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Seasonality, sociality, and reproduction: Long-term stressors of ring-tailed lemurs (*Lemur catta*)

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ABSTRACT

Fecal glucocorticoid (fGC) concentrations are reliable, non-invasive indices of physiological stress that provide insight into an animal's energetic and social demands. To better characterize the long-term stressors in adult members of a female-dominant, seasonally breeding species – the ring-tailed lemur (*Lemur catta*) – we first validated fecal samples against serum samples and then examined the relationship between fGC concentrations and seasonal, social, demographic, genetic, and reproductive variables. Between 1999 and 2006, we collected 1386 fecal samples from 32 adult, semi-free-ranging animals of both sexes. In males and non-pregnant, non-lactating females, fGC concentrations were significantly elevated during the breeding season, specifically during periods surrounding known conceptions. Moreover, group composition (e.g., multi-male versus one-male) significantly predicted the fGC concentrations of males and females in all reproductive states. In particular, the social instability introduced by intra-male competition likely created a stressor for all animals. We found no relationship, however, between fGC and the sex, age, or heterozygosity of animals. In reproducing females, fGC concentrations were significantly greater during lactation than during the pre-breeding period. During pregnancy, fGC concentrations were elevated in mid-ranking dams, relative to dominant or subordinate dams, and significantly greater during the third trimester than during the first or second trimesters. Thus, in the absence of nutritional stressors, social dominance was a relatively poor predictor of fGC in this female-dominant species. Instead, the animals were maximally challenged by their social circumstances and reproductive events—males by competition for mating opportunities and females by late-term gestation and lactation.

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Introduction

The normal mammalian stress response involves a cascade of neuroendocrine events that mobilize energy reserves necessary to cope with immediate challenges, in part by inhibiting nonessential physiological processes and diverting resources from 'maintenance' activities (Moberg, 2000; Munck et al., 1984; Sapolsky, 2002). A major component of this response involves the secretion of glucocorticoids (GC, primarily cortisol and corticosterone) from the adrenal cortex (Nelson, 2000; Sapolsky, 2002). A stressor can be any event, stimulus, or context that produces a physiological stress response, as revealed by an increase in GC concentrations (Creel, 2001; Sapolsky, 2002). While short-term GC elevation is an adaptive response to an acute stressor (Wingfield et al., 1998), chronic activation of the hypothalamic–pituitary–adrenal axis is physiologically costly and has a broad range of deleterious consequences (Sapolsky, 2002). Therefore, researchers frequently rely on GC concentrations, particularly through non-invasive fecal sampling (Whitten et al., 1998), to assess the well being of animals. Such studies have revealed significant inter-individual variation within species in the manner in which animals cope with stressors, which can be shaped, for instance, by social standing, age, or genetic constitution (Abbott et al., 2003; Moberg, 2000; Sapolsky, 2005). Detecting such variation, however, can require long-term, comparative studies that are challenging to conduct under field conditions. As researchers routinely focus on only one social group, one sex, or one portion of the year, we often lack an integrated overview of a species' long-term stressors.

Here, we measured fecal glucocorticoid (fGC) concentrations, over a 7-year period, in the adult members of a captive population of ring-tailed lemurs (*Lemur catta*), comprising several different social groups.

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We monitored both sexes, concurrently, during all phases of their annual reproductive cycle. By examining provisioned, semi-free-ranging animals, we minimized potential nutritional stressors to focus instead on the long-term seasonal, social, demographic, genetic, and reproductive stressors. Our aim was to test for variation in the physiological consequences of strict seasonal reproduction in an unambiguously female-dominant species.

Changes in GC concentrations within species have been linked to environmental factors, such as temperature (Weingrill et al., 2004), time of year (Perret and Predine, 1984), circadian rhythm (Coe and Levine, 1995; Smith and French, 1997a; Sousa and Ziegler, 1998), and food availability (Sapolsky, 1986), as well as to demographic factors, such as sex and age (Gesquière et al., 2005; Mateo, 2006; Robbins and Czekala, 1997). Although rarely explored, there is some evidence of a relationship between genetic constitution (e.g., homozygosity and ploidy) and the stress response (Garner et al., 2008; Kosowska and Zdrojewicz, 1991). GC concentrations also vary by the reproductive state of the individual (Pepe and Albrecht, 1995). Female mammals, in particular, incur substantial costs during reproduction, although this effect can be moderated by increased maternal experience (Bales et al., 2005).

For group-living animals, stressors also can include social factors, such as aggressive interaction (Sapolsky, 1982). More specifically, in gregarious species that are organized by dominance hierarchies, such as primates and social carnivores, stress physiology is often predicted by social status, although the relationship expressed both within and across species is highly variable (Abbott et al., 2003; Creel, 2001). In a meta-analysis of the primate literature, Abbott and colleagues (2003) identified the primary determinants of rank-related patterns in GC concentrations as the frequency of exposure to stressors and the availability of family social support, whereas Goymann and Wingfield (2004) related these patterns to the animals' different levels of allostatic load. Whatever the explanation, the focus of study often has been on male members of male-dominated societies (for an exception, see Pride, 2005a), although a number of researchers have explored similar questions in the members of female-dominated societies (lemurids: Cavigelli, 1999; Cavigelli et al., 2003; Gould et al., 2005; Pride, 2005c; hyenas: Goymann et al., 2001, 2003; Holekamp and Smale, 1998). Rarely, however, have intersexual dominance relations been considered in these analyses.

The ring-tailed lemur is a particularly interesting system in which to examine seasonal, social, and demographic stressors, as reproduction is highly seasonal and, unlike most mammals, the species is female dominant (Jolly, 1966). Consideration of these socioecological factors leads to different predictions about GC concentrations in the two sexes. As in many cercopithecines, lemur societies are characterized by female philopatry and male emigration (Jolly, 1966). Consequently, females have more kin present within their social group than do males. Unlike cercopithecines, however, lemur social status may not be maternally acquired and, within sexes, may not be linear or stable (Nakamichi and Koyama, 1997). Female rank, both within intra-sexual dominance hierarchies and relative to males, is aggressively mediated (Kappeler, 1990). If, as identified by Abbott et al. (2003), family ties influence the stress response of females and/or GCs reflect the physiological translation of behavioral subordination in males, one might predict persistently elevated GC concentrations in male, as compared to female, *L. catta*.

