# Coping with extreme free cortisol levels: Seasonal stress axis changes in sympatric North American flying squirrels

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## ARTICLE INFO

## ABSTRACT

Most environments exhibit predictable yearly changes, permitting animals to anticipate them. The hypothalamic–pituitary–adrenal (HPA) axis is a key physiological pathway that enables animals to cope with such changes. Monitoring glucocorticoid (the end products of the HPA axis) levels in wild animals throughout the year can improve our understanding of how this pathway responds to different conditions. For this study, we collected 18 months of data on two species of North American flying squirrels (*Glaucomys sabrinus* and *G. volans*) living in a southern Ontario forest where temperature and food availability fluctuate dramatically throughout the year. These squirrels are active year-round, nest communally, and rely on scatter hoarded foods in the winter months. Flying squirrels have extremely high levels of free plasma cortisol relative to other mammals, but it is unknown how these levels are affected by environmental and reproductive factors. For both species, our goals were to (1) validate an enzyme immunoassay (EIA) to measure their fecal glucocorticoid metabolite (FGM) concentrations and (2) assess yearly differences, seasonal changes, and the influence of sex, reproduction, and ambient temperature on FGM concentrations in each species. In the lab, we successfully validated the use of antibody 5α-pregnane-3β, 11β, 21-triol-20-one EIA for FGM analysis in both species. In the field, neither sex nor reproductive status (breeding condition or not) were linked to FGM concentrations in either species. FGM concentrations were higher in autumn compared to the spring and summer. There were no other seasonal differences. We discuss possible explanations for the autumn peak in FGM concentrations (increased energy expenditure and social nesting changes), as well as outline possible avenues for future research. Understanding how individuals and populations respond to environmental change is a critical goal in evolutionary ecology, particularly in the context of a rapidly changing Anthropocene.

## 1. Introduction

Most animals inhabit environments with seasonal changes in abiotic factors, such as day length, precipitation, and temperature. These changes can influence various aspects of animals' daily lives, such as food and water availability, predation risk, and cold exposure. Some species respond by migrating to more favourable environments during challenging times of the year (Bauer and Klaassen, 2013; Boonstra, 2004), whereas less mobile species must confront these challenges through morphological, behavioural, and physiological modifications.

For instance, snowshoe hares (*Lepus americanus*) increase their hair density in the winter to improve coat insulation, ultimately reducing foraging requirements and predator exposure (Balluffi-Fry et al., 2022, Kennah et al., 2023, Sheriff et al., 2009). Large bands of colobus monkeys (*Colobus angolensis ruwenzorii*) fission into smaller groups during the dry season (when food availability is low) to alleviate feeding competition; in the wet season, they remain in larger groups, likely to reduce their predation risk (Adams et al., 2021). Golden spiny mice (*Acomys russatus*) spend more time in torpor during the summer compared to the winter to conserve water in dry conditions (Levy et al.,

2011).

The hypothalamic–pituitary–adrenal (HPA) axis is a key physiological pathway that serves as an interface between an animal's internal and external environment (Sapolsky et al., 2000). As such, it has received much research attention in the context of seasonal change (Landys et al., 2006, Romero, 2002). Activation of the HPA axis leads to the release of glucocorticoids (GCs) into the bloodstream, through which they travel to tissues throughout the body and enter cells to regulate gene expression (review: Sacta et al., 2016). GCs are catabolic hormones that function primarily to partition energy amongst competing physiological demands (Henley et al., 2016, Moisan, 2013). They influence the expression of genes involved in a variety of processes, such reproduction, growth, metabolism, somatic recovery, and immunity (Le et al., 2005). In addition to coordinating predictable and routine functions, they are secreted in response to unpredictable stressors and divert energy toward activities that are immediately essential to survival (McEwen and Wingfield, 2003, Sapolsky et al., 2000).

Seasonal rhythms in baseline glucocorticoid concentrations have been documented in a wide range of vertebrates (review: Romero, 2002). Seasonal increases in GCs can be explained by a variety of factors, including mating competition during reproduction (e.g., Boonstra et al., 2001, Bradley et al., 1980), cold temperatures (Naidenko et al., 2011, Weingrill et al., 2004), and low food availability (e.g., Ukonaho et al., 2023, Zhang and Buck, 2022). The monitoring of GCs in wild animals is crucial in deepening our understanding of how a species' life history and ecology drive changes in GC concentrations (Ricklefs and Wikelski, 2002) and one can approach the problem either by measuring levels directly (in plasma) or indirectly (in feces, urine, hair, feathers, etc.) (Sheriff et al., 2011).

In our study, we investigate seasonal patterns in GCs indirectly (fecal glucocorticoid metabolites, FGMs) in two sciurid species that are sympatric in regions of North America where the boreal forest meets the eastern deciduous forests: northern (*Glaucomys sabrinus*) and southern (*G. volans*) flying squirrels. These squirrels have GC concentrations (predominantly cortisol) that are among the highest in any vertebrate species (Desantis et al., 2013). Desantis et al., (2018a) found that southern flying squirrels have total plasma cortisol levels that were 1.7 × higher than northern flying squirrels, 2.4 × higher than red squirrels (*Tamiasciurus hudsonicus*), and 3.0 × higher than grey squirrels (*Sciurus carolinensis*). It is not known if other flying squirrel species have similarly high cortisol levels. In most mammals, blood GCs are approximately 90 % bound to corticosteroid-binding globulin (CBG), a glycoprotein synthesized mainly by the liver (Breuner et al., 2013, Perogamvros et al., 2012). CBG-bound GCs are not able to pass through cell membranes, causing them to remain in the blood and be biologically inactive (Henley et al., 2016, Moisan, 2013). When GC action is required, these steroid hormones are released from CBG and diffuse passively through lipophilic cell membranes into the cytoplasm where they bind to receptors and then together move to the nucleus to regulate gene expression (Sacta et al., 2016). North American flying squirrels have extremely low CBG binding affinities, leaving 90 % of their circulating GCs in an unbound state (Desantis et al., 2013). High GCs levels coupled with low CBG binding results in extremely high levels of free GCs.

