding effort was responsible for elevated cortisol levels, I conducted experimental manipulations of food accessibility with semi-free-ranging female ringtailed lemurs at the Duke University Primate Center (Chapter 4). The results from these experiments, coupled with the Madagascar results, suggest that cortisol levels do not simply increase as food becomes less accessible and animals spend more effort feeding. Rather, glucocorticoid secretion may occur only under specific conditions that involve the relationship among the various energy sources available to an organism.

To explore the dominance-related individual differences in mean cortisol excretion, I examined how cortisol levels related to individual social repertoires in 37 females across eight social groups (Chapter 5). The results of this work suggest that specific behavioral traits may account for the relationship often found between dominance status and cortisol secretion in social animals.

Findings from this work indicate that the physiological stress process may occur differently in animals living in their natural habitat than it does in caged animals where the response is most commonly studied. Studies of glucocorticoid production in free-ranging animals provide a means to assess the adaptive aspect of this response and to determine the complex set of stimuli that may elucidate the response. Species, group, and individual differences in cortisol excretion patterns suggest differential adaptive strategies for responding to challenging and changing environmental conditions in the natural habitat.

CHAPTER 2

TEST OF FECAL CORTISOL MEASURE WITH RINGTAILED LEMURS

Field biologists have shown increasing interest in the measurement of steroid levels in feces particularly those researchers interested in understanding the reproductive physiology of free-ranging animal populations. The methods for extracting steroids from feces have received both praise and criticism, as would be expected for methods still undergoing refinement. In this chapter I will review the advantages and disadvantages of fecal steroid extraction methods, and then I will present analyses conducted to determine whether the method can be used to estimate glucocorticoid production in ringtailed lemurs.

Advantages and disadvantages of fecal extraction methods

The major advantage of fecal steroid methods over the classic blood measures is the non-invasive nature of fecal measures. This advantage is clearest when glucocorticoid levels are measured. These hormones are sensitive to capture stress and can increase in the blood stream within the brief interval between capture and venipuncture. Another advantage of the fecal measure is that it allows for frequent and repeated sampling from the same individual over time without disrupting behavior and physiology. Another benefit is that fecal measures reflect blood steroid levels averaged over a relatively long period (hours). For example, an animal that defecates once a day will have in its feces an average steroid level from the entire previous day. This fecal measure cannot reflect the pulsatile nature of steroid secretion into the bloodstream (e.g. peaks every 20 minutes) nor the diurnal variations that occur in circulating steroid levels (e.g. increased glucocorticoid circulation just prior to daily activity bouts in many species). This averaging effect of fecal steroid measures can be an advantage for researchers interested in mean daily or seasonal steroid levels. In contrast, single serum samples can be highly variable from one moment to the next particularly if the samples are collected either at a peak or a trough in secretion pulses.

One of the disadvantages associated with the method is that short-term increases in circulating steroids can probably not be readily identified. This makes it difficult to assess cortisol responsiveness of an individual, which is often a distinguishing characteristic across individuals or within individuals across time. That is, two animals may display similar basal cortisol levels, but when challenged, one animal may display a faster and greater increase in cortisol levels, which is often interpreted as an increased capacity to respond to a challenge (Sapolsky 1982, Astheimer *et al.* 1995). In addition, the interval between a significant change in circulating steroid levels and a subsequent change in fecal steroid levels has not been established clearly. In fact, this time delay may differ among species and among individuals (e.g. Graham & Brown 1996). Another disadvantage to the method, as it stands, is that temporal patterns of steroid secretion cannot be estimated from fecal measures. In other words, we cannot tell whether a fecal sample of moderately high steroid hormone concentration reflects that the animal in question has secreted multiple and/or concentrated pulses of steroids into their blood or has secreted moderately high levels of steroid into the blood over a relatively long period. This is an important distinction as acute versus chronic secretion patterns may reflect two very different strategies with two different effects. In sum, the fecal steroid measure is probably best suited for field endocrinology in which researchers are most interested in seasonal changes in steroid metabolism.

Selection of fecal extraction method

Samuel Wasser at the University of Washington (Seattle) has done extensive work fine-tuning methods to analyze sex steroids in feces (Risler *et al.* 1987, Wasser *et al.* 1988, 1991, 1993, 1994, 1996, Brown *et al.* 1994), and recently he has been developing methods to analyze fecal glucocorticoids specifically. The validation methods he has used and published have been extremely comprehensive. For this reason, his most recent method of steroid extraction (Wasser *et al.* 1994) was tested with ringtailed lemurs.

METHODS

Pilot test

Using the 1994 method in a pilot test with ringtailed lemurs at the Duke University Primate Center, I was able to detect significant elevations in fecal cortisol levels in males on mating days. Mating days are extremely chaotic and appear energy consuming for males as they fight for access to a single estrous female, and one would expect some elevation in cortisol levels on these days. These initial results led me to run some more accurate tests of the fecal cortisol measure.

Comparison of fecal and serum cortisol

To determine the applicability of Wasser *et al.*'s (1994) fecal steroid extraction procedure to assess cortisol in ringtailed lemur feces, I compared individual lemur cortisol measures taken from fecal and serum samples. Blood samples and feces were collected from ringtailed lemurs at the Duke University Primate Center, each analyzed for cortisol concentration (using the RIA procedure described below), and then compared. To collect samples, semi-free-ranging lemurs were corralled into a holding cage within their normal forest enclosure and then caught and bled within the following 60 minutes. Prior to this protocol, these lemurs had been corralled monthly, and always given food rewards. Occasionally corralling was conducted with a food reward and without further manipulation, followed by release within one hour.

Lemurs detained in these holding cages were relatively calm and occupied eating supplied food. Upon arrival of an animal technician with a white capture net, the lemurs immediately changed their behavioral activity. Feeding stopped, movement throughout the cage increased as animals searched for a cage corner in which to hide. Based on this change in behavior, and because the white nets are thought to signal capture and handling for these animals, "capture onset" was defined as the arrival of technicians with nets at the holding cage. The interval between capture onset and venipuncture of each lemur was recorded to later determine and control for the effects of capture stress on cortisol levels. Using this corralling protocol, two to three ml of blood were collected from the femoral vein of 24 individuals. Fecal samples were collected during the corralling and bleeding procedure. Blood was collected into Vacutainer SST tubes (Becton Dickinson, Franklin Lakes, NJ), allowed to clot for approximately 45 minutes, then centrifuged at 1200*g* for 20 minutes. The resulting serum supernatant was decanted and stored at -80° C until analyzed. Fecal samples were collected and individually stored in Whirl-Pak bags (Nasco, Fort Atkinson, WI) at -20° C until analyzed.

Fecal cortisol extraction method

Fecal samples were dried in a centrifugal evaporator (Savant, Farmingdale, NY) and 0.2 g of dry dust-like fecal matter was extracted; seeds and fibrous material were avoided. Drying fecal samples has been shown to help control for effects of dietary fiber on fecal steroid concentrations in baboons (Wasser *et al.* 1993). Cortisol was extracted according to the "short method" of fecal sex steroid extraction described by Wasser *et al.* (1994). For the boiling step of the procedure, I used 95% ethanol.

Fecal cortisol measure

Cortisol concentration of fecal extracts were analyzed using a radioimmunoassay (RIA) protocol developed in the Laboratory of Comparative Human Biology at Emory University (J. Stallings, pers. comm.). The protocol involved modifications to a commercially available RIA kit designed for human serum cortisol analyses (Pantex CORTISOL (CORT) ^{125}I, Santa Monica, CA). 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-estradiol, estriol, estrone, and testosterone. Prior to assay analysis, I dried fecal extracts and reconstituted them in a buffer solution made of 0.1% bovine serum albumin (BSA) in phosphate buffer saline (pH 7.4). The same buffer was used to dilute kit standards, antibodies, controls and serum samples. For the RIA, 20 μl ^{125}I cortisol tracer and 100μl of diluted (1:5) antiserum were added 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, CA), diluted (1:5) serum samples, and reconstituted fecal samples. After vortexing and subsequent overnight incubation at room temperature, I added 500 µl of diluted (1:5) PEG second antibody, vortexed the solutions, and incubated them for one hour at room temperature. Solutions were then centrifuged (1800*g*) at room temperature for one hour, supernatants decanted, and radioactivity of the remaining precipitates measured using ten minute counts in an Apex automatic gamma counter (ICN Biomedicals, Inc., Horsham, PA) using AGC plus software (v. 2.06). Standards, controls, serum samples and fecal samples were run in duplicate. Cortisol concentrations are expressed as ng of cortisol/g of dry feces.

Tests of cortisol assay precision

To assess the accuracy of the RIA, I divided four dry fecal 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 extracted and assayed and the slope of their curve was calculated along with the percent recovery of the added cortisol. The degree of parallelism for the RIA was determined by serially diluting three fecal 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. To determine the degree to which two separate assays produce similar results, inter-assay coefficients of variation were calculated from repeated measures of three commercial control solutions (Lypocheck controls I. II, III) diluted with buffer. Alternatively, to compare the replicability of measures within an assay, intra-assay coefficients of variation were calculated from four within-assay repeated measures of the control solutions. Finally, steroid recovery during extraction was estimated by adding a known amount (approx. 1200 cpm) of radiolabeled cortisol to samples prior to the extraction procedure and then counting the radioactivity of extracts to determine how much of the radiolabeled cortisol had been recovered after the extraction process. This recovery procedure was conducted with over 500 samples.

Statistical analyses

Serum cortisol values for each lemur were compared to cortisol levels found in the extract of their feces collected during the blood collection procedure. Since serum cortisol levels can increase within minutes of encounter with a stressor such as capture (Sapolsky 1992), I plotted individual serum cortisol concentrations relative to the time from capture onset to the time of blood collection. Based on this scatterplot, I estimated a point at which circulating cortisol levels appeared reliably elevated, and compared cortisol concentrations in serum samples collected prior to and following this point using a Student's *t*-test (assuming unequal variances). If this comparison showed a significant increase in serum cortisol levels after the selected time delay, all samples collected beyond that point were eliminated from the comparison of individual serum and fecal cortisol concentrations. Two correlational analyses were conducted to compare serum and fecal cortisol levels. One was conducted only with those individuals for whom serum samples were collected prior to the time delay determined by the previous analysis. The second correlational analysis was conducted with only those animals from whom serum was collected in less than nine minutes from capture onset. This nine minute window was selected based on Sapolsky's (1982) conclusion from studies with baboons -- i.e. that blood samples collected within nine minutes of darting for blood collection probably represent near-basal cortisol levels in this primate.

RESULTS

Precision of cortisol assay

The slope of the curve determined from the four spiked fecal samples was 0.99 and the mean (± SD) recovery of the cortisol doses was 112.4 ± 16.9% ($N = 12$). Slopes calculated from serial dilutions were not significantly different from the standard curve slope. Inter-assay 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. The mean (± SD) intra-assay coefficient of variation was 6.05 ± 5.18% ($N = 9$). Mean (± SD) recovery of radiolabeled cortisol was 88.9 ± 7.5% ($N = 532$).

Serum cortisol levels relative to capture onset time

Serum cortisol levels increased significantly after 16 minutes from the onset of animal capture ($t_{20} = 3.56$, $P < 0.01$; Fig. 2-1), a time delay comparable to that seen in baboons (Sapolsky 1982).

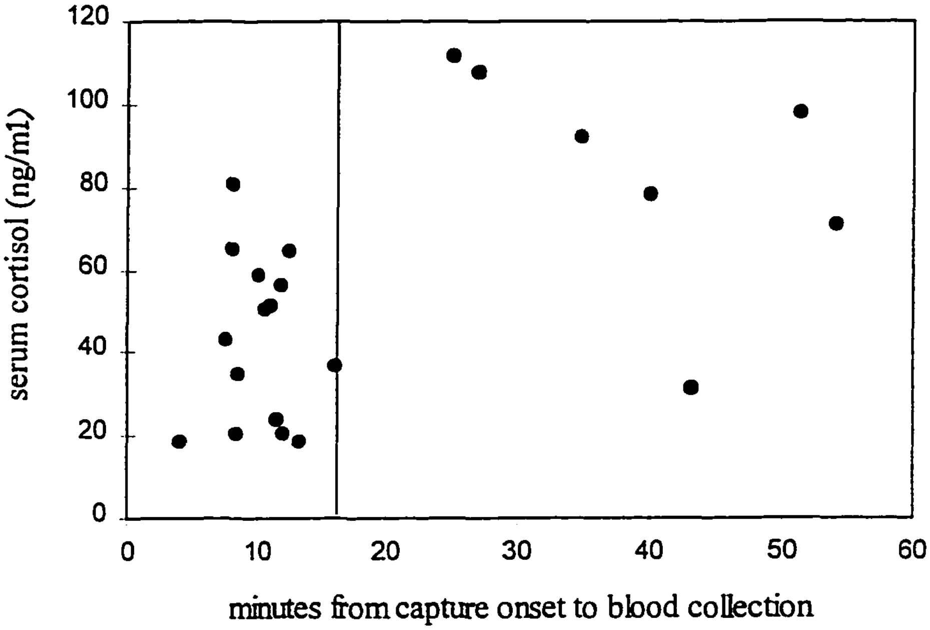

Figure 2-1. Serum cortisol levels relative to the time interval between capture onset and venipuncture for adult ringtailed lemurs. Each point represents one individual. The dashed line indicates the 16-minute mark.

Of the 15 animals for whom serum samples were collected within 16 minutes of capture onset, 13 produced fecal samples for comparison of fecal and serum cortisol measures. Serum and fecal cortisol levels for these 13 individuals were not statistically significantly correlated ($r = 0.51$, $N = 13$, $P < 0.10$; Fig. 2-2). However, when this analysis was limited to those serum samples collected within nine minutes of capture onset, serum and fecal cortisol concentrations were significantly positively correlated ($r = 0.97$, $N = 5$, $P < 0.01$; filled circles in Fig. 2-2). Of the eight serum samples collected between nine and 16 minutes (open circles in Fig. 2-2), six fell below the regression line calculated from the five samples collected within nine minutes of capture onset. This suggests that serum samples collected after nine minutes of capture onset may have included elevated cortisol levels relating to capture stress, and the elevation in serum cortisol levels could not have been reflected in feces collected at the same time as blood.

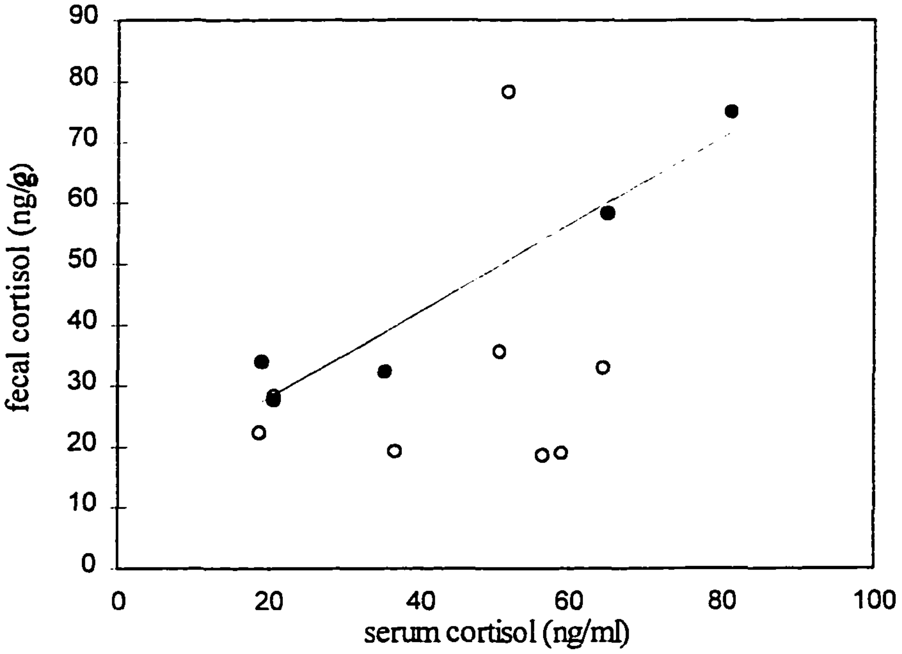

Figure 2-2. Fecal cortisol relative to serum cortisol concentrations in 13 adult ringtailed lemurs. Each point represents one individual. All blood samples were collected within 16 minutes of capture onset. The closed circles represent samples collected within nine minutes of capture onset. The regression line was calculated from the five individuals from whom blood was collected within nine minutes.

DISCUSSION

Within minutes of capture onset, a significant increase in serum cortisol concentrations occurred for adult ringtailed lemurs at the DUPC. The increase in circulating cortisol levels was similar to that seen in other species, in which an animal is exposed to either an acute (10-min) or chronic (1-h) stressor stimulus (e.g. electric shock, public speaking, darting, physical restraint, etc.) and blood or salivary glucocorticoid levels are significantly elevated within 10 to 15 minutes of stressor onset (e.g. olive baboons: Sapolsky 1982; Lapland longspurs: Astheimer *et al.* 1992; humans: Kirschbaum *et al.* 1992). These similarities indicate that the radioimmunoassay protocol used to analyze cortisol levels in the ringtailed lemurs is reliable. In addition to these findings, results from the tests of the cortisol assay precision indicate that the assay measures cortisol in serum and fecal extracts.