Additionally, the relationship between sex and GC concentrations may vary in relation to the annual reproductive cycle. Female ring-tailed lemurs are polyestrus, cycling up to three times per breeding season (Drea, 2007; Evans and Goy, 1968; Jolly, 1966; Van Horn and Resko, 1977). A female's cycle is roughly synchronous with that of the other female members of her group, such that group members cycle within 1–3 weeks of each other (Drea, 2007; Jolly, 1966; Pereira, 1991; Sauther, 1991). Thus, female receptivity to the male is restricted to three brief periods, with most conceptions occurring during the first cycle. Highly seasonal breeding may have evolved so that births (a typical stressor for females) are timed with favorable environmental conditions; nevertheless, by effectively narrowing the window of sexual receptivity, female synchrony also intensifies the males' competitive burden for reproduction (a typical stressor for males). Although breeding seasons may be predictable stressors for which animals are well prepared (e.g., Wingfield and Ramenofsky, 1997), the actual timing of female fertility within the breeding season may vary somewhat. Coupling extreme intra-sexual male competition with rebukes from unreceptive females, males receive the most aggression during the breeding season (Drea, 2007), which may significantly activate their endocrine stress response at that time.

Genetic diversity (i.e., neutral heterozygosity) might also predict fGC concentrations in both sexes, as inbreeding in lemurs can profoundly impact several fitness estimates, including survival (Charpentier et al., 2008a). Any such relationship might be most pronounced in males, however, as heterozygosity also predicts the seasonal quality of lemur olfactory signals (males: Charpentier et al., 2008b; females: Charpentier et al., submitted for publication), with the greatest loss in olfactory complexity being expressed by inbred males. The latter suggests that intense reproductive competition (experienced as stressors) might take the greatest toll on inbred males.

While we expected male ring-tailed lemurs to be most sensitive to social condition and possibly genetic diversity, particularly during the breeding season, we expected females to be influenced primarily by their own reproductive state, physiological condition, and maternal experience. Cavigelli (1999) found higher fGC concentrations during late gestation than during early lactation in free-ranging female ring-tailed lemurs; however, other periods were unavailable for comparison. With year-round monitoring, we further expected pregnant and lactating females, particularly primipares or females bearing twins, to experience greater fGC concentrations than non-pregnant females at other times of the year. Also, depending on the sex of their fetus, pregnant female lemurids show substantial variation in their sex steroid concentrations, with estrogens increasing dramatically in females bearing sons (Drea, unpublished data; Gerber et al., 2004; Ostner and Heistermann, 2003; Shideler et al., 1983). As circulating or exogenous estrogens can be associated with elevated GC (Kitay, 1963; Phillips and Poolsanguan, 1978; Viau and Meaney, 1991), we expected that dams bearing sons might show substantially higher fGC concentrations than dams bearing daughters.

Methods

Subjects and study periods

The subjects were 32 captive-born, gonadally intact and naturally cycling ring-tailed lemurs, including 13 females (age range: 2–21 years) and 19 males (age range: 2–20 years). These animals were the adult members of three semi-free-ranging social groups housed at the Duke Lemur Center (DLC), in Durham, NC (see below for housing details). In the wild, males and females typically mate for the first time at about 2.5–3 years of age (Sussman, 1991), but conceptions and sirings occur at younger ages in captivity (DLC unpublished records; Parga and Lessnau, 2005). Thus, we considered animals to be mature at about 2 years (Drea, 2007). All the animals were individually known (many additionally had unique collars or shave marks to facilitate recognition from afar). We studied them during three periods over a 7-year span: an 8-month period from December 1999 to July 2000, a 6-month period from October 2000 to March 2001, and a 37-month period from August 2003 to October 2006.

Housing and husbandry

The animals were housed socially in groups that occupied separate forested enclosures (1.5–5.8 ha) with access to indoor areas: During

bouts of inclement weather, the groups were kept in heated indoor areas (110–246 ft^2), which typically had an attached outdoor run (278–347 ft^2) enclosed by chain-link fencing and containing branch supports. By convention, the top-ranking male of multi-male groups remains with the adult females and infants during these temporary winter lock-ups, while the other adult males of the group are housed in adjacent runs (about 100 ft^2), often with shared fencing that permits visual and olfactory access between the animals. The separation serves to mitigate the intense male–male aggression that erupts during the breeding season and might otherwise escalate in confined quarters. These procedures are routine: Release to the outdoors and reunion of the social group are uneventful for the lemurs or, if anything, entail an increase in foraging.

Over the years of study, the composition and sex ratio of the adult members varied within groups, owing to the removal, death, or introduction of animals, as well as the maturation of younger members. We defined three types of social groups, as follows, by their male composition: 'multi-male' groups contained two to four adult males, as well as multiple adult females and their offspring; 'one-male' groups contained one adult male and multiple adult females with their offspring; and 'male-only' groups contained adult males only. Intra-sexual dominance status was assigned to each subject based on the outcome of aggressive interactions observed year round (e.g., Drea and Scordato, 2008; Scordato and Drea, 2007).

The subjects were fed daily rations of a commercially available primate diet (Purina® Monkey Diet 5038, PMI Nutrition International, Inc., Brentwood, MO), supplemented with fresh fruit and vegetables. When the animals were free ranging, they additionally supplemented their diet with food foraged from the forest. Water was always freely available. The animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All research protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Duke University (protocols #A457-99-09, A245-03-07).

Reproduction

At the DLC, the subjects' breeding season is shifted by 6 months from that in Madagascar (Van Horn, 1975) and begins in late October, with a peak in conceptions occurring in early November (i.e., during the females' first of potentially three consecutive and relatively synchronized estrous cycles). Thus, the breeding season typically refers to the span of time encompassing several breeding peaks, rather than representing one continuous season (Drea, 2007). Here, we more specifically define a behavioral 'breeding period' as the cumulative 2-week periods of the year during which mating and conceptions occurred in any group. These three periods included early November, early December, and early February. Accordingly, the 'nonbreeding period' included all other times of the year. As gestation lasts approximately 135 days, most births occurred in March, but some occurred as late as June. We defined the period of lactation as the 3 months immediately following parturition, when infants obtained nearly all of their nutrients by nursing. After 3 months, infants began to eat solid food and increasingly were being weaned by their mothers.

