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Fecal cortisol metabolite levels in free-ranging North American red squirrels: Assay validation and the effects of reproductive condition

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ABSTRACT

Patterns in stress hormone (glucocorticoid: GC) levels and their relationship to reproductive condition in natural populations are rarely investigated. In this study, we (1) validate an enzyme-immunoassay to measure fecal cortisol metabolite (FCM) levels in North American red squirrels (*Tamiasciurus hudsonicus*), and (2) examine relationships between FCM levels and reproductive condition in a free-ranging red squirrel population. Injected radiolabeled cortisol was entirely metabolized and excreted in both the urine (mean ± SE; 70.3 ± 0.02%) and feces (29.7 ± 0.02%), with a lag time to peak excretion in the feces of 10.9 ± 2.3 h. Our antibody reacted with several cortisol metabolites, and an adrenocorticotropic injection significantly increased FCM levels above baseline levels at 8 h post-injection. Relative to baseline levels, manipulation by handling also tended to increase FCM levels at 8 h post-manipulation, but this difference was not significant. FCM levels did not differ significantly between samples frozen immediately and 5 h after collection. Reproductive condition significantly affected FCM levels in free-ranging females (pregnant > lactating > post-lactating > non-breeding) but not males (scrotal testes vs. abdominal testes). Among females with known parturition dates, FCM levels increased during gestation, peaked at parturition, and declined during lactation. The difference between pregnant and lactating females was therefore dependent upon when the fecal samples were obtained during these periods, suggesting caution in categorizing reproductive stages. This study demonstrates the utility of fecal hormone metabolite assays to document patterns of glucocorticoid levels in free-ranging animals.

1. Introduction

The hypothalamic–pituitary–adrenal (HPA) axis plays a central role in enabling organisms to cope with and adapt to their external environment (e.g., Darlington et al., 1990). Unpredictable adverse stimuli that disrupt an organism from physiological homeostasis or its normal routine generally cause activation of the HPA axis (Levine and Ursin, 1991; Reeder and Kramer, 2005). Such stressors can potentiate the release of glucocorticoids (GCs) from the adrenal cortex downstream of the HPA stimulation (Sapolsky et al., 2000). Because GCs are released following HPA stimulation, levels of circulating GCs are often used as an index of the degree of stress experienced by laboratory and free-ranging animals (Möstl and Palme, 2002).

Laboratory animals have provided much insight into the functions of GCs and the effects of sex and reproductive condition on GC levels. Perhaps because laboratory studies are unable to replicate the multi-faceted contributors to abiotic and biotic seasonality in natural environments, many laboratory animals have much less or no seasonal variation in GC levels than free-ranging animals (Romero, 2002). Few studies have examined basic relationships between reproductive status and GC levels in free-ranging animals (Reeder and Kramer, 2005; Romero, 2002), which is surprising given that GCs and HPA activity can influence survival probability in the wild (e.g., Blas et al., 2007; Romero and Wikelski, 2001).

With the rapid development of non-invasive techniques to measure GC metabolites in feces and urine, determining the levels of GCs in free-ranging animals has become more feasible (Palme et al., 2005). Fecal sampling offers a number of advantages over blood sampling in natural populations. It is non-invasive as it does not require blood-drawing and often does not require trapping and immobilization. This is especially pertinent in study populations in which repeated blood sampling is not possible either during certain time periods (e.g., pregnancy) or because of small body size.

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Furthermore, fecal hormone metabolite levels represent an integrated average measure (depending on gut passage time: Palme et al., 2005) and are therefore less affected by transient increases in GC levels (Touma and Palme, 2005).

While the utility of fecal hormone metabolite assays in natural populations is clear, their use should not be indiscriminate (Touma and Palme, 2005). Only metabolites of glucocorticoids are excreted in feces and the metabolism of unbound plasma hormones in the liver and route of excretion (urine or feces) is species-specific (Palme et al., 1996, 2005). As such, immunoassays for measuring fecal hormone metabolite levels need to be carefully validated in a species-specific manner to ensure that the hormone of interest is being properly measured as a metabolite in the feces (Palme, 2005; Touma and Palme, 2005).

Our major aim in this study was to validate an immunoassay for measuring fecal GC/(cortisol: Boonstra and McColl, 2000) metabolites in North American red squirrels (*Tamiasciurus hudsonicus*). We also documented the effects of sex and reproductive condition on fecal cortisol metabolite (FCM) levels in a completely enumerated population of free-ranging red squirrels. Red squirrels are an excellent study species to examine how reproductive condition affects FCM levels in free-ranging mammals because repeated live-trapping of individually-marked red squirrels provides both fecal samples for GC quantification and also detailed reproductive information about males (when testes ascend and descend) and females (day of estrous, parturition, lactation, and weaning).

