Frequent serial fecal corticoid measures from rats reflect circadian and ovarian corticosterone rhythms

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Abstract

The circadian glucocorticoid rhythm provides important information on the functioning of the hypothalamic-pituitary-adrenal axis in individuals. Frequent repeated blood sampling can limit the kinds of studies conducted on this rhythm, particularly in small laboratory rodents that have limited blood volumes and are easily stressed by handling. We developed an extraction and assay protocol to measure fecal corticosterone metabolites in repeated samples collected from undisturbed male and female adult Sprague–Dawley rats. This fecal measure provides a non-invasive method to assess changes in corticosterone within a single animal over time, with sufficient temporal acuity to quantify several characteristics of the circadian rhythm: e.g. the nadir, acrophase, and asymmetry (saw-tooth) of the rhythm. Males excreted more immunoreactive fecal corticoids than did females. Across the estrous cycle, females produced more fecal corticoids on proestrus (the day of the preovulatory luteinizing hormone surge) than during estrus or metestrus. These results establish a baseline from which to study environmental, psychological, and physiological disturbances of the circadian corticosterone rhythm within individual rats.

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Introduction

The hypothalamic-pituitary-adrenal axis is exquisitely sensitive to environmental perturbations. Given this highly adaptive sensitivity, measuring hypothalamic-pituitary-adrenal axis function in undisturbed subjects can be problematic, particularly in small laboratory rodents. The simple act of handling, restraining, or collecting a blood sample from the tail vein in small rodents can significantly elevate glucocorticoid levels within minutes (Smith & Gala 1977, Tuli *et al.* 1995, Haemisch *et al.* 1999). Thus, even mildly invasive repeated sampling limits studies on the temporal secretory dynamics of the hypothalamic-pituitary-adrenal axis as they normally occur in the absence of human contact. The circadian glucocorticoid rhythm is altered in several pathological states; e.g. major depressive disorder (Sachar *et al.* 1973, Linkowski *et al.* 1985, Pfohl *et al.* 1985), Alzheimer's disease, sleep deprivation (Spiegel *et al.* 1999), and normal aging (van Cauter *et al.* 1996, Kern *et al.* 1996). Identifying such disruptions in a rodent model can be difficult given the problems involved in measuring the glucocorticoid rhythm within an individual over time. As a means of assessing daily temporal dynamics of corticosterone production non-invasively, we have applied newly developed techniques for measuring corticosteroid metabolites in feces to document continuous corticosterone production over time in minimally disturbed male and female laboratory rats.

In small rodents with limited blood volumes, fecal pellets are particularly effective for measuring the cirdadian rhythms of hormones. In contrast to larger mammals, which defecate only once or twice a day, small rodents defecate several pellets every 1–2 h (Kishibayashi *et al.* 1995), affording the higher temporal resolution needed to detect circadian rhythms and perturbations by a stressor. Fecal corticoid measures are also effective for documenting changes in glucocorticoid production over days if not months. Small rodents have small blood volumes, thereby limiting the frequency, volume, and/or duration with which blood samples can be collected for repeated measures within a single individual. Furthermore, jugular cannulation, which is typically used for repeated blood sampling, has been associated with high failure and complication rates after a week (Gans & McClintock 1993). In addition, the cannulation is a stressor, necessarily limiting

an animal's mobility and activity levels, and altering normal glucocorticoid levels and circadian rhythms (Royo *et al.* 2004). Finally, fecal pellets have relatively high concentrations of corticosteroid metabolites (e.g. male Sprague–Dawley rats excrete 80% of exogenously administered radiolabeled corticosterone in the feces (Bamberg *et al.* 2001)), and recent research has shown that a single antibody cross-reacts with a broad spectrum of corticosteroid metabolites, and therefore can be useful in a host of species (Goymann *et al.* 1999, Wasser *et al.* 2000), including rodents (Harper & Austad 2000, Ponzio *et al.* 2004).

In a first study, we assessed the functionality of fecal corticoid sampling by quantifying the temporal characteristics of its circadian rhythm and comparing them with those established from blood samples. Most mammalian species experience a marked circadian rhythm in circulating glucocorticoid concentrations, with peak circulating concentrations in the order of 5–10 times more concentrated than trough levels, and occurring just prior to the daily active period, i.e. in the early morning for diurnal species and at the end of the light period in nocturnal species. In many mammalian species, including rats, this rhythm is asymmetrical: circulating glucocorticoid concentrations rise rapidly at the end of the inactive sleep period, peak just prior to the active period, and then levels slowly decrease across the day to the nadir at the end of the active period (e.g. Guilleman *et al.* 1959, Retiene *et al.* 1968, Krieger 1973, Dahl *et al.* 1991, Frank *et al.* 1995, Rohatagi *et al.* 1996, Atkinson & Waddell 1997).

In a second study, we determined if the fecal corticoid measure could detect changes in glucocorticoid production across the estrous cycle in female rats. Female corticosterone production is highest on proestrus (prior to ovulation) and drops to its minimum during estrus and metestrus (Atkinson & Waddell 1997). On proestrus, the circadian peak in circulating glucocorticoid concentrations is twice that of estrus or metestrus.

The third study compared male and female rat fecal corticoid production. Males and females differ in liver function, produce a different array of fecal corticosterone metabolites, maintain different levels of corticosterone and corticosteroid-binding globulin in circulation, have differential plasma corticosterone-binding capacity, and metabolize corticosterone at different rates (Gala & Westphal 1965, Eriksson & Gustafsson 1970, Ottenweller *et al.* 1979, Woodward *et al.* 1991). Given these large sex differences, we expected sex differences in corticoid metabolite excretion based on HPLC co-chromatography and liquid chromatography-mass spectrometry (LC-MS) analyses.