The critical question is how these high levels of free GCs act in North American flying squirrels in comparison to other mammals. Desantis et al., (2018b) assessed plasma GC levels of southern flying squirrels at two points in the year: March (breeding period) and November (nonbreeding period). Total GC concentrations were greater in November compared to March, whereas CBG concentrations did not change (Desantis et al., 2018b). Thus, free GC concentrations are likely greater in November compared to March. Continuous data on GC concentrations throughout the year are needed to determine how the HPA axis responds to different conditions in North American flying squirrels. We had two objectives for each species: first, to validate a non-invasive GC measure (FGMs) and second, to assess seasonal changes in FGMs. We analyzed fecal samples collected from both species across an 18-month period to assess yearly differences, seasonal changes, and the impacts of reproduction, sex, body mass, and ambient temperature.

## 2. Methods

### 2.1. Study species

Northern (*Glaucomys sabrinus*) and southern (*G. volans*) flying squirrels are arboreal rodents known widely for their ability to glide between trees. Northern flying squirrels inhabit boreal and mixed-conifer-hardwood forests across Alaska, Canada, the northeastern US, and high elevation regions in the US, such as the Appalachian and Rocky Mountains (Wells-Gosling and Heaney, 1984). Southern flying squirrels inhabit deciduous forests across southeastern Canada, the eastern US, and high elevation regions in Mexico and Central America (Dolan and Carter, 1977). In recent years, climate change has caused a northward expansion of the southern species, leading to new regions of sympatry in eastern Canada and the northeastern US (Bowman et al., 2005).

Both species are nocturnal-crepuscular and active year-round (they do not hibernate). During the day, they nest in tree cavities, leaf nests (dreys), and occasionally subterranean nests (Minns et al., 2023, O'Brien et al., 2021). At night, they forage and cache food. Northern flying squirrels feed predominantly on fungi and lichens (Dubay et al., 2008, Vernes, 2004), whereas their southern counterparts feed predominantly on nuts and seeds (Dolan and Carter, 1977). Both species are highly social; individuals have overlapping home ranges (Holloway and Malcolm, 2007, Lavers, 2004, Persad, 2023), regularly nest in groups (Bakker and Hastings, 2002, Garroway et al., 2013, Maser et al., 1981), and have been observed to communicate with one another using ultrasonic vocalizations (Diggins, 2021). Heterospecific social nesting likely occurs on occasion (Olson et al., 2018). Reproduction typically takes place throughout March to the end of August, with some females producing two litters per year (Dolan and Carter, 1977, Smith et al., 2011). In both species, gestation lasts ~40 days, lactation around 6–8 weeks, and litter size averages 3 neonates (Dolan and Carter, 1977, Ferron and Ouellet, 1985, Hayssen et al., 1993, Stapp and Mautz, 1991).

### 2.2. Trapping

Field work took place from March 2021 to September 2022 in a mixed-conifer-hardwood forest at the southern end of Mississauga Lake in Ontario, Canada (Kawartha field site: 44°41′21.0″N 78°20′03.2″W). Flying squirrel research at this site has been ongoing since 2001 (J. Bowman, personal communication). This forest consists of deciduous trees, such as sugar maple (*Acer saccharum*), red oak (*Quercus rubra*), and trembling aspen (*Populus tremuloides*), as well as coniferous trees, such as eastern hemlock (*Tsuga canadensis*), white pine (*Pinus strobus*), and balsam fir (*Abies balsamea*). The site is located at the southern edge of the Canadian Shield and has a rocky terrain with varied elevation.

Flying squirrels were trapped using Tomahawk model 102 live traps that were strapped to wooden platforms mounted to trees ~2 m above the ground. Traps were baited with peanut butter and peanuts or sunflower seeds, set at dusk, and checked hourly throughout the night; squirrels were usually in the trap for less than 1 h, and never more than 2 h. Upon capture, we recorded sex, age (adult or juvenile), and weight using a Pesola spring scale. Fecal samples for FGM analysis were collected from the platform upon checking the traps and from squirrels directly if they defecated during processing. Only samples that had no visible urine contamination were collected. Samples were kept on ice packs in a cooler until they could be stored in a −20 °C freezer at Trent University that same night (1–6 h later). New squirrels were injected subcutaneously with a passive integrated transponder (PIT) tags (model TX1411SST, 12.50 mm × 2.07 mm, 134.2 kHz ISO,0.1020 g, Biomark Inc.) for future identification. A squirrel was considered to be in breeding condition if the nipples were visible in females and if the testes

were large in males. Unfortunately, we were not able to differentiate between pregnant and lactating squirrels. Species were identified in the field using morphological characteristics. Southern flying squirrels have pure white ventral fur, whereas northern flying squirrels have ventral fur with dark grey at the base and white tips (Dolan and Carter, 1977, Wells-Gosling and Heaney, 1984). All animal handling procedures were approved by the Animal Care Committees of Trent University (Protocol #: #25668) and the University of Toronto (Protocol #:20012684).

### 2.3. Validation

To assess whether fecal glucocorticoid metabolite concentrations reflect endogenous plasma concentrations, we carried out a rigorous validation (Palme, 2019). On 13 September 2021 (between 1900 h and 2200 h) nonreproductive adult squirrels (*G. sabrinus*: 3 males, 3 females; *G. volans*: 3 females, 2 males) were trapped at the Kawartha field site and transported to the Trent University Animal Care Facility. Squirrels were housed in an outdoor aviary, where they were exposed to ambient temperatures and lighting. They were fed apple, squirrel chow, and water *ad libitum*. Squirrels were individually housed in a polypropylene rodent housing cage (47 cm × 26 cm × 20 cm) that was nested within a polycarbonate rodent housing cage. The upper cage had a stainless-steel slatted floor, which permitted urine and feces to pass through and onto a metal mesh that was placed on the bottom of the lower cage. The metal mesh allowed urine to pass through, but captured feces, reducing urine contamination of the samples. Fecal samples that were visibly contaminated with urine were discarded. Squirrels were held in the aviary for four nights to acclimate to these conditions. On the fifth night, squirrels were injected intramuscularly with a synthetic adrenocorticotropic hormone (ACTH) CORTROSYN™ (Amphastar Pharmaceuticals), which was diluted with sterile physiological saline to obtain the appropriate dosage (12 IU/kg; 0.684 – 1.164 IU). This hormone triggers the adrenal release of GCs into the plasma and has been similarly used in other rodent species (*Tamiasciurus hudsonicus,* Dantzer et al., 2010; *Tamias alpinus*, *T. speciosus*, Hammond et a. 2015). Following the injection, fecal samples were collected every 2 h for 48 h. On 19 September, all squirrels were released at their site of capture.