Over the entire span of the study, we observed 18 pregnancies (experienced by 10 females), which involved 4 primipares and 14 multipares. Of these 18 pregnancies, 11 were conceived during the first estrous cycle, 4 during the second estrous cycle, and 3 during the third estrous cycle. The stages of gestation were defined as follows: first trimester, days 1–45; second trimester, days 46–90; third trimester, days 91–135. Eleven of these pregnancies resulted in singletons and seven in twins, the latter occurring more frequently in captivity than in the wild (Parga and Lessnau, 2005). Based on maternal sex hormone concentrations (Drea, unpublished data), we classified fetal sex according to whether or not a male fetus was present. Consequently, 'male fetal sex' included singleton males, twin males, and mixed-sex twins, whereas 'female fetal sex' included singleton females and twin females. Half of the pregnancies had at least one male fetus.

Blood and fecal sampling

The first two periods of study (1999–2001) involved blood and fecal sampling only, whereas during the last period (2003–2006), sampling was coupled with behavioral observation (see below). Using previously described procedures, we obtained one whole-blood sample (of 0.5–1.0 ml) per animal for DNA extraction (Charpentier et al., 2008a). Also using previously described procedures (Drea, 2007) and maintaining a minimum time delay between animal capture and a 3-ml blood draw (mean ± SEM = 5.31 ± 0.26 min), we obtained serum samples about once or twice monthly per subject throughout all stages of the study. For serum samples collected during the first two periods of study only, we collected concurrent fecal samples as part of our assay validation. Specifically, for each blood sample obtained for hormone analysis, we collected three consecutive fecal samples from the same subject, including on the day of blood sampling (day 0), the day following blood sampling (day 1), and the day after that (day 2).

Observations occurred only when animals were semi-free ranging. In addition, on days involving blood draws or group reunions we either avoided behavioral data collection altogether or postponed it until the afternoon, when grooming (post-blood draw) and foraging (post-release) resumed normal frequencies. We also obtained routine fecal samples during the period of behavioral study. Thus, between 1999 and 2006, we collected 1386 fresh fecal samples from adults and an additional 498 samples from infants and juveniles (<2 years), for a total of 1884 samples. We integrated the fecal analysis of adult and infant samples (as described below), but present data only on adult GC concentrations. We obtained most (92%) samples between 7:30 and 12:30 H and the remainder between 12:30 and 16:30 H. Thus, we considered time of collection as a potential explanatory variable in the modulation of fGC concentrations (see below). We stored the samples at −80 °C within 4 h of collection. We lyophilized, pulverized, and sifted the samples into a fine powder within 1 year of storage.

Genetic analyses

As part of several larger studies on DLC colony members (Charpentier et al., 2008a,b), we genotyped all of the 32 subjects in this study at 11–14 microsatellite loci. We calculated the mean neutral heterozygosity per individual (Ho) as the number of heterozygous loci divided by the number of genotyped loci, which ranged in the present subjects from 0.21 to 0.82 (mean ± SEM: 0.56 ± 0.02). As previously shown (Charpentier et al., 2008a), Ho was a good estimator of genome-wide inbreeding or genetic quality for this population.

Serum corticosterone and cortisol assays

Researchers examining stress in primates have reported on either corticosterone or cortisol. Following Wasser and colleagues (2000), we examined corticosterone, but expected a positive correlation between the two GCs. To verify this prediction in lemurs and facilitate comparisons across studies, we first evaluated both GCs in 48 serum samples obtained from a subset of four male subjects. These hormone assays were performed in duplicate at the Biomarkers Core Laboratory, Yerkes National Primate Research Center, using previously validated procedures. Serum corticosterone was determined using a commercial rat radioimmunoassay (RIA) kit (Diagnostic Products Corp., Los Angeles, CA) for which the sensitivity range is 5–2000 ng ml^{-1} at a 50 µl volume. The intra- and inter-assay coefficients of variation (CV) for corticosterone assays were 1.3% and 8.12%, respectively. Serum cortisol was determined using a commercial RIA kit (Diagnostic

Table 1
Effects of seasonal, social, demographic, and genetic explanatory variables on fGC concentrations in adult male and 'nonreproductive' female ring-tailed lemurs.

Explanatory variables	*df*	*F*	*P*	Summary
Breeding period	**1,829**	**13.56**	**0.0002**	**Breeding > nonbreeding**
Male group composition	**2,828**	**26.76**	**<0.0001**	**Multi-male > one-male > male-only**
Time of collection (AM/PM)	1,823	0.24	0.63	
Sex	1,829	0.25	0.61	
Age at sample (years)	1,829	1.55	0.21	
Ho	1,829	1.59	0.21	
Dominance rank	2,612	0.73	0.48	
Sex*breeding period	1,613	0.18	0.68	
Sex*Ho	1,829	0.33	0.56	

Model 1 is based on $n = 831$ data points and $n = 32$ animals. Effects included in the best-fit model are shown in bold. The degrees of freedom vary because of variation in sample size, owing to uncertain dominance ranks and unknown time of collection in a few cases. Three females and two males changed dominance status over the course of the study. The number of females of each dominance rank was as follows: 4 dominant, 6 intermediate, 4 subordinate. The number of males of each dominance rank was as follows: 4 dominant, 3 intermediate, 5 subordinate. Covariance parameter estimate for random effect of the individual (final model): 0.0561.

Systems Laboratories, Webster, TX) for which normal assay range is 0.5–60 μg dl^{-1} with a 25 μl volume. The intra- and inter-assay CVs for cortisol assays were 4.9% and 6.62%, respectively.