Methods

Animals

We collected fecal samples from young adult Sprague–Dawley rats: 10 adult males at 15 weeks of age and nine adult females at 11 weeks of age. All animals were housed singly in a reversed 14 h light/10 h darkness cycle with the dark phase beginning at 10:00 h (central daylight saving time, CDST). Temperature in the animal rooms was maintained at 21 ± 1 °C with 15 air changes per hour and food (Harlan Teklad Rodent Diet W, no. 8604) and water provided *ad libitum*. All animals lived in hanging cages (20 × 24 × 18 cm) with wire bottoms through which their feces dropped into a pan of standard wood-shaving bedding (Sani-Chips, laboratory grade). Animals had lived alone in these cages for more than 1 month at the time of this study.

Estrous-cycle days were monitored in females by measuring estrogen and progesterone metabolites in feces. Proestrus was defined as the day of maximal estrogen and progesterone metabolite excretion and these days were verified with vaginal cytology data (LeFevre & McClintock 1988).

Sample collection

Samples were collected for a full day from males to characterize the circadian rhythm, and across 4 days for females to characterize changes across the estrous cycle. Rats' cages did not have to be opened or disturbed during sample collection, thereby minimizing possible disruption of the corticoid rhythm. Samples were collected with forceps directly from the bedding under each hanging cage, and wetness (urine) surrounding any sample was noted. Collections occurred seven times a day at 6:00, 9:00, 12:00, 15:00, 18:00, 21:00, and 24:00 h. This sampling frequency was developed to maximize temporal acuity while maximizing the probability that at least one pellet would be defecated per sampling interval (Kishibayashi *et al.* 1995). Given that samples collected at 9:00 h could be defecated any time between 6:00 and 9:00 h CDST, we identified each sampling interval according to the midpoint time of the interval (e.g. samples collected at 9:00 h were identified as coming from the 7:30 h interval). The dark-phase samples (10:30, 13:30, 16:30, and 19:30 h intervals) occurred during the rats' active period, and the light-phase samples (22:30, 3:00, and 7:30 h intervals) occurred during the rats' inactive period. Samples were collected into Whirl-Pak bags (Nasco, Fort Atkinson, WI, USA), labeled, and stored at −30 °C until extracted. If a sample was collected from a urine-soaked area of bedding, the sample bag was marked as 'urine-contaminated'.

Fecal corticoid extraction

Fecal steroids were extracted using previously published methods (Wasser *et al.* 1994). Briefly, frozen samples were thawed, dried overnight in a centrifugal evaporator, crushed into a dust-like material, and 0·2 g weighed into

a 15 ml centrifuge tube. Ethanol 10 ml (100%) was added to each sample which was then boiled in a water bath for 20 min. Upon removal from the bath, tubes were centrifuged for 15 min and the supernatant was poured off into a glass tube. Ethanol 5 ml was added to the fecal sample tube, and the sample was then vortexed for 1 min, re-centrifuged for 15 min, and the supernatant added to the previous 10 ml of extract. Supernatants were evaporated under air then re-constituted with 1 ml methanol and stored at −80 °C until assay. To monitor procedural losses, radiolabeled corticosterone (1200 c.p.m.) was added to a subset of samples before extraction and post-extraction recovery was quantified by counting a portion of the extractant. Hormone mass was corrected by dividing by the percentage of isotope recovery.

To control for daily fluctuations in fecal mass, we present total fecal corticoids produced during each collection interval as opposed to the more traditional method of presenting concentration of corticoids in fecal samples. Thus, hormone concentrations were expressed as the total mass (ng) excreted per 3-h sampling interval. If a rat defecated 0·5 g fecal matter (dried) in a given 3-h interval, and the concentration of corticoids in that sample was 100 ng/g of dry fecal matter, then the total corticoid mass for that sampling interval would be 50 ng. Likewise, if that same animal had defecated 1·0 g during another interval, and the concentration during that interval was also 100 ng/g, the total mass for that interval would be 100 ng (see the Discussion for further details on methods of reporting steroid content).

RIA

A commercially available [^{125}I]RIA (ICN Biomedical, Costa Mesa, CA, USA) for rat and mouse serum/plasma corticosterone was used to quantify fecal corticoids in both males and females. To ensure antibody binding along the linear portion of the standard curve (20 and 80% binding), fecal extracts were diluted 1:25 (or more) with diluent provided with the RIA kit. All samples were assayed in duplicate and re-analyzed if the coefficient of variation exceeded 10%. Two control samples (each made by pooling fecal extracts from four or five animals) were analyzed in every assay (the 'low' pool with approximately 60% binding, and the 'high' pool with approximately 30% binding). Based on repeated analysis of these samples, intra- and inter-assay coefficients of variation for the assays of male samples were 5·23% ($n=5$) and 9·75% ($n=6$) for the low pool and 7·22% ($n=4$) and 8·35% ($n=5$) for the high pool. For the females, intra- and inter-assay coefficients of variation were 8·83% ($n=5$) and 17·81% ($n=5$) for the low pool and 8·00% ($n=5$) and 14·22% ($n=8$) for the high pool.

To determine the parallelism of diluted fecal extracts, a concentrated pooled extract made from nine individual fecal extracts was serially diluted (1:4 to 1:64) and the slope

of their antibody binding compared with that of the standards supplied with the RIA kit. To determine if the extract medium interfered with specific antibody binding, recoveries of known concentrations of corticosterone were calculated after assay in the presence of fecal extracts containing low concentrations of endogenous hormone.

