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# How does diet affect fecal steroid hormone metabolite concentrations? An experimental examination in red squirrels

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ABSTRACT

A growing number of longitudinal studies in free-ranging animals are measuring fecal steroid hormone metabolite concentrations (FHM). Free-ranging animals can exhibit major seasonal changes in their diet, yet we know relatively little about how diet affects FHM. We experimentally manipulated the diets of female and male North American red squirrels (*Tamiasciurus hudsonicus*) to determine how diet affected fecal cortisol (FCM) and androgen (FAM) metabolite concentrations. We measured FCM using an enzyme immunoassay (EIA) that we have previously validated and measured FAM using an assay we have previously validated for use in females and validate for males herein. We validated our EIA to measure FAM in males by identifying that 44.5 ± 0.05% of recovered radiolabeled testosterone was excreted in the feces, our EIA antibody detected the fecal testosterone metabolites, and males with scrotal testes had significantly higher FAM (3.02 ± 0.06 ln ng/g dry feces) than those with abdominal testes (2.73 ± 0.06). We initially fed all squirrels the same diet, but then switched one group of squirrels to a diet consisting of conifer seed ($n = 4$ squirrels) whereas the other group was switched to peanut butter ($n = 7$). FCM and FAM in squirrels fed conifer seed significantly increased from 0 to 94 h after their diets were changed. FCM in squirrels fed peanut butter significantly declined, whereas FAM declined but not significantly. This demonstrates that change in dietary fiber consumption (peanut butter versus conifer seed) or even slight differences in diet (conifer versus sunflower seeds) can strongly influence FHM.



## 1. Introduction

The neuroendocrine system can enable a prompt, adaptive, and multifaceted response to environmental variation. Responses of the hypothalamic–pituitary–adrenal (HPA) and –gonadal (HPG) axes to environmental information have been particularly well studied [25,42]. Adrenal (glucocorticoids: GCs) or gonadal (androgens) steroid hormones are produced as a downstream response to activation of the HPA and HPG axes, respectively [25,38]. GCs and androgens are intrinsically linked to metabolism, energy allocation, and reproduction [25,38,44]. There is a growing interest in measuring associations among hormones, physiological, behavioral, and life history characteristics in free-ranging animals [2,3,12,17,22,35,36,43].

Measuring hormone concentrations in free-ranging animals has traditionally required obtaining plasma samples, which can be difficult and potentially harmful to study animals because it generally requires live-trapping and withdrawal of blood from temporarily restrained animals [41]. Unfortunately this approach may not be feasible for large or rare and endangered species. Additionally, trapping or temporary restraint can also introduce systematic biases in plasma GC and androgen concentrations [9,11,37].

Recent studies have increasingly measured steroid hormone metabolite concentrations in fecal samples collected from free-ranging animals. Fecal steroid hormone metabolite concentrations (FHM) are thought to reflect an integrated average of circulating unbound hormone levels over some time period rather than point estimates that are obtained from plasma samples [14,28,31,40]. FHM will typically be unaffected by trapping- or restraint-induced stress as long as the time of temporary captivity is less than the total time it takes for food to pass from the duodenum to the rectum [15,16,27,28]. For these reasons and because of the technique's relative non-invasiveness, the diversity of animals in which FHM have been measured is growing rapidly [23,31].

However, the ease of collection belies some of the difficulties associated with measuring FHM in free-ranging animals [5,23,41,46]. A particularly relevant issue for studies in free-ranging animals that has received little attention is the potential effects of diet on FHM [25,48,50]. In omnivores or herbivores,

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variability in consumption of plant fiber may have an important effect on FHM. For example, a high fiber diet can increase estrogen metabolite concentrations in the feces of humans [13,33] and can decrease progesterone metabolite concentrations in the feces of yellow baboons *Papio cynocephalus cynocephalus* [50]. The mechanisms by which diet induces differences in FHM are largely speculative but may be due to the effects of dietary fiber on fecal mass, gut passage time, biliary excretion of hormones, and enterohepatic circulation, or due to changes in microbial activity that alters steroid hormone metabolite structure [10,13,20,48]. For example, increased dietary fiber consumption may decrease reabsorption of steroid hormones from the intestine, which increases FHM and decreases plasma steroid hormone concentrations [13].

Diets of free-ranging animals are rarely uniform and therefore longitudinal monitoring of FHM could be influenced by seasonal changes in their diet. For example, in herbivores that live in environments with major seasonal shifts in food availability, observed increases in fecal glucocorticoid metabolite concentrations during periods of low food availability could represent true seasonal endocrine changes, indirect effects of malnourishment, dietary influences that affect the recovery of the metabolites such as changes in fiber consumption, or a combination of all three. However, examinations of the role of diet on FHM in free-ranging or non-traditional study animals are rare (but see [14,49,50]).