### 2.4. Extraction and quantification of fecal glucocorticoid metabolites

All fecal processing took place at the University of Toronto Scarborough. Fecal samples were stored at −20 °C until steroid hormone extraction took place. Samples were freeze-dried for 16 h using a lyophilizer (LabConco, Missouri, USA) to remove water. Dried samples were then homogenized using a mortar and pestle and any undigested material was removed. Liquid nitrogen was added to the sample to facilitate sample homogenization. Samples were weighed to 0.05 g and added to 1 mL of 80 % methanol. If a sample was lighter than 0.05 g, the methanol volume was adjusted (e.g., 0.04 g of dried feces was added to 0.8 mL of 80 % methanol). Sample-methanol mixtures were shaken on a multi-vortexer (IKA Vibrax, Staufen, Germany, 1500 rpm) for 1 h and then centrifuged (2500 RPM, relative centrifugal force = 1428) for 15 min. Supernatant was aliquoted into a new 2 mL polypropylene tube and diluted in assay buffer to 1:500.

Validation sample extracts were analyzed with two EIAs: 5α-pregnane-3β, 11β, 21-triol-20-one EIA (Touma et al., 2003) and 11-oxoaetiocholanolone EIA (Möstl et al., 2002). These assays were tested because they have been used to measure glucocorticoid metabolites in other Sciuridae species (*Sciurus carolinensis*, Bosson et al., 2013; *Urocitellus columbianus*, Bosson et al., 2009; *Tamiasciurus hudsonicus*, Dantzer et al., 2010; *S. vulgaris*, Dantzer et al., 2016; *Tamias alpinus, T. speciosus*, Hammond et al., 2015; *T. striatus,* Montiglio et al., 2012; *U. parryii*, Sheriff et al., 2012; *Spermophilus citellus*, Strauss et al., 2007). The assay procedure is described in detail by Touma et al. (2003). Only the 5α-pregnane-3β,11β,21-triol-20-one EIA showed a spike in FGM concentrations in samples collected after the ACTH injection and so this

immunoassay was subsequently used to analyze field samples.

Parallel displacement between the standard curve and serial dilutions of fecal extract was used to detect immunological similarities between the standard and sample hormones metabolites. Five representative test samples were diluted at 1:50, 1:100, 1:200, 1:500, 1:800, 1:1000, 1:2000 in assay buffer and EIAs were run alongside the standard curve. A graph of sample hormone metabolite concentration vs. percent antibody confirmed parallelism. Sample dilution was selected based on 50 % binding of the sample curves.

### 2.5. Data analysis

#### 2.5.1. Validation

Fecal glucocorticoid metabolite (FGM) concentrations were measured in samples collected from 48 h before to 48 h after ACTH injection. In both species, FGM concentrations were log transformed so that model residuals fit assumptions of normality. Changes in FGM concentrations following ACTH injection were assessed using linear mixed-effect models (LMM) with restricted maximum likelihood (REML). Log FGM (log ng FGM/g feces) was the response variable, and time intervals, squirrel sex, collection time, and fecal sample size were the fixed effects. Fecal samples were binned into the following time intervals: 0–48 pre-ACTH injection, and into the following hourly intervals post-injection: 1–3, 4–6, 7–9, 10–12, 22–24, 25–27, 28–30, 31–33, 46–48. Fecal sample collection time was included as a fixed effect to account for a possible circadian rhythm. Squirrel ID was included as a random effect to account for repeated measures of FGM concentrations from the same individual. Sex was not significant in any of the models and so males and females were analyzed together. Species were analyzed separately due to possible species-specific metabolism (Palme, 2019).

#### 2.5.2. Field data

LMMs with REML were used to assess relationships between FGM concentrations and several predictor variables: year, season, sex, reproductive state, body mass, and nightly temperature. The response variable, FGM concentration, was log transformed to satisfy the LMM assumption that residuals are normally distributed. Due to a low number of fecal samples collected in certain months, we binned samples into four seasons (winter: Dec 21-Mar 20; spring: Mar 21-June 20; summer: Jun 21-Sep 20; autumn: Sep 21-Dec 20). Fixed effects were year, season, sex, reproductive state (breeding condition or not), body mass, minimum ambient temperature, and sample collection time, as well as interactions between reproductive status and sex, season and sex, and minimum temperature and season. Squirrel ID was included as a random effect to account for repeated measures of FGM concentrations from the same individual. Weather data were acquired from Haliburton Environment Canada Station (45°01′56.094″ N, 78°31′52.014″ W), which is located approximately 41 km northwest of the field site. To determine how body masses changed throughout the year, we ran LMMs with REML. The response variable was body mass, which was log transformed. The random effect variable was squirrel ID and the fixed effects were year, season, sex, reproductive state (breeding condition or not), and minimum ambient temperature, as well as interactions between sex and reproductive status, and minimum temperature and season.

