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TECHNICAL COMMENT

Assessment of the Stress Response in Columbian Ground Squirrels: Laboratory and Field Validation of an Enzyme Immunoassay for Fecal Cortisol Metabolites

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ABSTRACT

Stress responses play a critical role in the ecology and demography of wild animals, and the analysis of fecal hormone metabolites is a powerful noninvasive method to assess the role of stress. We characterized the metabolites of injected radiolabeled cortisol in the urine and feces of Columbian ground squirrels and validated an enzyme immunoassay for measuring fecal cortisol metabolites (FCM) with a 5α-3β,11β-diol structure by stimulation and suppression of adrenocortical activity and by evaluation of the circadian pattern of FCM excretion. In addition, we also evaluated the impact of capture, handling, and acclimation to the laboratory on FCM. Cortisol is highly metabolized, with virtually none being excreted, and of the radiolabeled cortisol injected, 31% was recovered in urine and 6.5% in feces. The lag time between cortisol injection and its appearance in urine and feces was 4.5 ± 0.82 (SE) h and 7.0 ± 0.53 (SE) h, respectively. FCM levels varied over the day, reflecting circadian variation in endogenous cortisol. Dexamethasone decreased FCM levels by 33%, and ACTH increased them by 255%. Trapping and housing initially increased FCM levels and decreased body mass, but these reversed within 3–7 d, indicating acclimation. Finally, FCM levels were modestly repeatable over time ($r = 0.57$) in wild, livetrapped, nonbreeding animals, indicating that FCMs provide a measure of the squirrel's stress-axis state. This assay provides a robust noninvasive assessment of the stress response of the Columbian ground squirrel and will facilitate an integration of its life history and physiology.

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Introduction

Sciurids are highly visible members of the Holarctic mammalian community because of their relatively large size, diurnal activity, and accessible habitat, and this has made them excellent models for behavioral, ecological, and evolutionary studies (Murie and Michener 1984). Columbian ground squirrels (*Spermophilus columbianus*) are hibernating social rodents within the diverse ground-dwelling sciurid group (*Cynomys*, *Marmota*, and *Spermophilus*) that live in alpine and subalpine meadows of northwestern North America (Elliot and Flinders 1991). They have been extensively used as mammalian models of evolution, ecology, population regulation, and behavior. Recent research on Columbian ground squirrels has focused on spatial learning (Vlasak 2006*a*, 2006*b*), resource availability and population regulation (Broussard et al. 2005), and dispersal and vigilance behavior (Neuhaus 2006; Fairbanks and Dobson 2007). However, little is known of their stress physiology, with the exception of one study on females' stress response to a perceived predator (Hubbs et al. 2000). Our research will lay the foundation for subsequent research on aging and stress in this and related species.

The stress axis (the hypothalamic-pituitary-adrenal axis) is central to permitting organisms to cope with both predictable and unpredictable challenges in their environment, thus maintaining homeostasis and cognition (Sapolsky 2002; Wingfield 2005). It is closely tied to the physiological controls of reproduction, aging, and immunity, and thus it is of particular interest to researchers studying the physiological ecology of wild populations (Boonstra 2005; Reeder and Kramer 2005). Glucocorticoids (corticosterone and/or cortisol, depending on the species) are key hormones secreted by this axis. They vary over the day to facilitate arousal and feeding behavior (Reeder and Kramer 2005) and over the year to facilitate life-history activities such as breeding, migration, and hibernation (Romero 2002).

The study of the stress axis in wild vertebrates has relied primarily on the collection of blood samples and more recently of fecal samples (Mateo 2007; Strauss et al. 2007; Thiel et al. 2008). Although there are advantages to drawing blood, it is

invasive and immediately stresses the animal, causing glucocorticoid levels to increase (Kenagy and Place 2000; Romero and Romero 2002). Fecal sampling is noninvasive, potentially minimizing the stress response and permitting continuous longitudinal sampling while minimizing proximity and handling. Fecal glucocorticoid metabolites represent the integrated average of short-term fluctuations in the blood through the actions of bile and intestinal mixing (Palme et al. 2005) rather than the point estimates obtained from blood sampling. However, there are problems with fecal glucocorticoid samples as well. First, virtually no pure cortisol or corticosterone is present in the feces (Palme et al. 2005), and hence, one needs to determine which metabolic breakdown products appear in the feces and have antibodies against them. Second, it is critical to validate that these fecal metabolites change in lockstep with stressors experienced by the animal. Third, as taxonomic relatedness does little to predict the precise nature of metabolites or their relative routes of excretion (urine versus feces; Palme et al. 2005; Heistermann et al. 2006), validations have been recommended for each new species (Touma and Palme 2005). The best way to clarify these questions is to perform radiometabolism experiments and a physiological and biological validation of the immunoassay.

Our study had three objectives. First, we needed to determine the time delay, the routes of excretion, and the structure of the fecal cortisol metabolites (FCM) of Columbian ground squirrels. Second, we needed to validate an enzyme immunoassay (EIA) to detect whether changes in endogenous cortisol levels owing to circadian fluctuations, to the stress of capture and handling, to long-term acclimation to laboratory conditions, and to induced stimulation (with adrenocorticotrophic hormone [ACTH]) or inhibition (with dexamethasone) of adrenocortical activity are reflected in FCM concentrations. Third, we needed to assess whether fecal metabolite levels in nonbreeding wild squirrels are repeatable over time and thus are a measure of the state or quality of the animal in nature.

Material and Methods

Study Site, Trapping, and General Handling of Squirrels

Columbian ground squirrels were livetrapped during July 2005 in the Sheep River Wildlife Sanctuary 32 km west of Turner Valley, Alberta, Canada (50°38′N, 114°38′W, elev. 1,470 m). All trapping normally took place between 0700 and 1200 hours, when the ground squirrels were most active. Squirrels were trapped in Tomahawk live traps baited with peanut butter (14 cm × 14 cm × 40 cm, Tomahawk Live Trap, Tomahawk, WI), tagged with monel #1005-1 tags (National Band and Tag, Newport, KY) in both ears, weighed, and sexed. During the study, both males and females were preparing for immergence into hibernation. Immergence in this area occurs between July 15 and August 25, with adult males immerging first, followed by adult females, and finally by both female and male juveniles (Young 1990).

