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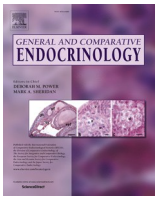

Assessing stress in a mammal from plasma and feces: A nutritional mismatch between the diet needed and the food -available

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ABSTRACT

A lack of agreement between the diet an animal needs for reproduction and survival and the food available in its environment has major impact on its fitness. The complexity of their digestive system is critical and may affect stress axis function. The squirrel family (sciurids) have a very simple gut and do not have the microbiome to digest high fiber foods well. Thus, they forage preferentially on forbs, seeds, and fungi, and avoid hard-to-digest grasses. We compared two measures of their stress axis – plasma free cortisol, a glucocorticoid (GC), and fecal cortisol metabolites (FCMs) – in Columbian ground squirrels *(Urocitellus columbianus)* from two meadows as they were building up body reserves in July for their pending hibernation. One meadow was natural with an abundance of forbs and the other unnatural with an abundance of grasses that had been seeded as a horse pasture. Our two measures went in the opposite direction: GC levels were lower on the natural meadow but FCMs were higher, whereas GC levels were higher on the grass meadow but FCMs lower. The fecal fiber content was lower on the natural meadow. Thus, when interpreting FCM levels, it is critical to understand both the digestive system of the study mammal and its diet to interpret stress axis function.

1. Introduction

The hypothalamic-pituitary-adrenocortical axis (HPA) – the stress axis – is a pivotal component of the neuroendocrine system in mammals and plays a key role in coordinating successful adaptation to the environment. The study of this axis is one of the best windows we have to 'see' under the surface of a wild mammal into the functional mechanisms it uses to cope, survive, and reproduce. The axis mediates the relationship of the mammal to its environment at four levels: in response to the sleep-wake cycle (the circadian rhythm) and food consumption (Oster et al., 2017), in response to short-term stressors (e.g. Wingfield et al., 2011), in response to chronic stressors (Boonstra, 2013), and in response to longer-term adaptations to particular ecological and evolutionary challenges (Wingfield and Romero, 2001; Reeder and Kramer, 2005; Boonstra et al., 2007).

Though there are four matrices to quantify stress axis function – plasma, feces [and urine], saliva, and hair – the dominant ones used in field studies are the first two (Sheriff et al., 2011). Plasma levels give an instantaneous measure of GCs when collected within 3 min but, when collected afterwards, will reflect the stress of capture and handling, though levels typically stabilize 30 min to 3 h after capture. It is invasive and the animal may have to be restrained or anesthetized prior to collection. Plasma GCs have been used extensively to give insight into the animal's ecology, life history, and stress physiology (e.g. Crespi et al., 2013). Fecal cortisol/corticosterone metabolites (FCMs) are the metabolic products of plasma GCs formed by the liver, which are then deposited into the upper intestine where further metabolism can occur by bacterial enzymes before final excretion via the rectum. FCMs give an integrated measure of the stress axis and can be obtained either by observing the animal and collecting the feces when the animal defecates or by live-trapping the animal and collecting the feces in the trap. FCMs will not be affected immediately by the stress of capture and handling, as there is a time lag of several hours between metabolism by the liver and excretion from the body. Feces are thus easy to collect, but analysis also

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needs to be rigorous and validated. The measurement of FCMs is now widely used because collection is non-invasive (Millspaugh and Washburn, 2004; Touma and Palme, 2005; Goymann, 2012; Palme, 2019). However, there are many environmental factors that must be borne in mind when interpreting FCM differences among animals and populations.

A key evolutionary constraint of all herbivorous mammals is the complexity of their digestive system and their diet. However, in the vast literature using FCMs (approximately 2322 papers since 1990, Palme, 2019 – supplementary file updated October 2025), the digestive system is seldom discussed. It is a complicating factor that could affect FCM output, particularly when diet varies from optimal to suboptimal. Herbivorous mammals have evolved a number of strategies to extract energy, protein, fats, and nutrients, but their ability to do so is dependent on the complexity of their digestive system. Though they can readily obtain needed nutrients from cell contents, digesting the cell wall presents a problem. They do not have the enzymes to digest the fiber in plant cell walls, as these are composed of cellulose, hemicellulose, and lignin – the fiber found heavily in grasses (monocotyledons) (Hume, 2002). To digest fiber, they must rely on their gut microbiome and have evolved a commensal relationship with it. At one end of the spectrum are the foregut fermenters (ruminants) that are extremely efficient (~50 %) at digesting cell wall fiber from their grass diet (Clauss et al., 2010). At the other end are the hindgut fermenters (ranging from horses and pigs to lagomorphs and rodents) with lower efficiencies (~10–40 %) (Sakaguchi, 2003 – Table 1). Sciurids (squirrels) are at the extreme end of the hindgut fermenters, with a very specialized, simple digestive system, an inability (largely) to digest fiber in grass, and a brief retention time of forage in their gut (Hume, 1994). They have a very limited ability to selectively retain fine particles while excreting large fibrous ones (Hume et al., 1993). This limits what they can eat, where they can live, and their body size (< 5 kg – Demment and van Soest, 1985). Thus, they must rely on a diet that is highly digestible (70 % or more – Hume 1994) with the correct mixture of highly digestible nutritional components.