Ringtailed serum and fecal cortisol levels were significantly and positively correlated when these comparisons were limited to serum samples collected within nine minutes of capture onset. This correlation provides support for the use of the fecal cortisol method with free-ranging ringtailed lemurs. The results of these tests provide some of the first comparisons of serum and fecal glucocorticoid levels in animals. Only three other studies have reported measuring cortisol in feces, and only one of them has compared individual circulating and fecal cortisol levels (Miller *et al.*

1991, Graham & Brown 1996, Whitten *et al.* 1998). In the following chapters, I will describe how this assay method was used to assess cortisol levels, both in free-ranging ringtailed lemurs in Madagascar and in semi-free-ranging captive ringtails at the Duke University Primate Center (DUPC). The method allows for identification of seasonal changes and individual variability in cortisol production. Such variability in fecal cortisol levels enables researchers to identify possible environmental and behavioral conditions associated with increased HPA axis activity, to identify the challenges animals face in the natural habitat, to determine the functional significance of glucocorticoid secretion, and the ways in which this response promotes accomodation to and survival within changing environmental conditions. The main limitation to the method is that the dynamics of a cortisol response to a specific stimulus cannot be assessed.

CHAPTER 3

BEHAVIOR ASSOCIATED WITH FECAL CORTISOL LEVELS IN THE NATURAL HABITAT

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). Laboratory studies (Weiss 1970, Hanson *et al.* 1976) and recent reports by Wingfield and colleagues (e.g. Wingfield & Ramenofsky 1997) emphasize that the "predictability" or "controllability" of stimuli are the prime characteristics that determine the degree of challenge associated with them. 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 which are unpredictable or uncontrollable. In this chapter I will document glucocorticoid excretion patterns for female ringtailed lemurs living in two free-ranging social groups in a forest reserve in southwestern Madagascar. To determine the possible context for elevated glucocorticoid secretion in these females, their fecal cortisol levels were related to behavioral estimates of predation threat, food accessibility, and dominance status.

Glucocorticoid responses to naturalistic variables

Past studies with both laboratory and free-ranging animals suggest several potential stressor stimuli that could stimulate increased glucocorticoid production in 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 post-partum 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). Several studies done with free-ranging avian, rodent and primate species have shown clear circannual and individual variability in circulating glucocorticoid levels. 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 that during stable periods, either low- or high-ranking individuals exhibit 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 and rhesus macaques respectively. In the only known studies in which free-ranging animal fecal glucocorticoid measures are reported, Creel *et al.* (1996 & 1997) found elevated glucocorticoid levels in high-ranking female African wild dogs. 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.

Purpose

The goals for the study presented here were: a) to determine if fecal cortisol levels fluctuate in synchrony with the common challenges of late gestation, predation threat, food accessibility, and/or dominance status, and b) to determine if fecal cortisol levels relate to either to the time of day samples are collected or to the weight of samples. Based on past research results, one can predict that fecal cortisol levels would be elevated during late gestation as compared to post-parturition, and during periods of increased anti-predatory behavior and decreased food accessibility. However, if decreased food accessibility is a predictable phenomenon, as it may be for the lemurs experiencing a yearly dry period they may be able to predict and physiologically prepare for these periods so that decreased food accessibility may not be a significant challenge (e.g. Pereira *et al.*, in press). 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 fecal cortisol measures. Finally, fecal weight may affect cortisol measures in that increased weight may dilute fecal cortisol concentrations which would primarily reflect diet bulk and not changes in circulating cortisol levels.

Study population

Behavioral observations were used to assess possibly challenging stimuli (gestation, predation threat, food accessibility, and dominance status) and Wasser and colleagues (1994) fecal steroid extraction method was used to monitor daily cortisol levels in free-ranging female ringtailed lemurs (Lemur catta) 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). Also, female agonistic interactions are relatively frequent so that dominance positions can be identified reliably by human observers (Kappeler 1993). In addition, ringtailed lemurs are diurnal, terrestrial, and live in large groups, which facilitates behavioral observations and fecal sampling.

METHODS

Subjects

Ten females from two social groups of ringtailed lemurs in the Beza Mahafaly Special Reserve in south-western Madagascar (23° 39'S lat., 44° 37'E long., Sauther 1992) were studied from August 1995 to January 1996 (Fig. 3-1). Each study group consisted of five adult females, three to four adult males, two to three juveniles and three to four infants. The Beza Mahafaly reserve consists of riverine forest which undergoes a marked annual dry season accompanied by a period of decreased food variability in October and November (Sauther 1992). Prior to this study, females were 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 ten meters of the forest floor.

Each social group was followed for seven six-day observation sessions, and sessions were alternated between the two groups throughout the study. During this time, all but one of these seasonally-reproducing females began the study in a late gestational state and ended the study in a mid-lactational state. In group 2, the one exceptional female gave birth to a seemingly healthy offspring in synchrony with the other females, then lost the infant between the eighth and tenth weeks of observation after it had received a severe wound to the head. Females in group 1

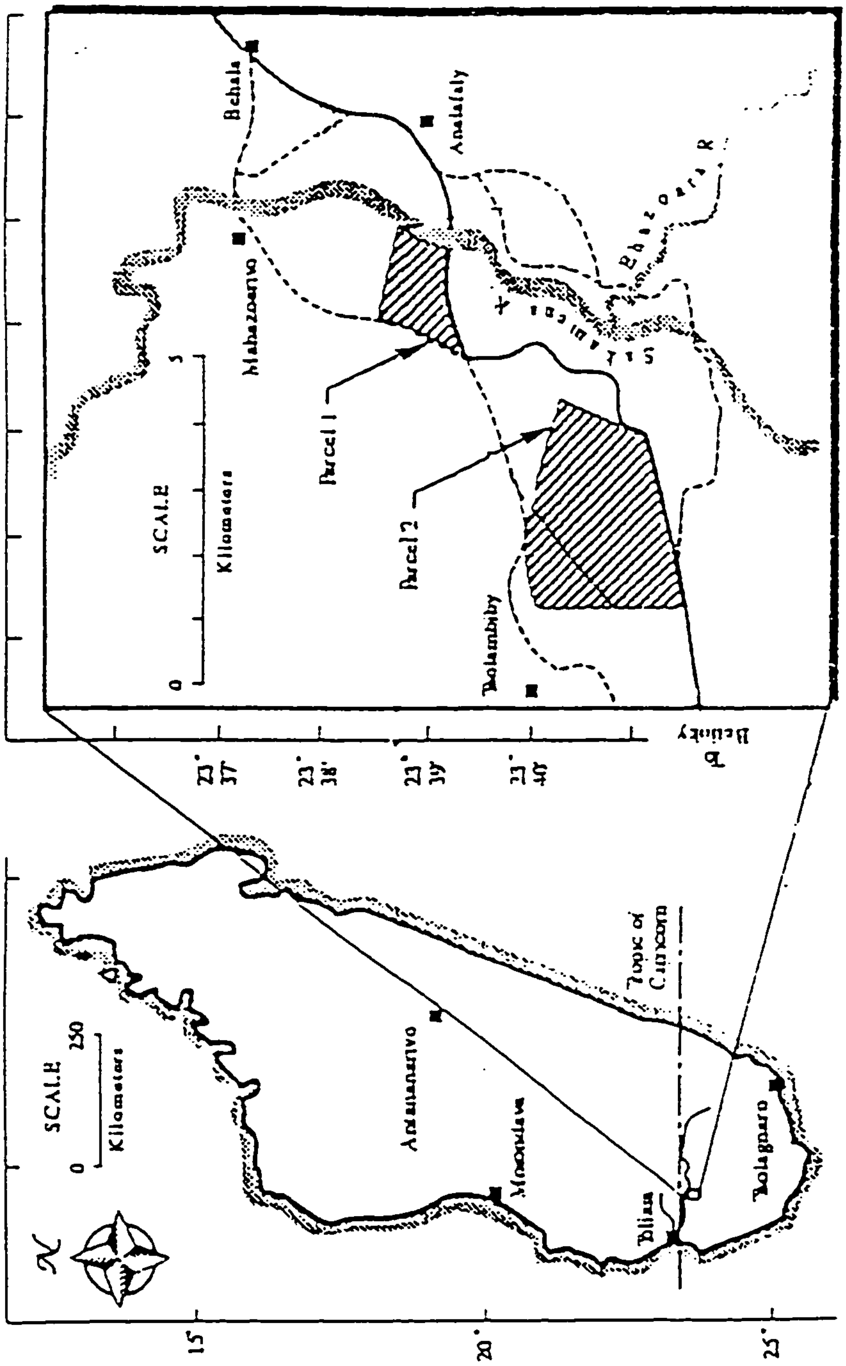

Figure 3-1. Map of Madagascar and Beza Mahafaly Special Reserve study site (from Sauther, 1992).

exhibited qualities of a fission-fusion social organization; two females, along with three or four males, would occasionally travel, feed and sleep separated from the rest of the group. Shortly after completion of this study, group 2 showed similar signs of group flux when two low-ranking females were observed separate from their original group (L. Gould, pers. comm.).

Throughout this chapter, behavioral and fecal cortisol means are reported for "observation weeks" (i.e. six-day follows). Fourteen weeks of data were collected; seven weeks from each group. Observation weeks were chronologically alternated between the two groups. Thus, means from "week 1" refer to the first week of data collected from group 1, means from "week 2" refer to the first week of data collected from group 2, etc. Landmark events during these weeks are outlined below and in Fig. 3-2.

The 14 observation weeks occurred over a period of 4.5 months (August 21, 1995 to January 6, 1996). Births occurred in September (weeks 3 and 4). The dry season lasted until mid-November (weeks 1-10), during which time less than 10 mm of rain fell. Mid-November to early January (weeks 11-14) was the beginning of the wet season, during which time over 100 mm of rain fell. A rush of new vegetation appeared in the reserve at the end of December (weeks 13 and 14). The end of the dry season and the transition into the beginning of the wet season provided an ideal opportunity to compare fecal cortisol levels from low to high food accessibility.

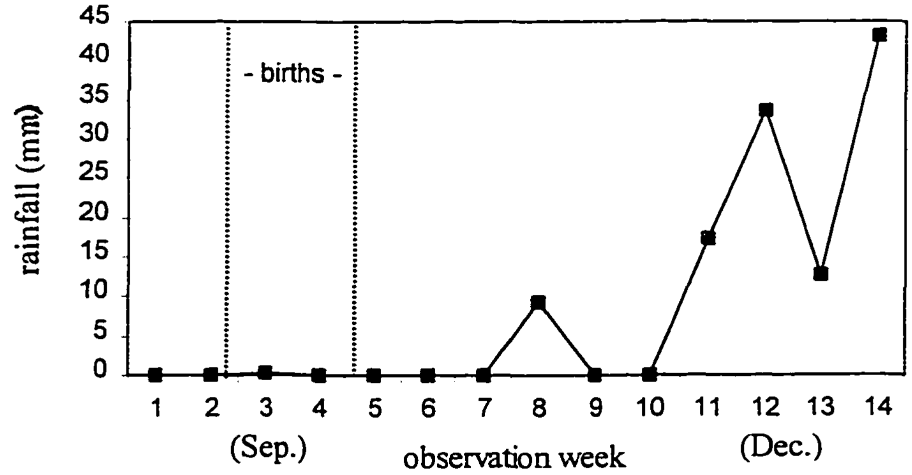

Figure 3-2. Time line of study identifying rainfall during each observation week (1-14), the period of births for all females, and the months when the study began and ended. Group 1 was observed during observation weeks 1, 3, 5, 7, 9, 11 and 13. Group 2 was observed during weeks 2, 4, 6, 8, 10, 12 and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

Behavioral observations

Behavioral data were collected to estimate the following factors: degree of predation threat, relative food accessibility, and dominance indices. Observations were conducted 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. Observations were conducted for a mean (± SD) of 8.3 ± 2.1 hours per day ($N = 85$). During the study, group 1 was observed for 378 hours and group 2 was observed for 330 hours.

Predation threat was estimated from all-occurrence records of "high intensity" anti-predatory responses. "High intensity" responses were identified as the highly conspicuous "yap" and "shriek" vocalizations defined by Jolly (1966) and Macedonia (1990). These vocalizations were exhibited by the group as a whole, and the calls were extremely loud and were associated with a rapid cessation of ongoing activity; these responses were very conspicuous during observation sessions. Focal animal samples (Altmann 1974) were collected daily to calculate feeding effort and dominance indices based on agonistic behavior. Two nine-minute 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 one-hour block within observation sessions. Behavioral states recorded during focal samples included resting, scanning, traveling, and feeding. Definitions of these behavioral states were adapted from Jolly (1966) and Pereira & Kappeler (1997). Aggressive and submissive acts, as defined by Pereira & Kappeler (1997), were also recorded during focal samples. All behavioral definitions are given in the Appendix.

Finally, to estimate predictability of food resource locations, all food sources, other than grasses, were tagged and marked with a unique number code. Scan samples were collected every 15 minutes during observation sessions. The following information was recorded during these samples: the group's location within the forest and the number codes for all non-grass food sources from which the lemurs were feeding. Fecal samples were collected opportunistically throughout observation days.

Weekly behavioral means were calculated to estimate changes in environmental conditions during the study. Weekly predation threat was estimated as the mean frequency of high intensity anti-predatory calling bouts per hour of observation. Feeding effort for each female was estimated as a ratio of time spent traveling and "actively foraging" divided by time spent sleeping, resting, and "passively foraging". "Active foraging" was defined as foraging from a standing position, "passive foraging" as foraging from a sitting position. Active foraging was used most often when food items were located at the ends of branches, and passive foraging was used primarily when feeding on grasses, leaves or large fruit. "Foraging" included chewing and drinking. Predictability of food resource location was estimated as the percentage of food sources visited more than once during a week of focal samples, and also as the number of repeat visits to sources per hour of observation in a week. Dominance indices for each female were calculated 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. This method of estimating relative dominance was used in place of the more traditional method of developing a dominance hierarchy from agonistic interaction matrices because the indices could provide a more accurate estimate of an animal's relative dominance level. Indices provided the proportion of times an individual won agonistic interactions as opposed to a simple rank of how many individuals a female could dominate.

Fecal cortisol measures

Fecal samples were collected during behavioral observation sessions and were stored in 40 ml screw-top polypropylene vials (Curtis Matheson Scientific, Inc., Norcross, GA) containing 15 ml of a 0.3% sodium azide in 100% ethanol solution. For each female, a mean (± SD) of 1.5 ± 0.4 samples ($N = 10$) were analyzed per observation day, with a total of 742 samples analyzed from all females. Storage ethanol was removed from samples with a centrifugal evaporator (Savant, Farmingdale, New York). Cortisol was extracted from dry feces and concentrations measured using the extraction and assay protocols described in Chapter 2.

Statistical Analyses:

Mean cortisol levels and behavioral measures were calculated for each female week. Bartletts' test of homogeneity of variance (Sokal & Rohlf 1995) was used to test whether data across weeks were homoscedastic. Feeding effort estimates were heteroscedastic and transformed using a square-root transformation. Fecal 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 three weeks, whereas group 1 females all gave birth within a period of eight days which occurred when observations were not being conducted on them. With births in group 2 spread out over a three-week period there was less possibility of having an environmental condition that was synchronous with all the births and could have accounted for possible changes in cortisol levels from late gestation to early lactation. For this reason, pre- and post-gestational cortisol levels were compared for group 2 females only. Paired *t*-tests were used to compare mean cortisol levels during the seven days prior to and the seven days following parturition. Single factor ANOVAs were used to determine if weekly behavioral means (anti-predatory behavior, feeding effort) differed across non-gestational weeks (weeks 5-14). A nested ANOVA was used to compare fecal cortisol levels, time of sample collection, and mean weight of samples across these same weeks. When an ANOVA indicated a significant difference among weeks, a Tukey-Kramer multiple comparisons or a Welsch step-up procedure was used to determine weeks that were significantly different from others (Sokal & Rohlf 1995). The 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: a) reproductive status, b) anti-predatory behavior, c) feeding effort, d) fecal sample weights, e) individual dominance indices, and f) days into the study. Reproductive status was categorized simply as gestation ("1") or post-parturition ("0"). Variables that produced *P*-values greater than 0.10 were removed from the regression model one-by-one.

RESULTS

Gestation and Fecal Cortisol Levels:

In group 2 females, cortisol levels 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 two times greater than levels just after parturition (Fig. 3-3).

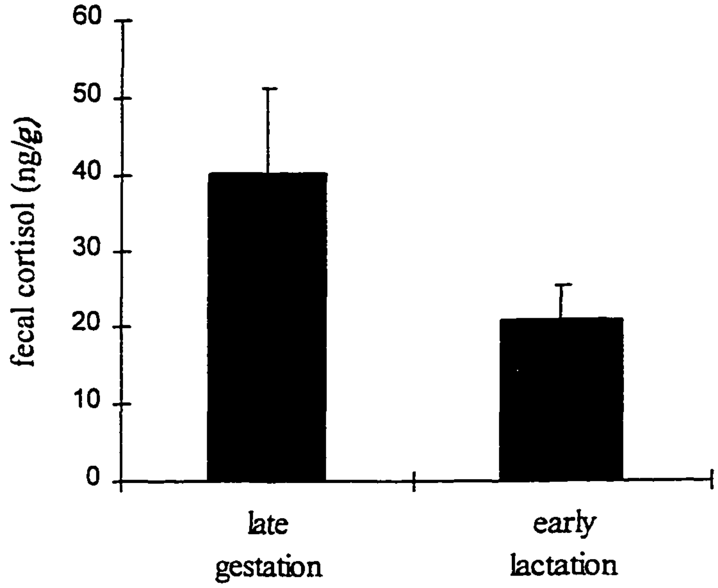

Figure 3-3. Mean fecal cortisol levels for females from group 2 during the last seven days of gestation and the first seven days of lactation. Bars indicate standard errors.