Laboratory Validation

To validate the FCM EIA and to determine which of the two potential antibodies best reflect changes in the stress response, we carried out a laboratory study on squirrels obtained from an untrapped meadow. Potential burrow sites were prebaited with live traps and peanut butter for 3 d before trapping. All squirrels were captured on July 8, held in captivity for 14 d, and then released. Five male (734 ± 26.5 g) and five female (526 ± 13.8 g) untagged squirrels >1 yr old were captured, immediately moved to the field laboratory, and held in cages on a regime of natural light and ambient temperature. They were fed an ad lib. horse ration in a dish (EQuisine Sweet Show Horse Ration, Unifeed, Okotoks, Alberta, Canada; a mixture of grains and molasses with a minimum of 13% crude protein, 2% crude fat, and 12% crude fiber) and lettuce and apple (for water).

To collect all of the feces excreted, squirrels were put in radiometabolism cages during the day (0700–1900 hours). The cages separated urine from feces and consisted of a polypropylene rodent housing cage (47 cm × 26 cm × 20 cm) nested within a polycarbonate rodent housing cage. The floor of the upper cage was cut out and replaced with a 1 × 1-cm wire mesh. Nested near the bottom of the lower cage was a 0.5 × 0.5-cm metal wire mesh. Most feces passed through the upper mesh but not through the lower mesh. Urine passed through both and was collected on the floor of the lower cage. During the night (1900–0700 hours), squirrels were moved to polycarbonate cages lined with 4 cm of wood chips provided for warmth and to ensure their survival and health. Fecal samples from overnight cages were collected from the wood-chip bedding each morning. Each morning and evening, animals were rapidly transferred to and from the radiometabolism cages using a pillowcase. All were weighed each morning. For the experiments that required injections, squirrels were lightly anesthetized using isoflurane (Abbott Laboratories, Saint-Laurent, Quebec) delivered at 3.5% in air from a purpose-built portable anesthetic delivery unit. This permitted ease of handling and prevented injury to both the squirrel and us.

During all treatments, fecal samples were recovered from the radiometabolism chambers by lifting the upper cage with the squirrel free from the lower cage and placing the former into a temporary holding cage. To avoid contamination with urine, we collected only the fecal pellets that did not pass through the 0.5 × 0.5 cm wire mesh. Any fecal sample contaminated with urine was discarded. One exception to this was the pellets collected following injection with ^{3}H-cortisol, which were all collected to determine the total recovery of ^{3}H-cortisol. Urine was collected with a 1-mL pipette and stored in 6-mL plastic scintillation vials. To collect all urine and residue, the floor of the radiometabolism cage was rinsed with 3–4 mL 100% ethanol, which was added to the urine sample. All feces and urine samples were frozen at −20°C within 15 min after collection.

All squirrels were subject to treatments in sequence (Table 1) to determine (1) the impact of trapping and housing on FCM, (2) the time delay and route of excretion in the urine and feces following ^{3}H cortisol injection, (3) the characteri-

Table 1: Chronology of experimental treatments to validate a fecal cortisol metabolite enzyme immunoassays in Columbian ground squirrels

Date	Experiment	Treatment	Sample Collection Times (h post)
July 8–10	Acclimation	Trapping and impact of housing	Baseline, 0, 4, 8, 10, 12, 24, 30, 36
July 11–14	Radiometabolism	Injected with ^{3}H-cortisol	0, 2, 4, 6, 8, 12, 24, 30, 35, 48, 60, 72, 82
July 15–16	Circadian variation	Circadian variation	0, 3, 6, 9, 12, 24, 27, 30, 33, 36
July 17–18	Adrenal suppression	Injection with dexamethasone	0, 2, 4, 6, 8, 10, 12, 24, 30, 36
July 19–20	Adrenal stimulation	Injection with ACTH	0, 2, 4, 6, 8, 10, 12, 24, 30, 36

Note. $n = 5$ males and 5 females. Experiments began at 0700 hours (hour 0); h post = time after initiation of experiment. ACTH = adrenocorticotropic hormone.

zation of the ^{3}H-cortisol metabolites, (4) the circadian pattern of FCM, (5) the impact of dexamethasone suppression on FCM, and (6) the impact of ACTH stimulation on FCM. This protocol was approved by the University of Toronto Animal Use Committee (protocol 20005660).

To evaluate the impact of trapping and housing on squirrels, we compared baseline FCM levels from feces collected beneath the live traps in the field on the day of capture with levels from feces collected from the same squirrels in the lab 1 d later (Table 1). Only squirrels captured in the field between 0900 and 1200 hours and producing feces at that time in both the field and the lab were used ($n = 8$).

To determine the predominant route of excretion (feces or urine), the time lag, and the metabolic products of cortisol, squirrels were injected with radioactive cortisol. On day 3 of captivity, squirrels were injected intraperitoneally at 0730 hours with 12.5 μCi (= 462.5 kBq) radiolabeled cortisol (Amersham Biosciences; 1,2,6,7-[^{3}H], specific activity: 1.55 TBq/mmol; dissolved in 0.25 mL physiological saline containing 5% [v/v] ethanol and 4.5% [v/v] toluene). Urine and feces were collected at regular intervals over the next 4 d as indicated in Table 1.

To determine circadian variation in FCM, fecal samples were collected on days 7 and 8 of captivity every 3 h beginning at 0700 hours and until 1900 hours (Table 1). Nighttime samples represented the pooled sample from 1900 to 0700 hours and collected the first thing in the morning.

