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Variation in fecal glucocorticoid concentrations in captive red-shanked douc langurs (*Pygathrix nemaeus*)

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Key words: douc langur, glucocorticoid, captivity

Summary

The goal of the current study was to gather baseline glucocorticoid data from red-shanked douc langurs (*Pygathrix nemaeus*) housed at the Endangered Primate Rescue Center (EPRC), Cuc Phuong National Park, Vietnam. We quantified fecal glucocorticoid concentrations in both males and females, and examined variation in levels in relationship to environmental variables (temperature, weather, housing condition). Samples were collected from four animals, two male and two female, over a three-month period. The results of this study suggest significant inter-individual differences in glucocorticoid levels, and while patterns of fecal glucocorticoids among individual animals showed varying degrees of fluctuation, the significance or underlying cause of these patterns remains unclear.

Thay đổi hàm lượng glucocorticoid trong phân của Chà vá chân nâu (*Pygathrix nemaeus*) trong điều kiện nuôi nốt

Tóm tắt

Mục đích của nghiên cứu này nhằm thu nhận các dữ liệu cơ bản về glucocorticoid ở loài chà vá chân nâu (*Pygathrix nemaeus*) được nuôi tại Trung tâm cứu hộ Linh trưởng Cúc Phương, Việt Nam. Chúng tôi đã tiến hành đo hàm lượng glucocorticoid trong phân của cả hai giới tính và kiểm tra mức độ thay đổi trong mối tương quan với các thông số về môi trường (nhiệt độ, thời tiết, điều kiện chuồng trại). Mẫu phân tích được thu từ 4 cá thể, hai đực và hai cái trong khoảng thời gian 3 tháng. Kết quả nghiên cứu cho thấy có những sự khác biệt có ý nghĩa về mức độ glucocorticoid giữa các cá thể, và trong khi lượng glucocorticoid trong phân thay đổi khác nhau giữa các cá thể thì nguyên nhân của những khác biệt này vẫn còn chưa rõ.

Introduction

Primates living in captive conditions often exhibit signs of stress, including stereotypic movement, hair-plucking, huddling, pacing, and rocking (Boinski et al., 1999). The physiological conditions underlying these responses are complex and are not fully understood, but they are known to involve increases in the amounts of glucocorticoid hormones, including cortisol and corticosterone (Axelrod & Reisman, 1984). Increased levels of these hormones can have both short-term benefits, and long-term costs. In the short-term, increases in these hormones prepare the body for the 'fight or flight' response, by increasing oxygen intake, and increasing immediate availability of energy. In the long-term, prolonged high glucocorticoid levels results in the pathological effects of stress (Sapolsky, 1994). While not all primates living in captivity show outward signs of stress, sustained elevated glucocorticoid concentrations may nevertheless result in detrimental physiological effects.

These issues are important for the management of captive animals in general, and of primates in particular. Past studies have successfully monitored the concentrations of glucocorticoids to assess relative stress levels in captive animal populations with regard to enclosure characteristics, husbandry techniques, and stimuli from other animals, and human interaction (e.g., Davis et al., 2005; Wielebnowski et al., 2002). The results of these studies have been used to recommend changes in the captive habitats of these animals as well as exhibition practices, usually with success. Lowering pathologically high and sustained glucocorticoid levels increases immune function and reproductive capacity, both important factors in the conservation and management of endangered species.

The goal of the current study was to gather baseline glucocorticoid data from red-shanked douc langurs (*Pygathrix nemaeus*) housed at the Endangered Primate Rescue Center (EPRC), Cuc Phuong, Vietnam. By gathering data on glucocorticoids from this population, future comparisons can be made with conspecific individuals housed at zoological institutions in the United States – where it has proven challenging to maintain this species (Edwards & Killmar, 2004).