2. Materials and methods

2.1. Capture and husbandry of squirrels for laboratory validation experiments

We captured 11 red squirrels (5 females, 6 males) from 4 to 5 January 2008 in Algonquin Provincial Park (APP: 45° 30′, 78° 40′) using Tomahawk live-traps (Tomahawk Live Trap Co., Tomahawk, WI, USA). Frequent trap checking prevented any squirrel from spending greater than 2 h in a trap. Squirrels were transported to the Wildlife Research Facility at the University of Toronto Scarborough. At the facility, each squirrel was placed into its own radiometabolism cage (91.5 × 61 × 46 cm) that contained a stainless-steel nest box (with 1 × 1 cm mesh floor), cotton bedding, and provided with ad libitum food (apples, sunflower seeds, and peanut butter) and water (bottle with stainless-steel nipple). Squirrels habituated to these conditions for 5 days before we initiated any manipulations. Floors of the radiometabolism cages were slatted to allow urine and feces to fall freely to a pan underneath each cage. Pans were covered with metal screen (0.5 × 0.5 cm) to prevent feces and urine from contaminating each other. Squirrels were maintained at a temperature of ~10 °C and on a photoperiod that was changed weekly to correspond to the natural fluctuation in photoperiod in the location of capture at that time of year. All squirrels were, reproductively, quiescent upon capture, but the 6 males became scrotal within 26 days of capture. Upon completion of this study, all 11 squirrels had gained weight (mean ± SE: 15 ± 4.66 g) and were returned to the site of capture. Red squirrels were captured in APP under permit #AP-08-0 and our protocol for the capture and housing of red squirrels from APP was approved in accordance with the guidelines of the Canadian Council on Animal Care by the University of Toronto Institutional Animal Care and Use Committee (#20006991).

2.2. Field methods for free-ranging red squirrels

Fecal samples from red squirrels were collected during April–July 2006, January–August 2007, and February–August 2008 from a natural population near Kluane Lake in the southwest Yukon, Canada (61° N, 138° W) that has been monitored continuously since 1987 (see McAdam et al. (2007) for details). In most years, female red squirrels here are in estrous for 1 day, and produce 1 litter after a ~35 days gestation and a ~70 days lactation period (Boutin et al., unpublished data; Steele, 1998). In years of high food abundance, some females produce 2 litters (Boutin et al., 2006). Upon first capture, all squirrels were tagged with uniquely numbered metal ear tags (National Band and Tag, Newport, KY, USA). We determined the reproductive status of males (abdominal vs. scrotal testes) and females (pregnant, lactating, or neither) via palpation during live-trapping (McAdam et al., 2007). Fecal samples were collected from underneath the traps using forceps and placed individually into 1.5 mL vials. Upon parturition, we back-calculated the day of estrous to obtain estimates of days post-conception. Females were categorized as non-breeding if they did not become pregnant during that year. We only used fecal samples from squirrels that had not previously been trapped or handled within the previous 72 h prior to capture. Our protocol for capturing and handling red squirrels in the Yukon was approved by the Michigan State University Institutional Animal Care and Use Committee (#04/08-046-00).

2.3. Radiometabolism study in captive squirrels

We injected 8 captive squirrels (4 females and 4 males) intraperitoneally with 1110 kBq of radiolabeled cortisol (1,2,6,7-[^{3}H]; Amersham Biosciences, Quebec, Canada; specific activity = 1.55 TBq/mmol) dissolved in 0.1 mL physiological saline containing 5% ethanol and 5% toluene at 0800 h on day 1 of this study. We attempted to collect all urine and feces every 2 h (except from 2200 to 0800 h) until 54 h post-injection, but in some cases at the 2-h interval there was either no urine or no fecal samples to collect (i.e., at 1000 h and 1400 h on day 1, at 1400 h and 1800 h on day 2, and at 1000 h on day 3). To collect urine, we first aspirated any urine from the surface of the pan with a 1 mL pipette and then rinsed the pan with 4 mL of 80% methanol and added this to the urine sample. Between sampling periods, we rinsed the pans twice with a radioactive decontamination solution.

2.4. Monitoring of baseline patterns and manipulation of adrenocortical activity in captive squirrels

To obtain baseline FCM levels, we collected fecal samples (n = 57) every 4 h for 48 h (except from 2000 to 0800 h) from 11 captive squirrels (5 females, 6 males) during a period in which squirrels were not manipulated. We pooled the samples collected at the same time in the 2 days into 5 sampling periods (0800, 1200, 1600, 2000, 0800 h). We used these as baseline FCM levels to compare how the two experimental manipulations (described below) affected FCM levels. To demonstrate that changes in adrenocortical activity are well reflected in FCM levels in red squirrels, we conducted two experimental manipulations on 8 squirrels (4 females, 4 males). First, we conducted a handling stress experiment ("handling stressor") where the squirrels were placed into a restraining bag, weighed, sexed, their reproductive status was determined, and placed back into their radiometabolism cage (~2 min/squirrel). Second, we injected squirrels with 4.0 IU/kg of synthetic adrenocorticotropic hormone (ACTH; Synacthen Depot, CIBA, Ontario, Canada), which increases adrenal cortisol production (Boonstra and McColl, 2000). For these two manipulations, squirrels were handled or injected at 0800 h on day 1 of the manipulation, and we collected fecal samples every 4 h (except from 2000 to 0800 h) for the following 36 h post-manipulation. The 3 collection periods for baseline values, handling stressor, and ACTH injection were separated by >3 days.

2.5. Collection and extraction of fecal samples from captive and free-ranging squirrels

All fecal samples from the captive squirrels were placed into 1.5 mL vials and then stored in a −20 °C freezer within 20 min of collection. To test the stability of FCM, we conducted an experiment to determine how the time from collection to freezing affects FCM levels in red squirrel fecal samples. Immediately upon defecation, individual fresh fecal samples were fully homogenized, separated into 2 equal mass aliquots (±0.001 g), and placed into a −20 °C freezer either immediately or after storing them at room temperature (~23 °C) for 5 h.

Fecal samples collected in the field were placed in 1.5 mL vials and then into a −20 °C freezer within 4–5 h after collection. During the winter months (<0 °C; January–April), when air temperatures are usually <0 °C, fecal samples are generally frozen upon collection and remain so while in the field (Dantzer, personal obs.). In the warmer months (May–September), fecal samples in 1.5 mL vials were placed into an insulated container with wet ice until they were placed into the freezer. Samples were then shipped to the University of Toronto Scarborough on dry ice and stored at −80 °C upon arrival.