Behavioral Measures:

During weeks 5-14, anti-predatory behavior was most frequent between weeks 7-11 (Fig. 3-4a). The frequency of anti-predatory behavior across weeks was significantly different for group 1 (ANOVA: $F_{4,25} = 2.90$, $P < 0.05$), but not significantly different across weeks for group 2 (ANOVA: $F_{4,25} = 0.491$, NS). For group 1, anti-predatory behavior 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 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. 3-4b). Weeks 9-12 coincide with the end of the dry season when food availability has been shown to decrease at Beza Mahafaly (Sauther 1992).

The lemur groups fed on a greater number of non-grass sources/h during weeks 7-10 (1.77-1.92 sources per hour during weeks 7-10 vs. 0.90-1.57 sources per hour in weeks 5, 6 and 11-14). The percentage of grass-like feeding resources were greatest during weeks 11-14 (57-74% during weeks 11-14 vs. 10-50% during weeks 5-10). The percentage of repeat visits to food sources was greatest during weeks 7-12 (compare 23-30% repeat visits during weeks 7-12 vs. 4-17% during weeks 5, 6, 13 and 14).

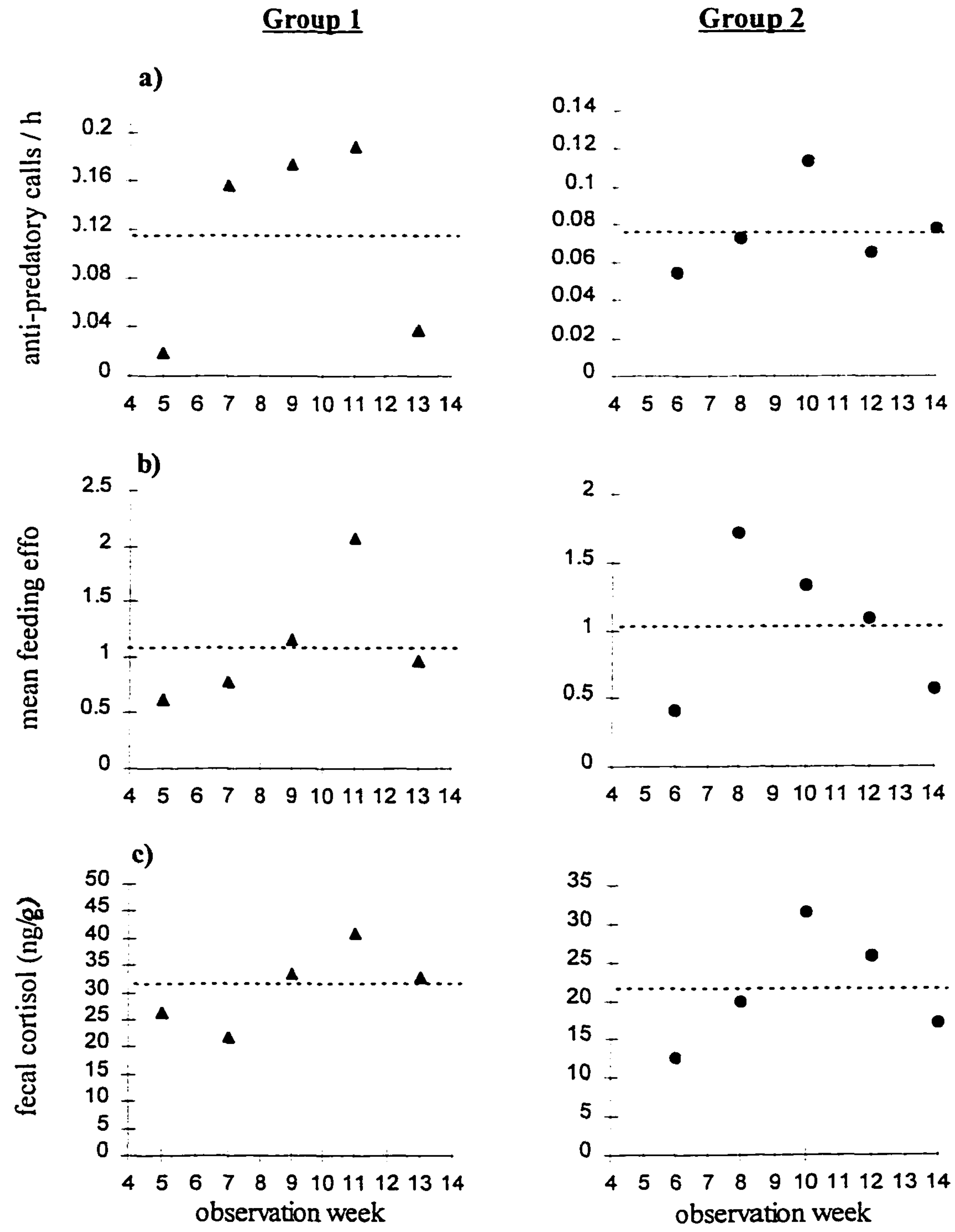

Figure 3-4 a,b,c. (a) Mean frequency of high-intensity anti-predatory vocalizations (b) mean feeding effort measure and (c) mean fecal cortisol levels for female ringtails during non-gestational weeks (weeks 5-14). The dashed horizontal lines represent across-week means for each measure.

Fecal Cortisol Levels:

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

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

Eight of the ten females had significantly different wet fecal weights across the ten weeks (ANOVA: $P < 0.05$), with the lowest weights occurring during weeks 9-12. Comparisons of fecal cortisol values and fecal weights within each female week indicated that 8 of 66 female weeks showed a significant ($P < 0.05$) negative correlation between cortisol values and dry fecal weight. To further evaluate the relative impact of fecal 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 during weeks 9-12. The second peak occurred at the end of the dry period when anti-predatory behavior and feeding effort measures were elevated (Fig. 3-4) and fecal weights were low. This cortisol pattern across weeks was apparent in all females, including the one female that lost her offspring between weeks 8 and 10.

Fecal Cortisol Levels Related to Behavioral Measures:

A multiple linear regression analysis was conducted to assess the relative relationships between weekly means of log-transformed fecal cortisol levels and the following the variables from each female-week: reproductive status (pregnant vs. post-parturition), anti-predatory behavior, feeding effort, fecal 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 3-1). A stepwise backward elimination of predictors with p-values of greater than 0.10 retained the reproductive status, feeding effort, fecal 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: $F_{6,30} = 8.11$, $P = 0.00003$; Table 3-1). A stepwise backward elimination procedure retained three of the predictors: reproductive status, dominance indices, and fecal weight. Together, these four variables accounted for 55.7% of the cortisol variability (ANOVA: $F_{3,33} = 16.09$, $P = 0.000001$). Further regression analyses were conducted without the fecal weight variable, since this variable relates to feeding effort measures 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 fecal weight variable was not included in the regression analysis.

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

Table 3-1. Results of multiple linear regression analyses for each social group. The dependent variable was log-transformed mean fecal cortisol levels and the predictor variables were reproductive status (pregnant = 1, post-parturition = 0), anti-predatory behavior, feeding effort, fecal weight, dominance indices, and days into study.

GROUP 1: **adjusted R^2 = 0.568**
$F_{6,25} = 7.81$
$P = 0.00084$

predictor	coefficient	t	p
reproductive	1.004	4.14	0.000
predators	0.839	0.80	0.430
effort	0.221	1.31	0.204
fecal weight	0.077	1.71	0.100
dominance	0.758	4.34	0.000
day	0.003	1.02	0.317

GROUP 2: **adjusted R^2 = 0.542**
$F_{6,30} = 8.11$
$P = 0.00003$

predictor	coefficient	t	p
reproductive	0.846	3.60	0.001
predators	0.223	0.13	0.901
effort	-0.077	-0.27	0.792
fecal weight	-0.094	-2.66	0.012
dominance	0.661	3.90	0.001
day	0.004	1.30	0.204

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, etc. Both female dominance indices and dominance ranks were significantly correlated with mean fecal cortisol measures ($r = 0.76$, $P < 0.05$; $r = 0.68$, $P < 0.05$; Fig. 3-5). Females with the highest dominance indices and ranks exhibited the highest fecal cortisol measures.

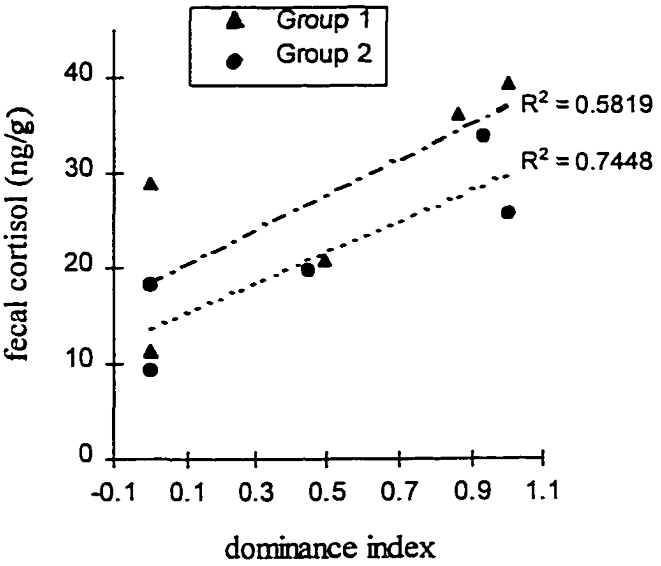

Figure 3-5. Mean individual fecal cortisol measures related to dominance indices for all females. Females with higher dominance indices (i.e. higher dominance rank) exhibit higher cortisol values than females with low dominance indices.

Female dominance indices also were significantly correlated with rates of initiating aggression ($r = 0.86$, $P < 0.01$). In other words, highly dominant females were more aggressive than were less dominant individuals. These relationships may begin to explain why high-index females exhibit high fecal cortisol values: high-index females appear to expend more effort on initiating aggressive interactions, and this increased energy expenditure may explain their elevated cortisol levels (e.g. Borer *et al.* 1992).

Another interesting pattern is that the two highest index females in each group had the largest increase in mean cortisol levels from weeks 5-8 to weeks 9-12 (from the middle to the end of the dry season, when feeding efforts were increasing). The two highest index females' cortisol increased over this period an average (± SD) of 18.7 ± 8.0 ng/g, whereas the three lowest index females during this time had a mean increase (± SD) of 8.9 ± 5.7 ng/g ($t_8 = 2.13$, $P < 0.05$). Higher mean cortisol levels in dominant females may reflect the fact that they respond to challenges with a greater cortisol response than do lower ranking females.

DISCUSSION

Fecal cortisol levels measured in free-ranging female ringtailed lemurs during this 14-week study are consistent with current beliefs that predation threat, decreased food accessibility and elevated social status present significant challenges to free-ranging animals. During the latter half of 1995, free-ranging female ringtailed lemurs at Beza Mahafaly Special Reserve experienced two periods of elevated fecal cortisol levels. One period at the end of gestation, a second period during the end of the dry season when behavioral repertoires indicated decreased food accessibility and increased predation risk. In addition, dominant females exhibited greater mean fecal cortisol levels than did their subordinate group mates. These results support the initial predictions of the study to varying degrees.

The prediction that cortisol levels would be elevated during periods of increased anti-predatory behavior was not well supported. Cortisol levels were elevated during weeks when anti-predatory behavior was elevated, but rates of anti-predatory behavior did not provide a strong predictor of cortisol variability. Lack of predictive power could suggest that predators do not present a significant challenge to the lemurs or that threats of predation are predictable and/or somehow controllable and therefore not stressful for the female ringtails. Alternatively, the lack of correspondence may suggest that anti-predatory behavior 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 anti-predatory behavior may be minimal, and only when risk is moderately high (e.g. predators are common but distant) would anti-predatory behavior be highest as a means of warding off predators. If this is the case, then rates of anti-predatory behavior may not be the best behavioral 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, or, that required responses to predators are short-lived and do not predict significant increases in cortisol levels.

The prediction that cortisol would be elevated when food accessibility was not well-supported. At the end of the annual dry season, when active foraging behavior increased, there was a measurable rise in fecal cortisol levels, but the feeding effort variable was not a reliable predictor of cortisol variability. 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. Alternatively, since repeat visits to non-grass food resources occurred more often during the dry season, food locations may have been more predictable during the dry season, which may reduce the challenge associated with locating, albeit decreased, food resources. Also, annual decreases in food accessibility may be predictable for the lemurs and therefore they may be able to prepare for the decrease (e.g. by fatting during the wet seasons, etc.) thereby minimizing the challenges of the dry season (Pereira *et al.* in press). One indicator that decreased food resources may be predictable is the fact that the timing and amount of rainfall at Beza Mahafaly in late 1995 were similar to those at Beza in 1987 and 1988 as reported by Sauther (1992; comparison of 1995 and 1987/88: < 5 vs. < 5 mm in September, 9 vs. 12 mm in October, 41 vs. 50 mm in November, and 73 vs. 85 mm in December).

Food access may be a more important challenge to the females in group 1 situated in a drier forest area. In the multiple linear regression analysis, feeding effort predicted a greater percentage of the cortisol variability for group 1 than for group 2. This difference may indicate that the two study groups were either differentially sensitive to the stimuli in their environment or that feeding efforts during the dry season were greater in group 1 and therefore posed a greater challenge. Group 1 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 riverbed in a more lush environment.

Elevated feeding effort and anti-predatory behavior appear to provide weak explanations for elevated cortisol levels during the end of the dry season. 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. Since all females gave birth within a three-week period, changing lactational demands were experienced synchronously, as were changing environmental conditions. Furthermore, since all females in this study gave birth to and lactated for one offspring, the influence of post-partum reproductive demands on cortisol levels are difficult to assess here. However, it can be noted that the one female that lost her offspring after observation week 8 exhibited an elevation in cortisol levels during weeks 10 and 12 similar to that seen in the continually lactating females. This elevation occurred two to four weeks after the female was last seen feeding her offspring. These results are difficult to interpret since there may be a confound from stressors associated with the loss of an infant.

Finally, the most reliable predictors of cortisol variability in the female ringtails of this study were reproductive status and dominance indices. Cortisol levels during late gestation were two times higher than 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). This replication of pre- versus post-gestational cortisol differences suggests that Wasser *et al.*'s (1994) fecal steroid extraction method provides a meaningful assessment of cortisol levels in female ringtailed lemurs.

Dominance indices provided a reliable predictor of cortisol variability. High-index females experienced high mean fecal cortisol levels and low-index females exhibited low mean levels. These findings coincide with Creel *et al.*'s (1996 & 1997) findings with female African wild dogs, although the social structure of ringtailed lemurs and African wild dogs present some clear differences (e.g. male vs. female dispersal, individual breeding vs. co-operative breeding, etc.). The results from the present study differ from those reported for free-ranging male baboons, where high-ranking individuals exhibit 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 ringtailed 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, wild dogs and male baboons. Another possibility may be that higher-ranking females respond to challenges with a greater cortisol increase than do lower-ranking females. Finally, a 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 exhibit higher cortisol titers (e.g. Sapolsky 1983). Recently analyzed data from three stable social groups at Berenty Private Reserve in southeastern Madagascar indicate that the relationship between dominance and cortisol seen at Beza Mahafaly also exist in the stable social groups at Berenty (S. Cavigelli, W. Levash, T. Dubovick & A. Jolly, unpublished data discussed in Chapter 5).

Finally, it must be stated that dominance ranks and dominance indices correlate with a number of behavioral categories which may best explain the relationship between cortisol and dominance. Glucocorticoid levels may relate best to factors other than, but associated with, dominance measures, such as age, rate of being attacked or attacking, quality of diet, number and quality of allies, differential costs of reproduction, etc. (e.g. Bernstein 1981, Creel *et al.* 1996, de Villiers *et al.* 1997). To sort out the reason for dominance rank effects on cortisol levels, we need more information on the relationships among dominance status, social behavior profiles, and glucocorticoid levels as well as other measures of physiological stress, in both male and female free-ranging animals.

Results from this study suggest that time of day does not significantly affect fecal cortisol measures in female ringtailed lemurs even though the circadian pattern in glucocorticoid secretion has been clearly documented in primates (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 feces accumulate hormones over several hours and therefore mute hormonal peaks and troughs, thereby making the difference between peaks and troughs less robust. Such issues need to be addressed in further fecal steroid analyses. The results of the multiple regression analyses also indicate that dry fecal weight should be taken into account when interpreting fecal 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 fecal weight increased, cortisol measures decreased) and for the second group the relationship was positive. Future resolution of this relationship will strengthen fecal glucocorticoid measures.

This chapter presents a preliminary application of a fecal steroid measure to assess cortisol levels in free-ranging primates and to determine the context for elevated glucocorticoid excretion. The results of the fecal cortisol analyses are consistent with ecological theories that suggest 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 than were reproductive state and dominance indices. The low degree of predictive power in the predation threat and feeding effort measures may reflect the fact that these variables may have fluctuated in a predictable fashion and were therefore negligible challenges. 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 predictable changes. 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 assessment of stimulus predictability and/or controllability. The findings from this field study, in concert with findings from other research studies, encourage further exploration of circannual and individual differences in fecal glucocorticoid patterns in free-ranging animals. Successful application of this method could open a new arena of research that can provide a base of knowledge about inter- and intra-specific differences in glucocorticoid responses to naturalistic environmental conditions, and can provide insight into strategies (species-specific, individual-specific, sex-specific, etc.) that promote survival in seasonally-variable habitats.

