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Fecal Corticoid Metabolites in Aged Male and Female Rats after Husbandry-related Disturbances in the Colony Room

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We applied novel noninvasive fecal steroid measures to characterize aged rats' responses to a series of common animal room disturbances, including a direct comparison of male and female immunoreactive corticosterone metabolites in feces. The fecal measure provides a unique method to measure the physiologic responses of laboratory animals to altered husbandry procedures. This assay is noninvasive and, because rodents produce fecal pellets throughout the day, long-term monitoring can be conducted to capture abnormal levels associated with alterations in husbandry procedures. Over a 3-h period, 10 male and 10 female Fischer 344 rats (age, 82 wk) were exposed to a series of events that can occur in a colony housing room (keys jingling, cage lids opening, alteration of the light cycle). Fecal samples were collected at timed intervals on the day before and several days after the exposure, extracted, and analyzed for fecal corticoid metabolites by use of a commercial enzyme immunoassay. Fecal metabolites in these aged rats were elevated 3- to 5-fold above baseline levels approximately 20 h after exposure to the experimental events. Overall, we detected more immunoreactive fecal corticoid metabolites in feces from male rats than female rats, even though female rats normally secrete greater amounts of glucocorticoids into circulation. Our results indicate that this assay can be used to identify marked elevations in corticoid metabolite levels after alterations in laboratory husbandry procedures. We discuss the implications of these findings for animal researchers and those involved in animal husbandry.

Abbreviation: EIA, enzyme immunoassay

Circulating steroid hormones are catabolized primarily in the liver by a series of enzymes including cortisone reductase, 11beta-hydroxysteroid dehydrogenase. These steroid metabolites are deposited in the duodenum and further degraded by bacteria until excreted in feces.[8,9] Researchers interested in measuring steroid hormone production non-invasively can take advantage of this metabolic process. Fecal metabolite measures have been used with subjects from which traditional blood samples may be difficult to collect (for example, field studies). Recently, this technology has been applied to laboratory animals, for which blood measures traditionally have been the standard means of assessing steroid hormones.[2,3,10,18] Advantages of the non-invasive fecal steroid measures with laboratory animals are many: a) repeated non-invasive sampling is possible indefinitely and therefore circadian and long-term production profiles can be assessed without the potential influence of chronic catheterization, b) because of the time lag required for steroids to pass from circulation into excreta, the glucocorticoid response to a given intervention can be assessed well after actual exposure to the intervention, and c) metabolized steroids in feces likely reflect the biologically active (that is, unbound) portion of circulating hormones.[21,22] Here we measure fecal steroid metabolites in laboratory rats to determine the effect of several common husbandry disturbances (for example, noise, cage opening, alterations in illumination schedule).

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To assess hypothalamic-pituitary-adrenal axis response to altered husbandry procedure, we quantified immunoreactive corticosterone metabolites in feces of aged rats before and after a complex alteration in their normal husbandry procedures. Past studies indicate that even seemingly inconsequential events in an animal colony room can influence rodent physiology, particularly affecting sympathetic function (for example, heart rate, blood pressure).[16,17] In the present study, we sought to quantify the effect of a considerable change in animal husbandry procedures on fecal excretion of corticoid metabolites. We designed a series of events that might occur in an animal colony room and repeatedly presented these events over a 3-h period. This study is the first in which fecal concentrations of corticoid metabolites were quantified before and after exposure of laboratory rodents to a series of environmental stressors. The results of the present study provide information regarding the extent to which animal room disturbances affect adrenal activity in laboratory rats.

Recent studies with young rats have determined that the time required for steroids to be metabolized and removed from circulation and for the metabolites to be passed in defecated feces can range from 6 to 24 h for young laboratory rats, with most studies identifying a lag time in the 6- to 18-h range.[2,3,6] Therefore, if a rat experiences a stressor, we can expect to see an elevation in fecal corticoid metabolites anywhere from 6 to 24 h after the initiation of this stressor. In light of this time lag, we quantified rat fecal corticoid metabolite levels at 3 to 7 h ('early') and 19 to 23 h ('late') after initial onset of husbandry alterations. Specifically, we compared fecal corticoid metabolite levels at these 2 sampling periods with those of time-matched samples collected

on the day before the disturbance. Using time-matched pre- and post-exposure comparisons, we controlled for circadian effects in our quantification of a potential increase in rat fecal corticosterone metabolites after a complex environmental disturbance. Fecal corticoid metabolite levels are thought to be an integrated index of prior circulating corticosterone levels.[10]