Male fecal extracts were assayed within 1 month of extraction. Female extracts were analyzed approximately 12 months from initial extraction. To evaluate any possible decay in the samples during this storage period, we compared corticoid concentrations in 20 fecal extracts diluted for assay within a month of extraction and the same extracts stored for 12 months before dilution and assay. The correspondence between these repeated measures was excellent ($R^2=0·934$), indicating a strong linear relationship. When we regressed results for the later analyzed samples on the immediately analyzed values, we found a slope of 1·045, indicating that high and low samples decayed at a similar rate over the delay period. Furthermore, an intercept of 60·10 ng/g indicated that the later-analyzed female samples, on average, were approximately 60 ng/g lower than if they had been analyzed immediately after extraction. This decay was significant ($t_{20}=4·68$, $P<0·001$) and was taken into account in the comparison of our male and female fecal corticoid values, which are presented as measured.

Co-chromatography analysis of commercial antibody binding

The number and relative proportions of immunoreactive fecal corticosteroid metabolites were determined by reverse-phase HPLC (Microsorb RP C-18; Rainen Instruments, Woburn, MA, USA) as described previously (Monfort *et al.* 1990). Before HPLC, fecal extracts were pre-processed using reverse-phase C$_{18}$ cartridges (SpiceTM; Analtech, Newark, DE, USA; Heikkinen *et al.* 1981, Monfort *et al.* 1990). A 55 μl portion of fecal extract, combined with ^{3}H-cortisol (2500 d.p.m.) and ^{3}H-corticosterone (2500 d.p.m.), was separated using a linear gradient of 20–100% methanol in water within 80 min (1 ml/min flow rate, 1·0 ml fractions). Separate aliquots of each eluate were counted directly to determine recovery and/or assayed to identify immunoreactive corticosteroid metabolites.

Identification of corticosterone metabolites

To determine the corticosterone metabolites binding to the ICN antibody, pooled immunoreactive HPLC eluates were subject to LC-MS analysis. Six corticosterone metabolites were identified for detection based on established metabolic pathways in the rat: (1) 11-dehydrocorticosterone, (2) 11β,21-dihydroxy-5β-pregnane-3, 20-dione, (3) 21-hydroxy-5β-pregnane-3,11,20-trione,

(4) tetrahydrocorticosterone, (5) 3α,21-dihydroxy-5β-pregnane-11,20-dione, and (6) 3α,20α,21-trihydroxy-5β-pregnane-11-one. Given that rats produce little or no cortisol (Bentley 1976), seven cortisol metabolites served as negative controls: (1) 11β,17α,21-trihydroxy-5β-pregnane-3,20-dione, (2) urocortisol, (3) cortol, (4) cortisone, (5) 17β,21-dihyroxy-5β-pregnane-3,11,20-trione, (6) urocortisone, and (7) cortolone.

For each sex, fractions 44–47 were analyzed because (a) these eluates represented the major immunoreactive peak, (b) they had similar polarity as immunoreactive peaks reported in other validation studies (Bamberg *et al.* 2001, Monfort *et al.* 1998), and (c) there was a clear sex difference in antibody binding to these eluates. Fractions 44–47 were combined and loaded on to a C_{18} cartridge (Waters Corporation, Milford, MA, USA). HPLC eluates that had been diluted with RIA diluent were dried down for transport, then reconstituted with methanol. To remove salt and impurities, samples were washed with loading buffer (5% methanol aqueous solution, containing 5 mM sodium acetate and 0·1% formic acid) at 2 µl/min flow rate, and then washed with elution buffer (95% methanol aqueous solution, containing 5 mM sodium acetate and 0·1% formic acid) at 2 µl/min flow rate. This cleaning method was validated with standard samples containing 0·5 pg of eight standards: (1) equilin (1,3,5[10], 7-estratetraen-3-ol-17-one), (2) 1,3,5[10]-estratrien-3-ol-17-one, (3) 5α-androstan-17-one, (4) dehydro-isoandrosterone (5-androsten-3β-ol-17-one), (5) androsterone (5α-androstan-3α-ol-17-one), (6) progesterone, (7) 11-dehydrocorticosterone (4-pregnen-21-ol-3,11,20-trione), and (8) corticosterone (4-pregnen-11β,21-diol-3, 20-dione).

Statistical analyses

To quantify the fecal corticoid circadian rhythm we calculated several characteristics of each animal's rhythm: peak value, trough value, acrophase, and nadir. In addition, to quantify the rate at which corticoid levels rise from trough to peak values and fall from peak to trough values we calculated the slopes of these functions (i.e. fecal corticoid value = slope × time + intercept). Slopes were calculated by regressing, for each animal, fecal corticoid values on time (in hours). Rising-slope calculations used all points from the trough to peak, falling-slope calculations included all points from the peak to the trough. To test the hypothesis that the rise in fecal corticoids occurs faster than the fall, we compared the absolute value of these two slopes within each individual with paired *t*-tests. Given the assymetrical (e.g. saw-toothed) circadian rhythm of circulating corticoids, the working hypothesis was that the slope of the rise would be steeper than the slope of the fall. Female fecal corticoid values (troughs, peaks, acrophase, nadir) were compared across days of the estrous cycle using repeated-measures ANOVAs. Male and female fecal corticoid values (troughs, peaks) were compared using *t*-tests.

Results

Defecation profile of adult Sprague–Dawley rats

The rats' defecation pattern permitted frequent daily sampling within individuals. Adult Sprague–Dawley rats defecated 46 ± 2 fecal pellets per day, with seven pellets (range, 0–16) defecated during each sampling period. The majority of pellets were defecated during the dark-phase sampling intervals (10:30, 13:30, 16:30, and 19:30 h CDST), but a significant portion (35%) was excreted during the light-phase sampling intervals (22:30, 3:00, and 7:30 h CDST). Across a complete day of fecal sampling (seven collection intervals), both sexes typically produced fecal pellets during six or seven intervals, and on average five or six samples were not contaminated with urine. The least-common intervals for defecation were at the beginning and end of the light phase (22:30 and 7:30 h).