To validate the assay by observing whether adrenal cortisol suppression or stimulation is mirrored by changes in FCM levels, the squirrels were injected first with a synthetic corticosteroid agonist (dexamethasone sodium phosphate [DEX]) and subsequently with synthetic adrenocorticotropic hormone (ACTH), using dosages that were effective in other squirrel species (Boonstra and McColl 2000). On day 9 of captivity, each squirrel was injected with 0.4 mg DEX (100 μL intraperitoneally at 0700 hours; Sabex, Montreal). On day 11, each squirrel was injected with ACTH (4.0 IU/kg intramuscularly at 0730 hours; Synacthen Depot, Ciba, Ontario; Table 1). Feces were collected at regular intervals for 2 d following each of these injections.

Field Validation

To assess whether point estimates of FCM levels obtained from livetrapped animals were repeatable over time and were thus indicative of individual profiles, we concurrently conducted an ecological validation by collecting fecal samples from the same nonbreeding squirrels caught on two different days. These animals represent a random cross section of animals of all ages (adults that had bred but were now nonreproductive and juveniles and yearlings that had not) and both sexes. On two meadows that had been livetrapped for up to 9 yr (F. S. Dobson and T. J. Karels, personal communication), we captured tagged squirrels from July 6 to July 26. Tomahawk live traps baited with peanut butter were set at 0730 hours and checked every hour until noon. On capture, tag number, sex, body mass, and location were recorded, and a fecal sample was collected from the ground under the trap. The samples were collected with a clean forceps, stored in 5-mL plastic vials, and kept on an ice pack until return to the lab, where they were stored at −20°C. The time intervals in days between collection of the paired samples were 1 ($n = 31$), 2 ($n = 3$), 4 ($n = 4$), 5 ($n = 1$), 7 ($n = 1$), 14 ($n = 1$), 16 ($n = 1$), 17 ($n = 1$), and 19 ($n = 3$). We assessed whether the length of the time interval between collections affected FCM levels. Repeatability was calculated by using the Pearson product-moment correlation coefficient (Krebs 1999).

Fecal Extraction

Fecal and urine samples were transported at −20°C to the University of Toronto. Feces were stored at −80°C until drying and extraction, and urine samples were stored at 4°C until analysis. To control for fiber and water content (Wasser et al. 1993), fecal samples were lyophilized (LabConco, MO) overnight for 18 h. Lyophilized fecal samples were frozen in liquid nitrogen and pulverized with a small grinding pestle. We weighed 0.15 ± 0.01 g of the pulverized sample and recorded the mass to the nearest 0.001 g. Samples smaller than 0.15 g were weighed and the mass recorded. The samples were extracted by vortexing (30 min at 1,450 rpm; Barnstead Thermolyne Maxi-Mix III, IA) with 5.0 mL 80% methanol (v/v) in a 15-mL Falcon tube (BD Biosciences). The samples were then immediately centrifuged (15 min at 2,500 *g*). Radioactivity was measured immediately in a scintillation counter. Aliquots of all other samples were stored at −80°C until shipped on dry ice to Vienna, Austria, for EIA analysis.

Determination of Radioactivity in Fecal and Urine Samples

One milliliter of each extracted fecal sample was added to 4 mL ACS scintillation fluid (Amersham), and its radioactivity was measured in a liquid scintillation counter (Packard Tri-Carb 2900TR, Boston) with quench correction. The remaining extract of the peak radioactive samples from one male and one female were dried down for reverse-phase high-performance liquid chromatography (RP-HPLC) analysis. Because many urine samples were opaque (which can affect scintillation counting), they were oxidized with 2–5 mL hydrogen peroxide (30%) before analysis. Consequently, they were dried in 20-mL glass scintillation vials in an oven (~75°C), reconstituted with 4 mL 80% methanol, divided into two parts to allow for proper dilution in scintillant, and then counted in 10-mL aqueous scintillation fluid (ACS; Amersham Biosciences). The amount of radioactivity recovered and the lag time—defined as the time after injection, when the highest concentration of radioactive metabolites was collected—were determined in the urine and feces for each animal.

RP-HPLC

To determine the polarity and general structure of cortisol metabolites in the feces and to determine whether native cortisol was excreted in the feces, we performed RP-HPLC separations on samples from one male and one female squirrel. The eluent was fractionized (for details of the HPLC separation, see Touma et al. 2003), and radioactivity and immunoreactivity in two different EIAs (see below) was measured in the fractions.

Determination of Fecal Cortisol Metabolites

To determine which of two antibodies cross-reacted with the FCM excreted by Columbian ground squirrels, we analyzed fecal extracts and RP-HPLC fractions using two EIAs. The first, an 11-oxoetiocholanolone EIA, measures metabolites with a 5β-3α-hydroxy-11-one structure (Möstl and Palme 2002), and the second, a 5α-pregnane-3β,11β,21-triol-20-one EIA, measures 5α-3β,11β-diol structure (Touma et al. 2003).

Statistical Analysis and Calculations

All data are expressed as means ± 1 SE unless stated otherwise, and comparisons of means were considered statistically significant at $P < 0.05$. FCM concentrations are given in ng/g feces. Statistics were performed with the JMP statistical program (ver. 5.0.1., SAS Institute, Cary, NC). Before analysis, we tested the assumption of normality using the Shapiro-Wilks test, and the FCM data were log transformed. The Mauchly criterion was used to determine the sphericity of the data (homogeneity of variance), and if statistical adjustment was required, the Greenhouse-Geisser adjustment for unequal variances was applied (Keselman et al. 1998). Because repeated-measures ANOVA (rmANOVA) requires balanced designs, 13 missing values (7.22%) were estimated by linear interpolation (using the values from adjacent hours of the same animal; treatments with missing values occurred in data sets involving circadian variation, DEX suppression, and ACTH stimulation).

Results

Route of Excretion and Time Lag

Of the tritiated cortisol injected, 38% was recovered (31.1% ± 3.67% in urine and 6.53% ± 0.53% in feces). There was no difference between males and females in total, in urine, or in fecal radioactivity recovery ($t_8 = -1.11$, $P = 0.30$; $t_8 = -0.93$, $P = 0.38$; $t_8 = -1.16$, $P = 0.28$, respectively). As missing data could inflate the time lag between injection and recovery, only animals that produced a sample at each sampling time were included ($n = 8$). Thus, two animals were excluded from this calculation. There was no difference between males and females in the time lag in either urine ($t_6 = 1.12$, $P = 0.31$) or feces ($t_6 = -1.60$, $P = 0.16$). The time lag was 4.5 ± 0.82 h for urine and 7.0 ± 0.53 h for feces (Table 2).