Here, we experimentally examined how diet affects fecal cortisol (FCM) and androgen (FAM) metabolite concentrations in captive North American red squirrels (*Tamiasciurus hudsonicus*). We measured FCM using an assay that we have previously validated for use in this species [7] and measured FAM using an enzyme immunoassay we have previously validated for use in female red squirrels [8] and validate for males herein. We validated the EIA to measure FAM in male squirrels by determining the route (urine or feces) and time course of excretion of radiolabeled testosterone metabolites, using reverse-phase high performance liquid chromatography (RP-HPLC) to characterize the structure of the testosterone metabolites and identify that our EIA antibody detects these metabolites, and finally showing that our EIA accurately reflects the reproductive condition (gonads active or quiescent) of free-ranging male squirrels. We then measured the changes in FCM and FAM as we manipulated the diets of female and male squirrels in captivity. All squirrels were initially fed the same diet (sunflower seeds, peanut butter, apples), but then we switched one group of squirrels to a diet consisting of conifer seed and apples whereas the other group was fed peanut butter and apple. We then measured any changes in FCM and FAM for 94 h after the manipulation started.

## 2. Materials and methods

### 2.1. Capture and husbandry of captive red squirrels

We captured 11 red squirrels (5 females, 6 males) in January 2008 in Algonquin Provincial Park (APP: 45°30′, 78°40′) using Tomahawk live-traps (Tomahawk Live Trap Co., Tomahawk, WI). Squirrels were transported to the Wildlife Research Facility at the University of Toronto Scarborough where they were maintained in captivity until their rerelease at their place of capture in March 2008. 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) and cotton bedding. Squirrels were maintained at a temperature of ~10 °C and on a photoperiod that was changed weekly to correspond to the seasonal change in photoperiod in the location of capture at that time of year. All squirrels were reproductively quiescent upon capture but within 26 days after capture, male squirrels developed scrotal testes reflecting gonadal recrudescence. Squirrels habituated to these conditions before we performed any procedures that we describe below and elsewhere [7,8]. Red squirrels were captured in APP under permit #AP-08-0 and our housing protocol 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. Radiometabolism of testosterone in males and RP-HPLC

The first step we took to validate our assay to measure FAM in male red squirrels was to identify the route of excretion (urine or feces) and the time delay of excretion of testosterone metabolites using a radiometabolism study [46], which we have previously performed in female red squirrels [8]. We injected six captive male red squirrels intraperitoneally with 1110 kBq of radiolabeled testosterone (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. We collected urine (0–52 h post-injection; $n = 50$ samples) and feces (0–120 h post-injection; $n = 75$ samples) every 4–8 h (except from 2000–0800 h: Table 1) from pans underneath the cages that were covered with metal screening (0.5 × 0.5 cm mesh) to prevent feces and urine from mixing. The floors of the radiometabolism cages were slatted (as well as the nest boxes) and therefore all excreta fell onto the pan (urine) or mesh screening (feces). All urine that was present at each sampling period was aspirated off of the surface of the pan using a pipette. The surface of the pans were then rinsed with 4 mL of 80% methanol and added to the urine sample. We rinsed the pans twice with a radioactive decontamination solution between sampling periods (Decon 75, Fisher Scientific, Pittsburgh, PA, USA). Fecal and urine samples were placed into a −20 °C freezer within 20 min of collection.

The second step we took to validate this assay to measure FAM in male red squirrels was to use reverse-phase high performance liquid chromatography (RP-HPLC) to characterize fecal $^3$H-testosterone metabolites and to demonstrate that our enzyme-immunoassay (EIA: see below) antibody detects the fecal testosterone metabolites. Fecal extracts of samples containing peak radioactivity (see below) from male ($n = 2$) squirrels were dried under air and then subjected to RP-HPLC. After separation, we measured both the radioactivity and immunoreactivity in the collected fractions. Details of this method can be found elsewhere [18,47].

### 2.3. Diet manipulation in captive squirrels

All captive squirrels were initially fed the same diet of ad libitum unroasted hulled sunflower seeds and all natural peanut butter (Kraft All Natural), as well as 1 apple every 48 h from 1 to 57 days post-capture. Squirrels readily drank water from a water bottle with a stainless steel nipple that was provided throughout captivity. All squirrels gained weight on this diet compared to their initial weight at capture (mean ± SE: 15 ± 4.66 g gained during entire period of captivity: Fig. 1).