Here we focus on an herbivorous sciurid. How do we rigorously quantify stress axis function in a mammal with a very simple digestive system – the Columbian ground squirrel? To survive hibernation, it must ingest high amounts of forbs to secure the needed nutrients, especially polyunsaturated fats (Frank, 1994; Florant, 1998). We assessed whether two key measures of the stress axis function – free cortisol from plasma and FCMs from feces – tell the same story when their habitat differs markedly. Samples were collected from a natural meadow providing an optimal forage (high amounts of forbs) and an unnatural one providing suboptimal forage (primarily grasses with high fiber from a meadow previously seeded as a horse pasture). Fiber in the grasses may cause an underestimation of FCM levels by diluting metabolites in the feces causing more rapid gut passage rates because of poor digestion (Hume, 1994). We collected these samples at a critical time in the squirrel life cycle – in mid-summer – when they were nonreproductive and preparing for hibernation. The main plasma and fecal samples were collected independently from two groups of squirrels (plasma: Boonstra et al. ms in review; fecal: Bosson et al., 2009), as we only realized subsequently of the power and value of such a simultaneous comparison. Nevertheless, our evidence here gives fundamental insight into the impact on how these two matrices differ when ground squirrels live in an optimal versus a suboptimal habitat.

2. Methods

2.1. Study species

Columbian ground squirrels are medium sized (400–900 g) hibernating, long-lived (up to 10 years), colonial burrowing rodents (Elliot and Flinders, 1991). They live in the alpine and sub-alpine meadows of western North America. They are only active for 3–4 months during which they breed, produce one litter, and then forage intensively to accumulate sufficient fat reserves prior to initiating their 8–9 month hibernation (Dobson et al., 1992). Immergence occurs between 15 July and 25 August, with adult males entering their hibernaculum first, followed by adult females and nonbreeding yearlings in early August, and finally by juveniles in mid to late August. During this narrow time frame, the meadow vegetation is maturing and declining in quality.

2.2. Study area

Our study took place in July 2005 on two meadows (elevation 1550 m) in the Sheep River Wildlife Sanctuary Provincial Park 32 km west of Diamond Valley, Alberta, Canada (50°38′10″N, 114°39′56″W). Hayfield was a 72 ha grassland and Meadow B (MB) a 2.6 ha natural meadow, 2.8 km west of Hayfield. Hayfield was a horse pasture (partially fenced) used by forest rangers up until the late 1990s (B. Mayer, Alberta Forest History Association of Alberta, pers. comm.). In 1963, a sod airstrip was built on Hayfield, not used, and then planted to non-native grasses (*Phleum pratense, Poa pratensis,* and *Bromus inermis*) over the entire meadow. Hayfield was cut and baled each autumn. A portion was still fenced in 2005 when we did our study. Cattle grazing was prohibited for ~ 25 years until 1998 (Bennett, 1997; Ruckstuhl pers. comm.). Thereafter, cattle grazing was permitted on both meadows, but only for short times each summer as they were being moved up the valley to the summer cattle allotments (Mayer pers. comm.; Murie pers. comm.). In 2005, ~ 300 cattle grazed both meadows for ~ two weeks in summer (Hayfield 1–15 July; MB 1–16 August) and several days in October. Though forage biomass was significantly higher on Hayfield (~145 – 160 g/m^2) than on MB (~70 – 100 g/m^2) (Bennett 1999), grass constituted > 90 % of the biomass on Hayfield and forbs constituted ~ 8 % (some sites had no forbs). In contrast, forbs constituted ~ 30 % on MB.

2.3. Field sampling procedures

We collected fecal and blood samples in July 2005 from three groups living on the two meadows. In the first and second groups, fecal and blood samples were collected from separate squirrels from 6 to 26 July; in the third group, we collected both fecal and blood samples from the same squirrel within the same capture event on 27 July. All animals were nonreproductive. We set live-traps (14 x 14 x 40 cm^3, Tomahawk Co., WI, USA) baited with peanut butter near burrows of individuals of known age between 0700–1100 h. Squirrels were removed from traps, flushed into a pillow case, identified by tag numbers, and weighed.