Red-shanked douc langurs (*Pygathrix nemaeus*) are Old World monkeys that are found only in Vietnam and Laos, and occupy primary and secondary forest habitats, at both medium and high altitudes (Fooden, 1996). They are diurnal primates, and spend at least 50% of their waking hours feeding on a variety of leaves (Pham Nhat et al., 1994). They live in multimale-multifemale groups of variable size (from 3-50 individuals), from which both males and females emigrate. With a life span of up to 30 years, their life history is divided into infant (birth-24 months), juvenile (24 months to 5 years), and adult (post-sexual maturity: 4-6 years in females, 4-9 years in males) phases (Ruempler, 1998). Unfortunately, these animals are endangered. One of the main reasons for their declining population numbers is habitat destruction, with hunting and the lasting effects of environmental disruption by the military during the Vietnam war also playing a role (Lippold, 1995).

Contributing to the difficulty in conserving this species are the problems associated with successful captive maintenance. Due to their highly specialized dietary needs and habitat requirements, zoological institutions have encountered serious challenges (Ensley et al., 1982). For example, of 28 offspring born at the San Diego Zoo, 8 died between the ages of 1.5-3.5 years (Lippold, 1989). Currently, there are only a low number captive individuals alive in zoological institutions worldwide. Only four are found in the United States (at the San Diego Zoo and the Philadelphia Zoo). However, there is a relatively substantial population housed at the EPRC. This rescue and rehabilitation center is home to over 150 animals from 16 taxa, including 29 red-shanked douc langurs. In contrast to the situation faced by the zoological institutions in the US, the population at the EPRC is thriving and reproducing.

A number of factors may contribute to the differences between the Vietnam and US groups, including ambient temperature, humidity, shade cover, and plant species available for consumption. It is possible that these factors, as well as other aspects of the captive environment, such as enclosure size, social groupings, and exposure to sights/sounds of other species (including humans), may play a role, through the physiological effects of stress, in the differential survivorship between these captive red-shanked douc langur populations. Monitoring of glucocorticoid concentrations (e.g., cortisol, corticosterone) can be used to gauge physiological stress, by detecting fluctuations in adrenal activity. The fact that glucocorticoids are secreted in a pulsatile manner in many mammals (Wasser et al., 2000), coupled with the confounding effects of darting an animal (in order to obtain blood samples) on the hypothalamic-pituitary axis, argues for the use of noninvasive sampling and measurement of glucocorticoid metabolites in studies that wish to explore the relationship between hormones and stress (Whitten, 1998; Wielebnowski et al., 2002).

One previous study measured fecal glucocorticoids in red-shanked douc langurs, in order to assess the physiological consequences of social changes on ovarian function in captive females (Heistermann et al., 2004). These researchers noted elevations in cortisol metabolites associated with changes in housing conditions and group compositions, some significant with much variation across different groups. That study did not employ an ACTH challenge and instead used the stress of anaesthesia associated with a dental operation for validation of their assay. The current study examines glucocorticoid metabolite excretion in relationship to differing social conditions, and employs an ACTH challenge in the development and validation of the cortisol assay.

Materials

Study animals

The animals at the EPRC are housed in outdoor enclosures constructed of wire mesh. The size of the cages is 10m x 5,5m x 3,5m. The cages are furnished with bamboo poles and horizontal bamboo construction. Animals are housed in mixed- and same-species groups, same- and mixed-sex groups. The animals included in this study were housed in one of two housing conditions: 1) same-sex, mixed-species and 2) mixed-sex, same-species. Fecal samples were collected from four individuals occupying two different enclosures (Table 1).

Table 1. Animals sampled in this study.

Individual	Sex	ID	Age (y)	Enclosure	# of fecal samples
Bruno	Male	#14	11	13A – Housed with two adult Hatinh langurs	45
Binh	Male	#28	11	5A – Housed with Jarra and Halffeet	59
Jarra	Female	#31	5	5A	59
Halffeet	Female	#46	5	5A	49

Sample collection

Sample collection began March 11, 2007. Daily fecal samples were collected between 9:00 and 11:30 a.m., in order to control for differences in diurnal fluctuation in hormone levels. At the time of collection, information about the animals' activities was recorded on data sheets (e.g., resting, grooming, feeding, sleeping), along with information about weather conditions (e.g., temperature, precipitation, amount of sunshine). Approximately 0.5 g of fecal material was transferred from the enclosure floor to a container marked with the animals' name and date. Samples were frozen at -20ºC until shipment to the United States. Samples were shipped frozen, by air courier and arrived in good condition.