All fecal samples were lyophilized (LabConco, Missouri, USA) for 14–16 h, frozen in liquid nitrogen, and then pulverized using a mortar and pestle. We then extracted 0.05 g of the dry ground feces by adding 1 mL of 80% methanol (Touma et al., 2003). The steroid metabolites were then extracted in a multi-tube vortexer at 1450 RPM for 30 min, and then centrifuged for 15 min at 2500*g* (Palme, 2005). We then took the resulting supernatant and either analyzed it immediately for the radioactive samples or stored at −80 °C for analysis via enzyme-immunoassay (EIA).

2.6. Determination of radioactivity in fecal and urine samples for captive squirrel radiometabolism study

Urine samples collected during the radiometabolism study were dried down under air until only ~1 mL remained. We added 4 mL of ACS scintillation fluid (Amersham Biosciences, Quebec, Canada) to the dried down urine or 100 μL of the fecal extract and determined its radioactivity using a liquid scintillation counter with quench correction (Packard Tri-Carb 2900TR, Boston, MA, USA).

2.7. Characterization of fecal ^{3}H-cortisol metabolites

Fecal extracts with peak radioactivity from female ($n = 2$) and male ($n = 2$) squirrels at the peak radioactivity excretion, and an extract from the ACTH stimulation test from each sex were dried down under air and sent to the University of Veterinary Medicine (Vienna, Austria). These fecal extracts were then subjected to reverse-phase high performance liquid chromatography (RP-HPLC). After separation, both the radioactivity and the immunoreactivity (see below) were measured in the fractions. Details of this method can be found in Lepschy et al. (2007) and Touma et al. (2003).

2.8. Determination of immunoreactivity

To quantify FCM levels, we used a 5α-pregnane-3β, 11β, 21-triol-20-one EIA, which measures GC metabolites with 5α-3β, 11β-diol structure (Touma et al., 2003), and has already been successfully validated for several species (Bosson et al., 2009; Lepschy et al., 2007; Nováková et al., 2008; Touma et al., 2004). Information regarding the cross-reactivity of the antibody used (Touma et al., 2003) and further details of the assay procedure can be found elsewhere (Möstl et al., 2005; Palme and Möstl, 1997). Intra-assay coefficient of variation (CV) was 7.6 ± 0.2% and the inter-assay CV for a high and low pooled fecal extracts were 17.9 ± 1.3% and 18.6 ± 1.1%, respectively ($n = 23$ plates). FCM levels are expressed as ln-transformed ng/g dry feces.

2.9. Statistical analyses

For the captive squirrels, we used linear mixed models (LMM) to examine (1) excretion patterns of ^{3}H-cortisol in urine and fecal samples (fixed effects: sex and sampling period), and (2) how the two treatments (handling stressor, ACTH) affected FCM levels within 24 h post-manipulation compared to the baseline FCM levels (fixed effects: treatment, sampling period, and treatment × sampling period interaction). Sex was not included in the latter two models because the sexes did not differ in FCM levels at the different sampling periods or in their responses to the treatments (for all sex × sampling period and sex × treatment comparisons, $P > 0.09$). We used paired *t*-tests to examine how the time from defecation to freezing affected FCM levels. Prior to conducting this *t*-test, we used a Shapiro–Wilk normality test to determine that the data were normally distributed ($W = 0.92$, $P = 0.31$).

For the analyses of fecal samples from free-ranging squirrels, we used LMM to examine how (1) FCM levels change with reproductive condition in males and females (fixed effect: reproductive condition), and (2) how female FCM levels vary prior to and after parturition (fixed effects: day post-conception and day post-conception^2). We used non-linear regression to assess non-linearities between days post-conception and FCM levels (general additive models: Hastie and Tibshirani, 1990). Following the LMM, we used Tukey's honest significant difference post hoc tests to determine differences among means of the six reproductive conditions.

We included squirrel identification (ID) as a random effect for all LMM because we had repeated measures on the same squirrels. We calculated the proportion of residual variance in the LMM that was due to the individual (i.e., repeatability: Lessels and Boag, 1987) and used likelihood ratio tests to determine if the random effects improved the fit of the models. In all the LMM that we conducted for analyzing FCM levels from captive squirrels, the random effects significantly improved the fit of the model ($P < 0.0001$ for all likelihood ratio tests).

Prior to analysis, FCM levels were ln-transformed to meet assumptions of normality. Diagnostic plots after the ln-transformation revealed that the residuals from all LMM were normally distributed, homoscedastic, and there were no outlying observations with high leverage. For all results below, we present mean ± standard error (SE) and considered differences statistically significant at $\alpha = 0.05$. All statistical analyses were conducted using R (R 2.9.0, R Development Core Team, 2009).

3. Results from captive squirrels

3.1. Route and time to peak excretion of ^{3}H-cortisol metabolites

We collected 97 fecal and 74 urine samples during 15 (feces) and 14 (urine) sampling periods over the 70 h following injection of ^{3}H-cortisol. We recovered 69.2 ± 0.06%, of the 1110 kBq of ^{3}H-cortisol injected, of which 70.3 ± 0.02% was in the urine and 29.7 ± 0.02% in the feces. There was no sex difference in the total amount of radioactivity recovered ($t_6 = 0.63$, $P = 0.63$).