Fecal corticosterone assays

To assay fecal samples, we used the methanol vortexing extraction method developed by Wasser and colleagues (2000). In short, to recover the steroids, we mixed ~0.2 g of dry fecal powder with 2.0 ml of 90% methanol and centrifuged the mixture twice, discarding the sediment each time. We stored the remaining methanol–steroid extract at −80 °C until assay. We had recorded the precise amount of each sample and later corrected its steroid concentration by this factor. If 0.2 g of dry sample were unavailable, we weighed the entire sample and then corrected the concentration for that amount.

We determined fGC concentrations in these samples using a Packard Cobra II Gamma Counter and the ImmuChem Double Antibody Corticosterone ^{125}I RIA kit (MP Biomedicals, Irvine, CA), validated for use in a variety of mammalian and avian species by Wasser and colleagues (2000). The primary glucocorticoid metabolite recovered in this assay is corticosterone, with the following known cross-reactivities: 0.34 desoxycorticosterone; 0.10 testosterone; 0.05 cortisol; 0.03 aldosterone; 0.02 progesterone; 0.01 androstenedione; 0.01 5α-dihydrotestosterone; <0.01 cholesterol, dehydroepiandrosterone, dehydroepiandrosterone-sulfate, 11-desoxycortisol, dexamethasone, 20α-dihydroprogesterone, estrone, estradiol-17-α, estradiol-17-β, estriol, pregnenolone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone. The minimum detectable dose was 7.7 ng ml^{-1}. We ran all volumes at one-half of the recommended volumes for the kit. For most samples, we added 10 μl of sample; if the initial concentration fell beyond the detection limits of the assay, we re-ran the samples at volumes of 5–50 μl.

We tested for parallelism in our fecal assay via a serial dilution procedure, as described by Khan et al. (2002), using dilutions of 1:2, 1:4, 1:8, 1:10, and 1:16. We compared the binding curves for serial dilutions of the corticosteroid standards ($y = -17.41\ln(x) + 138$; $R^2 = 0.9931$) to serial dilutions of material extracted from 10 fecal samples ($y = -17.484\ln(x) + 132.14$; $R^2 = 0.9867$). The binding curve from the serial dilutions could be superimposed upon the standard curve within the normal range of values, indicating that the amount of corticosteroid measured varied directly with the volume of extract.

We assayed all fecal samples in duplicate in 42 assays conducted over the course of the study. The maximum allowable limit for intra-assay variation was 6.0% between duplicates. The mean inter-assay variation over all 42 assays for high and low corticosterone controls was 8.0% and 13.0%, respectively. We excluded those samples with corrected corticosterone concentrations that fell beyond the standardized limits of the assay (<25 ng g^{-1} or >1000 ng g^{-1}). The number of samples excluded by this criterion was 15, i.e., less than 1% of the total 1884 samples assayed.

Statistical analyses and explanatory variables

We evaluated the relationship between serum concentrations of corticosterone and cortisol in male subjects, using Pearson correlations. Likewise, we evaluated the relationship between concentrations of serum and consecutive fecal corticosterone, as well as the relationship between serum cortisol and fecal corticosterone. We used general linear mixed models (Mixed procedure, SAS version 9) to assess the influence of social, demographic, genetic, and reproductive variables on the volume-corrected fecal corticosterone measures. We log-transformed fGC concentrations to achieve normality and used these values in all statistical analyses.

We used three separate models to examine the modulators of fecal glucocorticoid concentrations in ring-tailed lemurs. In all of these models, we entered individual identity as a random effect to correct for the non-independence of data points collected on a given individual. We used a backward model selection procedure to select a best-fit set of explanatory variables. Specifically, we started with all potential explanatory effects incorporated into the model, and then removed the effect with the highest *P* value from the model. We then refit the model to the corticosterone measures and repeated these steps until all *P* values for individual parameters remaining in the

Table 2
Effects of social, demographic, and reproductive explanatory variables on fGC concentrations in adult female ring-tailed lemurs.

Explanatory variables	*df*	*F*	*P*	Summary
Male group composition	**1,911**	**38.78**	**<0.0001**	**Multi-male > one-male**
Time of collection (AM/PM)	1,909	0.13	0.72	
Age at sample (years)	1,911	0.92	0.34	
Ho	1,911	1.06	0.30	
Dominance rank	2,767	0.10	0.90	
Reproductive condition	**2,910**	**3.07**	**0.047**	**Lactating > non-pregnant, non-lactating**

Model 2 is based on $n = 913$ data points and $n = 13$ females. Effects included in the best-fit model are shown in bold. The degrees of freedom vary because of variation in sample size owing to uncertain dominance ranks and unknown time of collection in a few cases.
Covariance parameter estimate for random effect of the individual (final model): 0.0374.

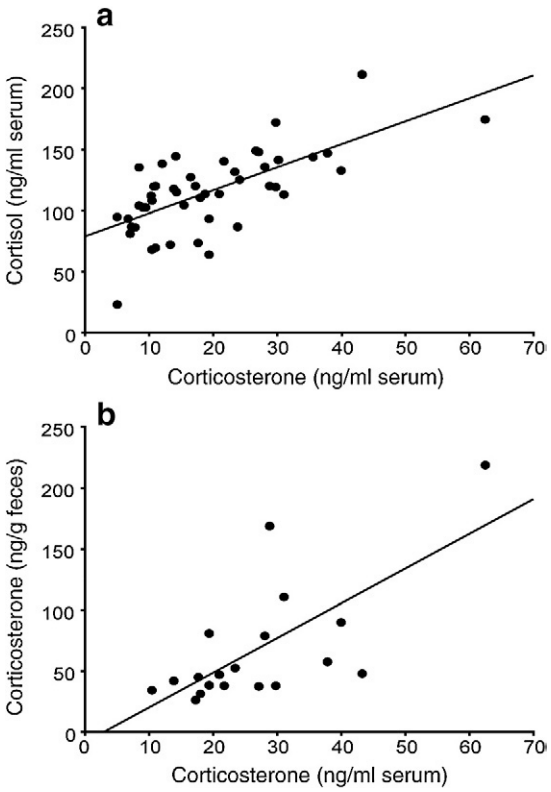

Fig. 1. Relationship between serum corticosterone and (a) serum cortisol and (b) fecal corticosterone in male ring-tailed lemurs. Represented in (a) are the averaged values for four males and in (b) are day 2 values for a single representative male.

model were less than 0.05. Lastly, when a significant effect obtained for a discrete variable with more than two classes, we used the least-squares means post-hoc procedure to ordinate the different classes (SAS version 9).

