TECHNICAL COMMENT

Noninvasive Monitoring of Fecal Cortisol Metabolites in the Eastern Chipmunk (*Tamias striatus*): Validation and Comparison of Two Enzyme Immunoassays

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ABSTRACT

Monitoring fecal glucocorticoid metabolites in wild animals, using enzyme immunoassays, enables the study of endocrinological patterns relevant to ecology and evolution. While some researchers use antibodies against the parent hormone (which is typically absent from fecal samples), others advocate the use of antibodies designed to detect glucocorticoid metabolites. We validated two assays to monitor fecal cortisol metabolites in the eastern chipmunk (*Tamias striatus*). We compared an antibody produced against cortisol and one produced against 5α-pregnane-3β, 11β, 21-triol-20-one using a radiometabolism study and an injection with adrenocorticotropic hormone (ACTH). Most cortisol metabolites were excreted in the urine (~83%). Peak excretion in the feces occurred 8 h after injection. Both assays detected an increase in fecal cortisol metabolite levels after injection of ACTH. Males, but not females, exhibited a circadian variation in metabolite levels. The sexes did not exhibit any difference over the time course and route of excretion or the relative increase in fecal cortisol metabolite levels after ACTH injection. The cortisol assay displayed higher reactivity to ACTH injection relative to baseline than did the metabolite assay. While both antibodies gave comparable results, the cortisol antibody was more sensitive to changes in plasma cortisol levels in eastern chipmunks.

Introduction

Studies of the hypothalamic-pituitary-adrenal (HPA) axis in free-ranging populations provide insights into how animals adjust their physiology, behavior, and life history to environmental perturbations (Boonstra 2005; Reeder and Kramer 2005; Wingfield 2005). A better understanding of this axis sheds light on central processes in ecology and evolution, such as the long-term consequences of predation on population dynamics (Sheriff et al. 2010*b*, 2011*b*), adaptive maternal effects (Breuner 2008; Love and Williams 2008), or the impact of human activities on animal populations (Thiel et al. 2008, 2011; Macbeth et al. 2010).

The activity of the HPA axis is classically studied by quantifying the secretion of its end products, glucocorticoids, in the blood. The last decades have witnessed important methodological improvements to monitor glucocorticoid levels in a less invasive manner, and it is now possible to quantify their metabolites in urine and feces (Palme 2005; Sheriff et al. 2011*a*). Fecal measurements may be preferable to blood measurements because they represent an integration of glucocorticoid levels in the blood over a few hours rather than the point estimates obtained from blood samples (Touma et al. 2003; Sheriff et al. 2010*a*). Thus, they are buffered against small fluctuations in glucocorticoid levels that are likely to occur with blood measurements and may reflect more accurately the baseline glu-

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cocorticoid level of the animal (Goymann 2005). Moreover, recent work has shown that the fecal glucocorticoid metabolite level of an animal may be a good index of an animal's capacity to secrete plasma glucocorticoids in response to an environmental perturbation (Sheriff et al. 2010*a*). Concentrations of fecal glucocorticoid metabolites thus enable studying not only HPA activity but also potentially its reactivity to environmental perturbations.

Measuring glucocorticoids in their metabolized form, however, introduces some complexities. First, the time over which blood levels of glucocorticoids are integrated in the fecal samples will depend on the species' biology and activity pattern (Palme 2005). Second, the proportion of glucocorticoid metabolites excreted via the feces or the urine may vary with the species or the sex of the animals (Touma et al. 2003; Palme et al. 2005). It is thus advisable to perform a radiometabolism study, where a known quantity of radiolabeled glucocorticoid is injected and traced in the feces as well as in the urine to assess the time lag between the secretion of glucocorticoids into the blood and the excretion of their metabolites in the feces and urine. Third, when measuring fecal glucocorticoid metabolites, one deals with a mixture of compounds displaying different molecular structures, in which little or no native (i.e., unmetabolized) glucocorticoid(s) is present (Möstl et al. 2005; Palme 2005; Bosson et al. 2009). It is thus necessary to ensure that the technique used to assess glucocorticoid reacts with an important portion of the glucocorticoid metabolites produced by the animal. It is also important to ensure that glucocorticoid secretion into the blood is well reflected in the fecal samples (Touma and Palme 2005).

Fecal glucocorticoid metabolites can be quantified with different approaches, including enzyme immunoassays (EIAs). Immunoassays originally designed to target native (i.e., nonmetabolized) glucocorticoids have been extensively used to quantify fecal glucocorticoid metabolites in an array of species (Harper and Austad 2000; Wasser et al. 2000; Young and Monfort 2009). These assays assume that the antibody has sufficient cross-reactivity to recognize (a group of) metabolites of the parent hormone and that an increase in blood glucocorticoid secretion leads to an increase in metabolite excretion in the feces. These assays initially were developed for blood measurements (Möstl et al. 2005). Although these assays have different cross-reactivities and hence different efficacy, they have been used with success to study relationships between fecal glucocorticoid metabolite levels and some aspects of the reproduction or the ecology of the animals in their natural environment (Jurke et al. 1997; Lynch et al. 2002; Mateo 2007; Young and Monfort 2009). Other studies use antibodies designed to recognize a group of fecal glucocorticoid metabolites sharing a common structure (Jurke et al. 1997; Lynch et al. 2002; Möstl and Palme 2002; Möstl et al. 2005; Mateo 2007; Young and Monfort 2009). These metabolite antibodies typically yield higher concentrations of measured metabolites and display a higher reactivity with the fecal glucocorticoid metabolites than antibodies targeting native glucocorticoids and are therefore more likely to detect small fluctuations in fecal glucocorticoid metabolites (Morrow et al. 2002; Frigerio et al. 2004).