On the first day of the diet manipulation experiment (58 days post-capture), we removed all remaining food from the radiometabolism cages. On this day and every 24 h thereafter, we provided squirrels in the "Peanut Butter treatment" (3 females and 4 males) with ~6 tablespoons of all natural peanut butter. Squirrels in the "Cones treatment" (2 females and 2 males) were provided with 40 white spruce (*Picea glauca*) cones per day. We considered this to be ad libitum provisioning of peanut butter and spruce seed as there was always peanut butter or whole cones remaining 24 h after providing them with the food. Squirrels in both treatments received access to ad libitum water and we also provided each

**Table 1**
Outline of radiometabolism (males) and diet manipulation (males and females) experiments in captive red squirrels.

| Date | Experiment | Treatment | $n$[a] | Collection periods for feces[b] (h) | Collection periods for urine (h) |
|---|---|---|---|---|---|
| 16–21 February | Radiometabolism | Injected with $^3$H-testosterone | 6 m | 0, 4, 8, 12, 24, 28, 32, 36, 48, 52, 60, 72, 80, 96, 104, 108, 120 | 4, 8, 12, 24, 28, 32, 36, 48, 52, 60, 72 |
| 3–7 March | Diet manipulation | Fed peanut butter | 3 f, 4 m | 2, 8, 10, 22, 26, 34, 46, 50, 58, 70, 74, 82, 94 | Not collected[c] |
| | | Fed spruce cones | 2 f, 2 m | 2, 8, 10, 22, 26, 34, 46, 50, 58, 70, 74, 82, 94 | Not collected[c] |

[a] m and f indicate males and females, respectively.
[b] Collection periods shown are for hours post-injection (radiometabolism) or -manipulation of diet.
[c] Urine samples were not collected for the diet manipulation experiment.

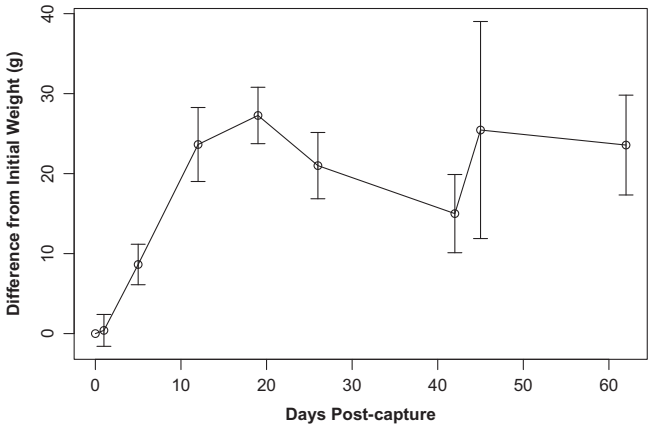


**Fig. 1.** Difference in weight (g) of captive red squirrels between initial capture (0 day post-capture) and up to 62 days post-capture. Squirrels were weighed at 0, 1, 5, 12, 19, 26, 42, 45, and 62 days post-capture.

squirrel with one apple every 48 h. We collected fecal samples from the screens of the radiometabolism cages every 4–12 h from 0 to 94 h after the diet manipulations started (Table 1). Fecal samples were placed into a −20 °C freezer within 20 min of collection.

### 2.4. Fecal sample collection from free-ranging male squirrels

We studied a free-ranging population of red squirrels in the Yukon, Canada (61°N, 138°W) that has been monitored continuously since 1987 [21]. Male red squirrels were routinely trapped on their territories using Tomahawk live-traps and handled using a canvas and mesh bag. All squirrels on these study areas were individually marked with uniquely numbered ear tags (National Band and Tag, Newport, KY, USA). During each capture, squirrels were identified by reading their ear tags, weighed, and their reproductive status (testes scrotal or abdominal) was determined by palpation. We collected fecal samples from free-ranging male red squirrels to demonstrate that our assay to measure FAM reliably distinguished between fecal samples from males with scrotal testes versus those with abdominal testes. Fecal samples were collected during capture from underneath the live-traps using forceps, placed individually into 1.5 mL vials, and then frozen at −20 °C within 4–5 h after collection. Fecal samples were generally frozen upon collection during winter trapping (February–April). During the warmer months (May–July), fecal samples were placed into an insulated container containing wet ice. This period of time when fecal samples are not completely frozen does not systematically affect FCM [7] or FAM [8]. All fecal samples were collected within 2 h of initial capture, which is not long enough for trapping-induced stress from the current capture to affect FCM [7] or FAM [8]. We also did not analyze any fecal samples from squirrels that were trapped within the previous 72 h. All of our live-trapping and handling procedures were approved by the Institutional Animal Care and Use Committee at Michigan State University (# 04/08-046-00).