Within 4 h of administration of ^{3}H-cortisol, radioactivity had significantly increased above background levels in both the urine (total kBq excreted; $t_{13} = 8.04$, $P < 0.0001$) and feces ((kBq/0.05 g dry feces; $t_{14} = 6.78$, $P < 0.0001$; Fig. 1). The mean time to peak excretion of ^{3}H-cortisol metabolites in the urine and feces was 7.1 ± 0.9 and 10.9 ± 2.3 h, respectively (Fig. 1). There were no sex differences in the time to peak excretion in neither the urine

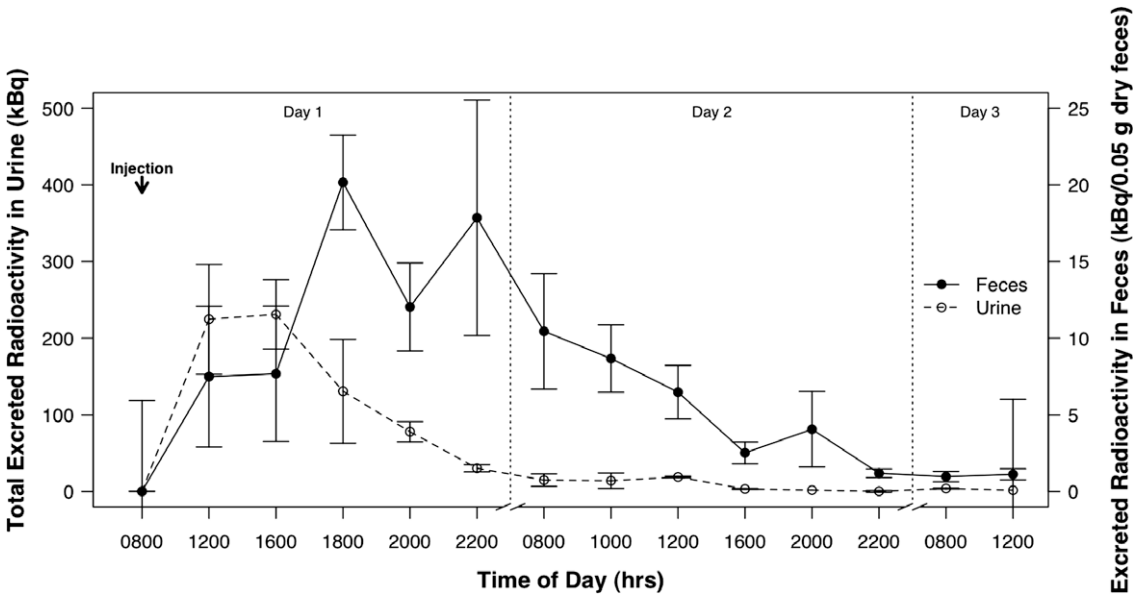

Fig. 1. Time course of excreted radioactivity in urine (kBq/sample) and feces (kBq/0.05 g dry feces) from North American red squirrels ($n = 8$) injected with ^{3}H-cortisol. Background fecal and urine samples were taken at the time of injection. Data are presented as means ± SE.

($t_7 = 0.24$, $P = 0.41$) nor the feces ($t_7 = -0.93$, $P = 0.19$). Radioactivity levels gradually decreased but remained significantly higher than background levels in the urine ($t_{13} = 5.46$, $P < 0.0001$) and the feces ($t_{14} = 7.41$, $P < 0.0001$) even 54 h post-injection of ^{3}H-cortisol (Fig. 1).

3.2. Characterization of fecal ^{3}H-cortisol metabolites

Cortisol was heavily metabolized with nearly no native cortisol present (Fig. 2). After the HPLC separations, there were several prominent radioactive peaks in the fecal extracts in both males ($n = 6$ peaks) and females ($n = 4$ peaks), with the major peak eluting fraction 20 (near the estrone glucuronide standard) in both sexes. There were both polar and non-polar metabolites in the fecal extracts and few metabolites were found beyond fraction 80. Inter-individual differences in cortisol metabolites occurred in females but not males. The 5α-pregnane-3β, 11β, 21-triol-20-one EIA detected several radioactive peaks in similar fractions (20–30, 40–50, 65–75) in both sexes (Fig. 2).

3.3. Effect of handling stressor on FCM levels

We collected 43 fecal samples during 8 sampling periods from 0 to 36 h after the handling stressor. Eight hours after the handling event, FCM levels had increased (7.03 ± 0.13 ln-transformed ng/g dry feces) from baseline FCM levels (6.04 ± 0.15) collected at the same time of day (1600 h), but this difference was only marginally significant ($t_4 = 2.04$, $P = 0.054$; Fig. 3).

3.4. Effect of ACTH on FCM levels

From 0 to 36 h post-injection of ACTH, we collected a total of 58 fecal samples during 8 sampling periods. Eight hours after the ACTH injection, FCM levels had significantly increased (7.2 ± 0.28) compared with baseline levels (6.04 ± 0.15) collected at the same time of day (1600 h; $t_4 = -2.93$, $P = 0.021$; Fig. 3). FCM levels were higher in response to the ACTH injection than FCM levels following the handling stressor collected at 8 h post-manipulation, but this difference was only marginally significant ($t_7 = -1.86$, $P = 0.053$; Fig. 3).

FCM levels at 32 h post-injection of ACTH (7.4 ± 0.34) were significantly higher compared with those at 32 h after the handling stressor (6.6 ± 0.22) collected at the same time of the day (1600 h; $t_7 = -2.56$, $P = 0.019$; Fig. 3).