In this study, we validate and compare two fecal assays to monitor HPA axis activity in the eastern chipmunk (*Tamias striatus*): one using an antibody raised against cortisol and another using an antibody raised against 5α-pregnane-3β, 11β, 21-triol-20-one. Cortisol is the main glucocorticoid secreted by all sciurid species studied to date, including the yellow-bellied marmot (*Marmota flaviventris*), California ground squirrel (*Spermophilus beecheyi*), golden-mantled ground squirrel (*Spermophilus saturatus*), red squirrels (*Tamiasciurus hudsonicus*), and yellow-pine chipmunks (*Tamias amoenus*; Boonstra and McColl 2000; Kenagy and Place 2000; Place and Kenagy 2000; Boonstra et al. 2001). First, we performed a radiometabolism study to analyze the time lag between cortisol secretion into the blood and its excretion in the feces as well as the relative importance of the routes of excretion (urine vs. feces). We measured the radioactivity as well as immunoreactivity of each fraction with a metabolite antibody recognizing steroids with a 5α-3β-11β-diol structure (Touma et al. 2003) as well as with a cortisol antibody validated in a diversity of species (Young et al. 2004). The metabolite antibody enables a reliable monitoring of the HPA axis in related species, such as Columbian ground squirrels (*Spermophilus columbianus*; Bosson et al. 2009) and red squirrels (*T. hudsonicus*; Dantzer et al. 2010). Second, we monitored baseline fecal cortisol metabolite levels of captive individuals over 2 d to determine the pattern of the circadian rhythm excretion. We also monitored fecal cortisol metabolite levels after stimulation of the adrenal by an ACTH injection to ensure that our method detects changes in blood cortisol levels. Finally, we compared both assays regarding their suitability to noninvasively measure adrenocortical activity in eastern chipmunks. While assays against the parent hormone and the assays against the metabolites have successfully been applied to study glucocorticoid levels in free-ranging populations, few studies compare these two types of assays. Consequently, our study will expand our knowledge on the relative advantages of these two types of assays applied to wild populations.

Methods

Capture and Housing of Chipmunks

We livetrapped nine chipmunks (five males and four females) on the campus of the University of Toronto at Scarborough (243°47′N, 79°11′W, elevation = 116 m) in August 2010. Longworth traps were baited with sunflower seeds, apples, and carrots; opened at dawn; inspected every 90 min; and closed around 15 h. Females showing signs of lactation were immediately released at the point of capture. We housed chipmunks individually in plastic cages (47 cm × 26 cm × 20 cm) equipped with a wire bottom and a water bottle with a stainless steel nipple. Cages were mounted within a second same-sized tray, equipped with a fine metal mesh. This system enabled the feces and urine to fall through the cage bottom, but the feces were caught on the fine wire mesh while the urine passed through, thus preventing contamination of the feces by the

urine. Animals sometimes urinated onto the feces, in which case the fecal pellets were discarded. Hence, we were able to collect the samples while minimizing disturbance of the chipmunks. Each chipmunk was provided with a plastic refuge, a piece of natural wood, and some cotton as bedding material. They had ad lib. access to rodent chow pellets, peanut butter, sunflower seeds, apples, carrots, and occasionally acorns. Chipmunks were kept at 20°C under natural photoperiod for the duration of the experiment. Except during collection of the samples, chipmunks were not exposed to human presence or noise. Chipmunks were left to acclimate for at least 5 d before beginning the trials. During that phase, feces were collected every 4 h between 0800 and 2200 hours to habituate them to the procedure (see table 1 for the chronology of the tests and the sampling times). Upon completion of the experiment, we inspected and weighed the chipmunks. Chipmunks did not lose weight or show any sign of deteriorating health related to the trials. However, we witnessed a decrease in activity during the last 3 d of the trials that paralleled the decrease in chipmunk aboveground activity frequently observed in nature during the late summer (Dunford 1970; Elliott 1978). All husbandry and manipulations were conducted under University of Toronto animal use protocol 20008380, issued in accordance with the Canadian Council on Animal Care guidelines.

Route and Time Course of Cortisol Excretion

To monitor the route and timing of cortisol excretion, we injected the nine chipmunks intraperitoneally with 1,110 kBq of radiolabeled cortisol (1,2,6,7-[3H]-cortisol; Amersham Biosciences; specific activity = 1.55 TBq/mmol). Before injection, radiolabeled cortisol was dried down under air and reconstituted in 10% ethanol and 90% sterile physiological saline solution in order to inject a volume of 300 μL. Chipmunks were injected between 0700 and 0800 hours, and urine and feces were then collected for 72 h (see table 1 for sampling intervals). We collected all dry (i.e., uncontaminated) feces pellets with forceps and collected all urine using a 1-mL pipette. We rinsed the collecting trays with 2 mL of methanol to collect the remaining radioactivity and added these to the urine sample. Feces mixed with urine were also collected and analyzed separately. All samples were immediately put on ice and frozen at −20°C within 20 min of collection. Urine samples were dried down under air and reconstituted in 1 mL of methanol (80%). We dried the fecal samples at 70°C for 3 d (until they reached constant mass; dried radioactive samples ranged from 5 to 50 mg). We then crushed the feces using a plunger and extracted each whole sample by vortexing the pulverized feces with 1 mL of methanol (80%). We quantified activity in the samples by adding 2.5 mL of scintillation fluid to 0.5 mL of urine or fecal extract and measured radioactivity with a scintillation counter with quench correction (Tri-Carb 2900TR, Boston, MA).