### 2.5. Extraction of hormone metabolites from feces

All fecal samples were stored at −80 °C until analysis except those collected in the field were stored at −20 °C until they were shipped to the University of Toronto Scarborough on dry ice and thereafter stored at −80 °C. Fecal samples were first lyophilized (LabConco, MO, USA) for 14–16 h to remove any potential differences in water content [50]. Next, samples were placed into liquid nitrogen and pulverized using a mortar and pestle. Between samples, we thoroughly rinsed and cleaned the mortar and pestle with 5 mL of 80% methanol. We then extracted 0.05 g of the dry ground feces by adding 1 mL of 80% methanol, shaking this solution on a multivortexer at 1450 rpm for 30 min, and then centrifuging for 15 min at 2500*g* [27,47]. The resulting supernatant containing the metabolites was stored at −80 °C until analysis via EIA.

### 2.6. Determination of radioactivity in fecal and urine samples from radiometabolism study

Metabolites were extracted from the fecal samples collected during the radiometabolism study as described above except that the mortar and pestle was also rinsed twice with a decontamination solution (Decon 75) between samples. Urine samples were dried down under air until only ~1 mL remained. To determine radioactivity in the urine and fecal extracts, we added 4 mL of ACS scintillation fluid (Amersham Biosciences, Quebec, Canada) to the concentrated urine or 100 μL of the fecal extract and quantified radioactivity using a liquid scintillation counter with quench correction (Packard Tri-Carb 2900TR, Boston, MA, USA).

### 2.7. Determination of immunoreactivity of fecal cortisol and androgen metabolites

To quantify FCM in the fecal samples collected during the diet study from captive squirrels, we used a 5α-pregnane-3β,11β,21-triol-20-one EIA, which measures GC metabolites with 5α-3β,11β-diol structure [47]. We have previously validated this antibody for use in this species [7]. Information regarding the cross-reactivity of the antibody used [47] and further details of the assay procedure can be found elsewhere [26,30]. Samples were run in duplicate and the intra- and inter-assay coefficients of variation were 6.2% and 9.1%, respectively ($n$ = 3 plates). The assay had a sensitivity of 0.82 pg per well.

To quantify FAM in fecal samples collected from captive males and females and free-ranging males, we used a testosterone EIA that measures 17β-OH androgen metabolites [29], which we have already validated for use in female red squirrels [8]. Details of this procedure [24] and cross-reactivity of the antibody can be found elsewhere [29]. It is likely that our EIA antibody detected FAM from

testosterone because it shows a high affinity to 17β-hydroxyandrogens and the cross-reactivity with 17-oxo- or 17α-hydroxyandrostanes androgen metabolites is below 0.1% [29]. Samples were run in duplicate and the intra- and inter-assay coefficients of variation were 5.5% and 16.2%, ($n$ = 14 plates). The assay had a sensitivity of 0.3 pg per well.

### 2.8. Statistical analyses

We used two separate linear mixed-effects models (LMMs) to determine how the diet manipulation in the captive male and female squirrels affected FCM and FAM. In both of these models, the fixed effects were diet treatment (Cones versus Peanut Butter), hours post-manipulation, and an interaction term between treatment and hours post-manipulation. We determined how male reproductive condition affected FAM in free-ranging squirrels using a linear mixed-effects model. The fixed effect in this model was male reproductive condition (scrotal or abdominal testes).

For both of our LMMs described above, we had repeated measures on the same squirrels so we included a random intercept term for the individual squirrel [32]. FCM and FAM were ln-transformed prior to analysis in our all our models. We used graphical inspection to ensure that the residuals from our LMMs were normally distributed, homoscedastic, and that there were linear relationships between our predictor and response variables. We calculated Cook's distances for all our observations to determine that there were no observations with high leverage in our LMMs. Below we describe mean ± SE and all FCM and FAM are given as ln ng/g dry feces.

## 3. Results

### 3.1. Route of excretion and time to peak excretion of radiolabeled testosterone in males

We recovered 42.6 ± 0.07% of the 1110 kBq of $^3$H-testosterone we administered to the captive male squirrels. Of the total radioactivity excreted, 55.5 ± 0.05% was recovered in the urine and 44.5 ± 0.05% in the feces. The time to peak excretion of $^3$H-testosterone was 6.6 ± 0.6 h in the urine and 19.8 ± 3.5 h in the feces (Fig. 2).

### 3.2. Characterization of $^3$H-testosterone metabolites by RP-HPLC analysis

Injected $^3$H-testosterone was heavily metabolized and polar metabolites that resembled conjugated steroids were the most common (Fig. 3). Radioactive peaks beyond fraction 60 were found and two of these peaks (eluting around fraction 83 and 87) yielded the highest immunoreactivity in the testosterone EIA. No radioactivity with corresponding testosterone immunoreactivity at the elution position of testosterone (near fraction 80) was present.