Characterization of Fecal Cortisol Metabolites

RP-HPLC revealed that several radioactive metabolites were formed in both sexes (Fig. 1), whereas pure ^{3}H-cortisol was almost absent. However, sex differences were observed. Several major radioactive peaks (>3 Bq) were present in the male, whereas only one peak was present in the female. In the male, the three main metabolites resembled nonpolar, unconjugated steroids, but more polar metabolites were also detected. In the female, the main radioactive peak eluted around fraction 5, indicating a high polarity (even higher than the standard E_2-diSO_4). Several smaller peaks of radioactivity (<3 Bq) were present as well, but more so in the male than in the female. Few radioactive metabolites were found in fractions 50–65 in the male, fractions 25–40 in the female, and beyond fraction 80 in both sexes (Fig. 1). The 11-oxoetiocholanolone EIA reacted with two of the largest radioactive peaks in the male (fractions 74 and 77). However, in the female, the main 11-oxoetiocholanolone EIA peak coincided only with a small radioactive one. Despite the difference in the pattern of radio-

Table 2: Relative portion of excreted cortisol metabolites in urine and feces and time lag between injection and recovery of peak excretion of ^{3}H-cortisol (12.5 μCi) in Columbian ground squirrels

	Males		Females		Combined	
	Mean	SE	Mean	SE	Mean	SE
^{3}H recovery (%):						
Urine + Feces	41.64	6.27	33.6	3.70	37.62	3.67
Urine	34.51	6.52	27.67	3.47	31.08	3.66
Feces	7.17	.75	5.93	.73	6.53	.53
Lag time (h):						
Urine	3.33	.67	5.50	1.20	4.50	.82
Feces	7.60	.40	6.00	1.15	7.00	.53

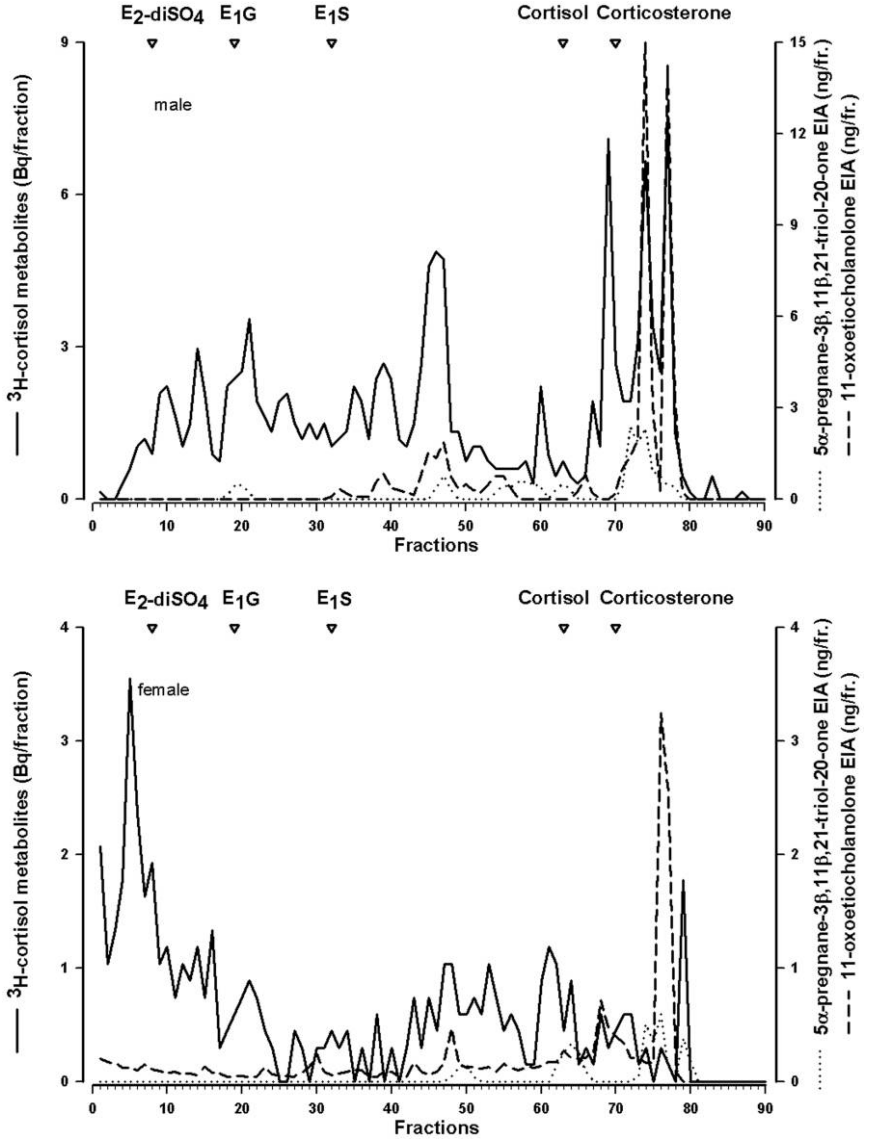

Figure 1. Reverse-phase high-performance liquid chromatographic radioimmunogram of peak radioactive fecal supernatant from Columbian ground squirrels. Samples were analyzed from a representative male (*top*) and a female (*bottom*) injected with ^{3}H cortisol. In each fraction, the presence of ^{3}H metabolites (*solid line*) and metabolites reacting with the 11-oxoetiocholanolone EIA (*dashed line*) and the 5α-pregnane-3β,11β,21-triol-20-one EIA (*dotted line*) is shown. The approximate elution time of standards are also marked with triangles of respective standards (E_2-diSO_4: oestradiol disulphate; E_1G: estrone glucuronide; E_1S: estrone sulfate, cortisol and corticosterone, respectively). The 11-oxoetiocholanolone EIA shows two large peaks coinciding with radioactive peaks in the male (*top*) but only one peak unrelated to a radioactive peak in the female (*bottom*). The 5α-pregnane-3β,11β,21-triol-20-one EIA shows a peak in fractions 70–80 in both males and females. The female shows a higher proportion of polar metabolites than the male. Note that on the *Y*-axis, different scales are used in the male and female.

active peaks between the sexes, the 5α-pregnane-3β,11β,21-triol-20-one EIA reacted with several radioactive metabolites and revealed a very similar pattern of peaks across fractions in both sexes (Fig. 1).