To determine which variables to include in the final models, we performed stepwise selection using Akaike information criterion (AIC) values. We used a conditional model averaging procedure for all models within 2 AIC units of the top-supported model (lowest AIC value) (Arnold, 2010, Burnham and Anderson, 2002). This average model was then used in subsequent post-hoc analyses. To check LMM assumptions for all models, we used diagnostic plots to confirm that residuals were normally distributed, homoscedastic, and that there were no outliers with high leverage. All analyses were carried out in the R environment (R Core Team, 2022); plots were constructed using 'ggplot2′ and LMMs were run using 'lme4′ (Bates et al., 2015). Effect sizes (β) were estimated

using the LMMs. Coefficients of significance for the LMMs were calculated using Satterthwaite's approximations for degrees of freedom using 'lmerTest' (Kuznetsova et al., 2017). If an independent factor with more than two levels was significant, we used 'emmeans' to run a Tukey post hoc test to assess which levels were statistically different (Lenth, 2023). Conditional model averaging was done using 'MuMIn' (Bartoń 2023).

## 3. Results

### 3.1. Fecal glucocorticoid metabolites

#### 3.1.1. Validation

Out of the 11 adult nonreproductive flying squirrels included in the validation, we were only able to include 8 in the analysis (4 northern and 4 southern flying squirrels). We were not able to collect sufficient fecal samples following ACTH injection from 3 individuals (1 northern male, 1 northern female, 1 southern female) and so they were excluded.

For the validation using 5α-pregnane-3β, 11β, 21-triol-20-one EIA, the average intra-assay coefficients of variation (CV) of pools run at the beginning and end of each plate were 4.9 % and 12.1 % for the low- and high-value pools, respectively. The average inter-assay CVs of low- and high-value pools were 3.8 % and 12.2 %, respectively (n = 6 plates). The average CV for sample replicates was 4.7 %. For the 8 squirrels included in the analysis, 7 showed an increase in FGM concentration post-ACTH injection using the 5α-pregnane-3β, 11β, 21-triol-20-one EIA, whereas 1 showed no change. Linear mixed-effect models of fecal samples from these 7 squirrels showed that sample collection time had a positive relationship with FGM concentration in both species, with concentrations increasing throughout the night (southern: β = 0.13 ± 0.05, t = 2.8, p = 0.008; northern: β = 0.09 ± 0.04, t = 2.4, p = 0.02). This trend was also observed with samples collected from the field (see below). ACTH injections resulted in an increase in FGM concentration 4–6 h after injection in both species (southern: β = 1.45 ± 0.31, t = 4.72, p = 0.002, northern: β = 1.16 ± 0.33, t = 3.48, p = 0.048, Fig. 1) and thereafter, a decrease within 48 h in the southern species (β = -2.0 ± 0.5, t = 3.6, p = 0.03) and within 33 h in the northern species (β = -1.6 ± 0.4, t = 3.5, p = 0.04, Fig. 1).

For the validation using the 11-oxoaetiocholanolone EIA, the average intra-assay CV of pools run at the beginning and end of each plate were 13.1 % and 9.5 % for the low- and high-value pools, respectively. The average inter-assay CVs of low- and high-value pools were 7.2 % and 10.9 %, respectively (n = 5 plates). The average CV for sample replicates was 5.1 %. There were no significant differences between pre-injection FGM concentration and FGM concentrations in any of the post-injection collection times. Thus, this antibody did not reflect endogenous changes in GC concentrations and was not used in our seasonal study.

#### 3.1.2. Field data: body mass

Throughout the study period, we monitored the body mass of 28 northern flying squirrels (9 females, 19 males) and 48 southern flying squirrels (19 females, 29 males) with measurements taken an average of 9 times per squirrel (range = 1–43) (Fig. 2). We trapped mostly adult squirrels, with only 3 northern and 11 southern juveniles trapped throughout the study.

For both species, we report the full model, the best model for predicting weights (lowest AIC value), and all models that are within 2 AIC units (Table 1). Information on all candidate models can be found in Supplementary Material. In the southern species, reproductive females were significantly heavier than non-reproductive females (β = 0.07 ± 0.01, t.ratio = 6.4, p < 0.0001). Season was significant, with lower weights in autumn; the only significant difference between seasons was between autumn and spring (β = 0.04 ± 0.01, t.ratio = 3.3, p = 0.006, Fig. 3). There was also a significant interaction effect between season and minimum ambient temperature in the southern species. During spring, minimum temperature had a positive relationship with body
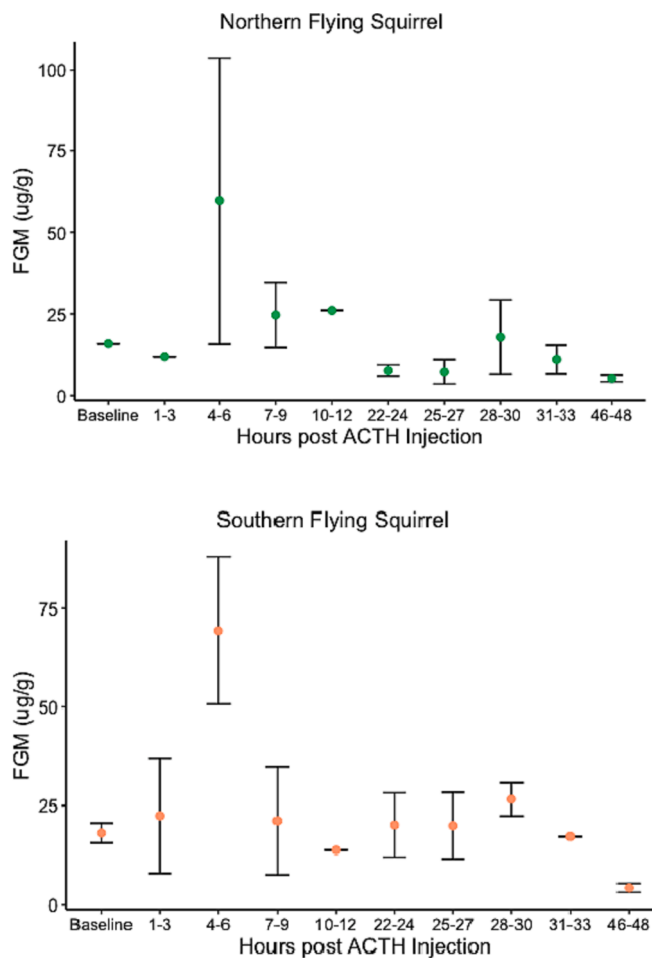


**Fig. 1.** Concentrations (μg/g dried feces) of fecal glucocorticoid metabolites (FGMs) during an ACTH challenge for northern (n = 38 fecal samples; top) and southern (n = 41 fecal samples; bottom) flying squirrels. Concentrations were determined using a 5α-pregnane-3β, 11β, 21-triol-20-one EIA. FGM concentrations were significantly higher 4–6 h after the ACTH injection compared to pre-injection levels (i.e. 48–0 h before). Circles represent means ± SE.

mass (β = 0.001 ± 0.0006, confidence limits: 0.0003 to 0.002, Fig. 3).