Methods

ACTH challenge

Adrenocorticotropic hormone (ACTH) is the pituitary peptide hormone that regulates glucocorticoid release from the adrenal cortex. In order to show that the glucocorticoid metabolites measured in fecal samples are a reliable indicator of physiological stress, the "ACTH challenge" is used. This quantifies the relationship between behavioral or environmental variables and stress hormones. Fecal and urine samples were collected starting one month prior to the challenge in order to determine baseline hormone concentrations. Then, ~2 IU/kg ACTH (Synacthen Depot, 100 IU/ml, Novartis Pharma SA, Vilvoorde, Belgium) was administered via blowpipe (Table 2). The ACTH challenge was performed on April 16 for all study animals. Fecal and urine samples continued to be collected for two months following the challenge.

Table 2. ACTH administration for each individual.

Individual	Body weigh	Time of injection	Amount of ACTH administered
Bruno	12 kg	8:00 a.m.	20 IU
Binh	12 kg	10:00 a.m.	20 IU
Jarra	5 kg	9:35 a.m.	10 IU
Halffeet	6.5 kg	9:35 a.m.	12 IU

Extraction of steroids from feces

Frozen faecal samples were lyophilized, pulverized using a rubber mallet and processed as described by Young et al. (2004), except that a shaking extraction method was used instead of boiling. Briefly, add 0.5 ml distilled water and 4.5 ml ethanol to ~0.1 g well-mixed dried feces, cap tightly and place on a multi-tube vortexer and vortex for 30 min. After centrifugation (500 g, 20 min), the supernatant was transferred into a glass tube and the pellet resuspended in an additional 5 ml 90% ethanol, vortexed for 1 min and recentrifuged for 20 min at 500 g. Combined ethanol supernatants were dried under air and resuspended in 1 ml 100% methanol. Methanol extractants were vortexed (1 min), sonicated (15 min) and revortexed (30 sec) prior to decanting into a plastic tube for storage at -20°C until assayed. The efficiency of steroid extraction from feces of each species was evaluated by adding ^{3}H-cortisol (~4,000 dpm) to faecal samples before extraction. Mean extraction efficiency was 90.3 ± 0.7%.

Faecal and urinary glucocorticoid metabolite analyses

Cortisol enzyme immunoassay

A cortisol EIA was used to analyze extracted feces by a modification of methods (Young et al., 2004) developed by Munro & Lasely (1988). The assay employed a cortisol-horseradish peroxidase ligand and antiserum (No. R4866; C.J. Munro, University of California, Davis, CA) and cortisol standards (hydrocortisone; Sigma-Aldrich Inc., St. Louis, MO). The polyclonal antiserum was raised in rabbits against cortisol-3-carboxymethyloxime linked to bovine serum albumin and cross-reacts with cortisol 100%, prednisolone 9.9%, prednisone 6.3%, cortisone 5% and <1% with corticosterone, desoxycorticosterone, 21-desoxycortisone, testosterone, androstenedione, androsterone and 11-desoxycortisol (C.J. Munro, pers. comm.). Faecal extracts were evaporated to dryness and diluted 1:16-1:50 in steroid buffer (0.1 M $NaPO_4$, 0.149 M NaCl, pH 7.0). All samples were assayed in duplicate. The EIA was performed in 96-well microtiter plates (Nunc-Immuno™, Maxisorp™ Surface; Fisher Scientific, Pittsburgh, PA) coated 14-18 h previously with cortisol antiserum (50 µl per well; diluted 1:20,000 in coating buffer; 0.05 M $NaHCO_3$, pH 9.6). Cortisol standards (50 µl, range 3.9-1000 pg/well, diluted in assay buffer, 0.1 M NaPO4, 0.149 M NaCl, 0.1% bovine serum albumin, pH 7.0) and sample (50 µl) were combined with cortisol-horseradish peroxidase (50 µl, 1:8,500 dilution in assay buffer). Following incubation at room temperature for 1 h, plates were washed five times before 100 µl substrate buffer [0.4 mM 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt, 1.6 mM H2O2, 0.05 M citrate, pH 4.0] was added to each well. After incubation for 10-15 min, the absorbance was measured at 405 nm. Parallel displacement curves were obtained by comparing serial dilutions of pooled fecal extracts (1:8 - 1:256) with the cortisol standard preparation. Intra- and interassay coefficients of variation were <10% and 15%, respectively. Assay sensitivity was 3.9 pg/well at 90% binding. Glucocorticoid metabolite concentrations are expressed as nanograms per gram dry fecal weight (ng/g).