CHAPTER 4

EFFECT OF FOOD ACCESSIBILITY ON FECAL CORTISOL LEVELS: EXPERIMENTAL TESTS IN A NATURALISTIC SETTING

Food accessibility is thought to be one of the major challenges facing free-ranging animals. One method for coping with predictable, seasonal decreases in food availability may be to prepare for the decrease by storing excess energy when food is readily accessible and then secreting glucocorticoids when food supply is decreased to mobilize the energy stored previously. Recent results by Pereira *et al.* (in press) suggest that this may be a strategy used by ringtailed lemurs. Results from Chapter 3, on free-ranging female ringtailed lemurs, showed that during the end of the dry season, when food accessibility was low, that females excreted more cortisol than during either the beginning of the wet or dry seasons. A possible causal relationship between food accessibility and cortisol levels could not be assessed from these observation field data. This chapter will explore the hypothesis that a relatively short-term decrease in food accessibility, associated with increased feeding effort, may activate the hypothalamic-pituitary-adrenal axis in a free-ranging primate. This work was conducted with female ringtails at the Duke

University Primate Center (DUPC) by simulating food accessibility conditions observed in Madagascar.

Glucocorticoids, energy metabolism, and food accessibility

Glucocorticoid secretion must be affected by food accessibility since these hormones are involved in stimulating catabolic processes such as inhibition of energy storage and mobilization of stored energy. However, how glucocorticoid levels respond to changes in food accessibility is not clear. During a period of decreased food accessibility, an organism must spend proportionally more energy to acquire nutrients from its environment. At some point the organism presumably must be faced with the situation of not having enough energy input to maintain average activity levels. Such an energy debit may lead to a variety of possible changes in glucocorticoid secretion. For example, a short term increase in glucocorticoid secretion during decreased food access would help mobilize energy units stored within the organism, thereby making up for the energy deficit. This short-term secretion may also serve to stimulate foraging behavior as seen in certain bird species (Silverin 1986, Astheimer *et al.* 1992). In addition, increased glucocorticoid circulation may actually promote energy conservation by promoting nocturnal restfulness as seen in two species of passerine birds (Buttemer *et al.* 1991). Alternatively, if a newly encountered energy deficit signals possible future and longer-lasting deficits, an organism may be best served by decreasing its basal metabolic rate along with decreasing glucocorticoid circulation to minimize energy use and to defer breakdown of stored energy until absolutely necessary. By monitoring glucocorticoid secretion patterns in free-ranging animals that experience seasonal decreases in food accessibility, we may be able to understand one hormonal strategy to cope with predictable and relatively long-lasting decreases in food availability.

Experimental tests of food availability on glucocorticoid excretion

Many experimental tests of how food availability affects glucocorticoid secretion have been conducted with caged animals. One such study with adult female squirrel monkeys (Saimiri sciureus) involved hiding various amounts of food under wood shavings within the monkeys' home cage (Champoux *et al.* 1993). In two-week long trials of decreased food supplies, foraging activity increased in these females along with significant increases in plasma cortisol levels. In another study with Dark-eyed juncos (Junco hyemalis) and Pine Siskins (Carduelis pinus) in outdoor enclosures, Astheimer and colleagues (1992) found significantly elevated corticosterone levels in birds that had just experienced a 36-hour fast. In a review of bird studies conducted on how environmental stimuli influence adrenal responsiveness, Harvey and colleagues (1984) concluded that food deprivation led to elevated corticosterone levels in captive birds, particularly in those normally kept on an ad libitum feeding schedule. These studies all involved decreasing food quantity to manipulate food accessibility. Additionally, all of these studies were conducted in a captive setting.

One difficulty with studies of caged animals is the confound of increased exposure to conspecific attacks when the effort involved in food acquisition is increased. In addition, depriving animals of food, rather than simply increasing the cost of food, may represent a rather severe change in normal feeding schedules in which plenty of food is normally available. In these studies, the mere novelty or extreme nature of food deprivation, rather than the extra work involved in food acquisition, may be the primary stimulus that leads to activation of the HPA axis. Finally, studies that examine the influences of food deprivation on circulating glucocorticoid levels have not explored how changes in feeding effort affect cortisol levels. Is there a relationship between these two variables? If there is a relationship, is it that glucocorticoid levels increase or decrease as feeding effort increases? Or is it too simple to think that there could be a simple relationship between these two variables, and if the two are related in a more complex way, what contextual cues are involved? Finally, is it reasonable to think that glucocorticoids may provide one means through which animals cope with changes in food availability in their natural habitat.

Study populations

Female ringtails are an interesting species for testing how food accessibility affects glucocorticoid secretion since it has been hypothesized that these females experience significant decreases in food accessibility in their natural habitat. To test the notion that decreased food accessibility and associated increased feeding efforts lead to increased glucocorticoid secretion in free-ranging ringtailed lemurs, food manipulations were conducted with lemurs living in naturalistic conditions at the Duke University Primate Center (DUPC). To determine whether the Madagascar and DUPC populations were comparable, and therefore whether experimental data from the DUPC would apply to Madagascar populations, activity budgets were compared between the Beza Mahafaly females recorded in 1995 and the DUPC females observed during the summer of 1996, prior to any food manipulations.

METHODS

Subjects

During the summers of 1996 and 1997, I studied eight adult female ringtailed lemurs that lived within two social groups in a forested enclosure at the Duke University Primate Center (DUPC) in Durham, North Carolina. The forested enclosure is 9.1 hectares in size and contained plant species commonly found in the southern piedmont forests of North Carolina. The lemurs within the enclosure were provisioned with monkey chow daily and they also fed on plants within the enclosure. In the summer, the lemurs spend approximately equal amounts of time feeding on supplied chow biscuits and feeding on naturally-occurring food resources. The lemurs' daily activity in the forest enclosures is similar to that observed in two social groups at Beza Mahafaly in 1995 and similar to that seen in Berenty Private Reserve in southeast Madagascar by Jolly (1966). Both at the DUPC and in Madagascar, the lemurs engaged in morning feeding and sunning bouts, afternoon siestas, evening feeding bouts, and frequent agonistic interactions within and between social groups. The major differences between the DUPC and Madagascar sites are temperature and rainfall. During the summer observation sessions at the DUPC the mean (± SD) temperature at noon was 27.1 ± 3.1 °C in 1996 and 28.8 ± 3.7 °C in 1997. Total rainfall during these two six-week summer observation sessions were 163 mm in 1996 and 157 mm in 1997. Mean (± SD) noon-time temperature at Beza Mahafaly during the 1995 austral summer (October to December) was 35.5 ± 3.1 °C and total rainfall during these three months was 122 mm.

The DUPC groups studied in 1996 and 1997 contained four to six adult females, four to six adult males, and three to four juveniles. During the fall and winter months of 1996, females received Depo-Provera contraceptive (medroxyprogesterone) injections. Six females gave birth to offspring in April of 1996 and were still lactating during the summer of that year. No females gave birth in 1997.

Prior to the experimental manipulations, each DUPC ringtail group was fed 3.5 liters of monkey chow (Purina, #5038) daily. The chow was distributed to the lemurs at approximately noon every day and dispersed by a Primate Center caretaker over an area of approximately 20 meters in diameter on the forest floor. The daily feeding location was rotated pseudo-randomly amongst prescribed areas within the forest enclosures (Fig. 4-1). (Area locations and sizes within the two enclosures were designed to minimize within and between group competition during feeding.) To indicate feeding time to the lemurs, caretakers wore a white laboratory coat during chow distribution.

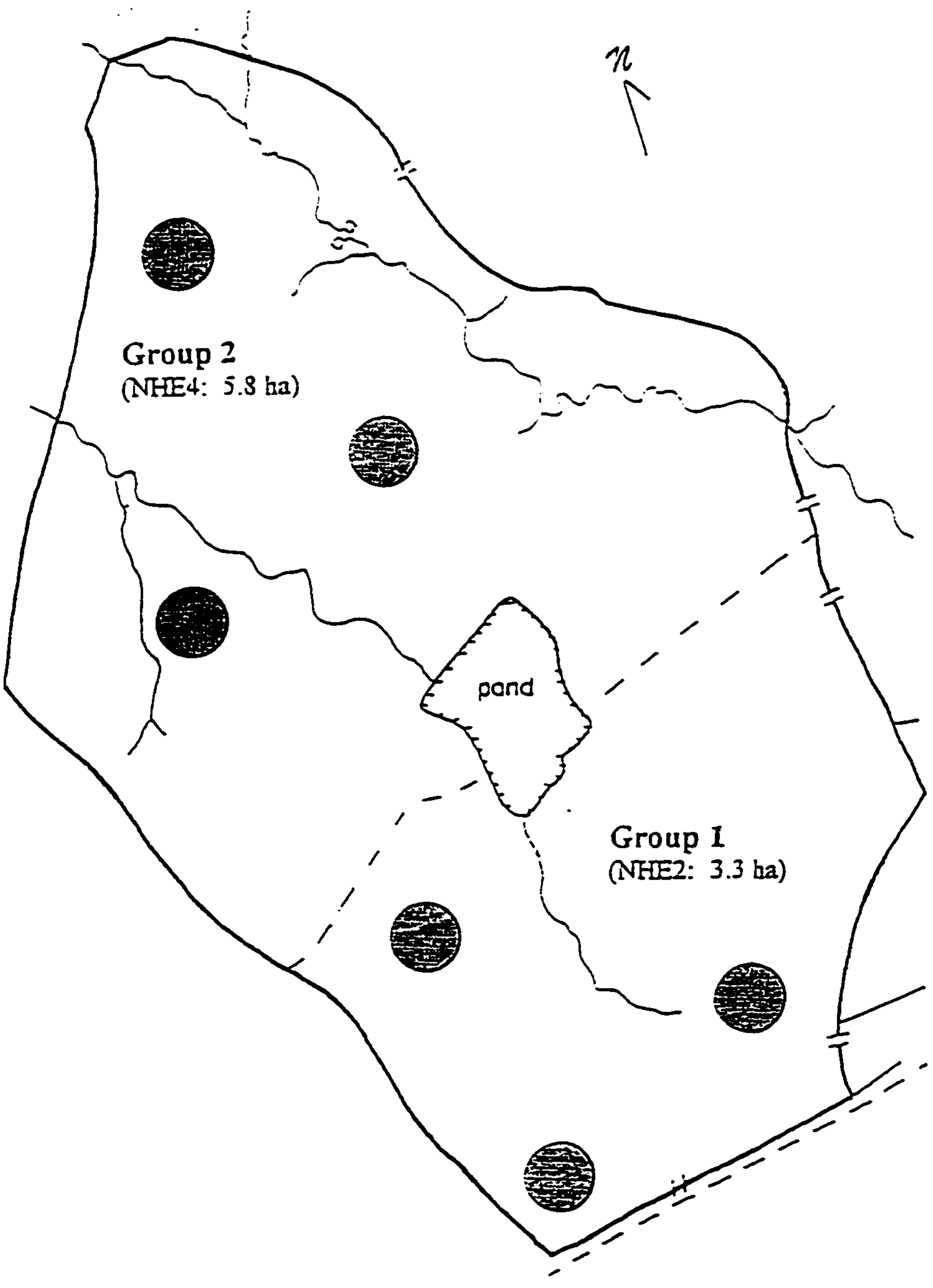

Figure 4-1. Map of the two adjacent DUPC natural habitat enclosures (NHE) where food manipulation trials were conducted with two ringtailed lemur groups. The circles indicate feeding sites and the dashed line identifies the defended limit of each group's homerange in the adjacent NHE's.

Comparison of behavioral activity budgets (Beza 1995 vs. DUPC 1996)

To assess any differences in DUPC and Madagascar lemur daily activity, the DUPC lemurs were observed during the summer of 1996 during six weeks of all-day observations, in a manner similar to that used in 1995 at Beza Mahafaly. For this behavioral comparison, the DUPC social groups were observed six days a week from July 16 to August 4 and from August 29 to September 25, 1996 (excluding one week when females were housed in cages after damage from Hurricane Fran). Observations were conducted from 0900 h to 1315 h, after which the lemurs settled down for their afternoon siesta, and observations resumed at the end of the siesta from 1600 to 1845 h. This observation schedule focused on the active parts of the ringtailed lemurs' days and was similar to that used with the Beza Mahafaly social groups. The one difference between the two sites was that observation sessions at the DUPC were alternated between the two social groups on a daily basis, whereas with the Madagascar groups, observation sessions were alternated between the two groups on a weekly basis.

Throughout the observation sessions, for both the DUPC and Madagascar groups, activity budgets were calculated from instantaneous scan samples collected every 15 minutes (Altmann 1974). The behavioral activity of each adult female was recorded during each scan sample. Six mutually-exclusive behavioral activities, as defined in the Appendix, were recorded: (1) feeding and foraging,

(2) resting, (3) scanning, (4) traveling, (5) grooming, and (6) other (which included scentmarking, defecating, urinating and playing).

The percentage of point samples that each female spent in each behavioral category was calculated from the six weeks of data. Based on all female means, I calculated the mean percentage of point samples spent within each behavioral category for the DUPC and Beza Mahafaly groups.

Food accessibility manipulations (DUPC 1997)

Three food accessibility conditions were designed: (1) high, (2) mid, and (3) low (Table 4-1). During all three conditions, the feeding amount, the feeding time and the feeding locations described above were retained. The only two variables manipulated within the conditions were the size of chow biscuits and the number of sites used to distribute food during daily feedings. The high accessibility condition involved distributing whole monkey chow biscuits greater than lemur fist size (30-40 mm) at one of the three possible feed site within each social group's range. For the mid accessibility condition, biscuits were crushed to approximately fist size (i.e. larger than bite size: 15-25 mm) and distributed over two feed sites. For the low accessibility condition, biscuits were crushed to approximately bite size (less than 10 mm) and distributed over three feed sites. Chow biscuit sizes and distribution patterns were selected based on food sizes and associated behavior seen at Beza Mahafaly during the wet and dry seasons in 1995.

Under the high accessibility condition, females could collect a chow biscuit and then sit and eat it over a period of one to two minutes, then move on to find the next biscuit. Whole biscuits appeared relatively easy to locate in the grassy cover of the forest floor. On the other hand, crushed chow pieces used in the low accessibility condition led females to remain standing, usually searching while simultaneously chewing, during the entire feeding/foraging period.

Manipulations were conducted from July 7 to August 16, 1997. Each condition lasted two weeks, and to minimize effects of uncontrollable environmental conditions the two groups never experienced the same condition at the same time. The order of conditions for one group was high- to mid- to low-food accessibility, and the order for the other group was low- to high- to mid-food accessibility.

Table 4-1. Food locations and sizes used in each of the three food accessibility manipulations.

	Experimental Manipulations:		
food accessibility condition:	high	mid	low
location:	1 site	2 sites	3 sites
food size:	greater than fist size ←——→ (30-40 mm)	(15-25 mm)	bite size (< 10 mm)

Behavioral measures (DUPC 1997)

Feeding effort, rates of initiating aggression, and rates of receiving aggression were calculated from focal animal sampling records collected between 1115 and 1330. Within observation periods, the order of focal animals was pseudo-randomized so that each female was observed twice within each 45-minute block per condition. Feeding effort was calculated the same way as described in Chapter 3 for the Beza Mahafaly females. Rates of initiating aggression and of receiving aggression were calculated for each adult female based on total focal animal sampling hours, similar to that used with the Beza Mahafaly females.

Cortisol measure (DUPC 1997)

Fecal samples were collected during observation sessions and stored in Whirl-Pak bags at -20°C until analyzed. Samples were frozen within two hours of collection and later extracted using the same methods described in Chapter 2.

Re-examination of feeding effort and cortisol levels in Madagascar females

In Chapter 3, I found a weak association between feeding effort and fecal cortisol measures for the females at Beza Mahafaly. In this chapter, I explore this relationship more closely to determine whether there existed a non-linear relationship between these two variables.

Mean female cortisol levels were compared to mean feeding effort levels during six observational weeks (Beza: September 24 to November 17, 1995; DUPC: July 7 to August 16, 1997) to determine whether the relationship between the 2 variables was best described with a linear or simple non-linear polynomial (quadratic) function. The R^2 values calculated for a linear function and for a quadratic function were compared.

Statistical analyses

Activity budgets measured from ten weeks of data collection with the Beza Mahafaly females in 1995 and six weeks of data from the DUPC females in 1996 were compared. Proportions of point samples spent within each activity category were compared between Madagascar and DUPC females using Student's *t*-tests.

Feeding effort measures between the high and low accessibility conditions were compared using Student's *t*-test for paired samples. Mean fecal cortisol levels across the three feeding conditions were compared using a repeated measures ANOVA with social group (1 or 2) as the factor and feeding condition (low, mid, high food accessibility) as levels. If the ANOVA results indicated a significant difference between feeding conditions, Student's *t*-tests were used to determine which conditions differed significantly.

The feeding manipulations used here may have indirectly shaped agonistic behavior which may have been a confounding variable in these tests. More feeding patches were available (three vs. one) in the low-accessibility condition and this may have allowed females to distance themselves from one another and avoid agonistic interactions. Also, increased effort spent on feeding in the low accessibility condition may have detracted from time and energy available for aggressive behavior. To determine if rates of female-female aggression changed significantly from the high- to low-food accessibility conditions a Student's *t*-test was used to compare these two rates.