The data indicate how a series of husbandry disturbances in an animal colony room affects fecal corticosterone metabolite levels in aged rats. We identified a window of time during which elevations in fecal corticoid metabolite levels might be evaluated in aged rats after exposure to common environmental disturbances and examined gender-associated differences in the amount of fecal corticoid metabolites as identified by use of a commercial enzyme immunoassay (EIA) kit previously used to quantify corticosterone metabolites in rat feces.[6,13]

Materials and Methods

To determine the change in fecal concentrations of corticoid metabolites after husbandry disturbances, we used a series of disturbances comparable to those that influence markers of physiologic activation in laboratory rodents (for example, heart rate and blood pressure).[16,17] We collected time-matched fecal samples before and after exposure to the disturbance to determine the extent to which fecal corticoid metabolite levels were elevated after exposure. Samples were collected: 1) 3 to 7 h after exposure to the disturbance (early) (a period during which we did not expect any change in fecal corticoid metabolites, given the time required for circulating steroids to be metabolized and excreted), and 2) 19 to 23 h after exposure to the disturbance (late) (a time at which peak corticoid metabolite levels might be expected in light of previous studies, which show a 6- to 24-h lag before metabolized steroids appear in the feces of young rats).[2,3,6]

Animals. The study population comprised 10 male and 10 female (age, 82 wk) Fischer 344 rats (Charles River Laboratories, Wilmington, MA). The rats were involved in a sentinel program to monitor colony health; based on results of sentinel testing, there were no significant health problems in the colony. All rats were housed individually in wire-bottom stainless-steel cages (47 × 38 × 21 cm) since their arrival at the animal facility several months prior to this study. The room was maintained at 22 ± 1 °C with relative humidity of 45% to 55%, 16 ± 1 air exchanges hourly, and a 12:12-h light:dark cycle with lights on at 0700. Water and food (Harlan Teklad Rodent Diet 8604, Madison, WI) were available ad libitum throughout the study.

Experimental design. Figure 1 presents the timeline of the experiment. To determine baseline (pre-exposure) concentrations of fecal corticoid metabolites, we collected feces from all subjects every 4 h, starting in the middle of the dark (active) phase (at 0200) on the day before exposure (Day 1) and continuing for 16 h. Samples were collected directly from absorbent paper-lined trays under each animal's cage, placed into a plastic bag, and stored at –80 °C until analyzed (see below). On the following day (Day 2), trays were cleaned at 0600, and exposure to the experimental intervention began at 0700 and ended at 1000. These interventions included: 1) opening and closing of cages every half hour from 0700 to 1000, 2) shaking of a metal cage containing keys for 30 s every half hour from 0700 to 1000, and 3) delaying the time when lights were turned on by 3 h (that is, lights on at 1000 instead of 0700). On Day 2, fecal samples were collected beginning at 1000 (at the end of the exposure protocol) and every 4 h thereafter for 20 h until 0600 on Day 3. The 4-h intervals were designed to maximize the probability

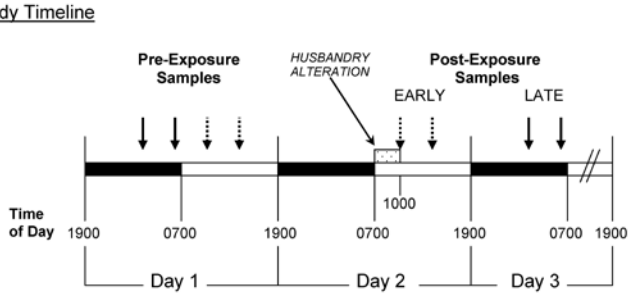

Figure 1. Study timeline. Black and white bars indicate the dark and light periods, respectively, of the photoperiod. The time of the stressor protocol is indicated by the stipled bar on day 2. Arrows indicate collection of fecal samples. Post-exposure samples collected early (that is, within 3 to 7 h) after exposure to husbandry alterations are indicated with dashed arrows, as are the time-matched pre-exposure samples. Post-exposure samples collected late (that is, at 19 to 23 h) after stressor onset are identified with solid arrows, as are the time-matched pre-exposure samples. We expected (and noted) a significant increase in fecal corticoid metabolite concentrations at the late sampling intervals but not necessarily at the early ones.

that: 1) each subject would defecate during each interval, and 2) we would capture an increase in post-exposure corticosterone metabolites in feces. However, only time-matched samples from before and after exposure were analyzed. All procedures were approved by the Institutional Animal Care and Use Committee at Hershey Medical School, The Pennsylvania State University, in accordance with *PHS Policy on Humane Care and Use of Laboratory Animals*.[12]