Characterization of Fecal Cortisol Metabolites

Six fecal extracts with maximum radioactivity (three samples from males and three from females) were dried down under air and sent to University of Veterinary Medicine, Vienna, Austria. The radioactive cortisol metabolites in these samples were separated according to their polarity by reverse-phase high-performance liquid chromatography (HPLC). We measured radioactivity and immunoreactivity in each fraction with the 5α-pregnane-3β, 11β, 21-triol-20-one EIA described below (for details see Touma et al. 2003; Lepschy et al. 2007). In addition, we quantified the amount of immunoreactive cortisol present in the fecal samples by measuring the immunoreactivity of each fraction with a cortisol EIA (see Palme and Möstl 1997 for additional details on the assay).

Monitoring Baseline and Stimulated Cortisol Secretion

To validate the ability of the EIAs to detect changes in cortisol secretion patterns, we injected animals with synthetic adrenocorticotropic hormone (4 IU/kg, ACTH; Synacthen Depot, CIBA). Fecal samples were collected to measure baseline levels of fecal cortisol metabolites 24 h before and 48 h after ACTH injection. Animals were injected between 0700 and 0800 hours. After injection, we collected samples from 0800 to 2200 hours for 48 h (see table 1 for frequency of sampling).

Extraction and Analysis of Fecal Cortisol Metabolites

We dried down fecal samples at 70°C for 72 h (samples typically reach constant mass after 48 h). Dried samples were then transferred to a 2-mL test tube and ground using a plunger. We extracted the fecal cortisol metabolite by vortexing 35 ± 5 mg of ground feces in 1 mL of methanol 80% for 30 min at 1,500 rpm and centrifuging (2,500 *g*; 20 min). We then analyzed the supernatant with a 5α-pregnane-3β, 11β, 21-triol-20-one EIA (Touma et al. 2003). Details about cross-reactivity of the an-

Table 1: Chronology of the manipulations carried out on nine Eastern chipmunks in August 2010

Date	Procedure	Treatment	Collection time for samples
August 7–8	Capture		
August 8–11	Acclimation	No manipulation	Every 4 h from 0800 to 2200 hours
August 12–15	Radiometabolism study	Injection of radiolabeled cortisol	Every 2 h from 0800 to 2200 hours
August 16	Baseline monitoring	No manipulation	Every 4 h from 0800 to 2200 hours
August 17–18	ACTH challenge	Injection of synthetic ACTH	Every 2 h from 0800 to 2200 hours
August 19	Baseline monitoring	No manipulation	Every 4 h from 0800 to 2200 hours

tibody and the assay procedure can be found in Touma et al. (2003). Mean intra- and interassay coefficients of variation of two pool samples were 11.5% and 16.5%, respectively.

To compare assays, we also analyzed the samples collected during baseline monitoring and after ACTH injection with a cortisol EIA. The cortisol EIA used a polyclonal cortisol antibody (R4866; C. J. Munro, University of California, Davis) with a horseradish peroxidase ligand (see Munro and Lasley 1988 for details on cross-reactivity; see Young et al. 2004 for details on EIA protocol). Mean intra- and interassay coefficients of variation were 9.7% and 9.9%, respectively.

Statistical Analysis

We tested for sex differences in time to peak excretion in feces and urine, percentage of injected radioactivity recovered, and the proportion of radioactivity excreted in the feces, using linear models. Fecal cortisol metabolite levels measured with the 5α-pregnane-3β, 11β, 21-triol-20-one and the cortisol assays were analyzed in a two-step manner. First, to explore the factor affecting cortisol baseline levels, we used a linear mixed model including hour of sample collection (categorical variable), sex, and their interaction as fixed effects. Individual identity was fitted as a random effect to account for repeated measurements of the same individuals over time (also known as pseudoreplication; Crawley 2007). A log-likelihood ratio test comparing a model including the random effect of interest and a model without it while holding the fixed effect structure constant was used to test for the significance of the random effect (Pinheiro and Bates 2000). For the individual random effect, we also report the repeatability (r), defined as the variance associated with the effect divided by the sum of this variance and the residual variance from the model (Pinheiro and Bates 2000). High r values are indicative of substantial, consistent differences among individuals. Second, to evaluate how ACTH injection affected fecal cortisol metabolite levels compared to baseline levels, we analyzed fecal cortisol metabolite levels in all collected samples (i.e., during baseline sampling and after ACTH injection) within one linear mixed model. This second model initially included hour of sample collection, time since ACTH injection, type of sampling (baseline monitoring or ACTH injection), sex, and their interactions as fixed explanatory variables. Again, we added chipmunk identity as a random effect to account for pseudoreplication and tested its significance using a log-likelihood ratio test as described above. All models were simplified by stepwise deletion of nonsignificant terms (Crawley 2007). To compare the results obtained with both assays, we computed Pearson's product-moment correlation coefficient for all samples analyzed. We also compared the relative increase in fecal cortisol metabolite level detected by each assay after ACTH injection using a linear model. The response variable was the increase in fecal cortisol metabolite level of each individual expressed as the percentage of baseline level at the same time of day (the percentage was then log transformed). The explanatory variables were number of hours since ACTH injection, assay used (5α-pregnane-3β, 11β, 21-triol-20-one or cortisol assay), sex of individuals, and their interactions. All fecal cortisol metabolite levels are expressed as log-transformed values (units are ng/g dry feces). Statistical analyses were performed using R 2.10.1 (R Development Core Team 2009). Means are reported with ± 1 SEM.