Because water content can vary from animal to animal, fecal mass is expressed as dry weight. On average, male rats defecated 6·88 ± 0·21 g per day and the females defecated 3·96 ± 0·18 g per day. Approximately 60% of the total daily fecal mass was excreted during the lights-off active dark phase (10:30, 13:30, 16:30, 19:30 h CDST). On average, the males defecated 1·17 ± 0·02 g/3 h during the active phase and 0·72 ± 0·18 g/3 h during the lights-off inactive phase (22:30, 3:00, 7:30 CDST). Females showed a similar pattern, with an average of 0·65 ± 0·03 g/3 h during the active phase and 0·34 ± 0·02 g/3 h during the inactive phase. The sex difference in fecal weight was comparable to sex differences in body weight (females weigh approximately 60% that of males). Even during the less-productive inactive phase, fecal mass was adequate for quantifying fecal steroids for both male and female adult rats. To account for the quantitative difference in fecal mass across the day, we expressed the amount of fecal corticoid metabolites in feces as a total mass excreted per 3-h sampling period. Fecal mass and fecal corticoid concentration were not correlated either within or between individuals, supporting the working hypothesis that the amount of corticoid metabolites in bile is not associated with the production of fecal pellets.

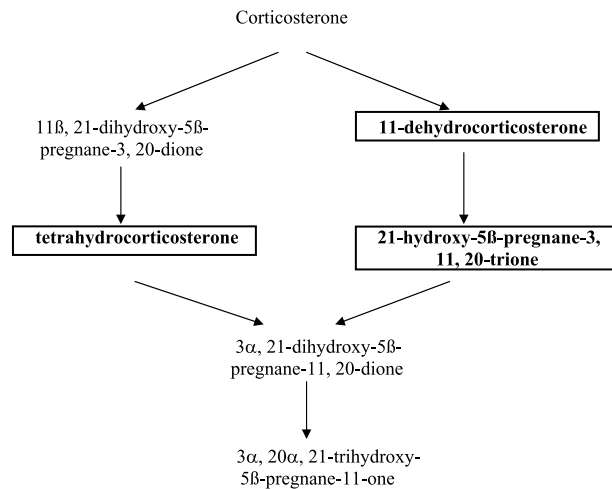### Biochemical validation of extraction and assay

Recovery of radioactively labeled corticosterone during the extraction procedure was 90% (n=72). Serial dilutions of pooled rat fecal extracts for males and females yielded displacement curves parallel to standard corticosterone. Mean proportional recovery of added corticosterone (range, 0·003–0·125 ng) for the female pool was 99 ± 6% ($y = 0·807x + 0·667$, $R^2 = 0·99$), and for the male pool it was

a)

b)
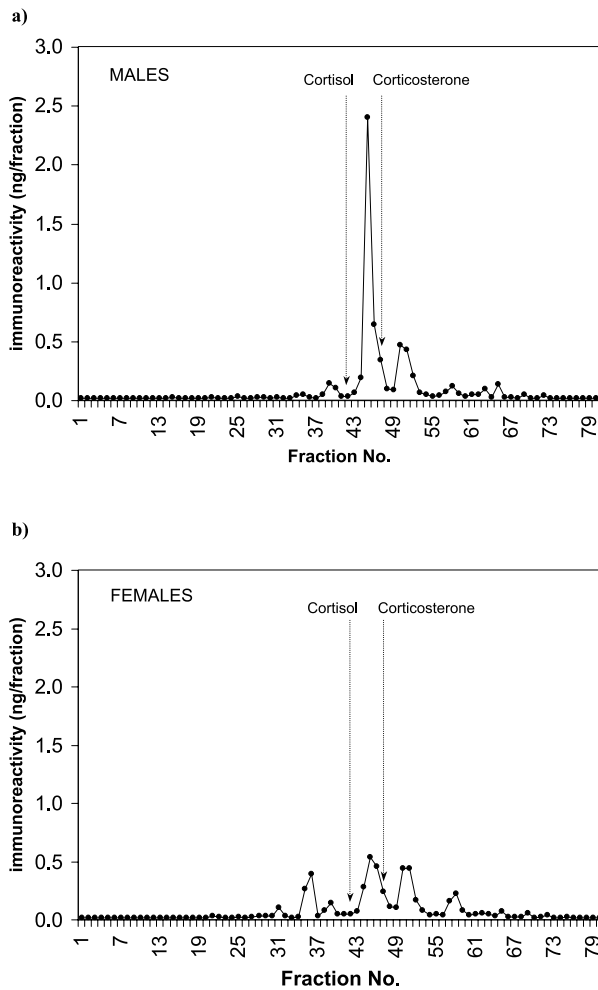
Figure 1 Immunoreactivity of (a) male and (b) female HPLC fractions to ICN corticosterone antibody.


Figure 2 Metabolic pathways of corticosterone degradation in the rat. Boxes indicate metabolites (identified by LC-MS) in the most immunoreactive HPLC fractions (Fig. 1, fractions 44–47).

$89 \pm 7\%$ ($y = 0 \cdot 941x + 0 \cdot 199$, $R^2 = 0 \cdot 99$). RIA of HPLC-separated fecal extracts from both males and females revealed a major immunoreactive peak that exhibited polarity intermediate between cortisol and corticosterone. Four additional minor immunoreactive peaks (two more polar than cortisol, and two less polar than corticosterone) were observed in both sexes (Fig. 1).