### 3.3. Biological validation of the EIA

We collected a total of 108 fecal samples from 65 males from 2007 to 2008. Male squirrels with scrotal testes had significantly higher FAM levels ($n$ = 46; 3.02 ± 0.06 ln ng/g dry feces) than those with abdominal testes ($n$ = 62; 2.73 ± 0.06; $t_{107}$ = 2.80, $P$ = 0.003; Fig. 4). This suggests that our EIA can reliably distinguish the gonadal status of male red squirrels.

### 3.4. Effects of diet manipulation on FCM and FAM in captive squirrels

In our diet manipulation experiment, whether captive squirrels were fed conifer seed or peanut butter had significant and opposing effects on FCM as the diet manipulation experiment proceeded. The initial FCM for squirrels fed conifer seed (6.28 ± 0.19 ln FCM; $n$ = 4) and peanut butter (7.06 ± 0.68 ln FCM; $n$ = 3) were similar ($t_{106}$ = 1.25, $P$ = 0.11), which is what we expected given they were initially fed the same diet. However, as the diet manipulation experiment proceeded, FCM in squirrels fed conifer seed significantly increased (slope for effects of hours post-manipulation on ln scale = 0.01 ± 0.003; $t_{106}$ = 3.75, $P$ = 0.0001; Fig. 5A) whereas those in squirrels fed peanut butter significantly declined (slope for hours post-manipulation on ln scale = −0.015 ± 0.003; $t_{106}$ = −4.39, $P$ < 0.0001; Fig. 5A). Mean FCM in samples taken 2 h after the start of the treatment (1200 h) compared to those taken at the same time of day (1200 h) but 74 h post-manipulation increased by 11% in squirrels fed conifer seed whereas they decreased by 14% in those squirrels fed peanut butter.

The diet manipulation had a similar effect on FAM. The initial FAM for squirrels fed conifer seed (4.04 ± 0.24 ln FAM; $n$ = 3) and peanut butter (4.33 ± 0.22 ln FAM; $n$ = 3) were also similar

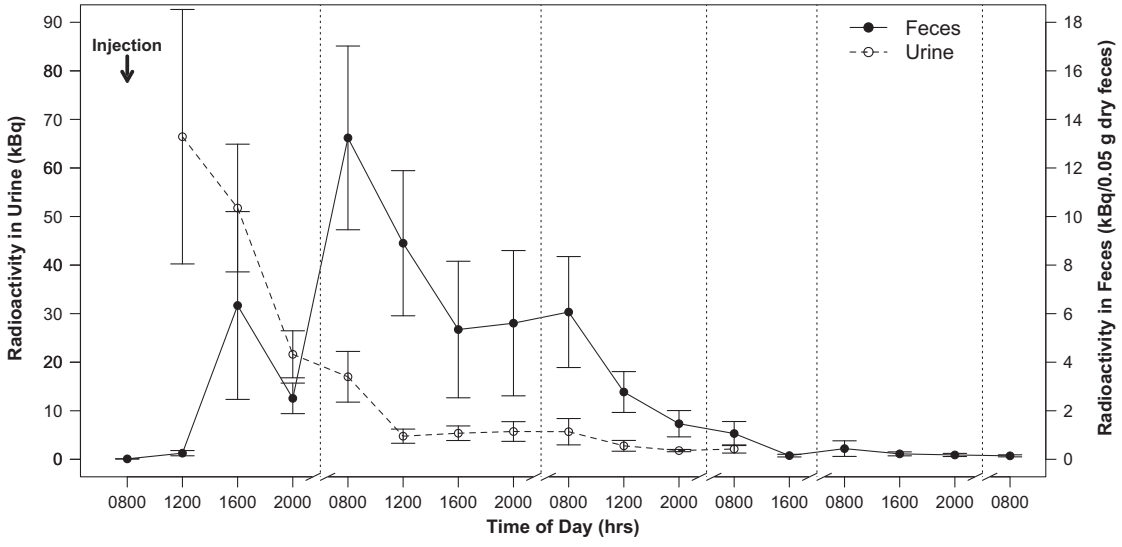


**Fig. 2.** Excretion of injected radiolabeled testosterone by captive male red squirrels ($n$ = 6) in urine (kBq/sample) and feces (kBq/0.05 g dry feces) over 72 and 120 h post-injection, respectively. Dashed vertical lines represent different days of study. Data shown are mean ± SE.