Fecal corticoid metabolite extraction and assay. Fecal pellets were dried and stored in labeled plastic bags at –80 °C until processed. The extraction protocol was modified from a previously published method used to measure corticoids in wild rhinoceros feces.[20] This extraction protocol was selected because we originally had planned to conduct high-performance liquid chromotography of extracted steroids. In brief, 0.2 to 0.5 g feces was removed from the stored sample, placed in preweighed filter centrifuge tubes (0.2 µm, Centrex MF, Schleicher and Schull, Einbeck, Germany), and homogenized by use of a tissue homogenizer after addition of 1 ml methanol:acetone (8:2 v/v). Samples were placed in the refrigerator for 1 h; after this incubation, 1.5 ml 80% methanol was added to each sample, which was mixed thoroughly and centrifuged for 20 min at 800 × *g*. The filter was removed and discarded (with solid debris), and each supernatant was transferred to a spin cartridge (3 ml/60 mg Nexus SPE, Varian, Palo Alto, CA). The extract was forced through the spin cartridges by low-pressure vacuum (about 254 mm Hg) and discarded, followed by elution of the steroid fraction with 1 ml methanol into glass vials. The eluate was dried over air at 40 °C in glass culture tubes and frozen at –80 °C until reconstituted for analysis.

Prior to assay, the dried extract was reconstituted with 400 µl 30% acetonitrile in glass vials and shipped in a coolbox with dry ice to the Biobehavioral Health Studies Laboratory at the Pennsylvania State University. The amount of fecal corticoid metabolites in each extract was measured with a commercial EIA kit designed to measure corticosterone in rodent plasma or serum (Correlate-EIA, Assay Designs, Ann Arbor, MI) and previously used to measure corticosterone metabolites in rodent feces.[6] At present, the specific immunoreactive corticosterone metabolites detected by this EIA kit are unknown, but this same EIA has been used to identify a rise in fecal corticoid metabolites following surgery, comparable to the rise found in circulat-

ing corticosterone levels after this procedure.[13] Prior to assay, extracts were diluted (1:40) with kit diluent (Assay Buffer 15). For each sample we calculated the concentration of corticoid metabolites as ng/g dry feces extracted. This conventional method was used instead of the 'total metabolites per time interval' method proposed recently[3,6] because these previous publications were unavailable prior to extraction of the current samples and because the fecal samples were discarded prior to measurement of total fecal weight, which would have enabled a more functionally accurate estimate of corticoid excretion.

Assay validation. To verify that the extract medium did not affect the functioning or accuracy of the assay, we conducted 2 validation procedures: a measure of parallelism and a measure of accuracy. To assess parallelism, we serially diluted (1:5) separate concentrated pools of samples of extracts from feces from male and female rats (5 to 6 samples in each pool) and compared the slope of their relative optical density with that of the standards supplied with the EIA kit. To assess accuracy, we determined the percentage recovery of known corticosterone amounts added to fecal extracts. Separate low-concentration pools were made for extracts of feces from male and female rats (5 to 6 samples in each pool). Increasing concentrations of corticosterone (0.01 to 1.00 ng; by use of the corticosterone standard supplied with the EIA kit) were added to a series of pool aliquots. Percentage corticosterone recovered was calculated for male and female rats. The intra- and interassay coefficients of variation were 7.63% and 9.14%, respectively.

Fecal corticoid metabolites before and after exposure to interventions. To control for time of day, we compared the pre- and post-exposure concentrations of fecal corticoid metabolites for early (1000 to 1400) and late (0200 to 0600) collection periods (Figure 1). These times were selected in light of reported lag times necessary for corticosterone metabolism in the liver and excretion of metabolites in feces.[2,3,6] The samples obtained at 1000 and 1400 on Day 2 were collected 3 to 7 h after exposure to interventions and were used to identify any unexpected early changes in fecal corticoid metabolites. Samples obtained from 0200 to 0600 on Day 3 were collected 19 to 23 h after exposure to interventions. Given the expected 6- to 24-h time lag required for circulating corticosterone to be metabolized and excreted in feces in young rats, we hypothesized that the maximal elevation in fecal corticoid metabolites in these aged rats would occur during the late time period (that is, 0200 to 0600, or 19 to 23 h after exposure to the intervention).

Analyses. Because fecal corticoid metabolite values during each collection period were skewed, we used log-transformed data in all statistical tests. To determine whether post-exposure fecal corticoid metabolite concentrations were higher than pre-exposure levels, we calculated the mean fecal corticoid metabolite concentrations at 2 time-matched points before and after the exposure period. Specifically, for each rat, we calculated mean fecal corticoid metabolite concentrations across the 1000 to 1400 intervals (that is, early) and across the 0200 to 0600 intervals (that is, late). Because early samples were collected only 3 to 7 h after the intervention, we did not expect to see a significant difference between pre- and post-exposure samples at this time point. In contrast, we expected a significant increase in the late post-exposure samples compared with their time-matched pre-exposure samples.