To isolate the effects of seasonality on both sexes, we first compared males to non-pregnant, non-lactating females (hereafter 'nonreproductive' females). Data points for the latter derived from females that cycled naturally, but did not get pregnant in a given year and from reproductive females prior to their conceptive cycle. Thus, Model 1 included the following variables: breeding period (two classes: breeding and nonbreeding), group composition (three classes: all-male, one-male, and multi-male), time of sampling (two classes: morning, 'AM,' and afternoon, 'PM'), sex (two classes: male, 'M,' and female, 'F'), age at sample (continuous variable, in years), genetic heterozygosity (continuous variable), and intra-sexual dominance status (three classes: dominant, intermediate, and subordinate). We also considered the possible interaction effects between sex and heterozygosity, and sex and breeding period (Table 1).

In two subsequent models, we assessed the effects of potential reproductive stressors on females, focusing on their general reproductive state in Model 2 and more specifically on gestation in Model 3. Model 2 included the previous variables (except breeding period and sex), plus reproductive state (three classes: pre-mating/non-pregnant, pregnant, or lactating, Table 2). Model 3 also included the variables considered in Model 1 (except breeding period and sex), plus the following variables: maternal experience (two classes: primiparous and multiparous), conception cycle (three classes, corresponding to the three potential estrous cycles), trimester of gestation (three classes: first, second, and third), litter size (two classes: singleton and twin), and fetal sex (two classes, 'male' for M, MM, and MF, and 'female' for F or FF).

Results

GC assay validation

As anticipated, we found a statistically significant, positive correlation between serum concentrations of corticosterone and cortisol ($r_{46}=0.675$, $P<0.001$; Fig. 1a). Moreover, serum and fecal concentrations of corticosterone were also significantly and positively correlated for day 2 of fecal collection ($r_{46}=0.253$, $P<0.05$). This pattern is illustrated for a single male subject ($r_{17}=0.696$, $P<0.001$; Fig. 1b) that also showed trends of positive correlations on day 0 ($r_{16}=0.337$, $P=0.085$) and day 1 ($r_{17}=0.359$, $P=0.066$). We could

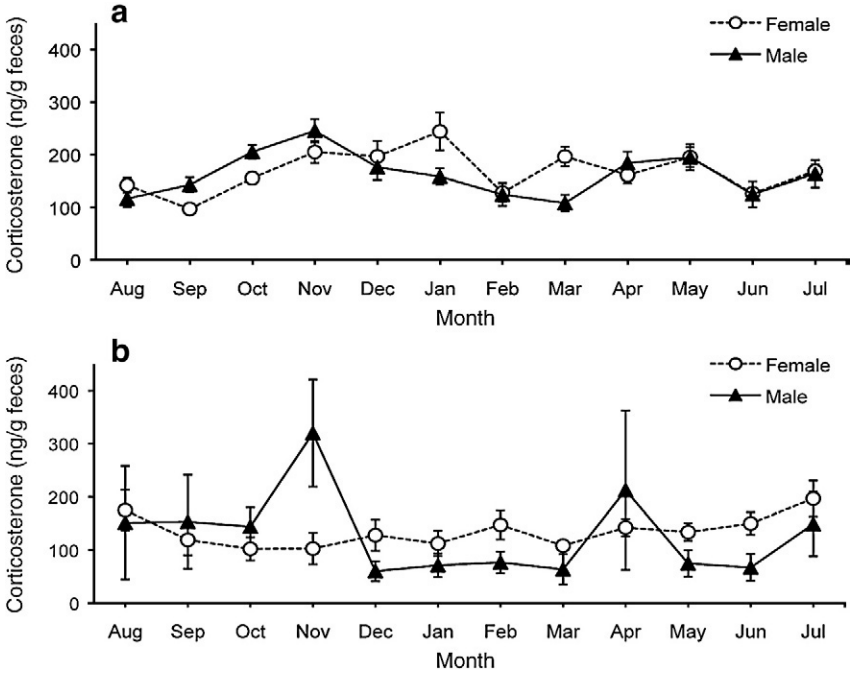

Fig. 2. Annual variation in fGC concentrations (averaged per month) in male and female ring-tailed lemurs living in (a) multi-male and (b) one-male groups.

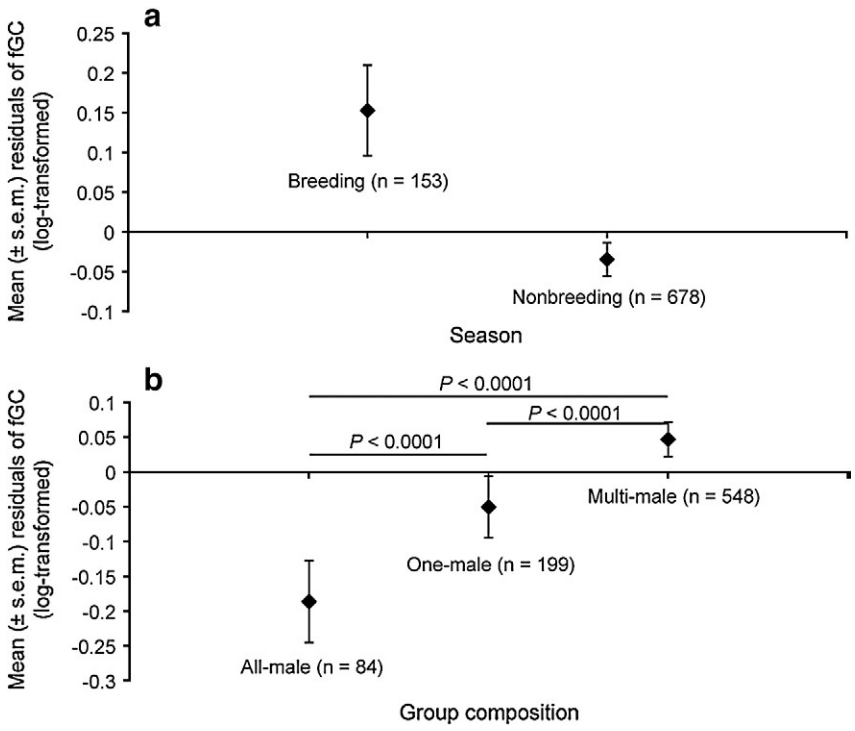

Fig. 3. Effect of (a) breeding period and (b) social context on fGC concentrations in adult male and nonreproductive female ring-tailed lemurs (Model 1).

find no correlation between serum cortisol and fecal corticosterone (e.g., day 2: $r_{42} = 0.077$, $P = 0.63$.).