Corticosterone radioimmunassay

Faecal extracts were also analyzed using a double-antibody 125I corticosterone RIA (MP Biomedicals, Costa Mesa, CA) shown effective in quantifying faecal glucocorticoids in diverse species (Wasser et al., 2000; Young et al., 2004). The polyclonal antiserum was raised in rabbits against corticosterone-3-carboxymethyloxime coupled to bovine serum albumin and cross-reacts with corticosterone 100%, desoxycorticosterone 0.34%, testosterone 0.1%, cortisol 0.05%, aldosterone 0.03%, progesterone 0.02%, androstenedione 0.01%, 5Δ-dihydrotestosterone 0.01% and <0.01% with all other steroids tested (manufacturer's data). Sensitivity of the assay at 90% binding was 12.5 ng/ml. There was no parallelism between serial dilutions of fecal extracts (neat - 1:64) and the corticosterone standard preparation. All dilutions bound at 60 - 80%.

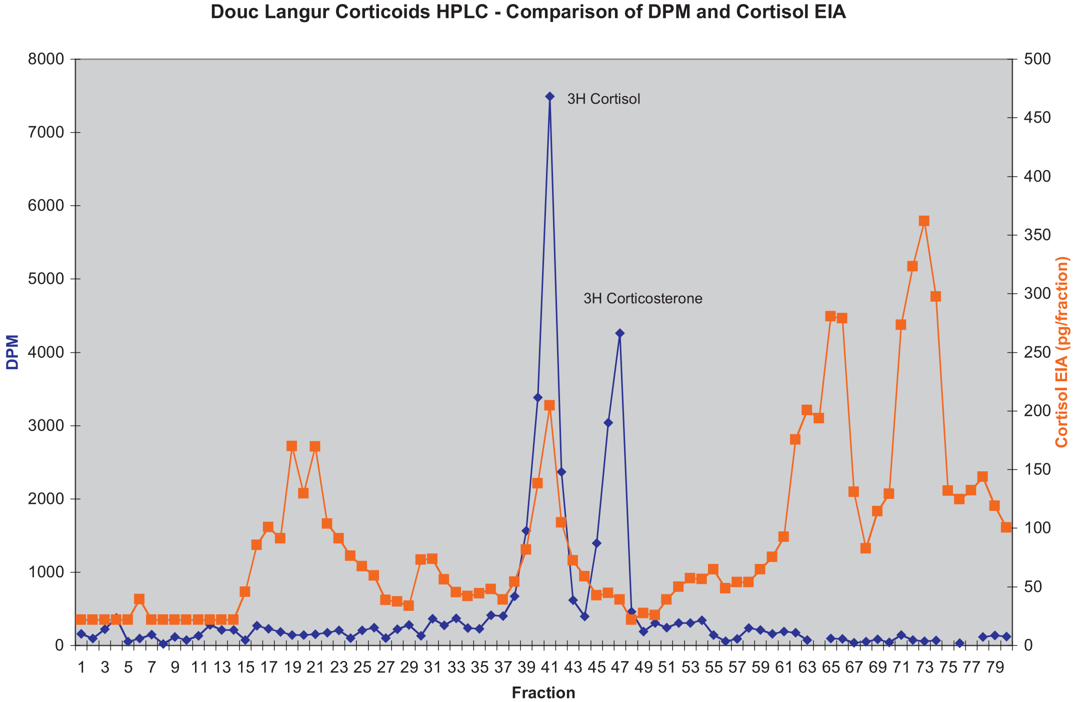
High-performance liquid chromatography (HPLC)