3.5. Effect of time between collection and freezing on FCM levels

Fecal subsamples stored at room temperature for 5 h tended to have higher FCM levels ($n = 6$; 6.6 ± 0.17; Fig. 4) than those paired subsamples frozen immediately ($n = 6$; 6.3 ± 0.28; Fig. 4), but this increase of 38 ± 19% in FCM levels was not significant ($t_{10} = -0.88$, $P = 0.39$).

4. Results from free-ranging squirrels

4.1. Effect of reproductive condition

Reproductive condition had a significant effect on FCM levels in male and female squirrels ($F_{5, 378} = 19.17$, $P < 0.0001$). Pregnant females ($n = 118$; 6.3 ± 0.08) had significantly higher FCM levels than those females that were lactating ($n = 198$; 5.8 ± 0.05; $P < 0.0001$), post-lactating ($n = 42$; 5.5 ± 0.1; $P < 0.0001$), or did not breed ($n = 101$; 5.1 ± 0.1; $P < 0.0001$; Fig. 5). Lactating females had significantly higher FCM levels than those that were post-lactating ($P < 0.0001$) or non-breeding females ($P = 0.001$; Fig. 5). Males with scrotal ($n = 19$; 5.9 ± 0.2) and abdominal ($n = 24$; 5.4 ± 0.2) testes did not differ in their FCM levels ($P = 0.36$; Fig. 5). Pregnant females had significantly higher FCM levels ($P = 0.025$; Fig. 5). The random effect for squirrel ID explained 58% of the residual variation (i.e., variation not explained by reproductive condition) and significantly improved the fit of the model ($\chi^2 = 115$, df = 1, $P < 0.0001$).

4.2. FCM levels during gestation and lactation

We collected fecal samples from 78 female squirrels in 2006 ($n = 12$ samples), 2007 ($n = 74$ samples), and 2008 ($n = 271$ samples). FCM levels significantly increased during gestation, peaked around parturition (35 days post-conception), and then significantly declined during lactation (36–104 days post-conception) and after

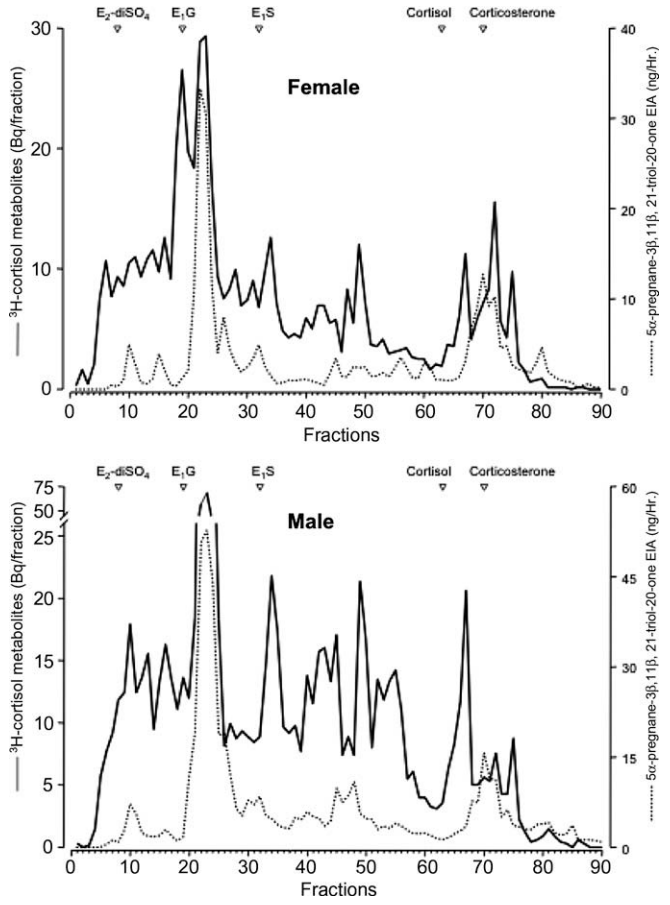

Fig. 2. Reverse-phase high performance liquid chromatography (RP-HPLC) radioimmunogram of peak radioactive fecal extracts from North American red squirrels. Samples shown are representative of female (upper figure) and male (lower figure) squirrels injected with radiolabeled cortisol. The solid line shows the ^{3}H-cortisol metabolites and the dotted line shows the metabolites reacting with the 5α-pregnane-3β, 11β, 21-triol-20-one antibody. Elution times of standards are marked with open triangles for estradiol disulphate (E_2-diSO_4), estrone glucuronide (E_1G), estrone sulfate (E_1S), cortisol, and corticosterone. Note the *y*-axes scale differences for males and females.

weaning (105 days post-conception; slope on ln-scale for quadratic term for days since conception = $-6.2 \times 10^{-5} \pm 2.1 \times 10^{-5}$; $t_{356} = -2.96$, $P = 0.0017$; Fig. 6). The random effect for ID explained 47% of the residual variation (i.e., variation not explained by days since conception) and significantly improved the fit of the model ($\chi^2 = 153.5$, df = 1, $P < 0.0001$).

5. Discussion

Our study validates an EIA to measure FCM levels with a 5α-3β, 11β-diol structure in North American red squirrels. We used a radiometabolism of cortisol and RP-HPLC to characterize the structure of the cortisol metabolites in the feces and showed that our antibody reacts with some of the main metabolites. Using a handling stressor and ACTH injection, we showed that adrenocortical activity is well reflected in FCM levels. Increased time from collection to freezing tended to increase FCM levels, but not significantly. Lastly, FCM levels in free-ranging female but not male red squirrels were significantly affected by reproductive condition, with pregnant squirrels just prior to parturition having the highest FCM levels overall, although there was substantial variation in FCM levels among individual free-ranging squirrels.