Results

Route and Time Course of ^{3}H-Cortisol Excretion

During the 72 h after injection of radiolabeled cortisol, we collected 151 and 180 urine and fecal samples, respectively. We recovered 48.33% $\pm$ 0.13% of the 1,110 kBq injected in each subject (range = 29.00–75.17). A total of 83.10% $\pm$ 6.67% and 16.21% $\pm$ 6.86% of the radioactivity recovered was found in the urine and the feces, respectively. The rest (0.69% $\pm$ 0.90%) was found in the feces contaminated with urine. There was no difference in the percentage of radioactivity recovered among sexes (effect = 12.85 $\pm$ 8.5, $t_7 = 1.51$, $P = 0.17$) or in the percentage of radiolabeled cortisol excreted via the feces (effect = -4.29 ± 4.65, $t_7 = -0.92$, $P = 0.39$). Median peak excretion of radioactivity in the urine occurred at 4 h after injection (range = 2–4, mean = 3.11 $\pm$ 1.05 h) and in feces 8 h after injection (range = 8–12, mean = 9.33 $\pm$ 1.73; see fig. 1). Time to peak excretion in urine ($t_7 = 0.27$, $P = 0.80$) or in feces ($t_7 = -1.04$, $P = 0.33$) was not affected by sex.

Characterization of Fecal Cortisol Metabolites

HPLC revealed that cortisol was almost completely metabolized in both females and males (see fig. 2). The most prominent radioactivity peaks eluted between fractions 35 and 50 for both sexes. There were differences between sexes regarding the formed metabolites and their immunoreactivity. Females produced more metabolites with higher polarity (fractions 5–20). The 5α-pregnane-3β, 11β, 21-triol-20-one assay detected more metabolites and had higher peak levels. However, also the cortisol EIA was able to react with some of the radioactive metabolites.

Monitoring Baseline and Stimulated (ACTH Injection) Cortisol Secretion

Baseline fecal cortisol metabolite levels ($N = 72$ samples) analyzed with the 5α-pregnane-3β, 11β, 21-triol-20-one EIA ranged from 1,720 to 15,000 ng/g of dry feces (median = 5,390 ng/g) and did not vary with sex in the sampling at 0800 hours (effect = -0.19 ± 0.19, $t_{57} = -0.96$, $P = 0.37$). However, males tended to show higher fecal cortisol metabolite levels during the rest of the day (all effects < 0.34, all $P < 0.04$; see fig. 3, *top*). Animals also displayed consistent individual differences regarding their fecal cortisol metabolite level (r = 51.22%, log-likelihood ratio = 25.67, $P < 0.001$). The ACTH injection ($N = 72$ samples) affected fecal cortisol metabolite levels ~8 h after the injection (effect = 0.51 $\pm$ 0.15, $t_{116} = 3.38$, $P = 0.001$; see fig. 3, *top*). Again, we detected important

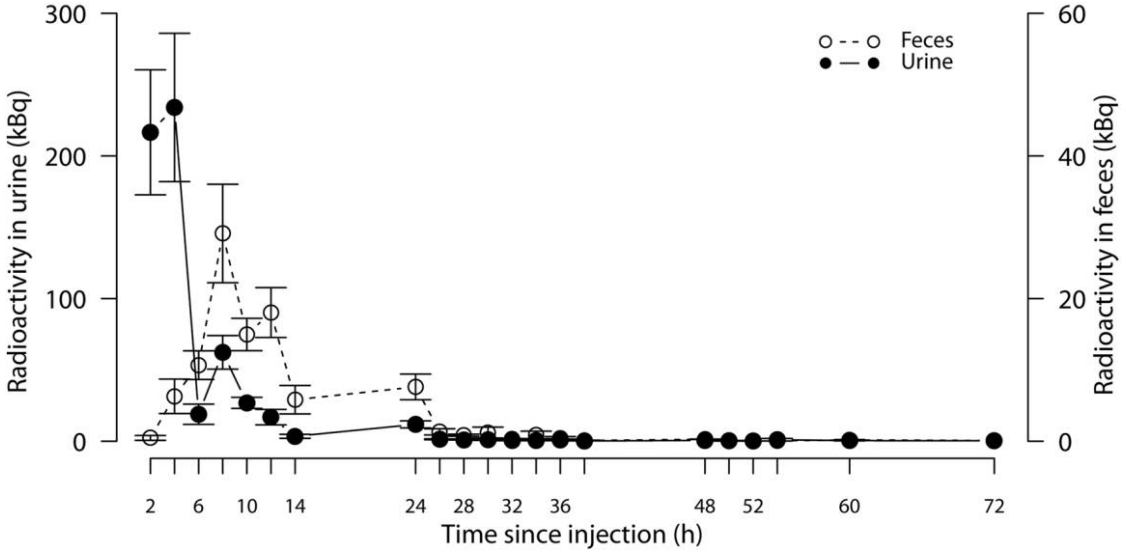

Figure 1. Radioactivity (mean ± SEM) excreted in urine (filled dots, solid line) and feces (open dots, dashed line) in nine eastern chipmunks after an injection of radiolabeled cortisol.

individual differences in the mean fecal cortisol metabolite levels ($r = 46.72\%$, log-likelihood ratio = 53.16, $P < 0.001$).