We used paired *t* tests to compare mean fecal immunoreactive corticosterone metabolite concentrations before and after exposure to a stressor at the 2 time points—early and late. We used time-matched comparisons to control for the daily circadian rhythm in corticosterone production.[3,6] Data from rats that did not produce a time-matched fecal sample pre- or post-exposure were not included in the paired *t* test analyses or in the figures. To compare fecal corticoid concentrations between genders, we used *t* tests to compare the 16-h means during the pre- and post-exposure periods. All *t* tests were 1-tailed. To account for the 2 repeated *t* tests used to compare fecal corticoid concentrations, the α level for statistical significance was set at 0.025 for these tests. For all other tests, statistical significance was set at $\alpha = 0.05$. Analyses were conducted with the Statview statistical program (SAS Institute Inc, Cary, NC). For clarity, the original, nontransformed values are presented in the figures.

Results

Assay validation. Serial dilutions of pooled extracts of rat feces yielded displacement curves parallel to that of the corticosterone standard (standard slope, –0.712; male rats, –0.763; female rats, –0.701). Mean percentage recovery of corticosterone added to pooled feces from male rats was 116% ± 24% ($y = 1.43x - 0.004$), and that for female rats was 106% ± 6% ($y = 0.922x - 0.010$).

Concentrations of fecal corticoid metabolites before and after exposure to husbandry disturbances. Fecal corticoid metabolite concentrations from male and female rats at 3 to 7 h after exposure were not different from those measured at the same diurnal time on the day before the disturbance (male rats: $t_8 = 1.30$, not significant; female rats: $t_8 = 0.82$, not significant; Figure 2 A, B). However, both genders had significantly higher concentrations at 19 to 23 h after exposure as compared with values from samples collected at the same time on the day before exposure (that is, pre- versus post-exposure at the 0200 and 0600 intervals; male rats: $t_7 = 7.92$, $P < 0.001$; female rats: $t_7 = 4.64$, $P < 0.01$; Figure 2 A, B). This pattern was seen for all 10 male rats and for all of the 9 female rats that contributed samples both before and after exposure. In fact, at the late time point, on average, male rats had a 5-fold increase and female rats a 3-fold increase in fecal corticosterone metabolites as compared with those measured at the same time on the day before exposure to disturbances.

Gender-associated differences. Overall, we detected approximately 30% less immunoreactive corticosterone metabolites in feces from female rats than male rats. This pattern was true during both the pre- and post-exposure periods (pre-exposure: $t_{18} = 1.89$, $P < 0.05$; post-exposure: $t_{18} = 3.10$, $P < 0.01$).

Discussion

We found that the concentration of fecal corticosterone at approximately 20 h after a series of husbandry disturbances (jingling keys, cage openings, altered light cycles) in an animal colony room was increased 3- to 5-fold in aged male and female Fischer 344 rats. This increase in concentration is less than that seen in the circulation of aged rats during a standard stress protocol (for example, restraint stress).[3,5,14] The lower magnitude we noted may reflect either decreased adrenal reactivity to husbandry disturbance (as compared with restraint stress), or most likely, that feces necessarily contain steroids metabolized over several minutes or hours and therefore provide an 'average' steroid level over time as opposed to absolute peak levels at any single point in time, as is available in blood measures. Both male and female rats excreted significantly more fecal immunoreactive corticosterone metabolites after exposure to the complex husbandry and laboratory interventions, as compared with before the disturbance. Future studies should compare fecal corticoid metabolite dynamics for young versus aged rodents. Because aged animals can have slower catabolism