5.1. Radiometabolism study

While the total percent recovery of injected radiolabeled cortisol (69.2 ± 0.06%) was within the range of previous studies (~38–95%: Bosson et al., 2009; Lepschy et al., 2007; Palme et al., 2005), we attribute the apparent loss of radioactivity to our urine collection procedure. During urine collection, some of the urine was left on the screen that covered the pans underneath the radiometabolism cages. We rinsed the screening with 80% methanol (described above), but apparently were not able to capture all of the urine. However, because we were able to collect and extract all of the feces, we think that our estimate of the amounts of radioactivity excreted in the feces is accurate.

Radioactivity first appeared in the feces 4 h after administration of ^{3}H-cortisol. The lag time from injection to peak concentration of radiolabeled cortisol (10.9 ± 2.3 h) was similar to that found in laboratory mice (8–12 h: Touma et al., 2003), but longer than that found in Columbian ground squirrels (7.03 ± 0.53 h: Bosson et al., 2009). Radiolabeled cortisol metabolites in both female and male squirrels were excreted mainly in the urine (70.3 ± 0.02% of recovered radioactivity). In previous studies of laboratory mice (*Mus musculus*) and rats (*Rattus norvegicus*), radiolabeled corticosterone metabolites were excreted mainly in the feces (Lepschy et al., 2007; Touma et al., 2003). While the percentage of radioactive cortisol metabolites recovered in the feces in this study is low compared with these latter studies, it is greater than those reported in Columbian ground squirrels (*Spermophilus columbianus*; Bosson et al., 2009) or brown hares (*Lepus europeaus*: Teskey-Gerstl et al., 2000).

As was found in almost all species investigated so far (Bosson et al., 2009; Palme et al., 2005), HPLC indicated that cortisol was extensively metabolized. However, several of the cortisol metabolites reacted with our EIA antibody. There were minor differences in cortisol metabolites among individual males. However, similarly to laboratory rats (Lepschy et al., 2007), we found large differences between the 2 females. Lepschy et al. (2007) hypothesized that these individual differences in formed metabolites in females might be due to the presence of differing gonadal steroids that rapidly change over the estrus cycle, which we did not measure in this study.

5.2. Physiological validation of the enzyme-immunoassay

We used a pharmacological treatment (ACTH stimulation test) and a handling stressor to demonstrate the physiological and biological validity of our EIA in red squirrels. Both the handling stressor and the ACTH stimulation test increased FCM levels 8 h after handling/injection compared with baseline FCM levels at the same time period, but only the results from the ACTH stimulation test were significant. Because changes in adrenocortical activity were well reflected in FCM levels, we successfully validated this EIA for use in North American red squirrels.

5.3. Patterns of FCM levels in free-ranging red squirrels

Documenting the effects of reproduction on GCs in small mammals can be difficult because of low recapture rates and the rarity of known dates of conception or parturition. Although pregnancy and lactation have major effects on GC levels in free-ranging mammals (see references below), there is little consistency in the detected direction and magnitude of these effects. For example, previous studies that grouped females into reproductive categories (i.e., pregnant vs. lactating) have found that GCs during pregnancy were either higher (Boonstra and Boag, 1992; Hunt et al., 2006; Reeder et al., 2004) or lower (Kenagy and Place, 2000) than during lactation. We found that pregnant females had significantly higher

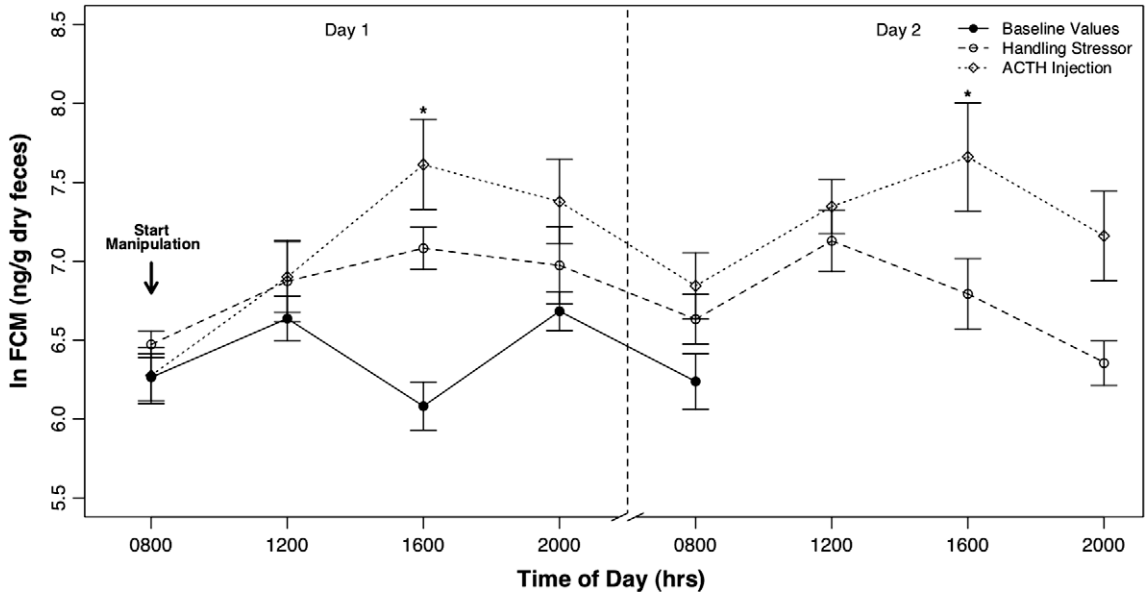

Fig. 3. Concentrations of fecal cortisol metabolites (FCM) in North American red squirrels in which squirrels ($n = 11$) were not manipulated ("Baseline Values"), and after squirrels ($n = 8$) were subjected to handling stressor and adrenocorticotropic (ACTH) stimulation tests. Manipulations were conducted on the same squirrels but on different days separated by >72 h. Asterisks denote significant differences ($P < 0.05$) between baseline FCM levels and the two treatments from 0 to 24 h post-manipulation and between FCM levels after the ACTH injection and handling stressor from 28 to 36 h post-manipulation. Data are expressed as means ± SE.