Analysis of the baseline fecal cortisol metabolite levels with the cortisol EIA ranged from 133 to 2,942 ng/g of dry feces (median = 700 ng/g) and revealed that males displayed higher fecal cortisol metabolite levels than females at 1200 hours (effect = 0.79 ± 0.30, $t_{51} = 2.59$, $P = 0.012$) and 1600 hours (effect = 1.01 ± 0.31, $t_{51} = -0.18$, $P = 0.002$; see fig. 3, *bottom*). The cortisol EIA also revealed important individual variation ($r = 72.20\%$, likelihood ratio = 50.04, $P < 0.001$). This EIA also detected an effect of ACTH injection on fecal cortisol metabolite levels with effects at 1600 hours (effect = 1.92 ± 0.46, $t_{51} = 4.14$, $P = 0.001$) and 2000 hours (effect = 1.67 ± 0.50, $t_{51} = 3.32$, $P = 0.001$). This EIA detected important individual variation in the mean fecal cortisol metabolite levels ($r = 40.26\%$, log-likelihood ratio = 41.05, $P < 0.001$). Concentrations of fecal cortisol metabolites measured with both EIAs were significantly correlated ($r = 0.45$, $N = 138$, $P < 0.001$).

Analysis of the relative increase in fecal cortisol metabolite detected by each assay (5α-pregnane-3β, 11β, 21-triol-20-one assay: median = 101%, range = 36–556; cortisol assay: median = 132%, range = 16–1,385) showed that the cortisol EIA measured a higher increase relative to the baseline samples 8 and 12 h after ACTH injection compared with the 5α-pregnane-3β, 11β, 21-triol-20-one EIA (8-h sampling: effect = 1.28 ± 0.39, $t_{106} = 3.25$, $P = 0.001$; 12-h sampling: effect = 1.11 ± 0.39, $t_{106} = 2.81$, $P = 0.005$; one outlier was excluded from the analysis; see fig. 4). Relative increase in measured fecal cortisol metabolites did not vary between both EIAs during all the remaining sampling times after ACTH injection (all effects < 0.50, all $P > 0.20$). We did not find any statistically significant difference between the sexes in the relative increase in fecal cortisol metabolites with either assay (all terms rejected with $P > 0.18$).

Discussion

The objective of this study was to assess glucocorticoid levels of eastern chipmunks noninvasively by validating the measurement of fecal cortisol metabolites using two different EIAs. The first used a cortisol antibody, while the second, using a 5α-pregnane-3β, 11β, 21-triol-20-one antibody, targeted a group of its metabolites. Our key conclusion is that both antibodies appear to accurately represent fecal metabolite levels but that the cortisol EIA is more sensitive. The radiometabolism experiment showed that only about 16% of the metabolites are excreted in feces and that the time lag between secretion of cortisol into the blood and the appearance of its metabolites in the feces was about 8 h. We found that males displayed a circadian rhythm, with a peak concentration at 1600 hours, whereas females did not. After an ACTH stimulation test, we found that both assays detected an increase in cortisol metabolites, which was expressed more strongly in the cortisol EIA. Finally, both assays detected ample individual variation, showing that animals consistently differed from each other regarding their fecal cortisol metabolite levels. We discuss each of these points below.

Our radiometabolism study showed that chipmunks excreted most of the injected cortisol in urine (83%) within the first 4 h after injection of radiolabeled cortisol. In contrast, we detected a peak in excretion in the feces 8 h after the injection. Blood cortisol is metabolized in the liver and excreted into the gut via the bile (Möstl et al. 2005). The time lag between radiolabeled cortisol injection and its peak excretion in the feces can be attributed mostly to the time needed for intestinal transit