Annual patterns in male and female fGC concentrations by social context

The mean monthly fGC concentrations for male and female ring-tailed lemurs (Fig. 2) illustrate overall seasonal, social, and sexual variation. As expected for a species with strictly seasonal reproduction, ring-tailed lemurs showed strong annual fluctuations in their fGC concentrations; however, these seasonal patterns appeared to differ between males and females depending on the social context (Fig. 2). For males housed with females (whether in multi-male or one-male groups), the highest peaks in fGC occurred in November, at the beginning of the breeding season, and again in April, during the birthing season. For females, however, a breeding-period rise in fGC concentrations occurred only in those females living in multi-male groups. For these females, the highest monthly fGC peak occurred in January, in the middle of the breeding season (Fig. 2a), whereas for females housed with only one male, the highest monthly fGC peak occurred in July, during the lactation period (Fig. 2b). In both sexes, animals appeared to have higher fGC concentrations in multi-male contexts than in one-male contexts. Below, we explore these and other variables in more detail using our three separate models.

Effect of breeding period and social context on male and female fGC concentrations

When males and 'nonreproductive' females were examined together (Table 1; Model 1), fGC concentrations were significantly greater during the peak mating periods within the breeding season (mean fGC concentrations ± SEM (ng/g): 181.6 ± 12.9) than they were during the remainder of the year (mean ± SEM (ng/g): 142.1 ± 3.8; Fig. 3a).

We also found a strong, significant effect of social context, as defined by a group's male composition, on the fGC concentrations of ring-tailed lemurs (Tables 1-3). For example, according to Model 1,

Table 3
Effects of social, demographic, and gestational explanatory variables on fGC concentrations in pregnant female ring-tailed lemurs.

Explanatory variables	*df*	*F*	*P*	Summary
Male group composition	**1,257**	**65.25**	**<0.0001**	**Multi-male > one-male**
Time of collection (AM/PM)	1,257	0.73	0.39	
Age at sample (years)	1,257	1.02	0.31	
Ho	1,257	0.36	0.55	
Dominance rank	**2,256**	**4.08**	**0.018**	**Intermediate > dominant, subordinate**
Maternal experience (parity)	1,257	1.99	0.16	
Conception cycle	2,256	0.47	0.63	
Trimester	**2,256**	**9.80**	**<0.0001**	**Third > first, second**
Litter size (singleton or twin)	1,257	0	1	
Fetal sex	1,257	1.25	0.26	

Model 3 is based on $n = 259$ data points and $n = 8$ of the 10 females (representing 16 pregnancies). Because the rank relations for two of these females could not be reliably differentiated between intermediate and subordinate, we excluded them from this analysis. Effects included in the best-fit model are shown in bold. The degrees of freedom vary because of variation in sample size owing to uncertain dominance ranks and unknown time of collection in a few cases. Covariance parameter estimate for random effect of the individual (final model): 0.

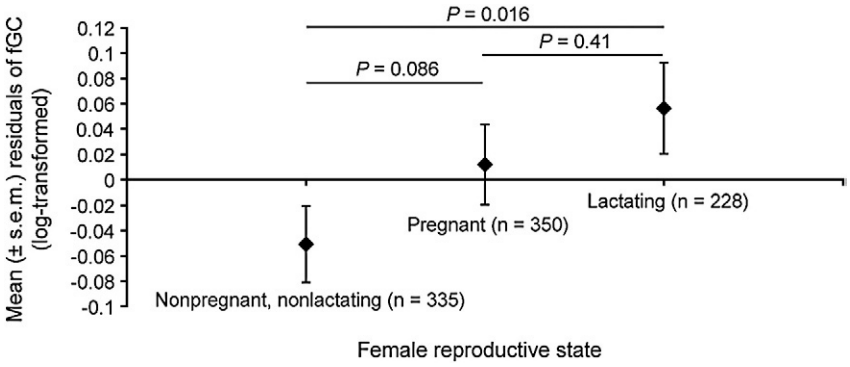

Fig. 4. Effect of reproductive state on fGC concentrations of adult female ring-tailed lemurs (Model 2).

the individuals in multi-male groups (including males and 'nonreproductive' females) had higher fGC concentrations than did the individuals in one-male groups (Table 1) or the males in all-male groups (Fig. 3b). Likewise, the members of one-male groups had higher fGC concentrations than did the members of all-male groups (Fig. 3b). The effect of the presence of multiple males on female fGC concentrations persisted for females in all reproductive states, as revealed by Models 2 and 3 (Tables 2 and 3, respectively).

Variables failing to predict male and female fGC concentrations

We found no relationship between sampling time and fGC concentrations in any of our three analytical models (Tables 1–3). Likewise, we found no effect of an individual's age or neutral heterozygosity on fGC concentrations in any of the three models (Tables 1–3). Although we found no effect of dominance rank in our first two models (Tables 1 and 2), we found a significant effect of dominance during pregnancy, as per our third model (see below; Table 3). Lastly, we found no effect of an individual's sex on overall fGC concentrations in Model 1 (Table 1). Although males and 'nonreproductive' females had overlapping fGC concentrations, males experienced their greatest stressors during the breeding period, whereas females also experienced other reproductive stressors throughout the year that rivaled their breeding-period stressors. Below, we turn to examining the specific reproductive stressors of females.