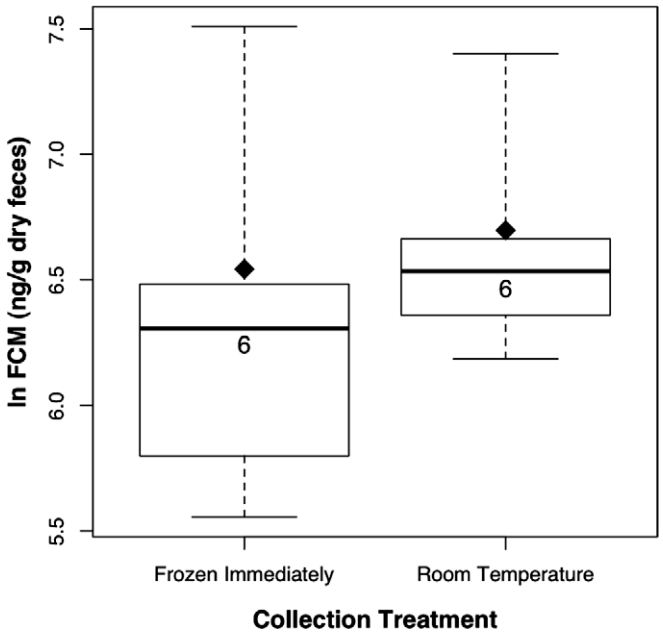

Fig. 4. Effect of time from collection to freezing on fecal cortisol metabolite (FCM) levels in North American red squirrel feces. "Frozen Immediately" indicates that the feces were frozen immediately upon collection. "Room Temperature" indicates that the paired subsamples of feces were left at room temperature (~23 °C) for 5 h and then frozen. Numbers inside boxes represent number of fecal samples. Box plots show 25–75% interquartile range (boxes), mean (filled diamonds), median (line within box), and the range (whiskers).

FCM levels than lactating, post-lactating, and non-breeding females. However, because we obtained repeated fecal samples from individually marked females whose day of conception was known, we were also able to examine how FCM levels varied over the course of gestation and lactation. In this analysis, we found that FCM levels increased throughout gestation, peaked around parturition, and then declined slightly throughout lactation. A similar situation was found in little brown myotis (*Myotis lucifugus*) where GC levels in lactating females were significantly lower than those in mid-to-late pregnancy females, but not females in early pregnancy (Reeder et al., 2004). Whereas the relationship between GCs and reproduction may be species-specific, available evidence suggests GC levels frequently vary during different stages of gestation and lactation. As a result, if animals are sorted into discrete categories of pregnancy and lactation, without references to days since conception or parturition, the category differences that are detected will be heavily influenced by the stage of gestation and lactation at which females happen to be sampled. We suggest that future studies exercise caution in interpreting comparisons of GCs among discrete reproductive categories.

5.4. Relationship between FCM levels, energy intake and expenditure

Among most mammals, lactation is the most energetically demanding stage of the annual cycle in general and of reproduction in particular (Kenagy et al., 1989 reviewed by Speakman, 2008)., In red squirrels, which do not hibernate (Pauls, 1978), daily energy expenditure (DEE) in lactating female red squirrels is significantly higher than in non-breeding females in the summer and non-breeding females in the winter (Fletcher et al., unpublished data; Humphries et al., 2005). According to the Energy Mobilization Hypothesis, GCs should be elevated during lactation because it is such an energetically demanding state (Romero, 2002). In partial support of this hypothesis, we found that FCM levels were significantly higher in lactating than in non-breeding female squirrels. However, we also observed significantly higher GC levels during late-pregnancy than the more energetically costly lactation period and this could be explained by a preparatory rather than responsive function of GCs. High GCs prior to parturition may motivate foraging (Dallman et al., 2007; Wingfield and Romero, 2001), which could allow pregnant females to accumulate energy reserves in preparation for the energetic demands during lactation (see Romero, 2002). Some but not all studies of small mammals have documented accumulation of energy reserves during pregnancy (Randolph et al., 1977; Millar, 1975; Gittleman and Thompson, 1988). Lactating red squirrels increase body fat levels during the early stages of lactation to match the increased reproductive demands in late lactation (Humphries and Boutin, 1996), but we