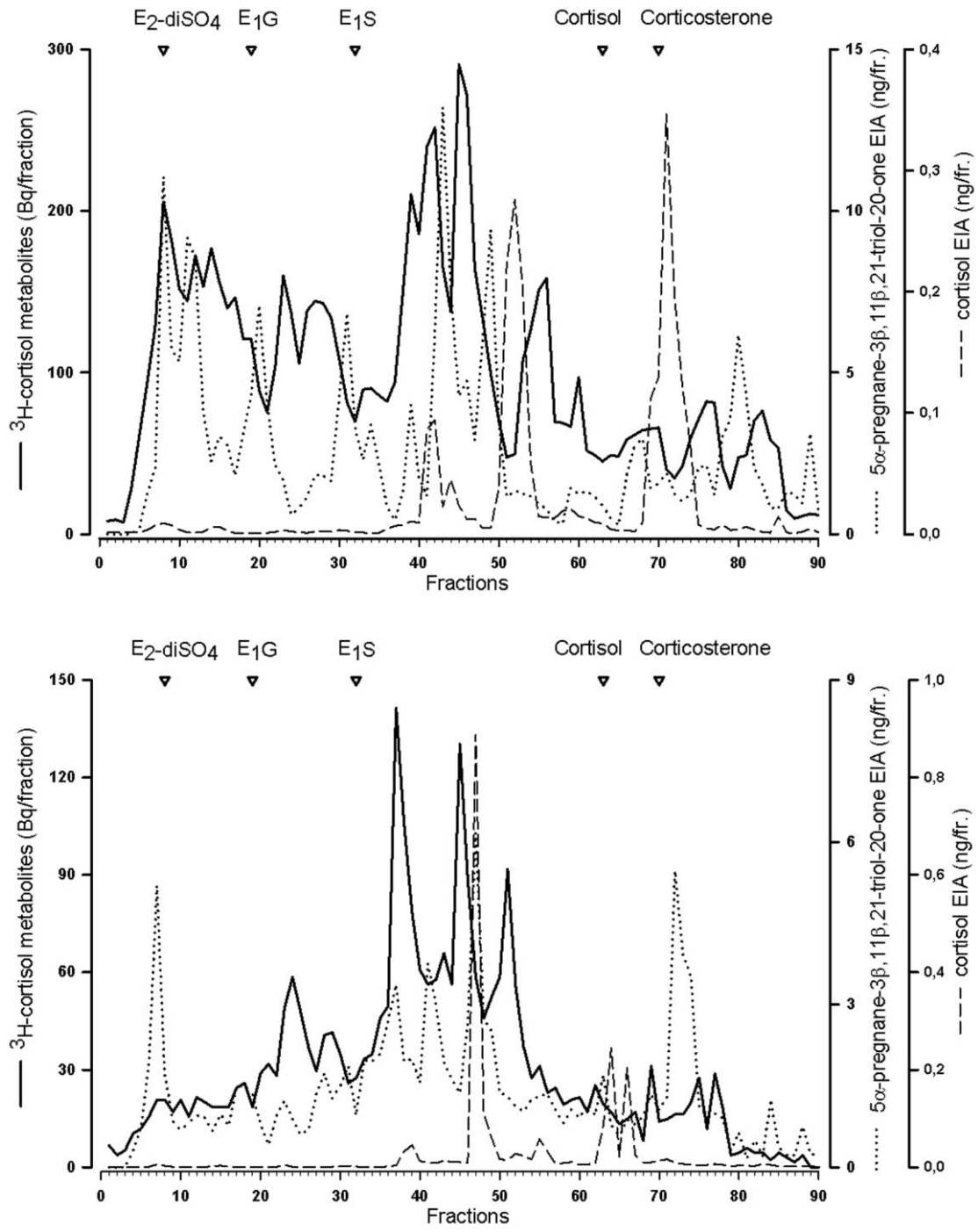

Figure 2. Radioactivity (solid line) and immunoreactivity determined by a cortisol (dashed line) and a 5α-pregnane-3β, 11β, 21-triol-20-one (dotted line) enzyme immunoassays of female (*top*) and male (*bottom*) eastern chipmunk fecal extracts fractionated by high-performance liquid chromatography. Open triangles mark the approximate elution time of estradiol disulphate (E2-dSO4), estrone glucuronide (E1G), estrone sulfate (E1S), cortisol, and corticosterone standards.

(Palme et al. 2005). Similar lag times have been described in Columbian ground squirrels (~7 h; Bosson et al. 2009). However, chipmunks exhibit a shorter time lag than red squirrels (10.9 h; Dantzer et al. 2010). We were surprised to recover only ~50% of the injected radioactivity, given that we collected all feces and urine puddles under as well as within the cage. This loss could be attributed to absorption of the radioactivity from the urine by the plastic of the cages because all other accessories in the cage had been removed for the duration of the radiometabolism study. If this is the case, our results would tend to

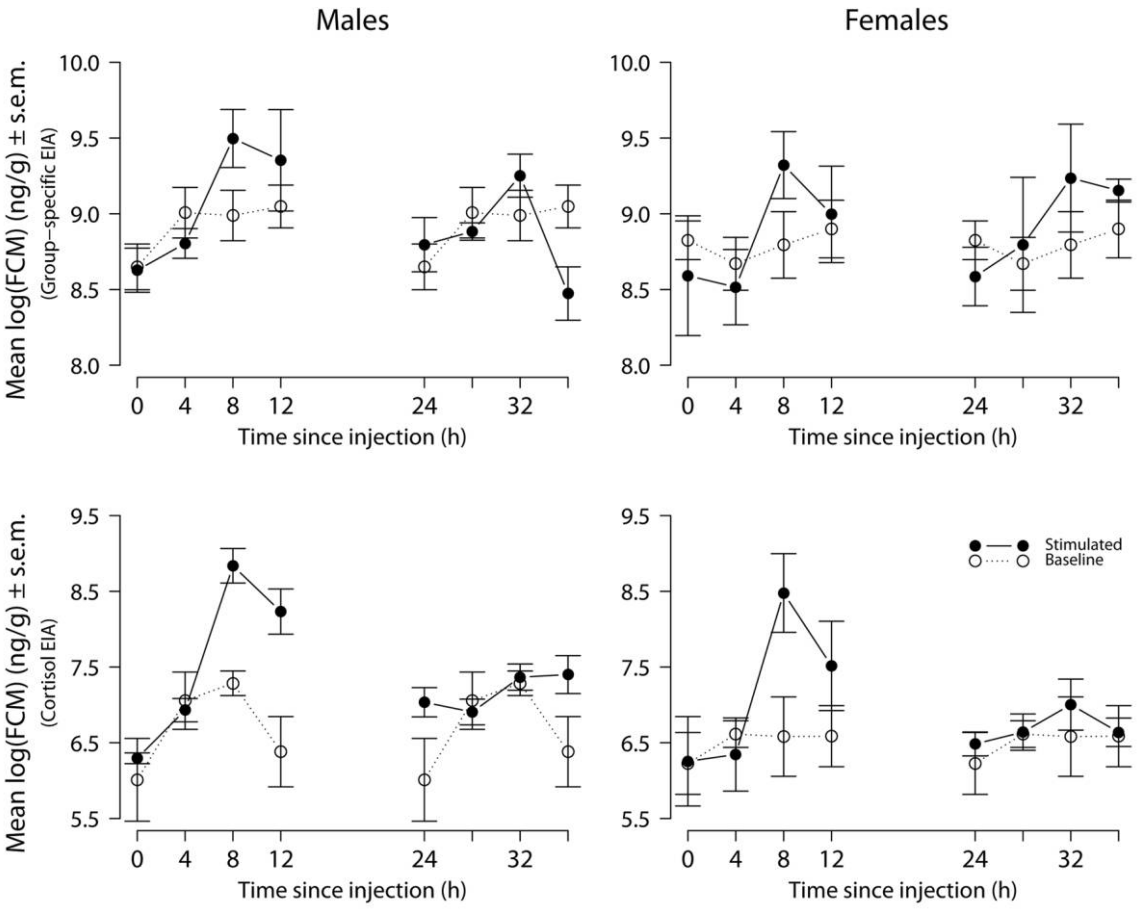

Figure 3. Concentrations of fecal cortisol metabolites (FCM) in male ($N = 5$) and female ($N = 4$) eastern chipmunks during baseline (open dots, dotted line) and after an adrenocorticotropic hormone injection (filled dots, solid line). FCM levels were measured using a 5α-pregnane-3β, 11β, 21-triol-20-one (*top*) and a cortisol (*bottom*) enzyme immunoassay.