The number and relative proportions of immunoreactive glucocorticoid metabolites in feces were determined by reverse-phase HPLC as previously described (Young et al., 2004). Six extracts were from post-ACTH faecal samples were pooled, evaporated to dryness and reconstituted in 0.5 ml phosphate-buffered saline (0.01 M NaPO4, 0.14 M NaCl, 0.5% bovine serum albumin, pH 5.0) before loading the total volume on a pre-conditioned C-18 matrix cartridge (Spice™ Cartridge; Analtech Inc., Newark, DE). The cartridge was washed with 5 ml distilled water and the total steroids eluted with 5 ml 100% methanol, evaporated to dryness, then reconstituted in 300 µl 100% methanol containing 3H-cortisol and 3H-corticosterone (~4,000-8,000 dpm for each radiolabeled glucocorticoid). Filtered faecal extracts (55 µl) were separated on a Microsorb C-18 column (Reverse Phase Microsorb™ MV 100 C18, 5 µm diameter particle size; Varian Inc., Woburn, MA) using a linear gradient of 20-100% methanol in water over 80 min (1 ml/min flow rate, 1 ml fractions). A subsample of each fraction (100 µl) was assayed for radioactivity to determine the retention times for the radiolabeled reference tracers. The remainder of each fraction (900 µl) was evaporated to dryness, reconstituted in 125 µl l steroid buffer and an aliquot (50 µl) assayed singly in the cortisol EIA and corticosterone RIA as described above.

Results

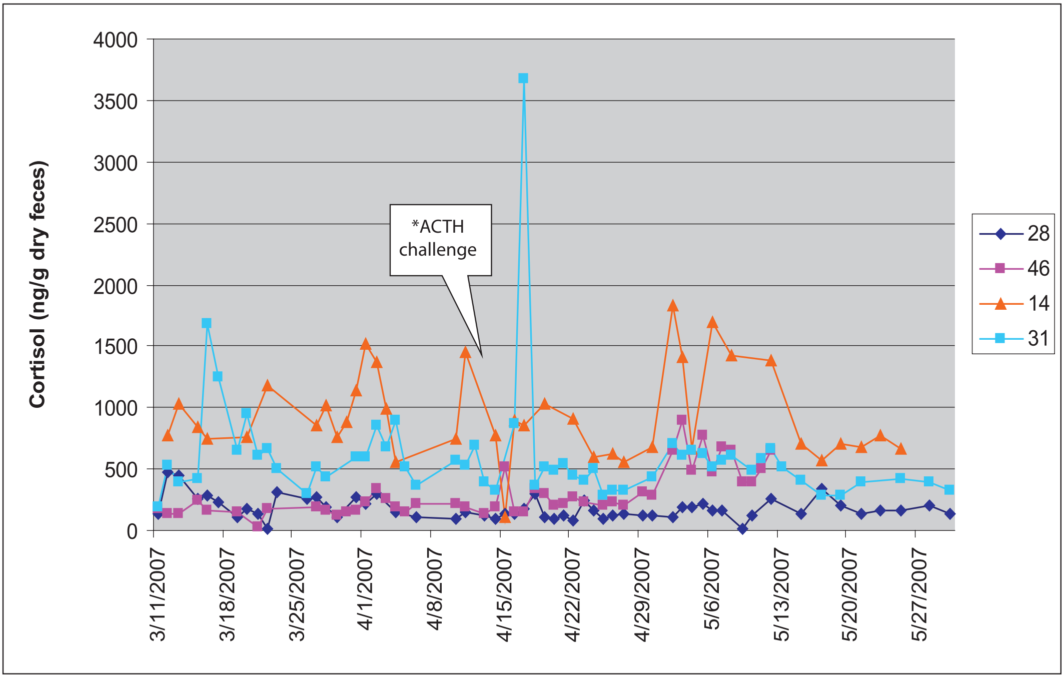
There was no immunoactivity in faecal extracts purified by HPLC using the corticosterone RIA. By contrast, analysis of HPLC fractions using the cortisol EIA detected several fecal metabolites, one of which corresponded with the ^{3}H-cortisol tracer (fractions 40-44) (Fig. 1). Three additional immunoreactive peaks were observed, one of which was less polar (fractions 18-29) and two that were more polar (fractions 63-69 and 73-77) than cortisol. No immunoactivity was associated with the ^{3}H-corticosterone reference tracer (fractions 46-48).