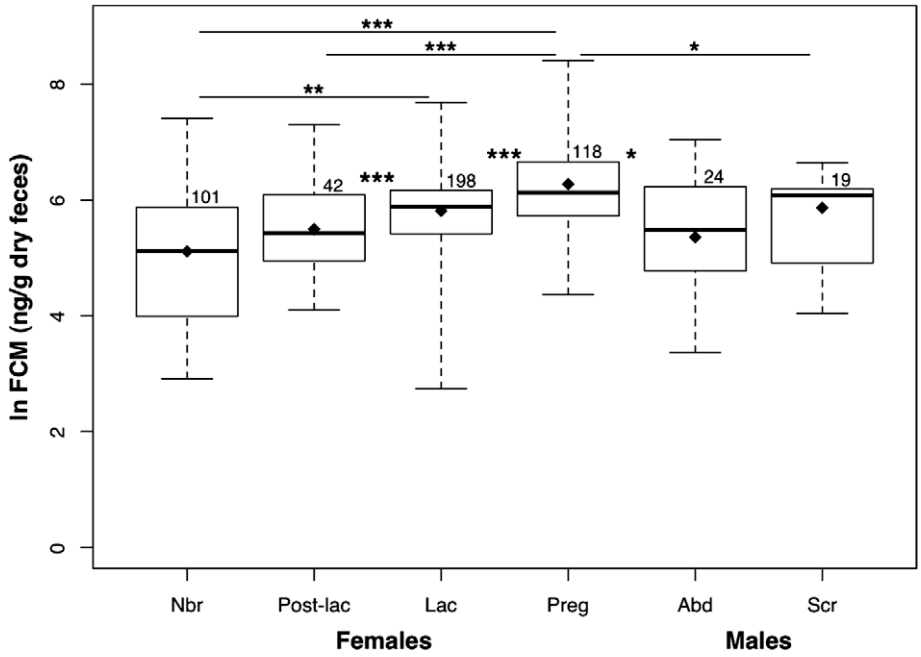

Fig. 5. Concentrations of fecal cortisol metabolites (FCM) in North American red squirrels of different reproductive stages (“Nbr”: non-breeding females; “Post-lac”: post-lactating; “Lac”: lactating; “Preg”: pregnant; “Abd”: males with abdominal testes; “Scr”: males with scrotal testes). Ln-transformed data are shown but we used linear mixed models individual identity (random effect) to determine significant differences among reproductive conditions. Significant differences are denoted by “*” ($P < 0.05$), “**” ($P < 0.01$), and “***” ($P < 0.0001$). Asterisks between boxes indicate significant differences between the two groups. Numbers above boxes are sample size of fecal samples analyzed. Box plots show 25–75% interquartile range (boxes), mean (filled diamonds), median (line within box), and the range (whiskers).

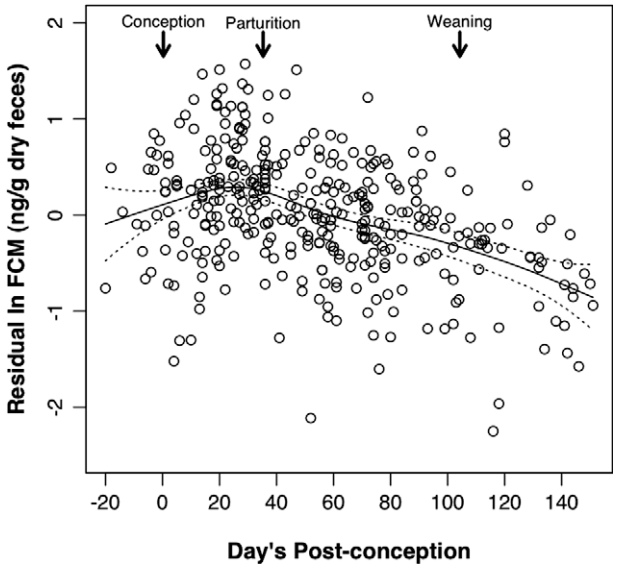

Fig. 6. Concentrations of fecal cortisol metabolite (FCM) levels in female North American red squirrels ($n = 78$ squirrels) prior to conception, during gestation and lactation, and after weaning ($n = 387$ samples). This significant non-linear relationship between FCM level and days post-conception was fit using a cubic spline. The quadratic effect of days post-conception on ln-transformed FCM level was significant in a linear mixed model with individual (random effect). Values on y-axis represent standardized residual ln-transformed FCM levels from this latter model.

do not know if the same occurs during pregnancy. Future studies will examine relationships between FCM levels, DEE, and food consumption in pregnant and lactating red squirrels.

5.5. Documenting inter-individual variation in FCM levels

Documenting the presence of individual variation in the hormonal traits of free-ranging organisms and how it affects fitness is critical to understand how natural selection acts upon hormonal traits. Recently, there has been growing interest in inter-individual variation in hormonal traits (reviewed by Williams (2008)). However, in many examinations of hormonal traits in free-ranging organisms, inter-individual differences are rarely well-documented. For example, trappability can reflect animal temperament on a shy-bold axis (Boon et al., 2008; Réale et al., 2007), which likely has a neuroendocrine basis (Koolhaas et al., 1999; Sih and Bell, 2008). Because those animals that have low capture probability are often not included in a repeated measures ANOVA due to the requirement of a balanced design (Gotelli and Ellison, 2004), estimates of both the central tendency and degree of variation of hormonal traits may be frequently biased in field studies.

In this study, we used linear mixed models (LMM) with identity as a random effect to deal with both an unbalanced design and repeated measures on the same individuals (Pinheiro and Bates, 2009). With this statistical approach, we were able to analyze all of the fecal samples collected from individual squirrels and to document that a large proportion of the residual variation in FCM levels in free-ranging squirrels was due to repeatable inter-individual differences (i.e., range 47–58%). If these inter-individual differences have a genetic basis, they could provide the raw material for natural selection. In order to make progress in understanding how natural selection acts upon hormonal traits, we suggest that future studies in free-ranging animals use similar statistical approaches to deal with unbalanced designs and report the presence of repeatable inter-individual variation in hormonal traits.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygcen.2010.03.024.

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