overestimate the proportion of radiolabeled cortisol excreted via the feces. A portion of this loss may also be attributed to radioactivity loss during metabolism. Indeed, some of the tritium incorporated into the cortisol molecules may be exchanged for nonradioactive hydrogen during metabolism. Using a more stable radioisotope (i.e., ^{14}C) to label the cortisol would have avoided this bias. However, ^{14}C-labeled glucocorticoids are very expensive because they require complex synthesis protocols (Möstl et al. 2005). In accordance with previous radiometabolism studies carried out in other species, our HPLC analyses of fecal samples show that ^{3}H-cortisol was extensively metabolized. Native cortisol was found only in small amounts, if at all, in the feces (Touma et al. 2003; Palme et al. 2005; Lepschy et al. 2007). Nevertheless, a cortisol EIA was able to react with some of the radioactive metabolites. As a result of the cross-reactions of the used antibody (antigen: cortisol-3-carboxymethyloxime linked to bovine serum albumin), these metabolites should have a 11a,17ß,21-triol-20-one structure (e.g., tetrahydrocortisol). The 5α-pregnane-3β, 11β, 21-triol-20-one assay detected more metabolites and had higher peak levels. Proper identification of the metabolites would require substantial efforts (including mass spectrometry and steroid standards no longer available), which are beyond the aim of this study. In addition, our results show once again that expressed differences in excreted fecal glucocorticoid metabolites are present even in closely related species (Bosson et al. 2009; Dantzer et al. 2010).

Both assays detected a significant increase in immunoreactivity after stimulation of the adrenals (with an injection of ACTH). Thus, our study validates both EIAs. Such a physiological validation is critical because it ensures that an assay is able to detect changes in endogenous cortisol production by the adrenal glands (Möstl et al. 2005; Palme et al. 2005; Touma and Palme 2005). Moreover, the time course of fecal cortisol metabolite levels after an ACTH injection was in agreement with the time course of cortisol excretion evidenced by the radiometabolism study, with a peak excretion occurring in the feces around 8 h after adrenal stimulation. Thus, both assays are suited to monitor cortisol production in the eastern chipmunk. We nevertheless detected important differences regarding the reactivity of each assay to an increase in blood cortisol. Indeed, whereas the 5α-pregnane-3β, 11β, 21-triol-20-one as-

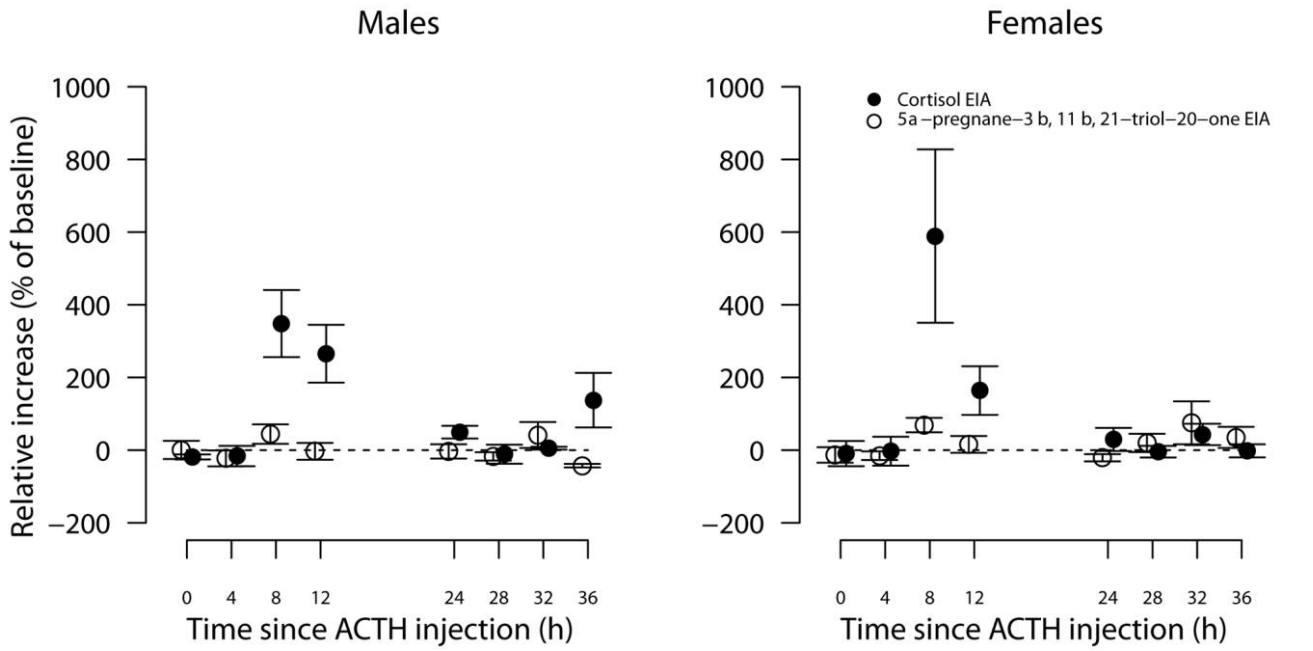

Figure 4. Fecal cortisol metabolites after an adrenocorticotropic hormone (ACTH) injection for male ($N = 4$) and female ($N = 4$) eastern chipmunks. Levels are expressed as a percentage of baseline levels (mean ± SEM) measured at the same time of day. Fecal cortisol metabolites were measured using a 5α-pregnane-3β, 11β, 21-triol-20-one (open dots) and a cortisol (filled dots) enzyme immunoassay. One outlier male was excluded from the analysis.