In the northern species, reproductive females were significantly heavier than non-reproductive females (β = 0.08 ± 0.01, t.ratio = 7.4, p < 0.0001). Season was significant, with lower weights in autumn; though none of the seasonal differences were significant (Fig. 4). There was also a significant interaction effect between season and minimum ambient temperature in the northern species. During autumn, minimum temperature had a negative relationship with body mass (β = −0.002 ± 0.0006, confidence limits: −0.004 to −0.001, Fig. 4).

#### 3.1.3. Field data: FGM concentrations

FGM concentration was measured in 150 samples from 26 different adult southern flying squirrels (10 males and 16 females) and in 140 samples from 16 different adult northern flying squirrels (8 males and 8 females). A mean of 7 (range: 1–34) samples were collected per individual. During the entire field season, we only acquired 16 fecal samples from 9 juveniles (1 northern, 8 southern), and so our analysis is restricted to adult squirrels. Mean FGM concentration for the southern species was 7055 ng/g of dried feces (range: 734–52,364 ng/g dried feces). Mean FGM concentration for the northern species was 4554 ng/g dried feces (range: 638–47795 ng/g dried feces). The mean intra-assay coefficients of variation (CV) of high- and low-value pools were 12.3 % and 9.0 %, respectively. The mean inter-assay CVs of high- and low-value pools were 14.3 % and 14.9 %, respectively (n = 15 plates). The
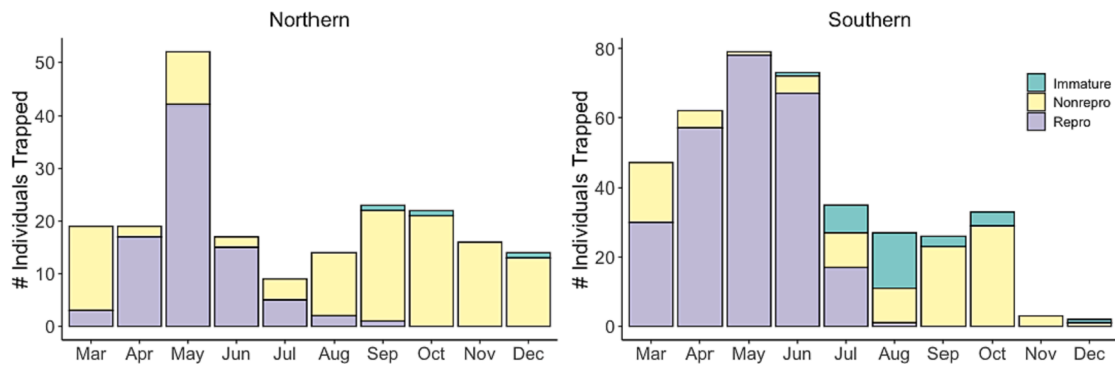
**Fig. 2.** Number of immature, reproductive, and nonreproductive flying squirrels caught each month of 2021 for northern (left) and southern flying squirrels (right). Numbers reflect multiple captures of the same squirrel.

**Table 1**

Model selection details for weight analyses. To determine which variables to include in the final models, we performed stepwise selection. Stepwise selection is a combination of forward and backward selection procedures that involves adding and removing variables at different steps. Among the available models for the specific data, the model with lowest Akaike information criterion (AIC) value is best. We included all models that are within 2 AIC units of the best model.

**Full model:** log(weight) ~ Repro.State + Sex + Repro.State*Sex + Season + Min. Temp + Season*Min.Temp + Year+ (1|Individual)

**Southern flying squirrels:**
  **Model 1:** log(weight) ~ Repro.State + Sex + Repro.State*Sex + Season + Min. Temp + Season*Min.Temp + (1|Individual)
    **AIC = -1175.7**
  **Model 2:** log(weight) ~ Repro.State + Sex + Repro.State*Sex + Season + Min. Temp + Season*Min.Temp + Year+ (1|Individual)
    **AIC = -1173.7**

**Northern flying squirrels:**
  **Model 1:** log(Weight) ~ Repro.State + Sex + Repro.State*Sex + Season + Min. Temp + Season*Min.Temp+ (1|Individual)
    **AIC = -804.85**
  **Model 2:** log(Weight) ~ Repro.State + Sex + Repro.State*Sex + Season + Min. Temp + Season*Min.Temp + Year+ (1|Individual)
    **AIC = -802.86**

mean CV for sample replicates was 5.6 %.

For both species, we report the full model, the best model for predicting FGM concentrations (lowest AIC value), and all models that are within 2 AIC units (Table 2). Information on all candidate models can be found in Supplementary Material. Southern flying squirrel FGM concentrations for both sexes were higher in autumn than in spring ($\beta = 0.8 \pm 0.2$, t.ratio = 3.7, p = 0.002, Fig. 5) and summer ($\beta = 1.1 \pm 0.2$, t.ratio = 5.3, p < 0.0001, Figs. 5, 6), and in spring than summer ($\beta = 0.4 \pm 0.1$, t.ratio = 3.2, p = 0.01, Figs. 5, 6). There were no other seasonal differences. FGM concentrations were also higher in 2022 compared to 2021 ($\beta = 0.3 \pm 0.1$, t.ratio = 2.1, p = 0.04). Sample collection time had a positive impact on FGM concentrations, with concentrations increasing from earlier in the evening to later in the evening ($\beta = 1.5e^{-1} \pm 5.4e^{-2}$, t.ratio = 2.8, p = 0.005). Sample collection time ranged from ~ 20:00 to 01:00 the next morning.