Fig. 1. Cortisol EIA immunoactivity of HPLC-separated fecal extracts.



Plotting individual fecal data by date illustrates differences in both concentrations of and patterns of glucocorticoid secretion (Fig. 2). Most notably, all animals except Jarra (#31) lacked a post-ACTH increase in glucocorticoid concentrations. Within a day of ACTH, there was a marked elevation in concentrations in that female. The lack in response in the other individuals might have been due to differences in efficacy of ACTH administration via blowpipe. Both Bruno (#14) and Jarra (#31) exhibited considerable fluctuations in glucocorticoid concentrations in the first half of the study period, and both also showed moderate glucocorticoid increases at the end of April, lasting roughly two weeks. Analyzing average glucocorticoid values across the study period, there was a nonsignificant trend towards increasing values in the third month of the study (Fig. 3). When individual trends were examined, two opposing patterns were noted: an increase in average glucocorticoid concentrations from the first to third month in #46 and #14, and a decrease in levels from the first to the third month in #28 and #31 (data not shown). There also was marked inter-individual variation in average fecal glucocorticoid concentrations (Fig. 4). Specifically, average

Fig. 2. Fecal glucocorticoid concentrations in study animals.



values for Bruno (#14) and Jarra (#31) were significantly higher than those for Binh (#28) and Halffeet (#46). In addition, Bruno's average glucocorticoid concentrations were significantly higher than those in the other animals (P < 0.001, Kruskal-Wallis). When analyzed according to housing condition (Fig. 5), Bruno (housed with two adult Hatinh langurs) had significantly higher glucocorticoid concentrations than the other three animals (housed with individuals of the same species) (P<0.001, Mann-Whitney U). Analyses of other variables (e.g. weather, temperature) in relationship to glucocorticoid concentrations did not reveal any significant relationships (Fig. 6, 7). Finally, there were no significant sex differences in glucocorticoid concentrations (Fig. 8).

Fig. 3. Average monthly glucocorticoid concentrations throughout the study period (1=March, 2=April, 3=May).

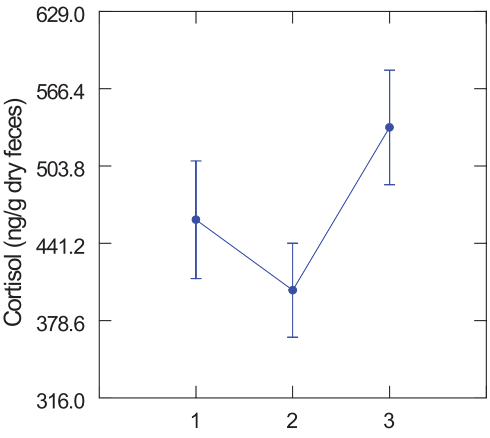


Fig. 4. Average glucocorticoid concentrations by individual.

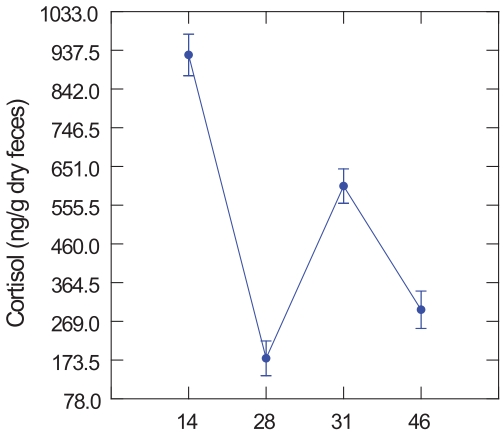


Fig. 5. Average glucocorticoid concentrations by housing condition (DS=different species (Hatinh langur), SS=same species).