say detected a mean relative increase of ~100% after ACTH injection, the cortisol EIA displayed a larger reactivity (relative increase of ~500% when the sexes are pooled). An increased reactivity is traditionally thought to result in a greater sensitivity (Morrow et al. 2002; Möstl et al. 2005). Therefore, the cortisol assay validated in this study should detect smaller fluctuations in fecal cortisol metabolite levels (Frigerio et al. 2004). These findings contrast with previous studies reporting a higher reactivity of metabolite assays compared to cortisol/corticosterone assays (Morrow et al. 2002; Frigerio et al. 2004). However, others also reported a cortisol EIA being better suited to some primate species (Heistermann et al. 2006). In addition, an assay targeting another group of fecal metabolites (11-oxoetiocholanolone EIA) turned out to be better suited than the 5α-pregnane-3β, 11β, 21-triol-20-one EIA to other squirrel species (Strauss et al. 2007; Sheriff et al. 2012). Thus, our results once more underline the importance of validating each assay in a given species (Palme 2005; Touma and Palme 2005; Sheriff et al. 2011*a*).

The assays we validated indicated significant gender differences in circadian patterns of baseline fecal cortisol metabolite levels. Circadian rhythms in fecal cortisol metabolite levels have been detected in Columbian ground squirrels (Bosson et al. 2009) but not in red squirrels (Dantzer et al. 2010). While male chipmunks displayed an increase in cortisol metabolite levels that peaked around 1600 hours each day, females did not. Circadian patterns in cortisol metabolite levels should reflect circadian fluctuation in blood levels. Animals typically display a peak in cortisol secretion into the blood at the beginning of their active phase, which is thought to enable increased locomotion and foraging behavior (Reeder and Kramer 2005). Accounting for the 8-h lag time, our results suggest that the peak cortisol secretion into the blood is located around dawn. The circadian variation in cortisol metabolites we observed in this study is thus in accordance with what we would predict. Studies reporting circadian cortisol variation in small mammals typically report more pronounced variation in females than in males (Touma et al. 2004; Cavigelli et al. 2005; Lepschy et al. 2007). In our case, we did not detect circadian variation in females. Sex differences over the circadian rhythm of fecal cortisol metabolites could arise from differences in activity patterns between the sexes or because of differences in metabolite composition between males and females. As suggested by Touma et al. (2003), activity pattern affects the timing and frequency of fecal excretion and might thus modify circadian fecal cortisol metabolite patterns. In our case, however, we did not detect any difference between males and females in the time course of excretion (see "Route and Time Course of ^{3}H-Cortisol Excretion"), suggesting that the differences we witnessed between males and females cannot be explained by sex differences in activity patterns. Males and females have already been shown to excrete different glucocorticoid metabolites in other rodent species (Touma et al. 2003; Lepschy et al. 2007). In our study, females produced more polar metabolites, as evidenced by the increased radioactivity eluted in fractions 10–20. In addition, the assays seem to recognize different metabolites in both sexes.

Finally, both assays detected ample individual variation. Such individual variation was absent in the radiometabolism study, and thus we cannot invoke individual differences in excretion patterns to explain individual variation detected by the assays. Consistent differences in fecal glucocorticoid levels among individuals are repeatedly reported in the literature (Touma and Palme 2005; Bosson et al. 2009). In our study, individuals were kept in a standardized environment, and such differences could reflect individual differences related to components of the HPA axis (e.g., some individuals could display an increased adrenal baseline activity). When encountered in the field, individual differences in fecal cortisol metabolite levels might also originate from reproductive or social status differences among animals and from environmental characteristics such as temperature or predation risk (Sapolsky and Ray 1989; Frigerio et al. 2004; Travers et al. 2010). Cortisol levels can also be associated with resource allocation decisions of animals relative to reproductive or survival functions (Ricklefs and Wikelski 2002). Individual differences in cortisol levels are related to continuous behavioral variation over the stress response, as well as individual variation in reproductive tactics or life history that characterize some species (Øverli et al. 2007; Lancaster et al. 2008; Koolhaas et al. 2010; Réale et al. 2010). Disentangling the effects of these factors on cortisol levels and explaining consistent individual variation in cortisol production require the monitoring of cortisol levels over extended periods of time within and among individuals and in natural environments. The assays we validated in this study will enable such studies in the chipmunk and will thus contribute to understanding the roles glucocorticoids play in the expression of adaptive individual variation (Wingfield et al. 2008).

Conclusion

In conclusion, our study investigated the timing and route of excretion of cortisol metabolites and validated two enzyme immunoassays to monitor adrenocortical activity in the eastern chipmunk. Both assays provide a valid method of measuring cortisol levels secreted by individuals around 8 h before sampling. The cortisol assay displayed higher reactivity. These immunoassays will enable longitudinal studies of the cortisol levels in free-ranging individuals, providing a valuable tool to study the physiological bases of individual variation in eastern chipmunks.

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