The averaged LC-MS spectra of male samples produced the following major ions: 331, 341, 351, 362, 373, 385, 333, 335, 337, 359, 310, 367, 369, 315, 397, and 325. Ions 373, 367, and 369 correspond to tetrahydrocorticosterone (or $3\alpha,20\alpha,21$-trihydroxy-5β-pregnane-11-one), 11-dehydrocorticosterone, and 21-hydroxy-5β-pregnane-3,11,20-trione, respectively (Fig. 2). Fewer major ions were detected in the female sample: 362, 333, 373, 310, 335, 367, and 369. Again, ions 373, 367, and 369 correspond to tetrahydrocorticosterone (or $3\alpha,20\alpha$, 21-trihydroxy-5β-pregnane-11-one), 11-dehydrocortico-

sterone, and 21-hydroxy-5β-pregnane-3,11,20-trione, respectively. Cortisol metabolites were not present in the analyzed eluates, nor were the remaining three corticosterone metabolites: 11β,21-dihydroxy-5β-pregnane-3,20-dione, 3α,21-dihydroxy-5β-pregnane-11,20-dione, and 3α,20α,21-trihydroxy-5β-pregnane-11-one.

Adjusting for urine contamination

Because rats excrete only 20% of corticoids in urine (Bamberg *et al.* 2001), it is possible that fecal samples soaked by urine may have their corticoids leached or diluted, leading to lowered fecal corticoid levels. Indeed, urine contamination did decrease fecal corticoid levels. When comparing samples contaminated and uncontaminated by urine, collected from the same individuals at the same time of day, contaminated samples had lower fecal corticoid values than uncontaminated samples (e.g. from 10:30 h collection: 110·4 versus 135·5 ng/3 h; $t_9 = 2 \cdot 60$, $P < 0 \cdot 05$).

Approximately one-quarter of the male samples and one-tenth of the female samples were found on a patch of urine-soaked bedding (identified as urine-contaminated or wet). We determined how much metabolites were leached by urine to adjust the corticoid values obtained from these wet samples. In 35 cases among the males, separately stored wet and dry (i.e. not urine-contaminated) samples were available for the same animal from the same collection period. To determine the relationship between wet and dry samples, we regressed the natural logarithm of the corticoid concentration for the dry samples on the logarithm of the concentration for the corresponding wet samples. The estimated intercept was 1·43 (S.E.M. = 0·38) and the estimated slope was 0·76 (S.E.M. = 0·07). A test of
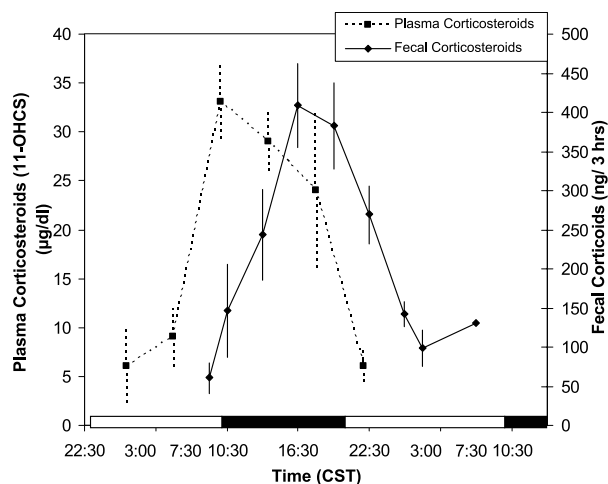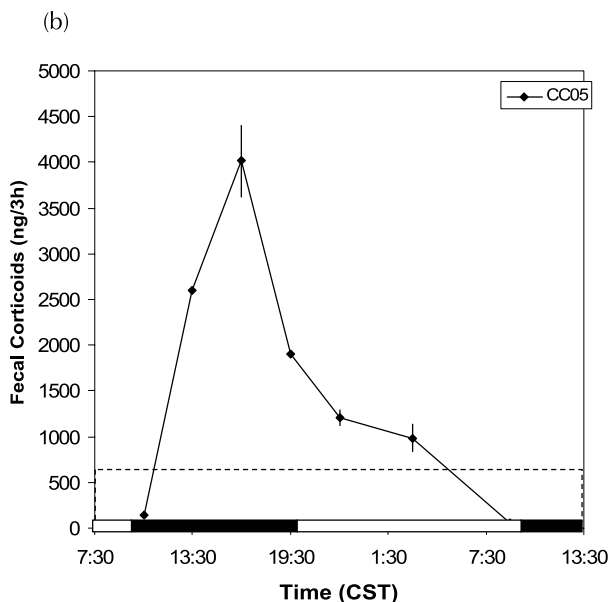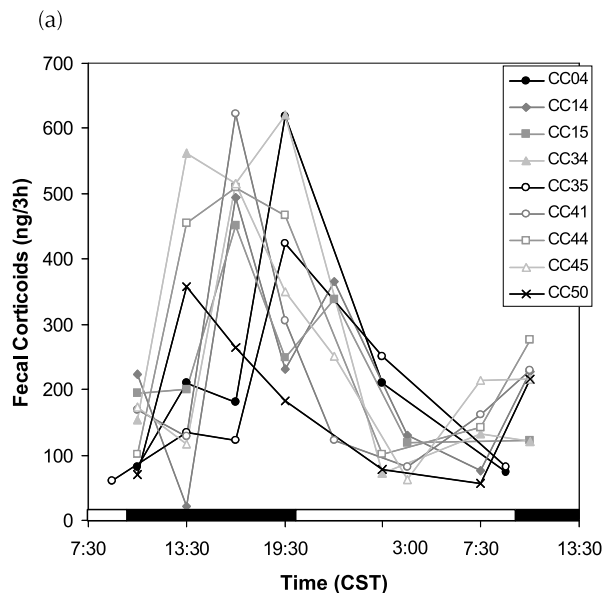
Figure 3 Mean (± S.E.M.) fecal corticoid values (solid line) at 3-h intervals for nine adult male Sprague–Dawley rats during a 24-h period, compared with previously published values of male and female circulating corticosteroid rhythm (dashed line) from Krieger (1973). Bars indicate S.E.M. The black areas on the abscissa indicate dark phases of the lighting cycle (the behavioral day in this nocturnal species).