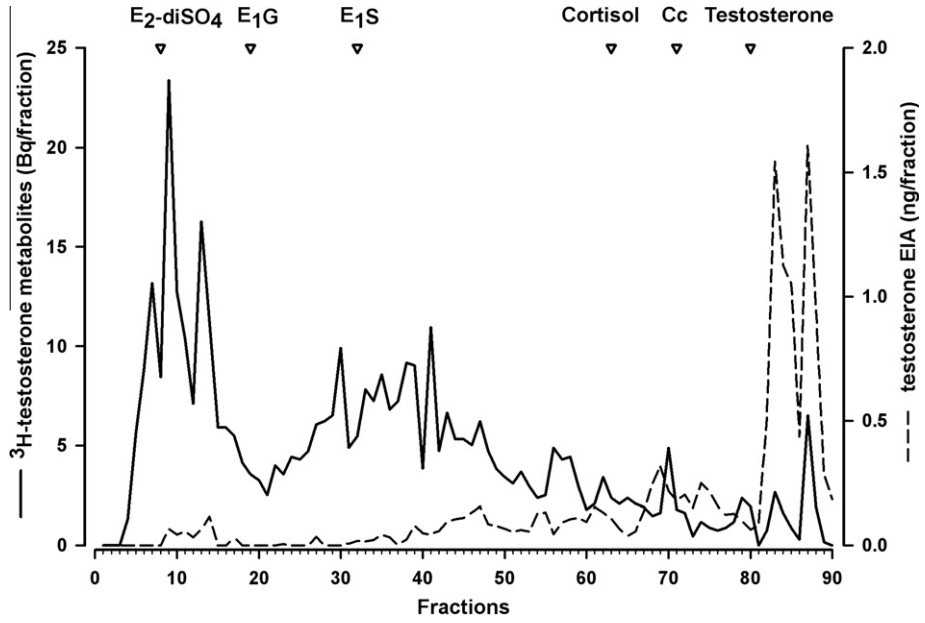


**Fig. 3.** Reverse-phase high performance liquid chromatographic (RP-HPLC) separation of fecal $^{3}$H-testosterone metabolites (peak sample) in the feces of captive male red squirrels. Open triangles mark the approximate elution positions of respective standards ($E_2$-di$SO_4$ = 17β-oestradiol-disulfate, $E_1$G = oestrone-glucuronide, $E_1$S = oestrone-sulfate, Cc = corticosterone).

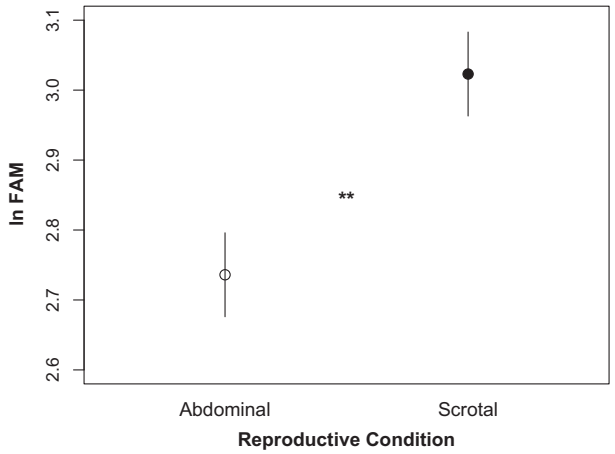


**Fig. 4.** Effect of reproductive condition (abdominal or scrotal testes) on fecal androgen metabolite (FAM) concentrations in free-ranging male red squirrels. Data shown are raw mean ± SE. Asterisks represent a significant difference from a linear mixed-effects model (see text) at $P < 0.01$ (**).