In group one, fecal samples were collected from a select number of squirrels each morning. The stress of capture and handling would not have affected FCM levels as it takes them ~ 7h for levels to increase in the feces (Bosson et al., 2009) and our feces collection occurred within 1 h of capture. We inspected traps frequently (every 10 min to 1 h) and, on capture, immediately identified, sexed, and weighed all animals. Squirrels readily defecate when trapped. We collected 5 fecal boli from under traps (those not contaminated by urine), placed them in 1.5 ml vials, stored them on ice in the field, then at −20℃ in the field lab, and then at −80℃ after transport to the University of Toronto. Published evidence indicates that under these conditions, FCM levels remain extremely stable (see review and references in Palme 2019). To avoid the possibility of carry-over effects from a previous capture-related stress on FCMs, we excluded samples from squirrels that had been live-trapped within the prior week.

In group two, blood samples were obtained from designated squirrels trapped at their burrows in the early morning just as they were emerging from their burrows. We continued trapping (1–2 h) until we had collected 6–8 squirrels for that day. Fecal samples were not collected. Squirrels were taken to a field laboratory in their livetraps, placed in a cool, quiet location, covered with pillowcases, and left for at least 1 h to habituate before collecting the blood. One animal at a time was then removed from its trap, ear tag read, weighed, anesthetized within 15–30

s with isoflurane (IsoFlo, Abbott Laboratories, Saint Laurent, QC, Canada), and a suborbital 0.6 mL blood sample collected. The sample was centrifuged at 8800g for 5 min and the plasma collected and frozen at −20 °C at the field site, and then, after transport to the university, at −80 °C until analysis. Steroid levels are extremely stable when kept under these conditions (e.g. up to decades from the biomedical literature − see review and references in Sheriff et al. 2011).

In group three, we compared true baseline levels of plasma cortisol at capture to their FCMs at capture. We live-trapped squirrels of known age on 27 July. Traps were strategically placed at burrows that squirrels were seen to have entered. Traps were monitored continuously and, when a squirrel was captured, it was immediately removed from the trap, transferred to a pillowcase, anesthetized within 15–30 s with isoflurane, and a suborbital 0.6 mL blood sample obtained. Thus, samples were obtained within 3 min of capture. We then read the ear tag, weighed and sexed the squirrel, and put it back in the livetrap. Approximately ~ 15–30 min later, 5 fecal boli were collected. Samples were processed as above.

2.4. Quantification of Steroid levels

Feces were lyophilized for 18 h, frozen in liquid nitrogen, pulverized into a fine powder with a small grinding pestle, and 0.15 ± 0.01 g extracted with 5.0 mL 80 % methanol. FCM levels were analyzed using a 5α-pregnane-3β,11β, 21-triol-20-one enzyme immunoassay that had been validated for Columbian ground squirrels (Bosson et al., 2009). FCM levels were determined in the lab in the fall and winter of 2005–6.

We quantified the amount of fecal fiber in a subset of samples from both meadows (N = 28: 10 juveniles from Hayfield, 10 juveniles from MB, and 8 adults from MB) using a modified version of the micro digestion neutral detergent fiber technique (Holechek and Vavra, 1982). Fiber content was expressed as percent fiber/0.15 g dry fecal mass after accounting for the mass of the filter paper.

Free cortisol levels were measured using the radioimmunoassay methods described in Delehanty and Boonstra (2023). To estimate free cortisol concentrations (not bound to its carrier protein − corticosteroid binding globulin) we measured the maximum corticosteroid binding capacity (MCBC) in plasma. Cortisol and MCBC levels were determined in the lab in late 2005-early 2006. The equilibrium dissociation constant (± SE) for Columbian ground squirrels for calculating MCBC is 4.73 ± 0.29 nM.

2.5. Data analysis

Data are reported as means ± 1 SE. For the analysis of age-related changes of free cortisol and FCM levels on the two meadows, we used ANOVA analysis with squirrels grouped into the two meadows and one of 4 age classes: juveniles (young-of-the-year), sub-adults (pre-breeding females aged 1 and males aged 1 and 2), young adults (females aged 2–5; males aged 3–5), and old adults (females and males aged >5). Power analysis was performed if the differences were not clear. When comparing a subset of these (e.g. juveniles on the two meadows), we used t tests. As our fecal collection methods was similar for the periods from 6 to 26 July and 27 July, all these results were pooled. However, as blood collection methods differed between these two periods (after 1 h and within 3 min, respectively), two separate analyses were carried out. Normality of the data was determined with the K-S normality test. Homogeneity of variances was determined with Bartlett's test. All analyses were done with StatView (SAS Institute, Cary, NC, USA, 1998).