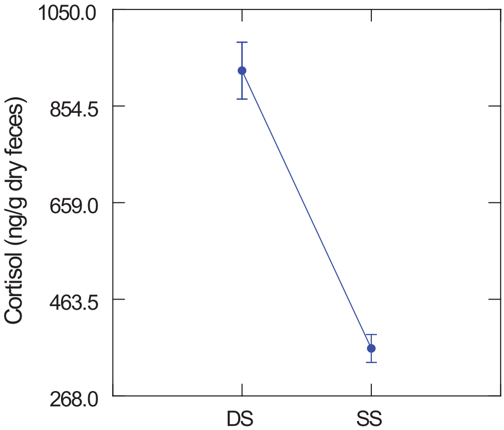


Fig. 6. Average glucocorticoid concentrations by weather; first letter indicates weather in first half of day, second letter indicates weather in second half of day (C=cloudy, R=rain, S=sunshine).

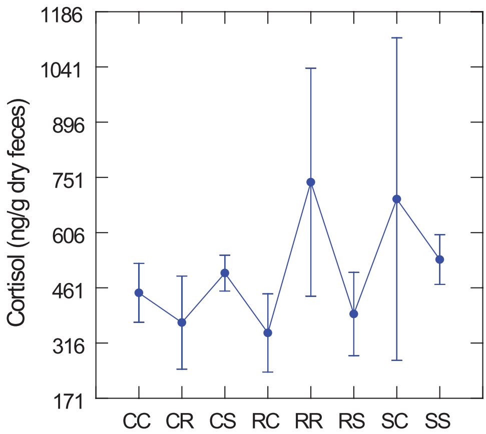


Fig. 7. Glucocorticoid concentrations plotted against average daily temperature, in degrees Celsius (average of morning, noon, and night temperatures).

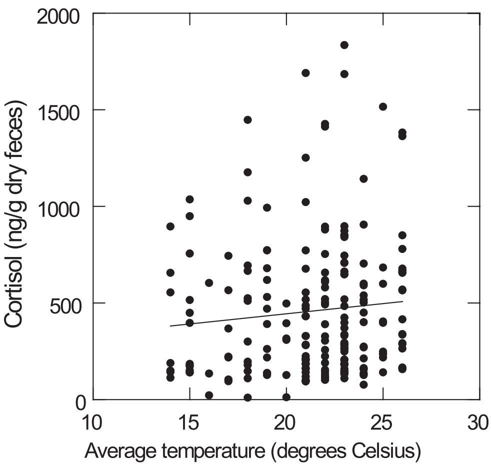
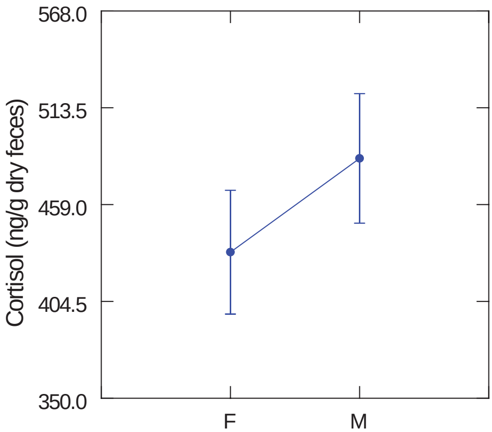


Fig. 8. Average glucocorticoid concentrations for females (F) and males (M).



Conclusions

The current study contributes data on fecal glucocorticoid levels in *Pygathrix nemeaus*, and adds to the one existing study on the subject (Heistermann et al., 2004) by examining concentrations in both males and females, and by looking at variation in relationship to environmental variables (temperature, weather, housing condition). The results of this study suggest significant inter-individual differences in glucocorticoid concentrations, and while patterns of fecal glucocorticoids among individual animals showed varying degrees of fluctuation, the significance or underlying causes of these patterns is unclear. Group housing with conspecifics versus members of a different species, particularly adult members of the same sex, may modulate glucocorticoid secretion. These results, while potentially indicative of "real" patterns, require further sampling over extended time periods to be substantiated. In particular, the lack of a rise in fecal glucocorticoids following ACTH administration in three of four individuals suggests that this component of the study may need to be repeated, perhaps utilizing a different route of administration or higher ACTH dose. Further studies should examine, in more detail, the relationship between seasonality (e.g., Fichtel et al., 2007), reproductive status (e.g., Lynch et al., 2002), and social and environmental factors (e.g., Weingrill et al., 2004) on fecal glucocorticoids in this species.

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