RESULTS

Comparison of activity budgets at DUPC and Madagascar

Female ringtailed lemur activity budgets in the DUPC enclosures during the summer of 1996 were very similar to those observed at the Beza Mahafaly Special Reserve in 1995. During active daytime hours, females at both sites spent approximately 23% of their time feeding/foraging, 12% sleeping, 35% scanning/resting, 20% traveling, 7% grooming and 3% in other activities (marking, urinating, etc.). For both the Madagascar and DUPC data, estimates of time spent sleeping during days are underestimated here since observations were not conducted during the daily afternoon siestas. The only significant difference in activity patterns between the two sites is that DUPC females spent significantly more time grooming than did the Madagascar females ($t_3 = 3.20$, $P < 0.05$).

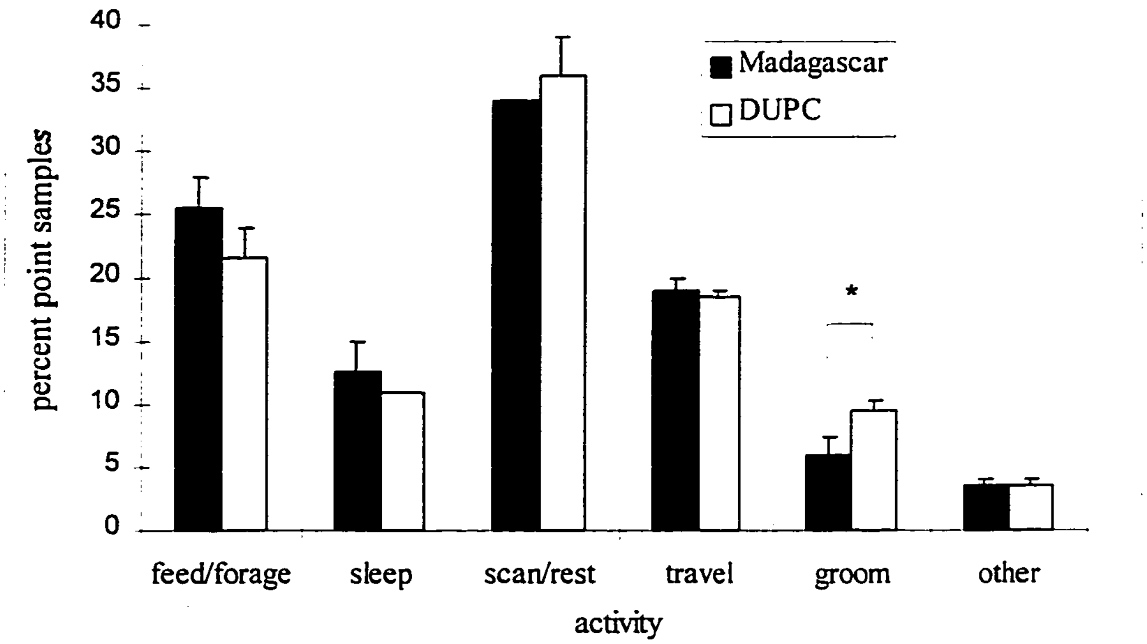

Figure 4-2. Percent of total point samples adult female ringtails spent in each behavioral activity in Madagascar (Beza Mahafaly) from September to December 1995 (filled bars) and at the DUPC from July to September 1996 (open bars). Error lines indicate standard errors.

Effect of food accessibility on feeding efforts

The manipulations used with the DUPC lemurs led to predicted changes in feeding effort levels: decreased food accessibility led to increased feeding effort by the adult female ringtails. Mean (± SD) feeding efforts increased from 0.35 ± 0.22 in the high accessibility condition to 1.05 ± 0.67 in the low accessibility condition (t_7 = 2.98, $P < 0.05$). This three-fold range of feeding efforts was similar to the range exhibited by females from the wet to dry seasons at Beza Mahafaly (Fig. 4-3). However, the food manipulations stimulated feeding efforts that were lower, on average, than those seen in Madagascar (Fig. 4-3).

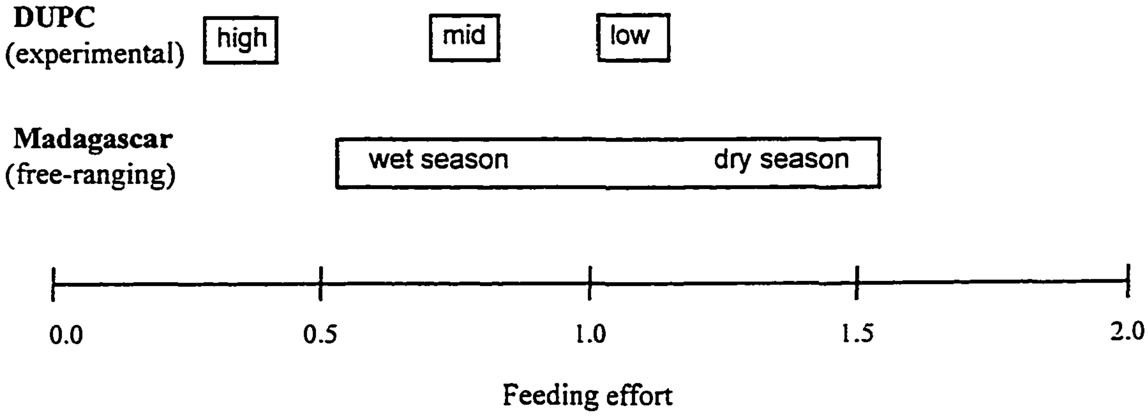

Figure 4-3. Mean feeding effort exhibited by females exposed to high, mid, and low food accessibility conditions in 1997 as compared to the range of mean feeding efforts observed in adult female ringtailed lemurs from dry to wet season in Madagascar (Beza Mahafaly) in 1995.

As feeding effort increased in DUPC females, mean fecal cortisol levels decreased significantly (ANOVA main effect of feeding condition: $F_{2,12} = 4.13$, $P = 0.043$; Figure 4-4). Mean (± SD) cortisol levels were significantly different from the high to low food accessibility conditions (24.3 ± 4.3 to 19.2 ± 7.3 ng/g; $t_7 = 2.72$, $P < 0.05$).

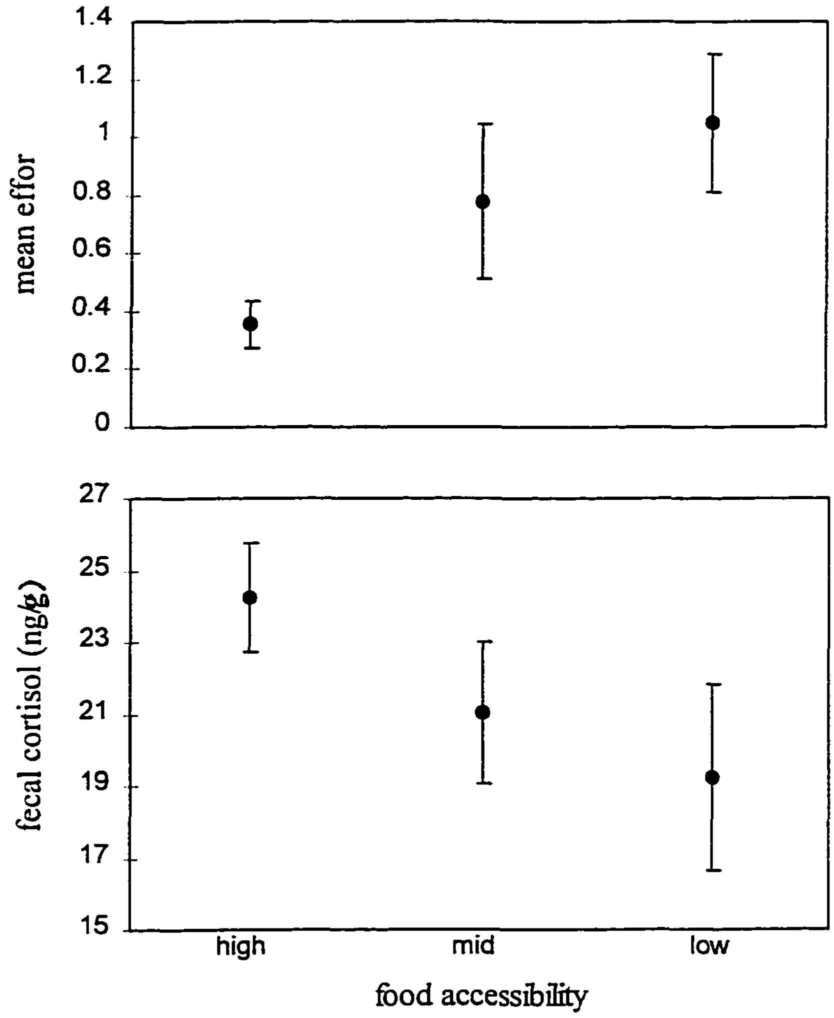

Figure 4-4. Mean feeding effort under each food accessibility condition and corresponding mean fecal cortisol levels in each condition for female semi-free-ranging ringtails at DUPC. Error bars indicate standard errors.

One possible explanation for these results is that during decreased food accessibility there may have been decreased female-female agonistic interactions, thereby leading to decreased cortisol levels. However, this was not the case for the DUPC females. The mean (± SD) rate of initiating female-female aggression was greatest in the low food accessibility condition (high vs. low access conditions aggression rates: 0.50 ± 0.57 vs. 0.67 ± 0.72 attacks/focal h; $t_7 = 1.66$, $P < 0.10$), although this increase from the high to low condition was not significant. Cortisol levels were lowest in the conditions when aggression rates were highest.

A re-examination of feeding effort levels in the Madagascar (Beza Mahafaly) females may clarify the unexpected cortisol levels observed during the food manipulations. The relationship between feeding effort and fecal cortisol levels may be non-linear, with mid-range feeding efforts associated with the lowest cortisol levels. Mean (± SD) weekly cortisol levels in the Madagascar female ringtailed lemurs were lowest (20.9 ± 8.9 ng/g) during the weeks when they displayed moderate feeding efforts, i.e. when they spent approximately four-fifths as much time actively foraging as they did passively foraging. These moderate effort levels are comparable to those seen in the low food accessibility condition at the DUPC. At very low feeding efforts in Madagascar (approx. 0.45), comparable to those seen in the high and mid-food accessibility conditions at the DUPC, Madagascar mean fecal cortisol levels were slightly greater than those seen at mid-range feeding efforts. The difference between the moderate effort and low effort cortisol levels was not statistically significant.

A comparison of individual mean feeding effort and cortisol levels also indicated a non-linear relationship between the two variables (Fig. 4-5). Females that exhibited, on average, moderate feeding effort (0.6 to 0.8) across food accessibility conditions at the DUPC or across observation weeks in Madagascar exhibited the lowest mean cortisol levels (approximately 20 ng/g). Females that exhibited, on average, very low mean feeding effort levels (0.4 to 0.6) had slightly greater mean cortisol levels (approximately 25 ng/g), and females that displayed the highest feeding efforts (0.8 to 1.2) had the highest mean cortisol levels (approximately 30 ng/g).

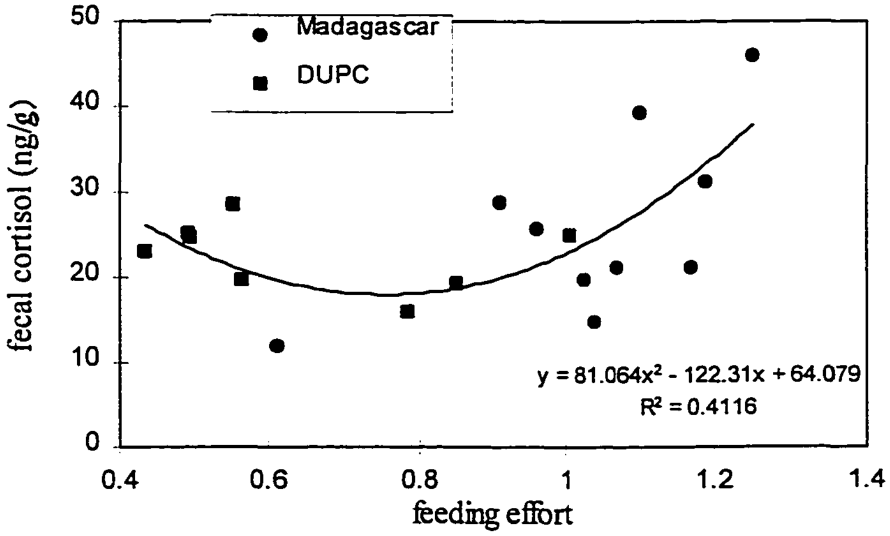

Figure 4-5. Mean fecal cortisol levels relative to mean feeding effort for adult female ringtailed lemurs in Madagascar (Beza Mahafaly, 1995) and at the DUPC (1997).

A linear regression line calculated from the mean DUPC and Madagascar effort and cortisol levels produced an R^2 value of 0.142 ($F_{1,16} = 2.66$, $P = 0.12$). A second-order polynomial fit to these data points produced an $R^2 = 0.412$ (line shown in Fig. 4-6). A similar comparative regression analysis was conducted with just the DUPC females, and a similar trend occurred with these females. A linear regression line fit to individual mean effort and cortisol levels produced an R^2 of 0.103 ($F_{1,6} = 0.69$, $P = 0.44$). A simple quadratic line led to an R^2 of 0.366, suggesting that the relationship between these two variables may be better described by a non-linear function than by a linear function.

DISCUSSION

The activity budget for female ringtailed lemurs at the DUPC was very similar to that of females at Beza Mahafaly in late 1995 and in 1987/88 (Sauther 1992). The similarity between the semi-provisioned DUPC and the unprovisioned Beza Mahafaly lemurs is unlike that seen in other species. Multiple studies with a variety of primate species have shown very distinct differences in activity budgets between unprovisioned and (semi-)provisioned groups. In most studies, unprovisioned groups spend two to three times more time in foraging and feeding than provisioned groups (olive baboons: Forthman-Quick 1984 & 1986, Musau &

Strum 1984; vervet monkeys: Brennan *et al.* 1984; rhesus monkeys: Malik 1986, Seth & Seth 1986; savannah baboons: Altmann & Muruthi 1988) -- even if provisioning involves something as simple as free-ranging animals eating resources found in a human garbage dumpster (Altmann & Muruthi 1988). The feeding regime and enclosure designs at the DUPC provide a unique captive setting that support behavioral repertoires similar to those seen in the natural habitat. The naturalistic enclosures provide an excellent setting for experimental manipulations and may produce findings that can apply to free-ranging populations with greater integrity than results from experimental tests conducted with caged populations. The one drawback to experimental tests conducted outdoors is the uncontrollable climatic conditions, however, these effects can be minimized by balancing experimental conditions across multiple social groups.

In the experimental food accessibility manipulations, female feeding effort levels increased and mean fecal cortisol levels decreased as food accessibility decreased. The relationship between feeding effort and cortisol levels is opposite in direction to that predicted from previous food accessibility manipulation studies conducted with caged animals (Harvey *et al.* 1984, Astheimer *et al.* 1992, Champoux *et al.* 1993). The negative relationship observed in this study may have emerged for several reasons, one of which does not include the possibility that decreased agonistic interactions accounted for decreased cortisol levels with decreasing food accessibility.

One explanation for the negative relationship between feeding effort and cortisol levels may be specific to animals living in highly predictable seasonal environments with relatively long periods of low food availability. For such animals, the most adaptive initial response to decreasing food accessibility may be to diminish metabolic rate and to decrease glucocorticoid secretion to avoid breaking down energy reserves too early into the season of decreased food supplies. What is unknown from the experimental data in this chapter is whether the lemurs use two, or more, strategies to survive their annual dry seasons. For example, they may use one strategy during the first signs of decreasing food availability (i.e. decrease glucocorticoid circulation to preserve energy stores) and another strategy at the end of a long period of decreased food supplies (i.e. increase glucocorticoid circulation to breakdown stored energy). Such a bimodal set of strategies may be most advantageous, particularly in species living in an environment in which the onset and duration of the low food accessibility period is relatively predictable. Based on this kind of scenario, the relationship between feeding effort and glucocorticoid secretion would be non-linear.

Another possible reason for a possibly non-linear relationship may be that individual lemurs can take one of two possible strategies when food quality and/or quantity are low. One strategy might be to decrease one's feeding effort, thereby conserving energy output when energy input is low. The other strategy might be to increase one's feeding effort with the possibility that increased effort may be rewarded with increased food quantity or quality. Such separate physical activity strategies during low food supplies have been observed among Lese horticulturists in northern Zaire (Jenike 1996). Both feeding strategies described above may lead to energy deficits during low food periods and thus explain the observed elevations in cortisol levels in female ringtailed lemurs that display either very high or very low feeding effort levels. At very low and very high feeding effort levels, food intake relative to food quantity or quality would be low and activation of the hypothalamic-pituitary-adrenal axis would stimulate the break down of stored energy reserves to support the organism.

This tradeoff between energy taken in and energy expended may explain the non-linear relationship between effort and cortisol in the Madagascar ringtail females. To determine how it may apply to the DUPC females that were fed the same amount of food in the high or low food accessibility conditions, we might consider the social dynamics between males and females during these feeding conditions. Under normal conditions, when the DUPC ringtailed lemurs were fed whole chow biscuits, males would approach the feeding site where biscuits had been scattered and quickly pluck a whole biscuit and then retreat to a more peripheral location, further from the feeding site, to sit and eat the biscuit for the next few minutes. In this way males accessed chow biscuits during the high accessibility condition and minimized aggression from females. When food accessibility was decreased, by distributing small biscuit pieces on the forest floor, the resource became easier for females to defend against males. Since food items were no bigger than a few bites worth, males could no longer easily enter the feeding area, snatch a large food item, and retreat to eat at a distance from females. This situation may have effectively decreased the amount of food consumed by males and therefore increased the amount of chow available to the females under the low accessibility conditions. This increased food intake for females may explain the decreased cortisol levels observed during the lower food accessibility conditions. Although the females worked more in these conditions, they may have been able to take in more food, and thereby greatly increased their food intake with relatively little increase in feeding effort.