Figure 4 (a) Individual adult male fecal corticoid values every 3–6 h over a 24-h period. Black bars on the abscissa indicate dark phases of the lighting cycle (rats' active period). (b) Male CC05 fecal corticoid values over 24-h period. The horizontal dashed line indicates the upper limit of values for the nine other males plotted in (a). Error bars indicate S.E.M. from repeated analyses of the same sample collected from this male.

the hypothesis that the true slope is equal to 1 (correspond-ing to a proportional relationship between the corticoid values for wet and dry samples) yielded a *P* value of 0·002. The R^2 value was 0·78, and a plot of the residuals indicated that the linear model fitted the data well. This estimated model was used to adjust corticoid concentrations in wet samples for both the males and females.

Fecal corticoid circadian rhythm in males

Males exhibited a distinct circadian rhythm in fecal corticoid excretion (Fig. 3). Peak corticoid levels occurred at the end of the active period and were approximately seven times greater than trough levels (512·6 ± 31·4 versus 74·5 ± 9·0 ng/3 h). The acrophase typically occurred during the 16:30 or 19:30 h intervals, corresponding to the end of the dark phase, approximately 6–9 h from the normal peak in circulating corticoids (Krieger 1973). Nine of the 10 males had their fecal corticoid acrophase during these two collection intervals. The nadir in fecal corticoid excretion generally occurred during the 3:00 or 7:30 h interval, corresponding to the middle and end of the light phase. Six of the 10 males excreted their lowest corticoid levels during these two intervals.

Males excreted corticoids in an asymmetrical (saw-toothed) circadian rhythm (Figs 3 and 4). Fecal corticoid concentrations in males rose from lowest to highest values within 9 h, with seven out of 10 males following this pattern. In contrast, it took approximately 15 h (or five collection intervals) for fecal corticoids to fall to nadir

concentrations. Thus, fecal corticoid levels rose from nadir to the peak in 6 h, less time than it took to decline from the peak to nadir levels (i.e. fecal corticoid values increased at a rate of approximately 12 ng/h and declined at a rate of approximately 8 ng/h). The absolute value of the

ascending slope was significantly greater than the absolute value of the descending slope ($t_8 = 2.25$, $P < 0.05$).

Differences in fecal corticoid excretion were quite marked among different individual males. The 24-h mean fecal corticoid value for one male was >30 standard deviations higher than for any other male (Fig. 4b). This male was excluded from the analysis of peaks, troughs, and slopes presented above because its corticoid concentrations were so dramatically atypical. Interestingly, this male was also behaviorally distinct from the other males: it was consistently slower to emerge from a familiar setting and slower to explore a complicated novel environment (S.A. Cavigelli & M.K. McClintock, unpublished observations). The average peak corticoid value for the nine males was 512·6 ng/3 h, whereas the peak corticoid value for the excluded male (Fig. 4b) was 6-fold higher than the next highest male (peak value, 4014·3 ng/3 h).

Fecal corticoid circadian rhythm in females

Fecal corticoids in females also were excreted in a circadian pattern, with peak values 6–18 times higher than trough values. However, key elements of this rhythm (e.g. peak and trough values) varied with the day of the estrous cycle (Fig. 5, upper panel). Daily corticoid means were lowest on the day of estrus and rose progressively during metestrus, diestrus, and proestrus ($F_{3,8} = 10.90$, $P < 0.0001$). Peak fecal corticoid levels on proestrus were two or three times higher than peaks during estrus or metestrus. Peak corticoid concentrations were lowest during estrus and sequentially higher during metestrus, diestrus, and proestrus ($F_{3,8} = 6.44$, $P < 0.01$). Average trough values were not significantly different across the days of the estrous cycle ($F_{3,8} = 0.53$, $P = 0.66$).

Throughout the estrous cycle, the acrophase and nadir of fecal corticoids for females were approximately 16:30 h and 7:30 h, respectively. As with the males, the majority of female acrophases (30/36) were in the 16:30 or 19:30 h intervals, corresponding to the end of the dark phase. The majority of nadirs (28/36) were during the same intervals as the males: the 3:00 and 7:30 h intervals, i.e. middle to end of the lights-on period.

Sex differences

On average, males excreted more immunoreactive fecal corticoids than did females. The daily mean fecal corticoid produced in males was greater than the female mean on all days of the estrous cycle ($t_{43} = 14.23$, $P < 0.0001$). Male trough and peak levels were repeatedly greater than female trough and peak levels (trough, $t_{43} = 12.92$, $P < 0.0001$; peak, $t_{43} = 9.19$, $P < 0.0001$). These differences did not disappear when females samples were adjusted to account for the storage decay (daily mean, $t_{43} = 13.39$, $P < 0.0001$; trough, $t_{43} = 11.25$, $P < 0.0001$; peak, $t_{43} = 8.04$,
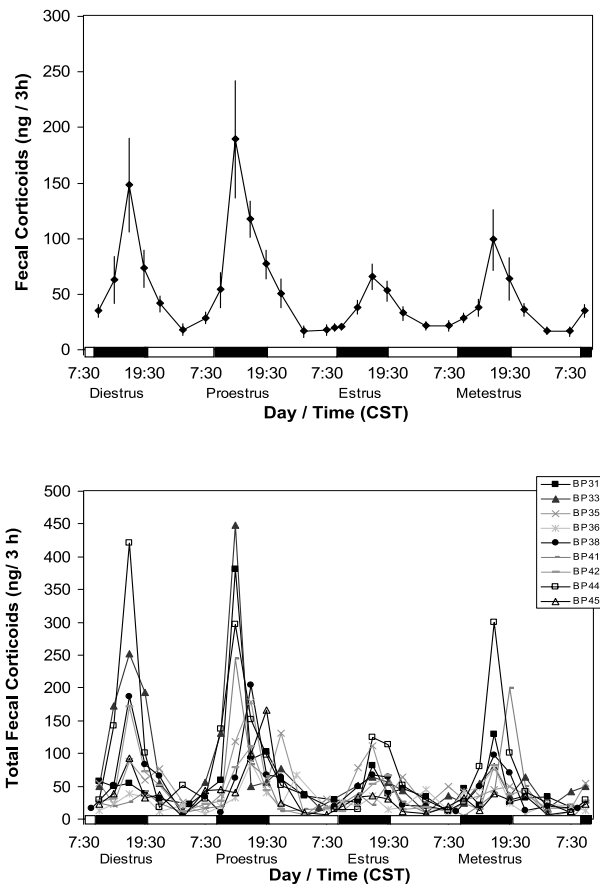
Figure 5 Upper panel: mean (± S.E.M.) fecal corticoid values over the 4-day estrous cycle for adult female rats. Black bars on the abscissa indicate dark phases of the lighting schedule (rats' active period). Lower panel: individual fecal corticoid values over a 4-day period for nine adult female Sprague–Dawley rats.