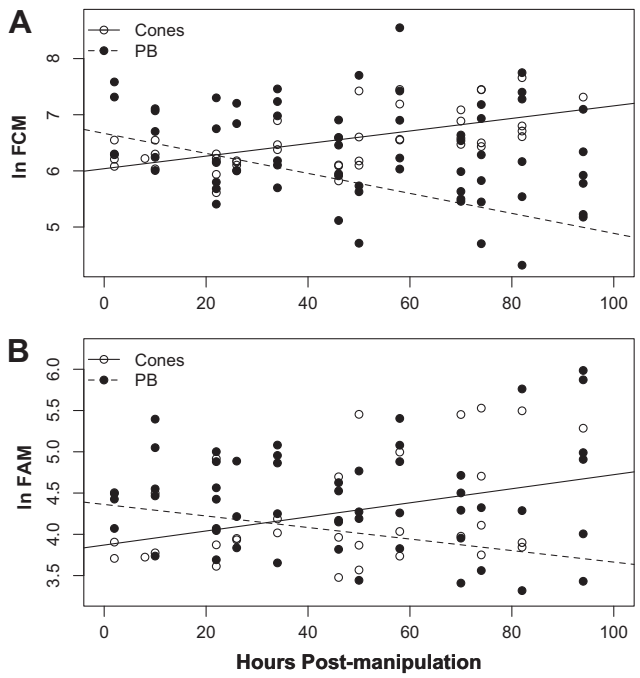


**Fig. 5.** Effects of diet on fecal (A) cortisol (FCM) and (B) androgen (FAM) metabolite concentrations in captive male ($n = 6$) and female ($n = 5$) red squirrels. Squirrels were all initially fed the same diet and then switched (0 h post-manipulation) to a diet of apple and either (1) peanut butter ("PB"; $n = 7$) or (2) spruce seed ("Cones"; $n = 4$). Ln-transformed FCM and FAM are shown on $y$-axes. Regression lines shown are from general linear models but statistical inferences were made from linear mixed-effects models (see text).

($t_{84} = 0.99$, $P = 0.16$), which again is what we expected because they were initially fed the same diet. However, as the diet manipulation experiment proceeded, FAM in squirrels fed conifer seed significantly increased (slope for hours post-manipulation on ln scale = 0.005 ± 0.002; $t_{84} = 2.41$, $P = 0.009$; Fig. 5B) whereas those in squirrels fed peanut butter declined but not significantly (slope for hours post-manipulation on ln scale = −0.003 ± 0.003; $t_{84} = -1.21$, $P = 0.11$; Fig. 5B). Mean FAM in samples taken 2 h after the start of the treatment (1200 h) compared to those taken at the same time of day (1200 h) but 74 h post-manipulation increased by 12% in squirrels fed conifer seed whereas they decreased by 9% in those squirrels fed peanut butter.

## 4. Discussion

Endocrine responses to environmental variation and their fitness consequences are increasingly being studied in free-ranging animals by longitudinal monitoring of FHM. However, seasonal changes in diet could systematically bias FHM across the monitoring period. In this study, we first validated an assay to measure FAM in male red squirrels. We initially fed all the captive squirrels the same diet and their FCM and FAM were similar immediately prior to when we switched their diets. We found that FCM and

FAM in captive female and male squirrels fed spruce conifer seed increased over the following 94 h after the manipulation started whereas those fed peanut butter declined over the same period. This study demonstrates that future studies monitoring FHM should carefully consider how seasonal changes in diet can influence FHM.

### 4.1. Validation of the EIA to measure FAM in male red squirrels

To demonstrate that this EIA provides biologically relevant measurements of androgen levels, we determined that (1) testosterone is heavily metabolized in male red squirrels, but that several testosterone metabolites were detected using our EIA and (2) FAM reflected gonadal status (scrotal or abdominal testes). We found that males with scrotal testes had significantly higher FAM than males with abdominal testes, which is typically a hallmark of physiological validation of an assay to detect FAM [1,24]. Although these differences were statistically significant, we expect that they are a conservative estimate of the difference in FAM between males with scrotal and abdominal testes. In a previous study [4], plasma androgen (testosterone and dihydrotestosterone) concentrations in male red squirrels with scrotal testes sampled in the early winter (February) prior to the start of the breeding season were significantly higher than those in males with abdominal testes sampled soon after the breeding season ended (June) and those in non-breeding condition (August). However, plasma androgen concentrations in males with scrotal testes sampled later in the breeding season (May) were not different than those sampled from males soon after the end of the breeding season (June) and in non-breeding condition (August). We found that FAM in males with scrotal testes that were mostly sampled later in the breeding season rather than prior to the start of the breeding season were significantly higher than those sampled from males soon after breeding and in non-breeding condition (June–July). We only had one fecal sample from a male with scrotal testes in the early breeding season (February). We predict that FAM sampled from males in the early breeding season would be even higher than what we found here for FAM in males with scrotal testes. However, this does not detract from our finding that our assay for FAM can reliably distinguish between males with scrotal and abdominal testes.

### 4.2. Excretion and characterization of radiolabeled testosterone metabolites

The metabolism and route of excretion of FHM is generally species-specific [28,31] and may also be sex-specific [14,31,47]. In female red squirrels, we have previously found [8] that the percentage of radiolabeled testosterone recovered in the feces (56.3 ± 10.4%) was greater than what we found in this study for males. However, post hoc analyses indicate that these differences between the sexes for the route of excretion were not significantly different (paired $t$-test: urine: $t_8 = 0.02$, $P = 0.98$; feces: $t_8 = 0.85$, $P = 0.41$). Thus, there do not appear to be any sex-specific differences in the route of excretion of testosterone metabolites in red squirrels.