This hypothesis involving alternative feeding effort strategies and how each may account for cortisol excretion patterns under different conditions of food accessibility requires better tests. Analyses of the relationship between energy spent on feeding and energy gained from feeding, and any relationship between these variables and an organism's glucocorticoid secretion strategy would be best tested with information on basal metabolic rates and food quality analyses. Depending on results from such analyses, the glucocorticoid measure may provide an interesting index of the ratio between energy consumed and energy expended for ringtailed lemurs and perhaps other animals.

CHAPTER 5

DOMINANCE STATUS AND FECAL CORTISOL LEVELS

In the field study described in Chapter 3, cortisol levels in free-ranging female ringtailed lemurs were significantly correlated with dominance status. The direction of the relationship (i.e. higher-ranking females exhibited higher mean cortisol levels) was opposite in direction to results from some previous studies with primates and other social animals (e.g. Manogue *et al.* 1975, Keverne *et al.* 1982, Sapolsky 1982). On the other hand, these results were similar to those recently reported on female African wild dogs and dwarf mongooses (Creel *et al.* 1996, 1997). In addition, the results support an early prediction made by Rowell (1974) that high-rankers should experience the highest stress levels within groups of free-ranging animals. Many animal researchers still use dominance ranks to provide some cross-specific characterization of individuals in a group. However, disparate results on the dominance-glucocorticoid relationship suggest that different dominance ranks may present different sets of challenges for each rank position among different animal species. Better comparative analyses of individual status in social groups require more specific descriptions of individual behavioral tendencies in the group (e.g. Smuts 1981).

The relationship between dominance and glucocorticoid secretion can be misleading or non-informative. What is it about high- or low-rank that makes such a position in a social network either more or less challenging? Dominance rank is often associated with several factors such as grooming rates, rates of giving and receiving aggression, access to food, exposure to predators, age, etc. Does one of these more molecular aspects of dominance status best explain the relationships that have been found between dominance and cortisol? In this chapter I will explore this question with female ringtailed lemurs. I will examine the relationship between dominance status and cortisol levels and go on to identify specific social behavioral traits that may best explain relationships found between rank and glucocorticoid levels. The results of these analyses support the notion that individual differences in glucocorticoid levels in social animals are best accounted for by tangible social behavioral differences specific to the group and/or species of study. In addition, I will propose some cross-specific predictions about challenges that may be associated with individual glucocorticoid differences.

Social dominance status and ranks

The concept of social dominance was developed to account for the frequently observed and reliable agonistic relationships found among members of a social group (Hausfater 1975, Bernstein 1981, Drews 1993). The classic example is that of the "pecking-order" found in chicken coups and identified by Schjelderupp-Ebbe (1931, as cited in Rowell 1974). The "pecking order" refers to the fact that it is readily possible to identify: (a) an alpha, or highest-ranking, individual whose presence elicits submissive signals from all group members, (b) a beta, or second-ranking, individual who elicits submission from all group members but the alpha individual, and (c) etc. Not all social groups produce such clear dominance hierarchies, and much controversy has developed around the concept and definition of dominance (Bernstein 1981, Drews 1993). However, the term is still used in animal behavior research, and the concept has regained momentum based on some recently published longitudinal data on benefits and costs of dominance rank positions.

Two longitudinal analyses of the costs and benefits of dominance have been presented recently with free-ranging baboons and chimpanzees in Gombe National Park, Tanzania (Packer *et al.* 1995, Pusey *et al.* 1997). Packer and colleagues examined indices of reproductive success in female baboons in Gombe National Park, Tanzania, over a period of 25 years. Regression analyses indicated that high rank was associated with significant benefits and costs, such as shorter interbirth intervals coupled with greater chances of miscarriages in top-rankers. Pusey and colleagues (1997) analyzed 35 years of data from female chimpanzees and noted primarily reproductive benefits associated with high rank, including increased growth rates in daughters and increased infant survival. However, it is important to note that these researchers indicated that the correlations between rank and reproductive success were so weak that if a non-reproductive top-ranking female were added to the analyses the significance of correlation coefficients disappear. In a cross-species review of dominance rank and mating success in male primates, Cowlishaw and Dunbar (1991) combined data from 32 primate studies, including 13 species, to examine whether rank was positively, negatively, or not at all correlated with reproductive success. After controlling for the confounding effect of age on male dominance rank, they found a significantly positive correlation between rank and measures of mating success such as: (1) number of copulations, (2) number of ejaculations, and (3) genetic paternity determinations. As Cowlishaw & Dunbar (1991) identified in their review, many studies report both costs and benefits associated with high rank, and some even identify both costs and benefits of high rank across years within the same study group. Different conclusions from a variety of studies suggest that dominance hierarchies may mean different things for different species, different groups, and different individuals. More molecular analyses of social behavior would be helpful in determining possible cost and benefits for different individuals within a social group.

Positive correlations between rank and reproductive success are often interpreted as indications of chronic stress in the less reproductively successful individuals (e.g. low-rankers) and negative correlations are usually interpreted as a reflection of stress involved in top-ranking positions. Studies that identify relationships between dominance rank and other cost/benefit measures such as food access and immune responsiveness tend to provide interpretations that include an underlying assumption of "stress" associated with one or another rank position. More direct assessments of this assumed stress involved in rank positions can now be examined in free-ranging animals with the fecal steroid methods described in the previous chapter.

Dominance status and glucocorticoid titers

To estimate costs and benefits of rank by measuring aspects of physiological stress many researchers study glucocorticoid (e.g. cortisol, corticosterone, etc.) levels among ranks. In general, results from studies on glucocorticoid levels in both caged and free-ranging animals have produced conflicting conclusions as to the potential costs and benefits associated with high and low rank.

Many studies with caged animals indicate that, if any rank differences occur in glucocorticoid levels, low-ranking animals within a stable social group exhibit the highest basal glucocorticoid levels (e.g. Manogue *et al.* 1975, Keverne *et al.* 1982, c.f. McGuire *et al.* 1984, Steklis *et al.* 1986, Saltzmann *et al.* 1994). Sometimes, researchers will associated these high glucocorticoid levels with decreased reproductive success and/or dampened immune responsiveness (e.g.

Blanchard *et al.* 1995). Although new findings with rodents clearly show that acute stress responses lead to redistribution of leukocytes that would make responding to environmental assault most effective and efficient (Dhabhar 1998). The high-glucocorticoid, low-immune function profile seen in low-rankers is frequently compared to those of chronically-depressed humans (e.g. Shively *et al.* 1997). Results from cage studies may provide interesting models of human depression; however, the applicability of these results to glucocorticoid function in free-ranging animals and/or non-depressed humans is not clear. A simple closed cage system, in which escape from aggressors is difficult or impossible, affects the challenges associated with rank positions in a way that differs from those seen in an open and complex natural habitat (Haemisch *et al.* 1994).

In addition to cage studies, field studies have been conducted on the association between dominance rank and glucocorticoid levels. Some identify patterns of elevated glucocorticoid levels in low-rankers, similar to that seen with caged animals (e.g. male baboons: Sapolsky 1982), others identify elevated glucocorticoid levels in high-rank individuals (e.g. female African wild dogs and dwarf mongooses: Creel *et al.* 1996, 1997), and yet others identify no reliable differences among ranks (e.g. white-browed sparrow weavers: Wingfield *et al.* 1991, male rhesus macaques: Bercovitch & Clarke 1995). Various explanations can be developed to explain these apparent differences among species and studies. One such explanation may have to do with sex differences since rank positions may infer differential costs and benefits for males and females. Another possible explanation may have to do with differential social behavior patterns of high- and low-rankers among different species.

Behavioral traits associated with rank and glucocorticoids

To account for individual differences in glucocorticoid levels among male baboons, Sapolsky and colleagues identified certain personality traits rather than ranks that associate with elevated serum cortisol levels in male baboons (Sapolsky & Ray 1989; Ray & Sapolsky 1992). These personality traits associated with elevated cortisol in male baboons are similar to those that are associated with elevated cortisol profiles in humans: inability to distinguish threatening and non-threatening situations and inability to redirect aggression. These personality analyses provide intriguing behavioral assays. At the same time, they beg the question of whether or not a more simple behavioral measure could provide clear predictions about individual glucocorticoid differences.

One common, although definitely not universal, difference between high- and low-rankers is in the frequency of agonistic behavior. Creel and colleagues (1996) suggested that frequent aggressive behavior displayed by high-ranking female African wild dogs may explain their high cortisol levels. Initiating aggression among carnivores may be a significant challenge because victims are equipped with flesh-damaging teeth and claws. In support of this idea, Sapolsky

(1993) and McGuire and colleagues (1984) have identified that only when a dominance hierarchy is unstable and high-ranking individuals show increased rates of aggression do high ranked individuals reliably exhibit elevated cortisol levels. However, the proposal that elevated aggression may explain elevated cortisol levels in high-ranking female African wild dogs does not take into account an important confounding variable present in these females: that is, the highest-ranking female in a group is usually the only reproducing individual in the communally-breeding species (Reich 1981, Burrows 1995). Saltzmann and colleagues (1994) have shown in marmosets that reproductively-active females exhibit greater cortisol levels than non-reproductively-active females, regardless of dominance rank. In African wild dogs, the physiological demands associated with reproductive activity, rather than aggression levels, may explain elevated cortisol levels in high-rank females. Further evidence that reproductive activity may confound Creel's hypothesis is the fact that high-rank male African wild dogs in his studies, although more aggressive than lower-ranking group mates, did not show significantly elevated glucocorticoid levels. The simple hypothesis that aggression levels may explain correlations between dominance ranks and glucocorticoid levels is attractive, but it needs further exploration in non-communally-breeding species.

This chapter explores simple behavioral assessments associated with rank and how they might account for observed dominance-glucocorticoid relationships found in female ringtailed lemurs. The goal is to determine if a simple behavioral characteristic, similar to that suggested by Creel and colleagues, can account for dominance-cortisol associations. If this is possible, then cross-specific predictions of individual cortisol profiles might be more feasible without reliance on the interspecifically-variable concept of dominance and/or personality. In addition. with such analyses, a more molecular description of potential social challenges facing free-ranging animals is possible. For example, instead of trying to determine whether low- or high-rank or whether certain personality traits are associated with increased glucocorticoid levels, we could show that rates of some certain social behavior such as receiving aggression or of mutual-grooming best predict glucocorticoid levels (e.g. Gust *et al.* 1993). In this way, we could better identify the social variables that present significant challenges to gregarious animals.

As a comparison species to free-ranging male baboons and female carnivores studied by Creel and Sapolsky, free-ranging female ringtailed lemurs provide an interesting complement. Ringtailed lemurs live in relatively large social groups similar in size to those of African wild dogs, although smaller in size than those of baboons. Female ringtails are the dominant sex of their species, as are male baboons and female African wild dogs. Also, within a social group, most female ringtails give birth each year. This fact removes the confound found in female African wild dogs where the top-ranking highly aggressive female who exhibits the highest glucocorticoid values within a group is also the only female that breeds. Finally, like baboons and carnivores, ringtails are relatively terrestrial animals.

Results from previous studies with other species provided the basis for selecting which patterns of social behavior to compare to rank and to cortisol levels in the female ringtailed lemurs. Five behavioral measures were compared that included both affiliative and agonistic (aggressive and submissive) behavior: (1) mean distance to nearest neighbor, (2) mean proportion of time spent auto-grooming (self-grooming), (3) mean proportion of time spent mutual-grooming (social grooming), (4) rate of initiating aggression, and (5) rate of receiving aggression.

Mean distance to nearest neighbor was selected as an index of the physical position of an animal within a social group. On average, high-ranking ringtails are located closer to their neighbors within a group that are low-rankers. Theoretically, decreased distance to neighbors represents a position of physical protection from predation (Hamilton 1971), which could make it a less-challenging position. Alternatively, this more central position may increase challenges associated with competition for food resources. Auto-grooming was selected as a classic indicator of anxiety in studies of animal well-being, and the measure is often used as such an index with free-ranging animals as well. Mutual-grooming was selected as the affiliative behavior, since grooming with a social partner has been shown to alleviate signs of physiological stress (Ray & Sapolsky 1992, Gust *et al.* 1993, Kamarck *et al.* 1995). Finally, rates of initiating and receiving aggression were selected as agonistic measures since they are two of the most salient behavioral traits that have been associated with physiological stress responses. Many studies show that initiating aggression in a staged fight arena can lead to elevated glucocorticoid levels, although receiving aggression in this same context is usually associated with a greater glucocorticoid response (e.g. Haemisch 1990, Fernandez *et al.* 1994, Schuett *et al.* 1996). The direction of any association between these five social behavioral measures and cortisol levels in female ringtailed lemurs was not predicted, since for many of the variables an argument could be made for a relationship in either direction.

METHODS

Female fecal cortisol values along the dominance hierarchy

In collaboration with W. Levash, A. Jolly, and T. Dubovick, fecal samples and agonistic interaction data were collected from eight social groups, six in Madagascar and two at the Duke University Primate Center (DUPC). Madagascar groups consisted of the two groups at Beza Mahafaly described in Chapter 3 and four groups at Berenty Private Reserve in the southeastern portion of the country near Taolagnaro (Fig. 3-1). Data from the Beza groups were taken from late

September to November 1995 and from the Berenty groups from late September to early October 1997. The two DUPC groups were studied from early July to mid-August 1997. Each of the six Madagascar groups consisted of five adult females. except for one of the Berenty groups, which had only three adult females. One DUPC group had five adult females, the other had four.

Dominance ranks were determined for each female by tabulating all non-aggressive agonistic interactions among females into a matrix, with "winners" plotted along the rows and "losers" plotted in the columns (e.g. Figure 5-1). To determine ranks, individuals in the matrix were re-ordered such that all agonistic interactions were above the diagonal.

UNORDERED

LOSERS

		93	139	143	419	459
WINNERS	93	—	20	5	15	6
	139	0	—	0	3	0
	143	0	31	—	15	2
	419	0	0	0	—	0
	459	0	13	0	8	—

- >

ORDERED

LOSERS

		93	143	459	139	419
WINNERS	93	—	5	6	20	15
	143	0	—	2	31	15
	459	0	0	—	13	8
	139	0	0	0	—	3
	419	0	0	0	0	—

Figure 5-1. Example of an unordered and an ordered agonistic interaction sociomatrix. The matrix on the left includes the five females within group 2 at Beza Mahafaly Special Reserve in Madagascar. Within any interaction, the "winner" is defined as the female that approached another female without showing any submissive signals and the "loser" is defined as the female that responded to an approaching female groupmate with a clear submissive signal, most usually the "spat" vocalization.

This method for determining dominance ranks is a common strategy for primatologists and other animal behaviorists (e.g. Hausfater 1975), and it produces clear results and rankings in species that form transitive dominance hierarchies. Transitive hierarchies refer to those in which the top-ranking individual can elicit submissive signals from all other group mates, and the second-highest ranked individual can elicit submission from all group mates but the top-ranker, and etc., down the line to the bottom-ranked individual. However, ringtailed lemurs do not always form such transitive dominance hierarchies (Jolly 1966, Pereira 1993). In other words, it happens that a female (female 'A') can elicit submissive signals from female 'B', who elicits submission from female 'C', who in turn can elicit submissive signals from female 'A'. For all eight groups studied here, there was no evidence that the dominance hierarchies were intransitive.

Behavioral characteristics associated with dominance and cortisol levels

Behavioral and fecal cortisol measures were collected from four of the groups described above: the two Beza groups and the two DUPC groups. Spacing behavior, affiliative, and agonistic behavioral measures were collected from these groups. Distance to nearest neighbor was recorded during instantaneous scan sampling; affiliative and agonistic interactions were recorded during focal animal sampling (Altmann 1974). Affiliative behavior recorded was mutual-grooming (i.e. grooming with a partner) and was expressed as the percentage of focal animal sampling time each female spent on this activity. The agonistic behavior recorded was initiating aggressive attacks and receiving aggression, and was expressed as the number of attacks per hour of focal animal sampling. Since ringtailed females in this study always responded to aggressive attacks with a submissive signal of some sort ("spat", cower, flee, etc.), "the receiving aggression" measure could also be called "the exhibiting submissive signals in response to aggression" measure. During focal samples, auto-grooming (i.e. self-grooming) was also recorded and expressed as the percentage of focal animal sampling time spent auto-grooming. The final behavior measured was distance to nearest neighbor, in meters, recorded during 15-minute instantaneous scan samples. Behavioral definitions can be found in the Appendix.

Individual mean cortisol levels were measured from fecal samples as described in Chapter 2.

Statistical analyses

Female cortisol values were skewed to the right. To satisfy the assumption of normal-distribution for the statistical analyses used here, cortisol values were log-transformed. To compare female cortisol levels across the dominance hierarchy in the eight social groups, a nested repeated measures ANOVA was conducted with females nested within groups nested within dominance rank. Student's *t*-tests were used to compare mean cortisol values between females of different ranks. Since *mean* cortisol levels may oversimplify a female's cortisol profile, the following additional analyses were conducted. Mean cortisol levels may be high in some individuals, not because their basal cortisol levels are actually elevated, but perhaps because they experience more peaks in cortisol levels and/or these peaks may be higher than females with lower mean cortisol levels. To test whether this is the case with high cortisol females studied here, *median* cortisol levels were compared among females, as well as the *minimum* cortisol measure from each female. If baseline cortisol levels are similar across females, this analysis might reveal either similar median and/or similar minimum cortisol levels across ranks. Student's *t*-tests were used to compare median and minimum cortisol levels between high- and low-mean cortisol females.