$P < 0.0001$), even when analyses were limited to proestrus, the day when females' corticoid values were highest (daily mean, $t_{16} = 7.58$, $P < 0.0001$; trough, $t_{16} = 6.22$, $P < 0.0001$; peak, $t_{16} = 4.83$, $P < 0.001$). There were no differences between male and female acrophases or nadirs (acrophase, $t_{43} = 0.91$, $P = 0.37$; nadir, $t_{43} = 0.73$, $P = 0.47$). For both the males and females, the shape of the circadian rhythm was similar, with a rapid rise in levels in 9 h and a slower return to trough levels in approximately 15 h (Figs 4 and 5). Finally, females produced fewer immunoreactive corticoids in the major HPLC-immunoreactive fractions (44–47; Fig. 1), and they had fewer major ions in these fractions compared with males.

Discussion

Fecal corticoids were excreted in a clear circadian rhythm with well-defined acrophases and nadirs. Sampling at

3-h intervals provided a high degree of temporal acuity for characterizing the circadian corticoid rhythm. Undisturbed male Sprague–Dawley rats excreted fecal corticoids in an asymmetrical pattern consisting of a rapid rise in corticoid excretion followed by a more gradual return to trough levels over the day. This function was not a by-product of averaging among many individuals, but was present within each individual. This asymmetrical rhythm was similar to that identified for circulating corticosteroids in rats (Krieger 1973) and humans (Dahl *et al.* 1991, Frank *et al.* 1995, Rohatagi *et al.* 1996).

Technical limitations associated with repeated blood sampling from small rodents over time likely hinder studies designed to evaluate the function and mechanisms of the circadian corticoid rhythm. The fecal corticoid measures provide an interesting complement to blood measures because feces can be collected with minimal animal disturbance over extended intervals without altering circadian rhythms – a concern that must be addressed when employing a chronic in-dwelling catheter. The asymmetrical excretory rhythms were consistent among animals, suggesting this method can be used for tracking circadian-rhythm alterations in individual animals. Finally, these results call into question the use of the cosinor analyses for estimating the corticoid circadian rhythms in rats (e.g. Atkinson & Waddell 1997). Our data suggest that more accurate methods for modeling this circadian rhythm need to be developed (e.g. Rohatagi *et al.* 1996, Wang & Brown 1996, Brown *et al.* 2001).

The amplitude of the fecal circadian corticoid rhythm is very similar to that for circulating corticosterone in Sprague–Dawley rats (Krieger 1973). In males, the peak-to-trough ratio of fecal corticoids is approximately 7:1 and the ratio for circulating corticosterone is approximately 10:1 (Krieger 1973). Given that fecal corticoid levels represent an integration of circulating corticoid levels, it might be surprising that the fecal corticoid amplitude does not show greater dampening relative to the circulating corticosterone amplitude. This lack of dampening, however, is likely due to the rats' high defecation rate within a day. The sampling density enables high temporal resolution with six or seven samples per day per animal. The integration of steroids in feces occurs only over relatively short intervals ranging from 3 to 4 h. This frequent, short-term temporal integration may be a distinct advantage for those interested in using a non-invasive method to document corticoid circadian rhythms or corticoid levels over extended periods.

The daily acrophase and nadir for fecal corticoid excretion occurred approximately 6–9 h later than the known acrophase and nadir for circulating corticosterone levels (Krieger 1973, Atkinson & Waddell 1997). This same lag time was true for both males and females and is similar to other time lags determined from studies that exogenously stimulated an elevation in circulating gluco-

corticoids (Bamberg *et al.* 2001, 12–18 h lag time; Touma *et al.* 2003, 4–8 h lag time; Royo *et al.* 2004, 12 h lag time). It should be noted that previously identified time lags greater than 9 h were found in studies that used infrequent sampling intervals (e.g. every 12 h), which gives greater credence to the 6–9 h lag period. By using the naturally occurring circadian rhythm to estimate the time lag between circulating and excreted corticoids, we avoided animal handling (i.e. required for exogenous corticotropin or corticosteroid administration), which has the potential to alter excretory lag time via sympathetic nervous system effects on gastrointestinal transit time. A well-defined time lag (6 to 9 h) provides another unique advantage to using fecal corticoid measures. This time lag permits researchers to assess adrenal responses to experimental manipulations after the manipulation has occurred, rather than disrupting the procedure to collect contemporaneous blood samples. Physiological responses to environmental stimuli or stressors can be evaluated well after the interaction of interest with a precise and integrated measure of adrenal activity.