Northern flying squirrel FGM concentrations for both sexes were higher in autumn than spring ($\beta = 0.7 \pm 0.1$, t.ratio = 4.8, p < 0.0001, Fig. 5) and summer ($\beta = 1.0 \pm 0.2$, t.ratio = 5.7, p < 0.0001, Figs. 5, 6). There were no other seasonal differences. Sample collection time had a positive impact on FGM concentrations, with concentrations increasing from earlier in the evening to later in the evening ($\beta = 0.1 \pm 0.04$, t.ratio = 2.8, p = 0.006).

## 4. Discussion

Our main findings were that for both species of flying squirrel: (1) a 5α-pregnane-3β, 11β, 21-triol-20-one EIA can be used to measure FGM concentrations, (2) FGM concentrations peaked in the autumn months, and (3) reproductive state and sex were not linked with FGM concentrations. Though our sampling was intense, our study was observational and encompassed just one winter season, and thus we are limited in the conclusions that can be drawn. One of the limitations to consider when using FGM measurements as a proxy for blood GC levels is that diet can impact the excretion of FGMs in feces through changes in metabolism and gut passage time (Dantzer et al., 2011, Palme, 2019). With this in mind, we must consider that seasonal differences in FGM concentrations could be due to a shift in diet at different times of the year. However, earlier work on the southern species supports our finding that free GC
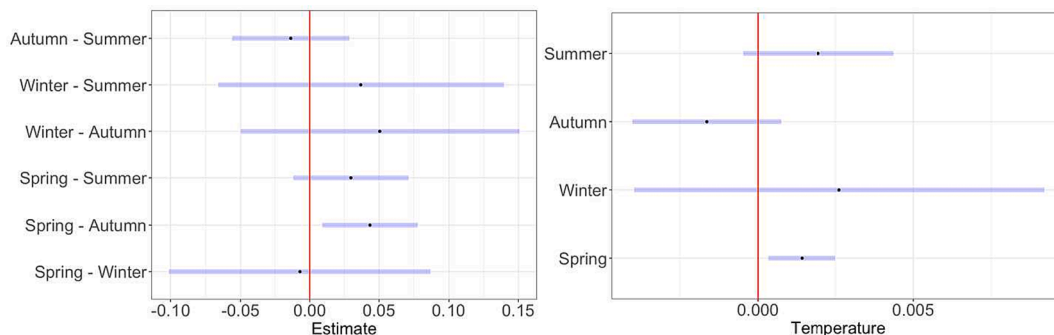


**Fig. 3.** Forest plots showing the estimates (black dot) and confidence intervals (blue bar) of the weight differences between seasons (left) and the impact of temperature on weights in each season (right) for southern flying squirrels. A confidence interval (blue bar) that does not overlap 0 indicates a significant effect. The only significant seasonal weight differences was that squirrels were heavier in spring compared to autumn (left). The only significant impact with respect to temperature and weight was that weight increased with temperature in spring (right).
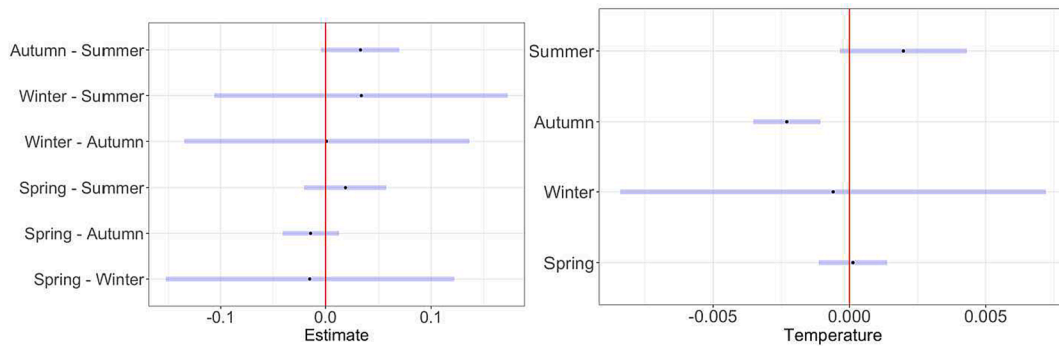
**Fig. 4.** Forest plots showing the estimates (black dot) and confidence intervals (blue bar) of the weight differences between seasons (left) and the impact of temperature on weights in each season (right) for northern flying squirrels. A confidence interval (blue bar) that does not overlap 0 indicates a significant effect. There were no significant seasonal weight differences (left). The only significant impact that we found with respect to temperature and weight was that weight decreased with temperature in autumn (right).

**Table 2**

Model selection details for fecal glucocorticoid metabolite (FGM) analysis. To determine which variables to include in the final models, we performed stepwise selection. Stepwise selection is a combination of forward and backward selection procedures that involves adding and removing variables at different steps. Among the available models for the specific data, the model with lowest Akaike information criterion (AIC) value is best. We included all models that are within 2 AIC units of the best model.

| |
|---|
| **Full model:** log(FGM) ~ Weight + Repro.State + Sex + Repro.State*Sex + Season + Min.Temp. + Season*Min.Temp + Year + Time + (1\|Individual) |
| **Southern flying squirrels:** |
|   **Model 1:** log(FGM) ~ Season + Year + Time + (1\|Individual) **AIC = 284.33** |
|   **Model 2:** log(FGM) ~ Weight + Season + Year + Time + (1\|Individual) **AIC = 286.23** |
| **Northern flying squirrels:** |
|   **Model 1:** log(FGM) ~ Repro.State + Sex + Repro.State*Sex + Season + Year + Time + (1\|Individual) **AIC = 256.68** |
|   **Model 2:** log(FGM) ~ Season + Time + (1\|Individual) **AIC = 256.80** |
|   **Model 3:** log(FGM) ~ Season + Year + Time + (1\|Individual) **AIC = 257.27** |
|   **Model 4:** log(FGM) ~ Weight + Repro.State + Sex + Repro.State*Sex + Season + Year + Time + (1\|Individual) **AIC = 258.38** |

concentrations in the blood are higher in November compared to March (Desantis et al., 2018b). Additionally, there are marked differences in the diets between these two species, but we still found similar relationships between season and FGM levels, suggesting that we are indeed detecting a real biological signature.