To compare behavioral traits that may account for cortisol differences among high- and low-ranking females mean behavioral measures were calculated for the two highest- and three lowest-ranking females in the Madagascar and DUPC groups. A Student's *t*-test, assuming unequal variance, was used to compare behavioral traits between the high- and low-rankers. To further explore relationships among dominance rank, behavioral traits and cortisol, correlation coefficients were calculated among the five social behavioral variables, cortisol, and rank. For this analysis, ranks were assigned in reverse order, such that the highest-ranking female in each group was assigned a "5", the next highest female a "4" and so on, so that a positive correlation between rank and any other trait would indicate that high-rankers had the highest measures within that variable. Finally, a multiple regression analysis, using a backwards stepwise elimination process, was used to determine the relative associations between each behavioral variable and cortisol levels among females. The dependent variable in these analyses was mean log-transformed fecal cortisol levels. The independent variables were: (1) social group identity, (2) mean nearest neighbor distance, (3) percent time spent auto-grooming, (4) percent time spent mutual-grooming, (5) rate of initiating aggression, and (6) rate of receiving aggression.

RESULTS

Female fecal cortisol values along the dominance hierarchy

Dominance ranks could be determined for 35 of the 37 adult females within the eight groups. In one of the DUPC groups, only one agonistic interaction was observed between two of the females, so their relative dominance ranks were not clear. Data from these two females were not used in the following analysis.

There was a significant difference in cortisol values among groups (ANOVA: $F_{7,27} = 2.38$, $P < .05$) and among the five rank positions (ANOVA: $F_{27,192} = 2.16$, $P <$ 0.001; Fig. 5-2). Cortisol concentrations in the two top-ranking females did not differ significantly from one another ($t_{13} = 0.97$, NS), and cortisol levels among the three lowest-ranks did not differ significantly (third- vs. fourth-rank: $t_{11} = 0.70$, NS; third-vs. fifth-rank: $t_{12} = 0.54$, NS ; and fourth- vs. fifth-rank: $t_{11} = 0.26$, NS). Mean cortisol levels for the two top-ranking females were significantly different from mean cortisol levels for the three bottom-ranking females cortisol ($t_{33} = 6.11$, $P < 0.001$). There is a distinct shift in cortisol concentrations from the two top-ranking females to the three bottom-ranking females in these ringtailed lemur groups. The mean cortisol level of high-rankers was almost twice that of bottom-rankers (top vs. bottom: 28.2 vs. 15.7 ng/g).

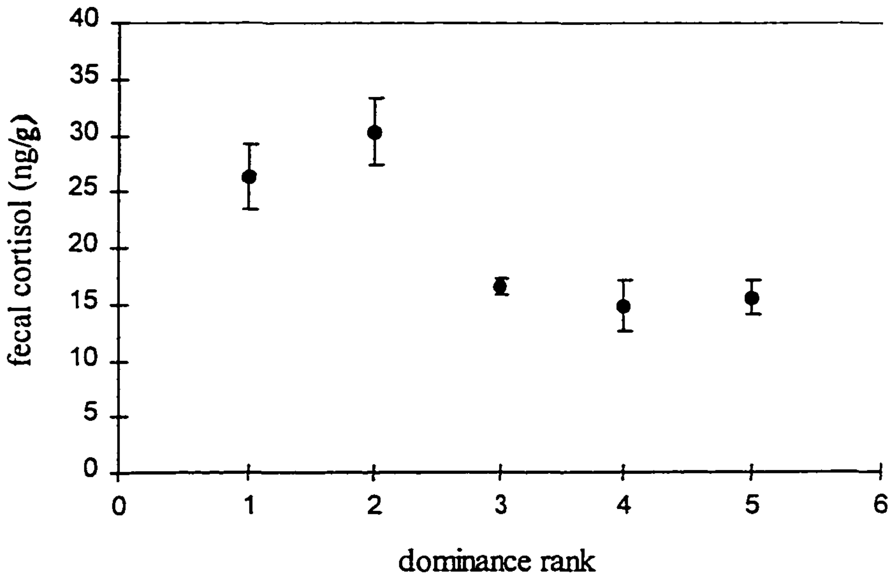

Figure 5-2. Mean fecal cortisol concentration relative to dominance rank position for adult female ringtails in eight social groups: six in Madagascar (two at Beza Mahafaly Special Reserve and four at Berenty Private Reserve) and two at the Duke University Primate Center.

The higher mean cortisol levels in top-ranking females may not reflect simply greater and/or more frequent cortisol peaks. The mean (± SD) median cortisol level for top-ranking females from all eight social groups was 24.2 ± 9.5 ng/g and for the bottom-ranking females it was 14.3 ± 4.3 ng/g ($t_{33} = 3.76$, $P < .01$). In addition, mean (± SD) minimum cortisol levels were significantly different between high- and low-rankers: 14.2 ± 7.1 ng/g vs. 7.9 ± 3.6 ng/g ($t_{33} = 3.14$, $P < .01$).

Behavioral characteristics associated with dominance and cortisol levels

Based on the apparent dichotomy in fecal cortisol concentrations among the two top-ranking and three-bottom ranking females within ringtailed lemur social groups, the following behavioral comparisons were made between high-rankers (top two) and low-rankers (bottom three). The results described below are based on data from the two Beza groups in Madagascar, studied in 1995, and the two groups at the DUPC, studied in 1997.

The only consistent behavioral trait that differed among top- and bottom-ranking females was the rate of being attacked. Bottom-ranking females were attacked significantly more often than top-rankers in both the Madagascar and DUPC groups (Table 5-1). No other behavioral traits are consistently different among high- and low-rankers except for rates of mutual-grooming among the DUPC females, where high-rankers mutually-groomed approximately twice as often as did low-rankers. Other trends included that low-rankers' nearest neighbors were usually situated further from their nearest neighbor than were high-rankers, and high-rankers initiated more aggressive interactions than did low-rankers. This second trend is not apparent in the means presented in Table 5-1 because one of the low-ranking female (third rank) was highly aggressive, and therefore elevated the mean for low-rankers. Finally, hypothesized differences in auto-grooming rates were not evident.

Table 5-1. Behavioral characteristics of two top-ranking and three bottom-ranking females in the Madagascar (Beza Mahafaly) groups and the DUPC groups.

	Madagascar			DUPC		
	HIGH-RANK (N = 4)	LOW-RANK (N = 6)		HIGH-RANK (N = 3)	LOW-RANK (N = 5)	
nearest neighbor distance (m)	2.6	3.2		1.4	1.8	
auto-groom (% total time)	4.9	5.1		6.9	4.1	
mutual-groom (% total time)	0.78	0.90		**3.6**	**1.3**	*
initiate aggression (attacks/h)	0.27	0.14		0.42	0.41	
receive aggression (attacks/h)	**0.01**	**0.25**	*	**0.0**	**0.89**	*
mean fecal cortisol (ng/g)	**33.4**	**18.1**	**	24.5	19.3	

results of *t*-test to compare high- and low-rank females:
* $P < 0.05$, ** $P < 0.01$

In a comparison of feeding effort across ranks, the two sites showed different trends; in the Madagascar groups, high-rank females displayed greater feeding effort than low-ranking females, and in the DUPC females the reverse was true, although for both sites these differences among rank were not significant.

Correlation coefficients among the various behavioral traits, dominance rank, and fecal cortisol measures identify trends similar to those identified from *t*-test analyses. For both the Madagascar and DUPC females, dominance rank was significantly and negatively correlated with rates of receiving aggression (i.e. lower-ranking females received aggression more often than higher-ranking females); (Madagascar: $r = -0.76$, $P < 0.05$; DUPC: $r = -0.94$, $P < 0.01$; Table 5-2 a,b). For the Madagascar groups, dominance rank also was significantly correlated with fecal cortisol levels ($r = 0.68$, $P < 0.05$; Table 5-2a). For the DUPC groups, fecal cortisol levels were also significantly and negatively correlated with the distance to nearest neighbors, such that more distantly spaced individuals (usually low-rankers) had lower cortisol measures ($r = -0.83$, $P < 0.05$; Table 5-2b). Neither rank nor mean cortisol levels were significantly correlated with any of the other behavioral measures. Scatterplots of behavioral data relative to both rank and mean cortisol levels for the Madagascar and DUPC females indicate that all relationships appear linear.

Table 5-2 a,b. Correlation coefficients among five behavioral variables, mean fecal cortisol levels, and dominance ranks for the Madagascar females and the DUPC females.

Madagascar	nearest neigh.	auto-groom	mutual-groom	initiate agg.	receive agg.	mean cortisol	rank
nearest neigh.	---	0.45	-0.01	-0.49	***0.90***	-0.39	-0.60
auto-groom		---	-0.23	-0.21	0.38	-0.14	0.03
mutual-groom			---	-0.40	0.10	0.25	-0.33
initiate agg.				---	-0.53	0.57	0.58
receive agg.					---	-0.60	**-0.76**
mean cortisol						---	**0.68**
rank							---

---

significance of correlation coefficients:
bold coefficients: $P < .05$
bold italicized coefficients: $P < .01$

DUPC	nearest neigh.	auto-groom	mutual-groom	initiate agg.	receive agg.	mean cortisol	rank
nearest neigh.	---	-0.25	-0.44	-0.25	0.66	**-0.83**	-0.72
auto-groom		---	***0.90***	-0.29	-0.47	0.04	0.57
mutual-groom			---	-0.13	-0.50	0.21	0.54
initiate agg.				---	-0.43	-0.12	0.31
receive agg.					---	-0.36	***-0.94***
mean cortisol						---	0.47
rank							---

---

significance of correlation coefficients:
bold coefficients: $P < .05$
bold italicized coefficients: $P < .01$

A multiple regression analysis was performed with the Madagascar females, using as the dependent variable mean log-transformed cortisol values and as the independent variables: (1) group identification, (2) mean nearest neighbor distance, (3) percent time auto-groom, (4) percent time mutual-groom, (5) rate of initiating aggressive attacks, and (6) rate of receiving aggression. The six variables accounted for 80.0% of the cortisol variability ($F_{6,3}$ = 6.32, P = 0.079; Table 5-3a) and the most salient predictor variables were mutual-grooming, initiating aggression, and receiving aggression. The relationship between mutual grooming and cortisol levels was positive (i.e. increased mutual-grooming by a female is associated with higher cortisol levels), as was the relationship between initiating aggression and cortisol. Receiving aggression was negatively associated with cortisol levels (i.e. those females that are frequently exposed to aggressive attacks are most likely to have the lowest cortisol levels).

A stepwise backwards elimination process excluded two variables, and the four remaining predictor variables together predicted 74.2% of the cortisol variability among females in Madagascar ($F_{4,5}$ = 7.47, $P < 0.05$). These variables were: (1) mean nearest neighbor distance, (2) percent time mutual-groom, (3) rate of initiating aggression, and (4) rate of receiving aggression.

In the DUPC groups, all six variables accounted for 86% of the cortisol variability ($F_{6,3}$ = 10.08, P = 0.042; Table 5-3b), and the most salient variables were group, and rate of initiating aggression. The rate of initiating aggression produced a negative coefficient, indicating that elevated rates of aggression predicted lower cortisol levels. A backward elimination process retained four of the six variables: (1) group identification, (2) percent mutual-grooming, (3) rate of initiating aggression, and (4) rate of receiving aggression. These four variables accounted for 91% of the cortisol variability among females ($F_{4,5} = 23.32$, $P = 0.002$). The relationship between mutual-grooming and cortisol was positive, as seen with Madagascar females, indicating that increased grooming was associated with increased cortisol levels. The relationship between initiating aggression and receiving aggression were both negative, indicating that as both behavioral measures increased cortisol levels decreased. The relationship between initiating aggression and cortisol for DUPC females is in a direction opposite to that seen with Madagascar females. The direction of relationships between all other social variables and cortisol were similar between the Madagascar and DUPC groups.

If rank is included as a predictor variable in the multiple regression analyses, along with the other behavioral variables, it is quickly eliminated in the backward elimination process for both the Madagscar and DUPC groups, suggesting that rank was a less reliable predictor of cortisol levels than were any of the behavioral variables. In particular, percent time spent mutual-grooming and rates of receiving aggression were better predictors of cortisol variability than was rank.

Table 5-3 a,b. Results of multiple linear regression analyses in which the dependent variable was mean log-transformed fecal cortisol level and the independent variables were group membership and the five behavioral measures described above. Separate regression analyses were conducted with (a) the Madagascar (Beza Mahafaly) females and (b) the DUPC females.

Madagascar:

adjusted R^2 = 0.800
$F_{6,3} = 6.32$
$P = 0.079$

predictor	**coefficient**	t	p
group	0.096	0.39	0.723
nearest neighbor	0.532	1.85	0.162
auto-grooming	0.141	1.67	0.193
mutual-grooming	0.471	3.39	0.043
initiate aggression	2.340	2.80	0.068
receive aggress.	-2.993	-2.49	0.088

DUPC:

adjusted R^2 = 0.858
$F_{6,3} = 10.08$
$P = 0.042$

predictor	**coefficient**	t	p
group	-0.482	-2.26	0.109
nearest neighbor	0.142	0.36	0.742
auto-grooming	0.006	0.15	0.889
mutual-grooming	0.033	1.27	0.295
initiate aggression	-0.194	-2.71	0.073
receive aggress.	-0.172	-1.59	0.210

DISCUSSION

The two top-ranking females in a group of ringtailed lemurs exhibited significantly greater fecal cortisol levels than did the three bottom-ranking females. As is suggested by differences in median and minimum cortisol levels, the difference in mean cortisol levels among ranks may reflect a difference in basal cortisol levels and not just a difference in the degree of cortisol responsiveness. (Individual differences in cortisol responsiveness will be better tested with experimental protocols that measure changes in circulating glucocorticoid levels after introduction of a noxious stimulus.) In addition, ringtailed lemur females that are frequently attacked and that mutual-groom least often within a group have the lowest mean fecal cortisol levels within a group. For ringtailed lemur females at Beza Mahafaly in 1995 and at the DUPC in 1997, these behavioral characteristics were seen in low-ranking individuals.

The fact that increased mutual-grooming predicted increased mean cortisol levels represents a relationship opposite in direction to those previously found with both caged animals and those in the field (Ray & Sapolsky 1992; Gust *et al.* 1993; Kamarck *et al.* 1995). The reason for this opposite effect may indicate that elevated cortisol levels drive increased mutual-grooming, such that high-rankers experience elevated cortisol levels for a reason other than their grooming profiles, and mutual-grooming may provide one way to reduce, perhaps minimally, cortisol levels. Tests with naturalistic social groups, in which glucocorticoids are experimentally elevated to levels within the range of those seen in free-ranging individuals, may help determine whether glucocorticoid hormones promote mutual-grooming.

Rates of receiving aggression provided the most reliable predictor of fecal cortisol levels for both the Madagascar and DUPC groups. The most common response to an aggressive attack was to emit a "spat" vocalization and to cower or flee. All of these responses must provide clear signals to an aggressor that the receiving party was submitting to their charges. These clear submissive signals and the fact that they are reliably shown in response to aggression have been identified by other researchers comparing ringtails and other primates (Kappeler 1993). These highly ritualized submissive signals and the reliability of their use by victims of aggression may explain why rates of receiving aggression provide one of the most reliable predictors of fecal cortisol levels in the female ringtailed lemurs. Females may avert or neutralize aggressive attacks by simply emitting the highly stereotyped submissive signal, thereby minimizing any possible challenge associated with being attacked. The signal may serve to "turn off" the aggressor, and it may be so effective that victims can be certain of avoiding any aggressive attacks by using this one signal. This "coping strategy" may serve to remove social challenges associated with low-rank. In other words, low-ranking females may essentially "take control" of aggressive attacks by emitting these signals, and therefore do not need to activate the hypothalamic-pituitary-adrenal axis.

On the other hand, aggressive females, often high-ranking females, may not have such a "coping strategy" for dealing with the challenge inherent in initiating aggression. The fact that high-rankers are highly aggressive suggests that their rank position may not be perceived as a stable position and that it must be continually reinforced through aggressive attacks. This would suggest lemurs assume that an aggressive attack could always be met with returned aggression. Returned aggression is rare, but it does occur when lower-ranking females begin to challenge the status of a higher-ranking female. The constant threat of returned aggression, beyond the physical requirements of lunging, chasing, or cuffing, could explain the increased cortisol levels associated with aggressive, high-ranking females.

Relationships previously found between dominance rank and glucocorticoid levels are not usually as striking as those seen among the eight ringtailed social groups described in this chapter. The clear dominance effects seen with the female ringtails may result from the fact that dominance relationships among female ringtails are relatively simple in comparison to other primates. For example, triadic interactions, often seen in higher primates, are rarely seen in ringtails (Kappeler 1993). In addition, agonistic interactions always involve one of the partners displaying a submissive posture rather quickly, and escalation of fights is extremely rare. Finally, social groups are relatively small, therefore minimizing the number of potentially agonistic partners.

Based on the studies of free-ranging social animals, we could formulate predictions as to which species might display a relationship between dominance rank positions and glucocorticoid levels. Clear dominance-cortisol relationships will probably emerge only in social groups that are relatively simple and in which highly stereotyped aggressive and submissive displays are used. In more complex social systems, the best way to understand individual differences in glucocorticoid levels and intra-specific variability in cortisol-dominance relationships would be to identify behavioral characteristics associated with dominance ranks and to examine relationships between these characteristics and cortisol levels. As has been discussed frequently by animal behaviorists, dominance status may provide only a superficial idea of the agonistic relationships within a social group.