Impact of Trapping and Housing

Trapping and housing caused a significant increase in FCM levels on day 1 in the lab (416.87 $\pm$ 57.78 ng/g) relative to their levels at capture in the field the previous day (263.03 $\pm$ 61.10; one-tailed paired *t*-test, $t_7 = 2.12$, $P = 0.036$). Another measure of the stress of housing was the change in body mass over the time in the lab (Fig. 2). We carried out a rmANOVA on body mass with sex as treatment. There was sex effect ($F_{1,96} = 37.79$, $P = 0.0003$) and a time effect ($F_{12,96} = 17.52$, $P < 0.0001$) but no interaction effect ($F_{12,96} = 0.99$, $P = 0.46$). The sex effect simply indicated that males (740.3 $\pm$ 7.8 g) were almost 200 g heavier than females (559.8 $\pm$ 5.5 g). The time effect indicated that body mass changed over the period they were confined in the lab. The lack of an interaction effect

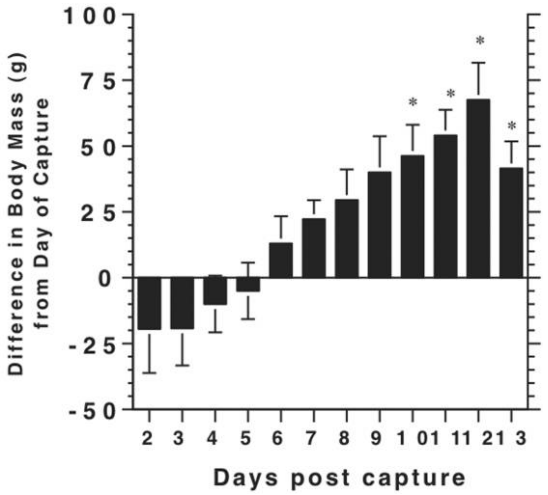

Figure 2. Impact of capture, housing, and subsequent treatments on Columbian ground squirrel mass ($n = 10$). Changes in mass are presented relative to the mass at capture. Data are presented as means (± 1 SE). Significance relative to the mass at capture was tested with a paired t-test with $P < 0.05$ and adjusted by a Bonferroni correction. Those means that differ from the mass at capture are indicated by an asterisk.

indicated that males and females responded similarly over time. All squirrels initially lost mass (about 20 g), but by day 4 in the lab, they were about the same mass as at capture, and by days 12–13, about 50 g heavier.

Circadian Pattern

To assess how FCM levels changed over time, we carried out a rmANOVA with sex as a treatment. For the 5α-pregnane-3β,11β,21-triol-20-one EIA, there was a marginal sex effect ($F_{1,40} = 4.51$, $P = 0.07$), a marked-time effect ($F_{5,40} = 8.97$, $P < 0.0001$), and no interaction effect ($F_{5,40} = 0.09$). For the 11-oxoetiocholanolone EIA, there was no sex effect ($F_{1,40} = 0.33$), no time effect ($F_{5,40} = 1.82$), and no interaction effect ($F_{5,40} = 0.63$). Thus, we focused only on the results from the former EIA. Over the entire 2-d period and all samples, males had 43% higher FCM levels than females (608.55 $\pm$ 37.79 ng/g, $n = 30$ vs. 422.98 $\pm$ 39.91 ng/g, $n = 30$, respectively; Fig. 3). The marked-time effect indicated that the former EIA was able to document a pronounced circadian pattern in FCM levels; the latter was not. For the former EIA, the last fecal sample in the evening (1900 hours) was approximately 55%–70% higher than the first sample in the morning (0700 hours), and in general, levels after 1200 hours were 45%–55% higher than the sample collected first thing in the morning (Fig. 3).

Suppression and Stimulation of Adrenal Glucocorticoid Production

To assess the efficacy of the two EIAs in documenting how FCM levels changed over time—first in response to DEX suppression and subsequently in response to ACTH stimulation—we carried out a rmANOVA with sex as a treatment. Both DEX and ACTH injections resulted in a significant change in FCM levels over time using the 5α-pregnane-3β,11β,21-triol-20-one EIA but not using the 11-oxoetiocholanolone EIA (Table 3). Hence, only the 5α-pregnane-3β,11β,21-triol-20-one EIA was successful validated. For DEX, although the main effect of sex was not significant, the significant interaction of time and sex indicated the sexes were not uniform in their response. DEX suppressed adrenal activity an average of ~33% at 12 h postinjection (at 1900 hours) relative to levels collected first thing in the morning (at 0700 hours, with FCM levels in females increasing by 1.6% and in males declining by 34.7%; Fig. 4). However, relative to the circadian pattern of change in FCM (documented above; Fig. 3), suppression occurred in both sexes but was not as pronounced in females. For ACTH, the main effect of sex was significant, but the interaction effect of time and sex was not, indicating that males and females differed and that this difference was consistent over the course of the experiment (Table 3; Fig. 4). ACTH simulated adrenal activity an average of ~255% at 12 h postinjection (i.e., FCM levels at 1900 hours relative to those at 0700 hours, with females showing an increase of 142% and males 677%; Fig. 4).

Repeatability of Field Measurements

FCM levels were repeatable over time ($r = 0.57$, $P < 0.0001$, $n = 46$ [26 females, 20 males]; Fig. 5). The remaining unexplained variance might be related to the length of time between the two estimates (ranging 2–19 d). We examined the relationship between the number of days between the samples and the difference between the samples and found none ($F_{1,45} = 0.054$, $P = 0.82$). Hence, FCM levels are modestly similar over time within squirrels in postbreeding condition in summer.