We successfully validated fecal glucocorticoid metabolite analysis in northern and southern flying squirrels. We analyzed samples using both

a 5α-pregnane-3β, 11β, 21-triol-20-one and a 11-oxoaetiocholanolone EIA, but only the former showed a significant increase in FGM concentrations after the ACTH injection. Specifically, FGM concentrations were elevated 4–6 h post-ACTH injection for both species and then declined to baseline levels within 33 and 48 h for the northern and southern species, respectively. Though we only found an increase in 7 out of 8 squirrels post ACTH injection, we believe that the results are compelling. It is possible that the ACTH was not properly injected into the muscle of the non-responding squirrel.

### 4.1. Seasonal changes

FGM concentrations increased significantly in autumn for both species. This aligns with previous research showing that southern flying squirrels' total blood GC concentrations were higher in November than March, whereas CBG concentrations did not change (Desantis et al., 2018b). Flying squirrels do not hibernate during the winter months, relying instead on other energy conservation strategies that may be linked to an autumn increase in circulating GCs.

Flying squirrels are scatter hoarders; they cache their food in a variety of different places within their habitat (Harlow and Doyle, 1990). Muul (1968) studied southern flying squirrels in Michigan and found that caching behaviour increased starting in October. Through a series of experiments, he showed that this increase was triggered by changes in photoperiod (independent of temperature). Though we didn't measure foraging activity for our study squirrels, they likely increased food caching around the same time. The increase in FGM concentrations in autumn could be due to the energy expenditure required for intense
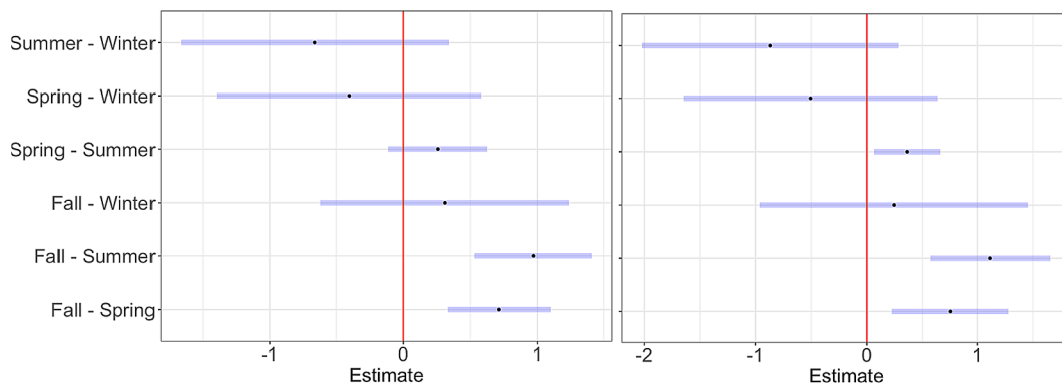


**Fig. 5.** Forest plots showing the estimates (black dot) and confidence intervals (blue bar) of the differences in fecal glucocorticoid metabolite (FGM) concentrations between seasons for northern (left) and southern (right) flying squirrels. A confidence interval (blue bar) that does not overlap 0 indicates a significant difference. FGM levels were significantly higher in fall compared to spring and summer for both species. FGM concentrations were significantly higher in spring compared to summer for the southern species (right).
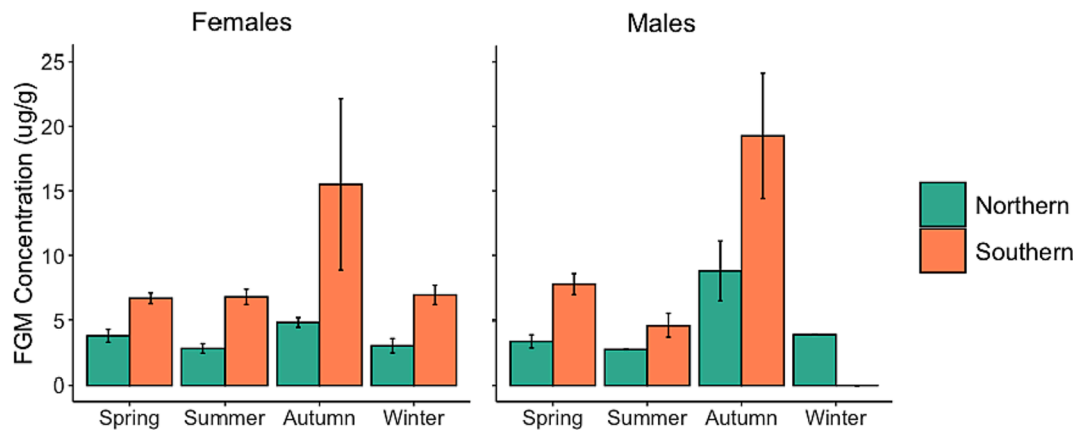
**Fig. 6.** Changes in fecal glucocorticoid metabolite concentrations in northern (*Glaucomys sabrinus*) and southern (*G. volans*) flying squirrels across seasons: winter: Dec 21-Mar 20; spring: Mar 21-June 20; summer: Jun 21-Sep 20; autumn: Sep 21-Dec 20. Bars represent means ± SE.

foraging and food caching during these months. Increased GC levels have not been linked to food caching in other sciurids, but data from another scatter hoarding sciurid (yellow-pine chipmunks, *Neotamias amoenus*) shows an increase in total plasma GC concentrations in the fall compared to the spring (Kenagy and Place, 2000; Place and Kenagy, 2000; Romero et al., 2008). Future research could use accelerometers to measure differences in foraging and food caching behaviours between individuals and see if this is associated with differences in FGM concentrations (Gleiss et al., 2011).