Individual differences in stimulation of the HPA axis in social animals may show superficial relationships to dominance ranks, merely because rank positions tend to be associated with certain behavioral traits. Interspecific variability in the relationship between glucocorticoids and rank suggest that further analyses of this simple relationship may be misguided. Rather, it may prove more informative to identify specific behavioral traits associated with glucocorticoid levels, rather than the common comparison of glucocorticoid levels along the dominance hierarchy. These behavioral traits need to be studied relative to the context of the species being studied as well as the group, sex and time of year. For example, for males within a sexually-dimorphic species, initiating aggression towards the same sex may present a greater risk than similar acts would for females attacking females since the risk involved in such actions would be greater due to increased fighting ability among males. The present study with female ringtails examined only five social behavior patterns as they relate to individual cortisol levels. Future studies that can address the relationship between glucocorticoid levels and a greater array of both behavioral and physiological traits (e.g. rates of inter-group aggression, age, reproductive success, feeding success, etc.) are prone to find more meaningful explanations for individual and species differences in glucocorticoid excretion patterns. Identification of specific traits that are related to specific glucocorticoid levels will provide greater insight into the challenges associated with a gregarious life. With more information we may find generalizable patterns across species and individuals.

CHAPTER 6

GENERAL CONCLUSIONS

Results from the studies discussed in the previous chapters suggest new ideas about the adaptive function of glucocorticoid hormones. In this final chapter, I will elaborate on the following ideas: (1) how our understanding of stress physiology may be affected by the environment in which we study it, (2) the functional significance of glucocorticoid secretion patterns in free-ranging animals, and (3) what species or individual differences in glucocorticoid titers may tell us about species and individual coping strategies.

Cage vs. free-ranging studies

The work discussed in previous chapters emphasizes the advantages of studying the glucocorticoid response in free-ranging, as well as caged, animals. Studying the stress response in animals housed in a cage tends to focus attention on chronic stress rather than acute stress. Animals in a simplified unchanging cage being exposed to noxious stimuli are probably more apt to activate the stress response in a chronic fashion even if the stimulus is not long-lasting, simply because the cage context may have become associated with the noxious stimulus. On the other hand, an animal in an open environment, experiencing multiple stimuli, some of which are potentially noxious, can flee or find alternative behavioral patterns to avoid or minimize the stressors, and therefore they may be more apt to activate short term glucocorticoid secretions. The comparison between cage and free-range settings suggests that studies of stress physiology with caged animals will tend to focus on responses to chronic challenges whereas studies with free-ranging animals will tend to focus on responses to acute challenges.

The glucocorticoid response has often been viewed in terms of its negative, pathological aspects -- e.g., damage to hippocampal cells (e.g. Uno *et al.* 1994, Landfield *et al.* 1996), inhibition of the immune system (Sapolsky 1992) and of reproductive behavior (e.g. Greenberg & Wingfield 1987). However, these studies have focused primarily on effects of chronic glucocorticoid secretion, for example that seen by injection of glucocorticoids or by chronic exposure to stressor stimuli (e.g. Blanchard *et al* 1995, Fuchs *et al.* 1995). These responses are of interest in determining the negative impact of chronic stress on health; however, a simple concentration on this aspect of stress tends to draw attention away from potentially positive aspects of acute stress on health (e.g. Dhabhar 1998). At present, we have much more information on the chronic stress condition. This work would be well complemented by more research on mechanisms and functions of acute stress. Researchers have shown distinct differences (often opposite) in the kind of effects these two kinds of stressors can have on performance, growth, health, etc. (e.g. Henry 1992). This suggests that the acute stress response may provide many benefits to organisms, the sum of which are yet unknown. In the future, I think we would be well-rewarded to focus more attention on functions of short-term physiological stress. One way to do this is to study glucocorticoid responsiveness in animals that live under naturalistic conditions and that experience stressors commonly found in the natural habitat.

Glucocorticoid secretion in organisms living in dynamic habitats

Animals that live in habitats where nutritional resources are seasonally scarce may use a system of fatting and decreased glucocorticoid secretion during times of high nutritional availability and then activation of glucocorticoid secretion during the lean times to break down energy reserves (fat, etc.) stored during times of abundance. If the low resource season is long-lasting, animals may be best served by initially decreasing glucocorticoid circulation at the first signs of decreased food supplies and only increasing glucocorticoid levels during the final, most severe, period of decreased food resources. With such strategies, organisms that live in environments in which resources are predictably and seasonally limited can prepare for such conditions during the time of plenty by storing energy in fat, etc. and only at the most severe periods of decreased food supplies, mobilize this stored energy by secreting glucocorticoids. Such organisms may be equipped to deal with negative ramifications of chronic glucocorticoid secretion during low food availability. Alternatively, these organisms may avoid problems associated with chronic glucocorticoid elevation by maintaining low basal levels and then secreting glucocorticoids more often in short bursts and/or in a more exaggerated fashion during harsh periods (e.g. Astheimer *et al.* 1995) to avoid negative effects of chronically-elevated circulating glucocorticoid levels. The conditions predicting increased glucocorticoid secretion in an organism are probably both species- and individual-specific. Non-invasive glucocorticoid measures enable us to study gross patterns of excretion of these hormones among various species and individuals in the natural habitat. Seasonal patterns of cortisol excretion in female ringtailed lemurs suggest that further research, using more fine-tuned and invasive methods, to assess seasonal and individual differences in glucocorticoid secretion patterns will provide insight into differential strategies used to survive in dynamic environments.

Species and individual differences

Seasonal glucocorticoid secretion patterns may differ across species and individuals, even among those that experience the same seasonal environmental changes. Consider for instance a potential difference between a dominant and subordinate individual within a social group. Within a group in which social competition exists, during a period of food abundance, a dominant individual may be more able to access higher quality or quantity of foods, allowing it to develop larger energy stores in its body than a subordinate individual. A subordinate individual, faced perhaps with greater social competition within the social group, may have less time to process and store as much energy as the dominant individual during this same period. During the time of nutritional scarcity, these two individuals may take different strategies. The dominant, well-fatted individual may begin to secrete increased levels of glucocorticoids early into this period to break down stored energy to survive through the lean time. The dominant animal may be able to continue with this strategy throughout the lean period, constantly dipping into the reserves stored during the time of abundance. On the other hand, the subordinate individual may also best survive by increasing glucocorticoid secretion during the scarce times, but may not be able to maintain this strategy throughout the season or at the same level as the dominant individual if it attained less food for storage during the abundant season. Another response by the subordinate may involve minimizing energy requirements through behavioral modifications. In this scenario, the more "stressed" or challenged animal would appear to be the subordinate individual. However, in terms of glucocorticoid levels, the subordinate individual may be the one with the lowest circulating glucocorticoid levels, similar to what was found with ringtailed females in Madagascar and at the Duke University Primate Center. This is just one of many possible scenarios that may account for individual differences. More data on both individual and species differences behavioral and glucocorticoid excretion profiles from the natural habitat will provide means for determining potentially varied strategies for survival in dynamic environments.

Another issue to address is the potential costs and benefits of increased and decreased glucocorticoid levels. When I compared cortisol levels among females within a group, I found a clear relationship between dominance status and fecal cortisol measures: high-ranking females had higher mean cortisol levels than low-ranking females. One interpretation of this result may be that high dominance status incurs many costs. Creel and colleagues (1996, 1997) have supported such an interpretation by citing evidence that high-rankers display increased aggression which may explain increased costs. However, another interpretation may be that increased glucocorticoid secretion, if frequent but not chronic, may incur benefits with limited costs. An animal that responds to challenges by activating the hypothalamic-pituitary-adrenal axis experiences an energy surge, and if the response is not prolonged, may avoid the negative aspects of chronic glucocorticoid secretion such as neuronal damage (Uno *et al.* 1994). An animal which does not respond to adversity with activation of the HPA axis may simply not perceive adversity very well or may not have adequate storage reserves that make such a response advantageous. An animal which maintains chronically elevated glucocorticoids and/or an inhibited glucocorticoid response (e.g. certain male baboons, Sapolsky 1982), may be at a disadvantage. Such cortisol responsiveness cannot be directly assessed using only fecal glucocorticoid measures. This issue will be best addressed using methods such as those described by Sapolsky (1982) and Astheimer *et al.* (1994) in which glucocorticoid secretion into the blood stream is measured in response to an acute stressor in animals in the natural habitat. Fecal steroid measures provide a productive way to document general patterns of glucocorticoid excretion in free-ranging animals. which can then lead to more specific questions about species and individual strategies of glucocorticoid secretion in dynamic habitats.

Lemur catta ETHOGRAM

Behavioral States

feed: ingest food item (feeding bout begins when animal actually begins to chew, and ends when chewing ceases for longer than 5 s)
passive feed: feeding with hind quarters on substrate
active feed: feeding while traveling or standing on substrate

rest: sitting (hind quarters touching substrate) and eyelids closed

scan: sitting (hind quarters touching substrate) and eyes open

travel: standing on all four limbs or walking or running

scent-mark: rub wrist or genital region on an inanimate object

groom self: repeated strokes over body surface with tooth comb or tongue
groom partner: unidirectional grooming of partner; partner identified
mutual groom: two animals grooming one another simultaneously; partner identified

defecate: crouch with tail elevated and hind quarters approximately 8 cm from substrate

urinate: crouch in with tail elevated and hind quarters nearly touching substrate

Affiliative events

approach (*app*): one animal approaches to within arm's length of stationary partner, and stays within this radius for > 5 s; not scored when animals traveling; may not be scored when another affiliative etc. behavior occurs within those 5s
depart (*dep*): move from within arm's length of partner to beyond this distance
sit-in-contact/huddle (*sc/hud*): > .25 of body in contact with partner; first letter in code refers to focal animal position, second letter refers to portion of partner's body in contact with focal

Appendix

Aggressive events *

push (*p*): with either one or two hands, press firmly against partner, maintaining contact for longer than 0.5 seconds

head lunge(*hl*): thrust only top portion of body toward nearby partner

lunge (*lg*): thrust whole body toward nearby partner

cuff (*cf*): manually strike partner (very quick motion, not the same as "hand on")

one-handed grab (*g1*): rapidly seize partner's pelage or body part using one hand

two-handed grab (*g2*): rapidly seize partner's pelage or body part using both hands

pin (*pin*): one or two hands hold partner stationary against an object (usually the ground) for longer than 0.5 seconds

bite (*bt*): orally seize partner for longer than 0.5 seconds

nip (*nip*): bite that lasts less than 0.5 seconds

charge (*cg*): suddenly sprint at partner over a distance of greater than one "lunge length" (behavior qualified by indicating distance over which the animal charged at the partner - e.g. "cg 3" = charged over distance of three meters)

chase (*cs*): sprint in pursuit of fleeing partner (qualify the same way as a chase)

rear (*rr*): stand on hind legs and raise arms slightly during an agonistic encounter (often precedes a lunge)

grapple (*gpl*): two animals locked in each other's grasp, usually on their sides, on the ground; often followed by one animal fleeing

feint to cuff (*fc*): rapidly raise hand, as if to cuff partner

Submissive events *

repeated glance (*rgl*): turn head and gaze toward partner, then turn away, and repeat more than once

cower (*cw*): rapidly lower body or pull body/head away from partner that is less than 1.5 meters away

walk away(*wa*): walk in one direction away from partner that is less than 2 meters away

trot away (*ta*): gallop or slow run in one direction away from partner; involves more bounce in the gate than during a flee

light spat (*lsp*): less audible version of the spat

spat (*sp*): rapid series of 2-10 staccato, high-pitched, noisy calls of moderate amplitude; typically accompanied by grimace and/or cower

Submissive events * (continued)

face spat (*fsp*): repeated spat with face positioned less than 5 cm from partner's face; often accompanied by crouched posture and swaying head laterally in front of partner
deep spat (*sp*): more guttural version of the spat
jump away(*ja*): one single leap away from partner (qualify the same way as a charge, usually only one or two meters)
flee (*fl*): run away from partner after gazing or engaging in social interaction
look away (*la*): turn head or body responsively to gaze fixedly at substrate or away from approaching or socially assertive partner without departing

Other events *

play (*py*): repetitive and highly stereotyped arched lunges and pounces, interspersed with chasing, head-first roll-over, and jumping away
pounce (*pc*): lunge that includes a vertical component greater than approximately 10 cm
take chow (*tc*): manually or orally seize chow biscuit from partner's hand or mouth
travel toward (*trt*): walk, run, etc in the direction of one or more animals, but does not approach within arm's length of animal(s)
anoint tail (*at*): rub brachial glands along tail, presumably saturating tail with glandular secretions
tail wave (*tw*): from a standing position, facing a partner, position tail horizontally above body so that tail tip reaches the vertical plane tangent to nose tip and gyrate tail tip, presumably to disperse scentmark molecules toward partner; often preceded by a tail anoint
crouch (*cr*): spread forelimbs and lower shoulders to ground as if to prepare for a lunge, charge or chase

* Definitions of aggressive, submissive, and "other" events are adapted from Pereira & Kappeler 1997.

Anti-predatory calls

click / click series: low-intensity calls generally given after ground disturbances, and usually accompanied by animals entering trees and visually searching ground (Andrews, 1963; Jolly, 1966; Sauther, 1989)

chirp / moan / gulps: low-intensity call generally given when avian predator perched nearby in a tree or when encountering another social group; usually accompanied by animals approaching bird (Andrews, 1963; Sauther, 1989)

yaps: high-intensity call generally given after ground disturbances after potential predator has been identified; usually accompanied by visually searching ground (Jolly, 1966; Sauther, 1989)

shriek / scream: high intensity, high pitch call emitted when avian predator flies in close proximity of group; accompanied by animals orienting toward and visually tracking predator (Jolly, 1966; Sauther, 1989)

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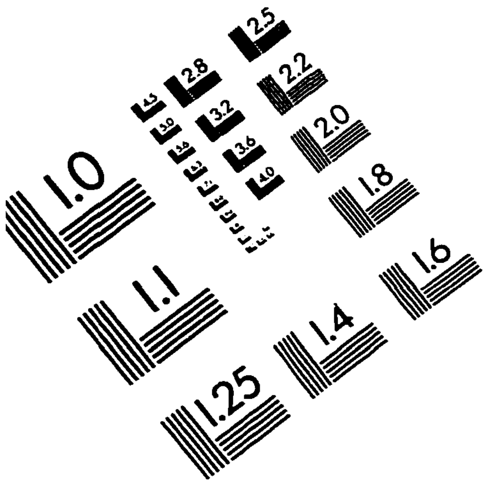

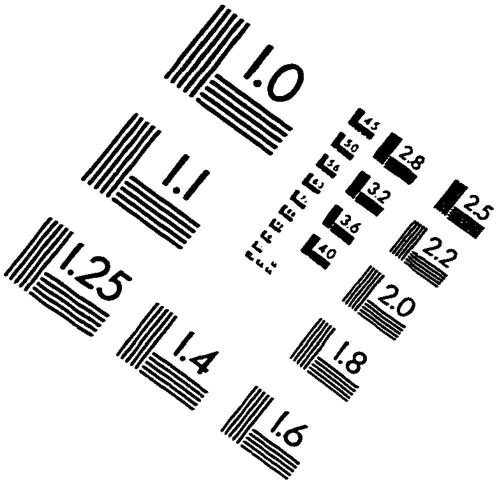

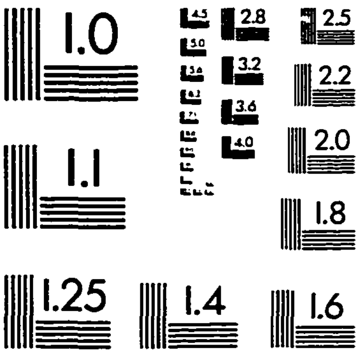

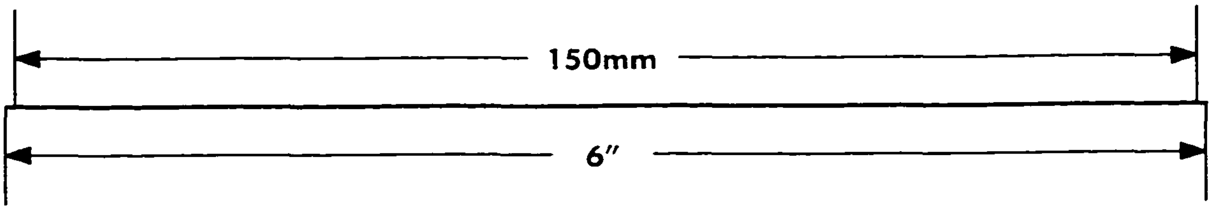

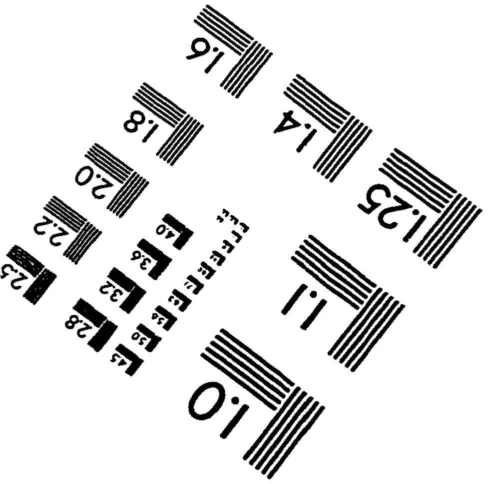

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