Discussion

Our studies characterize cortisol metabolism and validate an EIA to determine the FCM concentrations in Columbian ground squirrels. Taken together, they indicate that an EIA

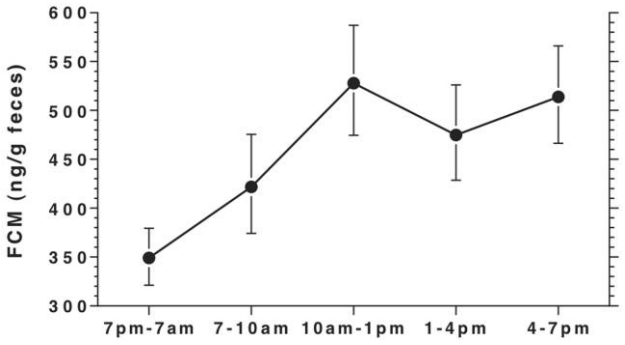

Figure 3. Circadian variation of fecal cortisol metabolites (FCM) over 24 h in Columbian ground squirrels. Each data point is the mean (± 1 SE) of 20 samples collected during that period pooled over two consecutive days.

Table 3: Repeated-measures ANOVA showing the effects of adrenal stimulation (adrenocorticotrophic hormone [ACTH]) and suppression (dexamethasone) measured with a 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay (EIA) and an 11-oxoetiocholanolone EIA over time in Columbian ground squirrels

Source	df	MS	*F*	*P*
ACTH:				
5α-pregnane-3β,11β,21-triol-20-one EIA:				
Sex	1	.41	10.04	.013
Subject	8	.04	…	…
Time	5	.42	11.77	<.0001
Time × Sex	5	.07	2.05	.09
Error	40	.04	…	…
11-oxoetiocholanolone EIA:				
Sex	1	.29	2.21	NS
Subject	8	.13	…	…
Time	5	.07	1.00	NS
Time × Sex	5	.14	.19	NS
Error	40	.07	…	…
Dexamethasone:				
5α-pregnane-3β,11β,21-triol-20-one EIA:				
Sex	1	.14	3.72	.09
Subject	8	.04	…	…
Time	5	.09	3.72	.007
Time × Sex	5	.06	2.58	.041
Error	40	.02	…	…
11-oxoetiocholanolone EIA:				
Sex	1	.003	.01	NS
Subject	8	.20	…	…
Time	5	.05	.80	NS
Time × Sex	5	.06	1.03	NS
Error	40	.06	…	…

Note. Samples were collected every 6 h during the day and pooled overnight (see Table 1 for collection times).

measuring FCM with a 5α-3β,11β-diol structure enables noninvasive monitoring of adrenocortical activity. There are six major findings in this study. First, the time delay of FCM is approximately 4.5 h in the urine and 7.0 h in the feces, with 6.6% of the metabolites being excreted in the feces (Table 2). Second, RP-HPLC analysis indicated that cortisol was almost absent in the feces and that the structure of the metabolites differed between the sexes (Fig. 1). Third, these squirrels rapidly adapt to laboratory conditions, being initially stressed for the first 3 d but then habituating, as indicated by increasing body mass (Fig. 2). Fourth, the 5α-pregnane-3β,11β,21-triol-20-one EIA revealed a pronounced circadian rhythm in FCM, with concentrations being lowest just after arousal in the morning and highest in the afternoon and early evening (Fig. 3), Fifth, this EIA was validated in both sexes as a tool to monitor the stress response, as FCM levels were suppressed by DEX and stimulated by ACTH (Fig. 4). Finally, within-animal FCM levels were modestly repeatable over time in the field, and thus, these estimates can serve to characterize individual differences (Fig. 5).

There is one caveat that must be addressed before discussion of our results. The recovery of the ^{3}H-cortisol in the urine and feces was low (~38%). There are two possible explanations for these low recoveries—either loss in urine and/or loss in feces. With respect to the urine, the squirrels produced copious amounts, which may have exceeded normal levels. This occurred because they would not readily drink from water bottles (at least over the 14 d we had them in the lab), and thus we were forced give them foods high in water content (apple and lettuce). There may have been adsorption of metabolites from the urine to the polycarbonate cages and/or spilled grain. Although the grain was rinsed with methanol and the wash was collected during sample collection, the grain may have absorbed a significant amount of the radioactivity and required more stringent extraction methods than we used. For feces, unlike urine, there were few places to lose radioactivity, because fecal collection is simple. Thus, the most likely explanation for the low recovery was loss of isotope from the urine owing to adsorption to food. Consequently, we assume that the percentage ^{3}H- of recovered radioactivity in the feces relative to the injected