Southern flying squirrels increase their nest group sizes in the winter to reduce thermoregulatory costs (Muul, 1968; Stapp et al., 1991; Thorington et al., 2010). Squirrels that nest in groups expend significantly less energy compared to those that nest individually (Stapp et al., 1991). Research near our study site showed that southern flying squirrels nest in mixed-sex groups of, on average, 2.4 (range = 1–10) squirrels in the summer months (1 Mar-16 Nov) and 6.2 (range 1–22) in the winter months (17 Nov-28 Feb) (Garroway et al., 2013). Summer nest groups began to associate with one another in November, with group sizes peaking in January (Garroway et al., 2013). Though these grouping changes are routine, it is possible that they are stressful due to changes in conspecific interactions. Future research could examine variation in nest group characteristics to determine if this is related to differences in FGM concentrations. Little is known about the grouping behaviour of northern flying squirrels across season, but they likely rely less on social thermoregulation than their southern counterparts, as they are more cold adapted.

### 4.2. Yearly change

FGM concentrations were higher in 2022 than in 2021 for the southern species. This difference could be due to the spongy moth (*Lymantria dispar*) outbreak that occurred during the summer of 2021 (Haq et al., 2021). This outbreak led to low acorn production during autumn 2021, leaving squirrels with lower food supplies over the winter (J. Bowman, unpublished data). In the winter, northern flying squirrels feed primarily on fungi (Currah et al., 2000, Maser et al., 1986, Vernes et al. 2004) whereas southern flying squirrels feed on hard mast (e.g., *Quercus* spp. acorns, *Fagus grandifolia* beechnuts, Harlow and Doyle, 1990, Ivan and Swihart, 2000, Weigl, 1978). Diet analysis of the squirrels at our site found that both species of flying squirrel fed on mushrooms and truffles throughout the winter 2021–2022 (Persad, 2023). It is possible that increased FGM concentrations in 2022 were a result of low food availability for the southern species in winter 2021, which led to energetic stress the following spring and summer. Long-term monitoring of flying squirrels' stress physiology before and after the spongy moth outbreak is needed to confirm this speculation.

### 4.3. Sex and reproduction

Sex and reproductive state did not significantly affect FGM concentrations in either species. Previous work showed that southern flying squirrel females had higher levels of both total cortisol and CBG than males (Desantis et al., 2018b). The authors suggest that free GC levels are likely similar in females and males, as the additional cortisol in females would be bound to the additional CBG. This interpretation aligns with our results, as FGM concentrations reflect free GC levels (Breuner et al., 2013, Fauteux et al., 2017, Sheriff et al., 2010). Research on Siberian flying squirrels (*Pteromys volans*) in Finland showed that during the breeding period, males had higher hair cortisol concentrations than females (Santangeli et al., 2019). These findings can be explained by the sex-specific reproductive strategies in this species: females are territorial and maintain the same home range throughout their lives, whereas males have home ranges that overlap with those of other males and females (Selonen et al., 2013). During the breeding period, males increase their movement and thus expend more energy to compete for mating opportunities with females, explaining the increase in cortisol concentrations (Selonen et al., 2013). In contrast, North American flying squirrels are highly social, with both species nesting in groups year-round (northern: Currah et al., 2000, Vernes, 2004, southern: Garroway et al., 2013; Ivan and Swihart, 2000, Weigl, 1978). Thus, we do not expect a significant increase in movement and effort to be associated with breeding for males in either species, particularly for the southern species, as breeding begins in March, when squirrels are nesting in their larger winter groups.

It is surprising that we did not see an increase in FGM concentrations in reproductive females compared to nonreproductive females given what we know about the role of glucocorticoids for female reproduction in other mammals (reviews: Edwards and Boonstra, 2018; Stead et al., 2022). It is possible that blood GC concentrations (and thus FGM concentrations) remain the same while GC action is modified at the cellular level. Though CBG is produced mostly by the liver, there is evidence that it can also be produced by other tissues, including the brain, liver, kidney, uterine tubes, endometrium, and adipose tissue (del Mar Grasa et al., 2001, Jirikowski et al., 2007, Misao et al., 1994, Miska et al., 2004, Möpert et al., 2006, Perrot-Applanat et al., 1984, Scrocchi et al., 1993). Though speculative, CBG may be acting as a GC buffer within the cells, just as it does in the blood (Sivukhina and Jirikowski, 2014). If this is the case, flying squirrel females may downregulating CBG expression to increase activity of GCs when they are reproducing. This would have no effect on blood free GC levels but allow for GC activity to increase in response to the demands of pregnancy and milk production. Research on changes in intracellular expression of CBG throughout the reproductive cycle is needed to better understand this phenomenon. It is also possible that we did not find an increase in FGM concentrations in reproductive

females due to insufficient samples collected around the time when reproductive differences in GC concentrations are most dramatic (Dantzer et al., 2016). For instance, FGM concentrations of females in the early to middle stages of pregnancy may not differ from concentrations in unreproductive females, whereas females in late stages of pregnancy are known to show a peak in GC secretion (review: Edwards and Boonstra, 2018)

## 5. Conclusions

We monitored northern and southern flying squirrels across an 18-month period and found that, for both species, FGM concentrations were highest in autumn, when squirrels are preparing for the cold, food-limited months ahead. Intense foraging and food caching and/or changes in social nesting groups during autumn months may underly this trend. In neither species did sex or reproductive status significantly impact FGM concentrations. More fine-grained and long-term data on FGM concentrations coupled with activity data will shed light on these trends.

## CRediT authorship contribution statement

**Samantha M. Stead:** . **Phoebe D. Edwards:** Writing – review & editing, Methodology, Formal analysis. **Rebekah Persad:** Writing – review & editing, Methodology. **Rudy Boonstra:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Julie A. Teichroeb:** Writing – review & editing. **Rupert Palme:** Writing – review & editing, Resources. **Jeff Bowman:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2024.114467.

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