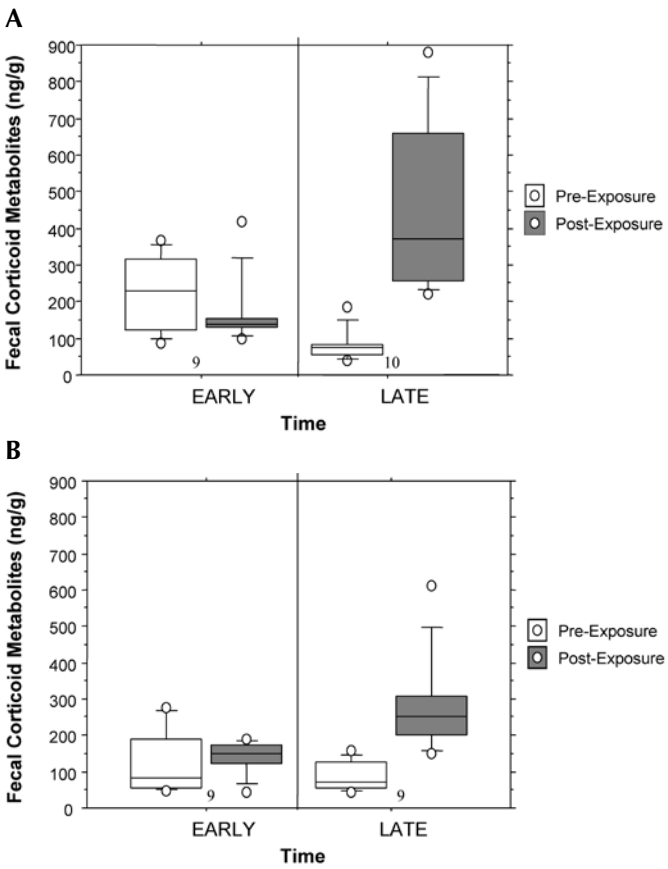

Figure 2. Concentrations of fecal corticoid metabolites at early and late time points before and after exposure to husbandry alterations. Early samples were collected 3 to 7 h after exposure onset; time-matched pre-exposure samples also were collected. Late samples reflect samples collected 19 to 23 h after exposure onset; time-matched pre-exposure samples also were collected. (A) Male rats. (B) Female rats. The median and 25th and 75th percentiles are shown by the line and box outline. The error bars indicate the 10th and 90th percentiles and the minimum and maximum values are given as open circles. The numbers above abscissa indicate the number of animals contributing time-matched pre- and post-exposure samples.

and gut motility[11] and more prolonged elevations in circulating corticosterone after challenge,[14,15] we could expect significant age-associated differences in both the magnitude of rise and duration of the rise in fecal corticoid metabolites after husbandry disturbances. The current results indicate that fecal sampling in aged rats probably should continue beyond 24 h after a stressor. The fecal corticoid measure may provide a unique method to assess the temporal dynamics of hypothalamic-pituitary-adrenal axis activity and to assess glucocorticoid negative feedback function in aged subjects.

Although female rats generally release more corticosterone into circulation than do males (both after exposure to a stressor and under baseline conditions),[1] our study confirms previous findings that female rats excrete less immunoreactive corticosterone metabolites in feces than do males.[3] These gender-associated differences likely reflect several factors. First, although female rats tend to produce more corticosterone than do male rats, female rats also have approximately twice the amount of corticosteroid-binding-globulin activity, which inhibits steroid breakdown.[9] Second, female rats excrete a different set of corticosterone metabolites than do male rats,[8] and these metabolites may bind less readily to the antibodies used in the corticosterone assay. Last and most importantly, female rodents may excrete relatively more glucocorticoids in their urine than feces;[4,8,19] male rats excrete 80% of metabolized corticosterone in feces.[2]

In this study, we found a 3- to 5-fold difference in corticosterone excretion after a series of disturbances in the colony room (noise, lighting alterations, cage openings). The fecal steroid measure provides a unique method to monitor the severity of colony room disturbances. The advantage of this method is that adrenal responses to husbandry disturbances can be assessed without stress associated with the manipulation typically associated with traditional blood collection methods (for example, restraint, invasive sample collection). In addition, because this fecal metabolite measure accommodates serial sampling, it is possible to collect an integrated, long-term measure of adrenal responses to long-term conditions, including estimates of how and whether glucocorticoid circadian rhythms have been altered by such events as changes in light schedules or severe colony room disturbances. In fact, the significant increase in fecal corticosterone metabolites in the present study may reflect a delay in the normal corticosterone diurnal peak in these rats due to the delayed initiation of the light cycle that was part of the complex husbandry disturbance. Finally, the results suggest that repeated long-lasting alterations in husbandry procedures have a significant influence on hypothalamic-pituitary-adrenal axis function in laboratory colony rodents.

We have shown the utility of collecting fecal samples after exposure to various interventions to identify a potential rise in fecal corticosterone metabolites. The appropriate lag time between the event and the time of measurement will depend on the metabolic rate of the study subjects. Factors that influence the metabolic rate include body size, weight, and perhaps age of the study animals, with older animals potentially requiring a longer post-exposure sampling window than younger animals.[11,14,15] In addition, we emphasize that excreted corticosterone metabolites should not be compared directly between genders as an approach to providing accurate indicators of circulating corticosterone levels.

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