Effect of reproductive and social variables on female fGC concentrations

A female's reproductive state significantly predicted her fGC concentrations (Table 2; Model 2): females showed significantly greater fGC concentrations during lactation (mean ± SEM (ng/g): 156.5 ± 6.0) than during the non-pregnant, non-lactating portion of the year (mean ± SEM (ng/g): 143.9 ± 5.5; Fig. 4). Greater fGC concentrations were also obtained during pregnancy (mean ± SEM (ng/g): 148.7 ± 5.7), compared to the non-pregnant, non-lactating portion of the year, but this trend was not statistically significant (Fig. 4).

Furthermore, within the subset of gestating females, we detected a relationship between intra-sexual dominance status and fGC concentrations (Table 3; Model 3): females of intermediate status had modestly higher fGC concentrations during pregnancy than did either dominant or subordinate females (Fig. 5a). Moreover, we found a significant effect of the trimester of pregnancy (Table 3; Model 3): fGC concentrations during the third trimester were greater than those

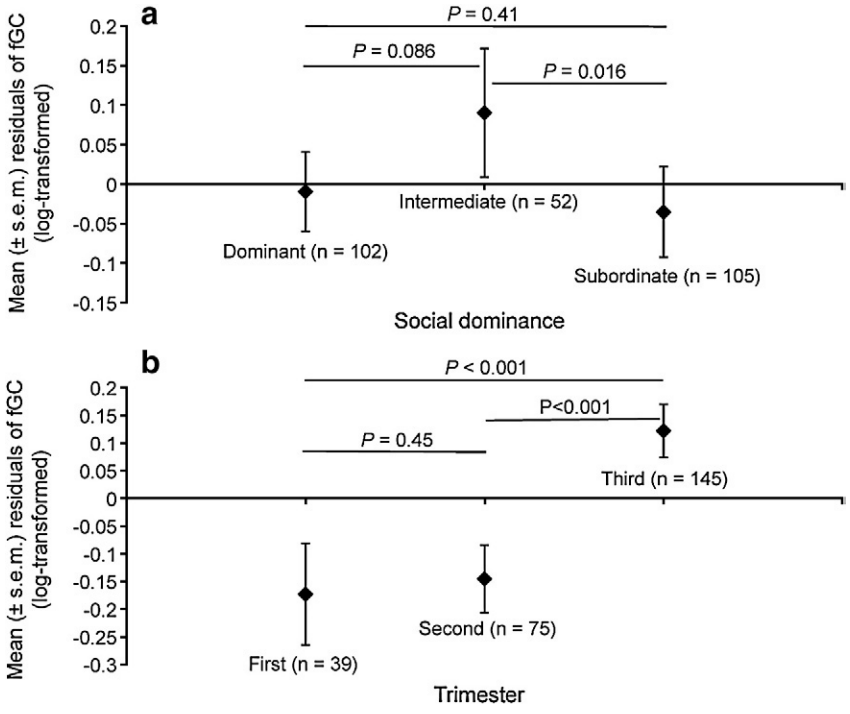

Fig. 5. Effect of (a) social dominance and (b) trimester on the fGC concentrations of pregnant ring-tailed lemurs (Model 3).

during either the first or second trimesters (Fig. 5b). We did not, however, find any effect of maternal experience, conception cycle, litter size, or fetal sex on the fGC concentrations of pregnant females (Table 3).

Discussion

As might be expected for a strictly seasonal breeder, ring-tailed lemurs living in semi-free-ranging social groups showed clear annual cycles in their fGC secretion, with peak concentrations generally coinciding with the peak in breeding, namely, the three 2-week periods surrounding conceptions. Perhaps more surprisingly for an unambiguously female-dominant species the sexes did not differ in their overall mean fGC concentrations. Nonetheless, annual fGC patterns varied in males and females, presumably reflecting sex-biased costs of reproduction. In particular, males were maximally stressed by social factors relating to competition for mating events, whereas females were maximally stressed by their physiological state (i.e., late-term pregnancy and lactation).

Our results on seasonal effects in male fGC concentrations differ from the negative findings reported by Gould and colleagues (2005), which we attribute to differing representations of the nonbreeding period(s) within the breeding season. Gould et al. (2005) examined fGC concentrations during a 20-day mating period and a 2-month 'post-mating' period that followed the first breeding cycle. Thus, their post-mating period encompassed a potential second breeding cycle. At this time, males are still in breeding condition, show heightened aggression, and maintain elevated testosterone concentrations (Drea, 2007). By comparison, we included all three mating periods (each shortened to 2 weeks of actual reproductive activity) in our estimate of the breeding period, and included all post-mating periods (shortened to exclude any portion of a subsequent breeding cycle), as well as the remainder of the year, in our estimate of the nonbreeding period. Moreover, we were able to precisely identify the dates of conception in all of our groups. This precision, coupled with year-round sampling, likely enhanced our ability to detect the anticipated effect of strict seasonal breeding on male physiology.

Interestingly, the reproductive stressors in both sexes appeared to be compounded by the social instability that seasonally characterizes this species (Cavigelli and Pereira, 2000; Drea, 2007; Jolly, 1966; Sauther, 1991; Vick and Pereira, 1989). A potent stressor to males and females alike derived from the group's social structure, notably the presence of multiple adult males within a mixed-sex group. In males, this social stressor was particularly pronounced during the aggressive breeding period, as evidenced by the sustained versus abrupt elevation of fGC concentrations in the male members of multi-male versus one-male groups, respectively.

Consistent with a previous report (Pride, 2005a), therefore, the presence of multiple males in a group increases male fGC concentrations. We additionally found that the presence of multiple males in a group also affected adult female fGC concentrations in a similar fashion. Were females of this species more synchronized in their cycles, this latter finding might suggest increased female stress owing to intra-specific competition over males; however, female group members are sufficiently asynchronous within a breeding cycle (Pereira, 1991) to presumably allow mated males to replenish sperm stores (Koyama, 1988). Limited evidence that female lemurs compete over males is also complemented by ample evidence that females mate multiply (Koyama, 1988; Sauther, 1991). Instead, the stress response of females suggests to us that, despite being the dominant sex, females experience negative consequences of inter-male competition or negative consequences of rebuking more numerous solicitors. Like females, male fGC concentrations were strongly influenced by the presence of the opposite sex, in that members of all-male groups had the lowest fGC concentrations of all.