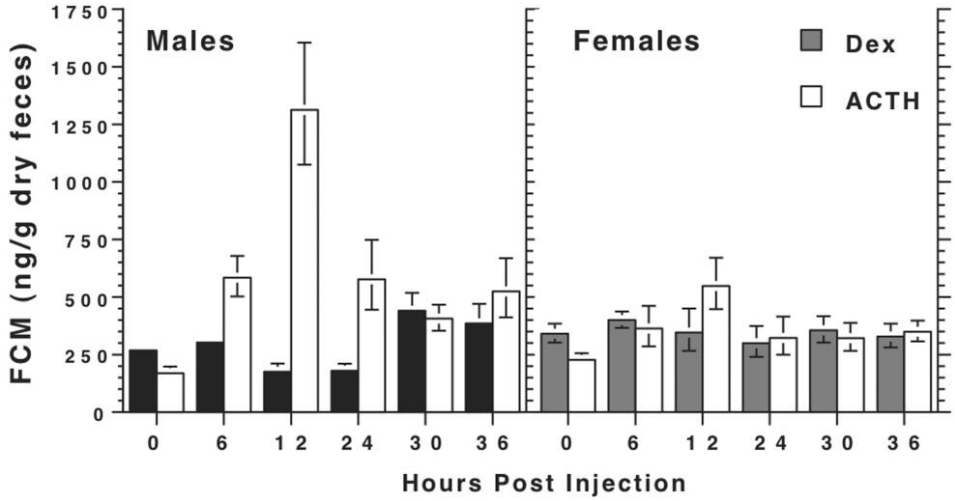

Figure 4. The response of male ($n = 5$) and female ($n = 5$) Columbian ground squirrels to an injection of dexamethasone (DEX; black bars in males, gray bars in females) and of adrenocorticotropic hormone (ACTH) using the 5α-pregnane-3β,11β,21-triol-20-one EIA to detect fecal cortisol metabolites (FCM). Injections were at 0700 hours (hour 0) on July 17 for DEX and on July 19 for ACTH. Bars represent the mean FCM concentrations during each sampling interval postinjection. Data are presented as means (± 1 SE).

^{3}H-cortisol is an accurate reflection of what was present. Low fecal excretion of glucocorticoid metabolites (about 8%) has also been found in European hares (*Lepus europaeus*; Teskey-Gerstl et al. 2000). However, for most rodents, excretion via feces ranges from about 73% in mice (Touma et al. 2003) to 80% in rats (Lepschy et al. 2007). Therefore, our values are the lowest in any rodent reported thus far. However, if the antibodies cross-react with excreted FCM, a low fecal excretion is not critical, as EIAs are highly sensitive assays with low (pg scale) detection limits (Möstl et al. 2005). Therefore, it was possible to measure FCM successfully in both hares (Teskey-Gerstl et al. 2000) and Columbian ground squirrels (this study).

The time lag between cortisol injection and peak appearance was 4.5 h in urine and 7.0 h in feces (Table 2). This indicates that the rate of glucocorticoid metabolism and excretion during the day (the normal active phase of these diurnal squirrels) is only a little longer than that in mice (about 4–6 h) injected during their active period in the evening (Touma et al. 2003). Similar delay times (7.5 h) of peak concentrations of FCM levels following a stressful event were found in European ground squirrels (Strauss et al. 2007). If the ^{3}H-cortisol injections in our squirrels had occurred in the evening, the time delay would be predicted to be on the order of about 12 h, because the rate of gut passage, which is affected by activity, is the main factor influencing lag times (Palme et al. 1996, 2005).

Very little, if any, native cortisol was present in the feces of ground squirrels (Fig. 1). This finding agrees with those of other studies in mammals; almost all excrete no native glucocorticoids in the feces (for reviews see Palme et al. 2005; Touma and Palme 2005). This indicates extensive metabolism of glucocorticoids by the liver. In addition, Columbian ground squirrels are hindgut fermenters that eat a variety of grasses and forbs with a lot of fiber. They do not retain food particles in a cecum but instead rely on microbial digestion of fiber in their hindgut (Hume et al. 1993). There is thus the potential for microbial alteration of steroid metabolites in the gut. In addition, the results of RP-HPLC analysis indicate that males and females produce markedly different metabolites of cortisol. Similar to laboratory rats (Lepschy et al. 2007), the female excreted more polar metabolites than the male. Sex differences in glucocorticoid metabolism also exist in mice (Touma et al. 2003) and birds (e.g., chicken [Rettenbacher et al. 2004] and black grouse [Baltic et al. 2005]).

Trapping and housing initially had negative effects on the squirrels, causing an increase in FCM levels (by 58.5% within 24 h and a 20-g loss in body mass within 2 d; Fig. 2). The

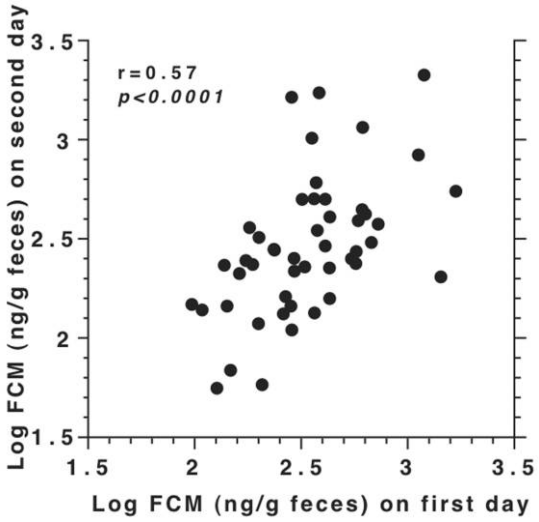

Figure 5. Repeatability of fecal cortisol metabolites (FCM) measured from the same field-caught Columbian ground squirrels on different days.

latter lasted only about 3 d, and by the end of the experiment, squirrels had gained an average of 50 g. The initial mass loss could have been a result of the change in diet, but it also was likely caused by the stress of confinement. Stress should result in an increase in cortisol levels (as reflected in higher FCM levels), and this promotes gluconeogenesis (production of new glucose through the breakdown of other body tissues (Miller and Tyrrell 1995). Stressors such as high predation risk are known to result in marked mass loss in species in nature as well (Hodges et al. 2006). Acclimation to the laboratory environment is desirable for most ecophysiological studies to reduce the effect of stress, as this can influence memory, metabolism, and susceptibility to disease (Sapolsky 2002). Hubbs et al. (2000) brought female Columbian ground squirrels into the lab and noted a marked reduction in alarm calling and piloerection within 2 d of captivity. Another consideration potentially influencing the rapidity of acclimation to the laboratory conditions was the overriding necessity in squirrels to gain mass before hibernation. Bachman (1994) found that when juvenile Belding's ground squirrels (*Spermophilus beldingi*) had restricted the foraging times just before hibernation, they lost fat-free mass but not fat, the critical necessity for surviving hibernation. Thus, our EIA measuring metabolites with a 5α-3β,11β-diol structure can detect the effects of capture, handling, and confinement stress.