Corticoid metabolites in male and female Sprague–Dawley rat fecal extracts were both more and less polar than corticosterone. The HPLC co-chromatography profile for the rat fecal extracts was comparable to other reports in rodents and other species (Bamberg *et al.* 2001, Monfort *et al.* 1998) in which the same ICN corticosterone antibody was used. This commercial antibody used in the present study is known to cross-react with a variety of corticosteroid metabolites, and is therefore referred to as a group-specific antibody that appears to be particularly sensitive to changes at sites C-11 and C-21 (Wasser *et al.* 2000). LC-MS analyses of the major immunoreactive HPLC eluates verified that the ICN antibody detects at least three of the six major corticosterone metabolites in rat feces (tetrahydrocorticosterone, 11-dehydrocorticosterone, and 21-hydroxy-5β-pregnane-3,11,20-trione). The remaining three metabolites may have been (1) present in untested eluates, (2) present in mass quantities that were below detection by either immunoassay of HPLC co-chromatography or LC-MS in tested eluates, or (3) metabolized to non-immunoreactive forms. Further studies are planned to address these questions. The laboratory validation clearly reveals that we can detect a group of fecal corticosterone metabolites, some of which are major metabolites of corticosterone. While unmetabolized corticosterone is not present in rat excreta, we have demonstrated the physiological validity of measuring a group of fecal corticoid metabolites to track adrenal rhythms in both sexes of the rat.

Co-chromatographic analysis revealed that the major immunoreactive metabolites detected by the ICN antibody had similar retention times for both sexes, although the degree of immunoreactivity for each fraction differed between the sexes. For example, the fraction of intermediate polarity between cortisol and corticosterone,

containing the three identified metabolites, represented the majority of binding for males but not for females. In addition, males excreted greater concentrations of immunoreactive corticoids than females, which is interesting because females are known to secrete more corticosterone than males (e.g. Atkinson & Waddell 1997). Sex-related differences in fecal corticoid concentrations may be partially explained by differences in steroid biosynthesis or catabolic pathways (Eriksson & Gustafsson 1970). Females may excrete less overall mass of corticoid metabolites from blood into feces than males because their plasma corticosterone-binding capacity exceeds that of males, and they have a slower fractional clearance rate than males (Ottenweller *et al.* 1979, Woodward *et al.* 1991). Thus, females may catabolize less corticosterone through the liver and/or excrete less mass of corticosterone metabolites in feces, and more in urine, than males. Further studies on steroid metabolism and/or with alternative antibodies may help elucidate these sex differences. Nonetheless, between-sex similarities in the rhythms of fecal corticoid excretion (i.e. acrophase and nadir) were clear, which confirms the usefulness of fecal measures evaluating adrenal status in both sexes, and the multiple immunoreactive peaks suggest that the commercial antibody has the potential to respond to many corticosterone metabolites.

Fecal steroid measures are most frequently presented as concentrations (e.g. ng/g of feces). However, total mass of corticoids in the feces is a more accurate measure of production than relative concentration. Concentration of corticosterone in blood correlates highly with corticosterone production from the adrenal because blood volume is relatively constant. In feces, corticosterone from the blood is metabolized by the liver and enters the small intestines through the bile duct, independently of fecal mass, which is regulated throughout the small and large intestine (Schwarzenberger *et al.* 1996). Unlike blood, however, the volume (mass) of fecal matter defecated can change significantly both within and between days. For example, during the inactive portion of the day, rats produce less fecal mass than during active periods. This reduced fecal mass during inactivity provides less 'diluent' or 'medium' for the metabolized corticosterone passing through the bile duct, potentially resulting in 'falsely' elevated concentration of corticoids in feces during inactive periods.

When corticoid values are presented as concentrations per gram of fecal matter, the same amount of corticoids metabolized and secreted in the bile during an inactive and active period leads to very different reported concentrations: higher concentrations are reported during the inactive period when fecal volume is reduced, due to the decreased volume of fecal 'diluent'. (When we analyzed the data as corticoid concentration per gram of fecal matter, corticoid values were greater than expected during the inactive low-fecal-defecation periods (data not presented).)

Thus, reporting total fecal corticoids (as opposed to concentration) excreted over a given time period provides a more accurate index of corticoid production and blood concentration than does a fecal concentration value which assumes constant fecal ('diluent') volume; this is particularly true when frequency of defecation and total mass of feces are known for the study animals. We recommend this format for reporting fecal steroid results in future studies.

Non-invasive fecal measures have generally been used to assess steroid excretion in wild animal populations (e.g. Creel *et al.* 1997, Cavigelli 1999, Wasser *et al.* 2000, Goymann *et al.* 2001). However, it is clear that these same methods have tremendous potential, and a number of important advantages for studying laboratory animals, including that (1) behavior is not disturbed by sampling, (2) the circadian glucocorticoid rhythm is not affected by the possible stress associated with handling, restraint, and blood sampling, (3) sampling frequency is limited only by defecation rates, (4) individual animals can be monitored for days, months, and throughout life, which permits manipulation and documentation of long-term endocrine rhythms, (5) fecal measures represent a complete and integrated measure of hormone production because hormones are 'pooled' in the gut before excretion, and (6) fecal steroids may reflect unbound (i.e. the biologically active portion) circulating glucocorticoid titers, since bound steroids are less readily metabolized by the liver (Westphal 1971, 1983, Leeper *et al.* 1988).

We demonstrated the utility of fecal corticoid measures for assessing the circadian corticosteroid rhythm and for assessing long-term changes in corticoid production in individual laboratory rats. This method may also have applications in other small laboratory animals that possess high metabolic and frequent defecation rates, characteristics that facilitate frequent, repeated non-invasive fecal sample collection. Among the most compelling uses of this method will be for conducting long-term studies to understand how external factors modulate circadian rhythmicity and/or produce chronic changes in corticoid production in laboratory rats.

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