These intergroup comparisons highlight the powerful modulatory effect of social context on an individual's experience of an otherwise consistent or predictable stressor. Social modulation also may help explain why the relationship between dominance status and GC concentration appears to vary, not only across species, but across studies of the same species. In some cases, subordinate animals show elevated baseline GC concentrations relative to dominant animals (NWM: Manogue, 1975; OWM: Sapolsky, 1982; hyenas: Goymann et al., 2001). In other cases, the reverse is true (lemurids: Cavigelli, 1999; Cavigelli et al., 2003; Fichtel et al., 2007; NWM: Bales et al., 2005; Ziegler et al., 1995; OWM: Weingrill et al., 2004; apes: Muller and Wrangham, 2004; hyenas: Holekamp and Smale, 1998; mongooses and wild dogs: Creel et al., 1996; wolves: Sands and Creel, 2004). Oftentimes, however, no rank relation with GC can be found (lemurids: Gould et al., 2005; Ostner et al., 2008; NWM: Smith and French, 1997b; Lynch et al., 2002; OWM: Bercovitch and Clarke, 1995; Stavisky et al., 2001; Weingrill et al., 2004; apes: Robbins and Czekala, 1997; hyenas: Holekamp and Smale, 1998).

As with intersexual comparisons, we found no evidence of intra-sexual dominance status on fGC concentrations in either adult male or 'nonreproductive' female ring-tailed lemurs, contrary to some studies (Cavigelli, 1999; Cavigelli et al., 2003), but in agreement with others (Gould et al., 2005; Pride, 2005c). Cavigelli (1999) and colleagues (2003) reported that dominant females had elevated fGC concentrations relative to subordinates and linked this finding to the purportedly 'cooperative' breeding system of this species, which has low reproductive skew. Although the cooperative nature of lemur breeding systems is a matter of debate (as relatively rare occurrences of allonursing appear to be facultative, rather than obligate), the authors further noted that this dominance effect was least pronounced in their DLC study group, attributing the difference to the potential fat reserves of provisioned, captive animals. Nevertheless, the results of other field studies do not necessarily support this interpretation. Although not focused on the question of dominance effects, Pride (2005b) reported similar fGC means for dominant and subordinate females, suggesting the absence of rank-related effects in a wild population.

Perhaps dominance effects on fGC concentrations in female lemurs reflect temporary social instabilities. It may also be the case that the analytical scale of long-term studies is relatively insensitive to transient social patterns in fGC concentrations. Alternately, although we found that fecal corticosterone significantly predicted serum corticosterone, there may be sufficient differences between the various fecal assays of glucocorticoids to explain some of the variation. While the reasons for the differences between studies remain obscure, it would appear that no one pattern can be considered as characteristic of the species: Ring-tailed lemurs may not necessarily show rank-related fGC patterns similar to those of cooperative breeding systems with high reproductive skew. Given that the intra-sexual dominance hierarchies of lemurs are neither necessarily linear nor stable, contradictory findings are not entirely unexpected for studies of different groups facing different social challenges.

Social instability may also explain why we found a modest rank-related effect of fGC concentrations in pregnant females. Notably, intermediate-ranking dams showed greater fGC concentrations than did dominant or subordinate dams. It is possible that changes in or challenges to one's dominance status, which occur more frequently among intermediate-ranking animals, are accompanied by frequent agonistic encounters that provoke elevations in fGC concentrations. These encounters may be more taxing on pregnant females that are already physiologically stressed.

In various primates, ovulation, pregnancy, and lactation have been linked to adrenal activation and to an increase in GC concentrations (lemurids: Cavigelli, 1999; Cavigelli et al., 2003; New World monkeys, NWM: Bales et al., 2005; Saltzman et al., 1994; Smith and French, 1997b; Ziegler et al., 1995; Old World monkeys, OWM: Altmann et al.,

2004; Weingrill et al., 2004; humans: Allolio et al., 1990; Lockwood et al., 1996). In this study, female ring-tailed lemurs experienced maximal stress as a result of a combination of social variables and reproductive physiology: Females housed with multiple males showed peak fGC concentrations in mid breeding season, when many were already pregnant, whereas females housed with only one male showed peak fGC concentrations during lactation. More specifically, fGC concentrations were equally elevated in females during late gestation and early lactation. Pride (2005c) also found no differences between female fGC concentrations before and after parturition, but Cavigelli (1999) found a 50% reduction in fGC after parturition. Lastly, we found a significant elevation of fGC in the third trimester of gestation as compared to the first or second trimester, consistent with patterns described previously (Allolio et al., 1990; Cavigelli, 1999; Lockwood et al., 1996). These data indicate that female lemurs, like many other species, experience increasing physiologic costs as their pregnancies progress and throughout the period of exclusive infant nursing (which we observed to be about 3 months following parturition).

In summary, male ring-tailed lemurs experience their greatest stressors during the breeding season, when male–male competition is at its peak. Female ring-tailed lemurs, although socially dominant, may not be sheltered from the social instability resulting from this male–male competition. Indeed, this social stressor may overshadow the physiological stress caused by pregnancy and lactation, as evidenced by the annual cycle of fGC secretion in females under different social conditions. In addition, other forms of social instability, such as intermediate rank, can strain the resources of females already bearing the physiological costs of pregnancy and result in elevated fGC concentrations over females in stable dominance positions. Our findings show that the strength of sexually differentiated, annual fGC patterns, at least in lemurs, can be modulated by a group's social structure. More broadly, however, these findings call attention to the importance of (a) documenting the social structure of a focal group, (b) evaluating both sexes concurrently, (c) conducting prolonged studies (spanning at least one annual cycle), and (d) following multiple social groups for comparative examination. Only through such data can we begin to understand the long-term stressors of social species.

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