The time to peak excretion of radiolabeled hormone metabolites may also be sex-specific [6]. We found that the time to peak excretion of radiolabeled testosterone in the feces of male red squirrels (19.8 ± 2.7 h) was longer than what we have previously reported [8] in females (10.3 ± 0.8 h). However, a post hoc analysis indicates that this difference was not significantly different (paired $t$-test: $t_8 = 2.14$, $P = 0.065$). Nonetheless, trapping-induced stress could not have influenced FAM from feces collected in the field as traps were checked every 2 h, whereas the peak in radioactive testosterone metabolites in males occurred nearly ~20 h after injection, which is similar to what we have found previously for excretion of radiolabeled cortisol in females and males [7] and testosterone in females [8].

Finally, the structure and type of steroid hormone metabolites excreted in the feces may also be sex-specific. For example, Goymann [14] found sex-differences in the testosterone metabolites in the excreta of European stonechats (*Saxicola torquata rubicola*) and suggested caution in comparing FAM between males and females. The type of testosterone metabolites excreted in the feces of red squirrels may also be sex-specific. In a previous study, we found three peaks of immunoreactive testosterone metabolites in the feces of female red squirrels [8] but in the present study in male squirrels, we only found two peaks (Fig. 3). Although these results urge caution in comparing FAM between females and males, they do not contradict the overall validations of our EIA to measure FAM in both female and male red squirrels.

### 4.3. Effects of diet manipulation on FCM and FAM in captive squirrels

After the diets of the squirrels were switched from the same diet of sunflower seeds, peanut butter, and apple, we found that FCM and FAM in squirrels fed spruce seed increased over the course of the diet manipulation, whereas those fed peanut butter declined during the same time period. The observed differences in FCM and FAM could have been caused by differences in dietary fiber content of sunflower seeds (21.5% acid detergent fiber content: [39]), spruce seeds (19.8% acid detergent fiber content: [19]), and peanut butter (6.6% crude fiber). Compared to squirrels fed peanut butter, those fed sunflower or spruce seeds consumed higher quantities of dietary fiber and also had increased excretion of FCM and FAM. Previous studies have found that increased consumption of fiber can cause the excretion of FHM to increase [13,33], decrease [14,49,50], or have no effect [14,34,49]. Our results agree with previous studies [13,33] in which higher fiber consumption increased the excretion of FHM.

The mechanisms by which dietary fiber consumption affects FHM are unknown [10,13,20,48]. Changes in FHM caused by increased dietary fiber consumption could be attributed to increased transition time of ingested materials from the duodenum to the rectum. Unbound hormones in the plasma are metabolized by the liver and excreted into the gut via the bile ducts [45]. Some of these hormone metabolites are reabsorbed via enterohepatic circulation [20,45]. Previous studies have speculated that an increase in the frequency of defecation due to increased consumption of dietary fiber could decrease reabsorption of hormone metabolites in the small intestine and therefore cause an increase in FHM excretion [13]. Although we did find that squirrels fed spruce seed (10 ± 2.3 fecal samples collected per individual over 94 h) defecated more frequently than those fed peanut butter (8 ± 1.4), it is unknown whether this caused the observed differences in FHM.

We found that a change from sunflower seeds to spruce seeds caused a significant increase in the excretion of FCM and FAM from 0 to 94 h after the diets were switched. This is surprising given that the fiber content of sunflower (21.5%) and spruce (19.8%) seeds were similar. This suggests that even the type of seeds (sunflower or spruce) squirrels were fed significantly influenced FHM. As a result, in addition to changes in consumption of dietary fiber, even subtle differences in the diets of free-ranging animals can influence FHM.

Previous studies have concluded that differences in FHM caused by variation in dietary fiber consumption are primarily due to the water content of feces. Increased fiber consumption can increase the water content of feces and therefore differences in FHM caused by changes in dietary fiber can be eliminated by removing the water via lyophilization [50]. Although we do not know if there were differences in the water content of feces of squirrels fed seeds

or peanut butter, we lyophilized all of our fecal samples and yet we still found persistent effects of diet on FCM and FAM. Future studies measuring FHM should consider monitoring the diets of their study animals in addition to lyophilization of fecal samples to eliminate any differences in FHM that are observed due to dietary changes.

Free-ranging animals often exhibit major seasonal shifts in the type and quantity of foods that they consume. As previous studies have found in humans and other animals, these changes in diet can have a major influence on patterns of FHM. We found that the consumption of dietary fiber and even consumption of similar types of food (seeds) with similar fiber content significantly altered the excretion of FCM and FAM. Future studies should perform careful experiments that manipulate the diets of their study animals to determine whether seasonal changes in FHM are due to ecological variation, reproductive condition, or changes in diet.

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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.2011.08.010.

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