There was a clear circadian rhythm to FCM levels (Fig. 3), and this pattern was as expected after accounting for the lag of 6–12 h in gut passage times (i.e., the low first-morning sample should reflect the nadir of the previous evening and night, and the high by midafternoon should reflect the peak at arousal; Reeder and Kramer 2005). Endogenous levels should be at their peak just before arousal from sleep and are involved in the normal day-to-day activities associated with the diurnal cycle of waking, such as increased locomotion, exploratory behavior, increased appetite, and food-seeking behavior (McEwen et al. 1988; Dallman et al. 1990). Most mammals vary steroid concentrations in the blood over the day, but only a few studies have looked at this in small mammals. Florant and Weitzman (1980) examined circadian rhythms in serum cortisol in woodchucks (*Marmota monax*) and found a spike just at dawn (lights on). Circadian variation in fecal glucocorticoid metabolites has also been observed in rats (Cavigelli et al. 2005; Lepschy et al. 2007) and mice (Touma et al. 2004).

Of the two EIAs we used, only the 5α-pregnane-3β,11β,21-triol-20-one EIA proved suitable to evaluate adrenocortical activity, as FCM levels declined following a DEX injection and, most importantly, increased following an ACTH injection (Fig. 4; Table 3). There was also a sex difference in the percentage of change after the administration of DEX and ACTH, as males showed a more pronounced decrease and increase, respectively, to these injections than did females. Thus, we conclude that in field studies on Columbian ground squirrels using this EIA, the effect of sex on FCM levels must first be examined. In squirrels, the main glucocorticoid is cortisol (Boonstra et al. 2001). However, this EIA has also been validated in mice (*Mus musculus f. domesticus*, strain C57BL/6J; Touma et al. 2003), a species that uses corticosterone as their primary glucocorticoid. Thus, both steroids must form FCM with a 5α-3β,11β-diol structure. A similar situation was reported by Nováková et al. (2008) in spiny mice, which also use cortisol as their main glucocorticoid. The 11-oxoetiocholanolone EIA showed no variation as a function of daily rhythm, of the DEX injection, or of the ACTH injection. However, this EIA works well in four other species that also have cortisol as their dominant or only glucocorticoid: European hares (Teskey-Gerstl et al. 2000), elephants (Ganswindt et al. 2003), cattle (Möstl and Palme 2002), and the European ground squirrel (Strauss et al. 2007). This underlies the necessity of validating each species before the application of such noninvasive methods (Touma and Palme 2005).

This is the first published extensive validation of a group-specific EIA measuring stress hormones in a ground squirrel species. However, Mateo and Cavigelli (2005) validated a generalized radioimmunoassay for fecal corticoid metabolites in Belding's ground squirrels, using commercially available radioimmunoassay kits (ICN Biomedicals, Cosa Mesa, CA) for cortisol and corticosterone. These kits use antisera that are specific for either cortisol or corticosterone but must have sufficient cross-reactivity to FCM to permit their detection (we assume that Belding's, as in Columbians [Fig. 1], excrete no native cortisol). They found the cortisol kit to have higher affinity for metabolites in the extracts than the corticosterone kit. However, both kits showed significant binding to extracts following DEX and ACTH injections. Unlike our study (Table 1), Mateo and Cavigelli collected feces every 12 h from 6 h onward, so the time resolution of their study was lower than ours. In addition, the concentrations of DEX and ACTH they used to successfully elicit a measured FCM response in the feces (Mateo and Cavigelli 2005, Fig. 8) were about 2.5 and 5 times greater, respectively, than what we used. Following the DEX injection, they found that FCM decreased between 6 and 18 h (a decrease of approximately 25%; Mateo and Cavigelli 2005, their fig. 8*b*), whereas we found the greatest suppression at 12 h (a decrease of 33%; Fig. 4). Following the ACTH injection, they found that FCM concentrations increased between 18 and 30 h (an increase of approximately 25%; Mateo and Cavigelli 2005, Fig. 8*a*), whereas we found the peak at 12 h (an increase of 255%; Fig. 4). Given that both DEX and especially ACTH rapidly cause changes in endogenous cortisol in squirrels (Boonstra and McColl 2000), we expect that these plasma changes should have been reflected in FCM changes within 7–12 h (our peak excretion of radioactive cortisol was at 7 h). Hence, we conclude that our validation results with 5α-pregnane-3β,11β,21-triol-20-one EIA are more sensitive and are probably a better reflection of endogenous cortisol changes than the ICN kits, given that this EIA is highly specific to the FCM actually produced (Möstl et al. 2005).

For many studies, the ultimate application of noninvasive fecal glucocorticoid analysis is in field-based studies (Mateo 2007; Charbonnel et al. 2008; Thiel et al. 2008). In Columbian ground squirrels, measurements from the same animal were repeatable over time and thus are a reasonably robust measure

of animal state during the summer following the breeding season. Obviously, at times of rapid environmental, developmental, or behavioral change, repeatability would be either not present or lower. One major argument for using an EIA for FCM to evaluate stress levels is that fecal metabolites should dampen pulsatile secretion of glucocorticoids and thus be a good measure of chronic stress (Boonstra 2005; Palme et al. 2005). However, fecal EIAs potentially introduce a whole set of other variables decreasing repeatability, such as variability in diet and fiber ingestion between days due to season and food availability.

In conclusion, our results provide an extensive validation of an EIA for noninvasive monitoring of adrenocortical activity, which now can be used in ecological studies of natural populations of the Columbian ground squirrel. The guiding rule of thumb has been that an EIA must be validated in each species (Palme 2005), but this is based on comparisons of members of different genera (reviewed in Touma and Palme 2005). That an 11-oxoetiocholanolone EIA was successfully used in European ground squirrels but was not suitable in Columbian ground squirrels underlines the need to validate the EIA for measuring FCM in each species, even if they are closely related. However, as these validations are time consuming, require wild animals to be confined in specialized conditions, and may not even be possible in many circumstances, it would be useful to find an EIA that would have wide applicability in related species. Therefore, the 5α-pregnane-3β,11β,21-triol-20-one EIA should also be tested in other ground squirrel species.

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