3. Results

As we obtained samples for both GC and FCM measures from both females and males, we first determined whether the data from the sexes could be pooled. There was neither a sex nor age effect on free cortisol levels (sex − $F_{1,133} = 0.05$, $P = 0.82$, *Power* = 0.06; age − $F_{3,133} = 1.65$, $P = 0.18$, *Power* = 0.31; interaction − $F_{3,133} = 1.92$, $P = 0.13$, *Power* = 0.48). The average free cortisol levels (ng/ml) in females and males were 133.5 ± 8.0 (N = 113) and 202.1 ± 32.0 (N = 28), respectively. There was no sex effect on the FCM levels, though there was an age effect (sex − $F_{1,136} = 0.35$, $P = 0.56$, *Power* = 0.09; age − $F_{3,136} = 8.03$, $P < 0.0001$, *Power* = 0.99; interaction − $F_{3,136} = 0.26$, $P = 0.86$, *Power* = 0.10). The average FCM levels (ng/g) in females and males were 402.9 ± 34.8 (N = 91) and 437.8 ± 39.9 (N = 54), respectively. Thus, for subsequent analyses, we combined data from both sexes for our corticosteroid measures.

The difference between the two meadows with respect to plasma free cortisol levels versus FCM levels was striking (Fig. 1). In plasma, there was a significant meadow effect, with squirrels on Hayfield having average free cortisol levels (ng/ml) 89 % higher (169.2 ± 11.8, N = 102) than those on MB (89.4 ± 6.7, N = 39), but there were no age or interaction effects (meadow − $F_{1,133} = 14.53$, $P = 0.0002$; age − $F_{3,133} = 0.45$, $P = 0.72$; interaction $F_{3,133} = 1.15$, $P = 0.33$).

In contrast, average FCM levels (ng/g) on Hayfield were 64 % lower (333.6 ± 28.2, N = 88) than those on MB (546.6 ± 46.7, N = 56). There was a meadow effect ($F_{1,136} = 8.56$, $P < 0.004$, *Power* = 0.84), an age effect ($F_{3,136} = 9.15$, $P < 0.0001$, *Power* = 1.00), but no interaction effect ($F_{3,136} = 0.16$, $P = 0.92$, *Power* = 0.08). The age effect occurred because juveniles had FCM levels (547.8 ± 42.7, N = 71) that were 89 % higher than the older age classes combined (yearlings, young adults, and old adults) (290.5 ng/g ± 24.0, N = 73). Juveniles from the two meadows differed significantly ($t_{70} = 2.47$, $P = 0.02$), with levels on MB being 202.2 ng/g higher than on Hayfield (664.5 ng/g ± 68.4, N = 30 vs 462.4 ng/g ± 51.0, N = 41). In the older age groups, the meadows also differed significantly, but the older age classes did not (meadow $F_{1,67} = 10.55$, $P < 0.002$, *Power* = 0.91; age $F_{3,67} = 1.71$, $P = 0.34$, *Power* = 0.34; interaction $F_{3,67} = 0.38$, $P = 0.69$, *Power* = 0.11). Thus, our two cortisol measures went in the opposite direction on these meadows: free plasma cortisol levels were lower on MB than on Hayfield, but FCM levels were higher on MB than on Hayfield.

The fecal fiber analysis indicated that MB squirrels had 15 % lower fiber content (33.75 % ± 2.33) than Hayfield squirrels (48.75 % ± 2.11) ($t_{26} = -4.36$, $P = 0.0002$). Fecal fiber levels from juveniles did not differ from older squirrels on MB ($t_{18} = -1.39$, $P = 0.18$).

In a sample of squirrels captured on 27 July from which we obtained both free cortisol plasma levels (within 3 min of capture) and FCM levels, no relationship was found between them ($r = 0.15$, $F_{1,15} = 0.32$, $P = 0.58$, Fig. 2).

4. Discussion

Fecal corticosteroid metabolites are often assumed to be a robust proxy for blood glucocorticoid levels and HPA function (e.g. Mateo and Cavigelli, 2005; Sheriff et al. 2010; Stead et al., 2024). However, our findings in Columbian ground squirrels challenge this assumption under conditions of varying diet quality. These two measures went in the opposite directions in these squirrels (Fig. 1): free cortisol levels were lower on the natural meadow (Meadow B) but FCM levels higher, whereas free cortisol levels were higher on the grass meadow (Hayfield), but FCM levels lower. Our measurement of fecal fiber content was lower on MB than on Hayfield. Thus, given that the plasma GC levels are the benchmark of the internal state of these squirrels, the FCM levels did not accurately reflect them. We postulate three potential explanations for our results.

First, perhaps the higher fiber in the diet in Hayfield squirrels resulted in a shorter gut retention time (i.e. they could not digest the higher grass fiber diet well) and hence lower FCM per unit mass of feces. In contrast, the lower fiber in diet of MB squirrels resulted in a longer retention time, and hence higher FCM per unit mass of feces. However, this explanation is unlikely given the simplicity of their intestines. Alternatively, the intestinal retention time was similar for squirrels on both meadows, but those on Hayfield produced many more feces per

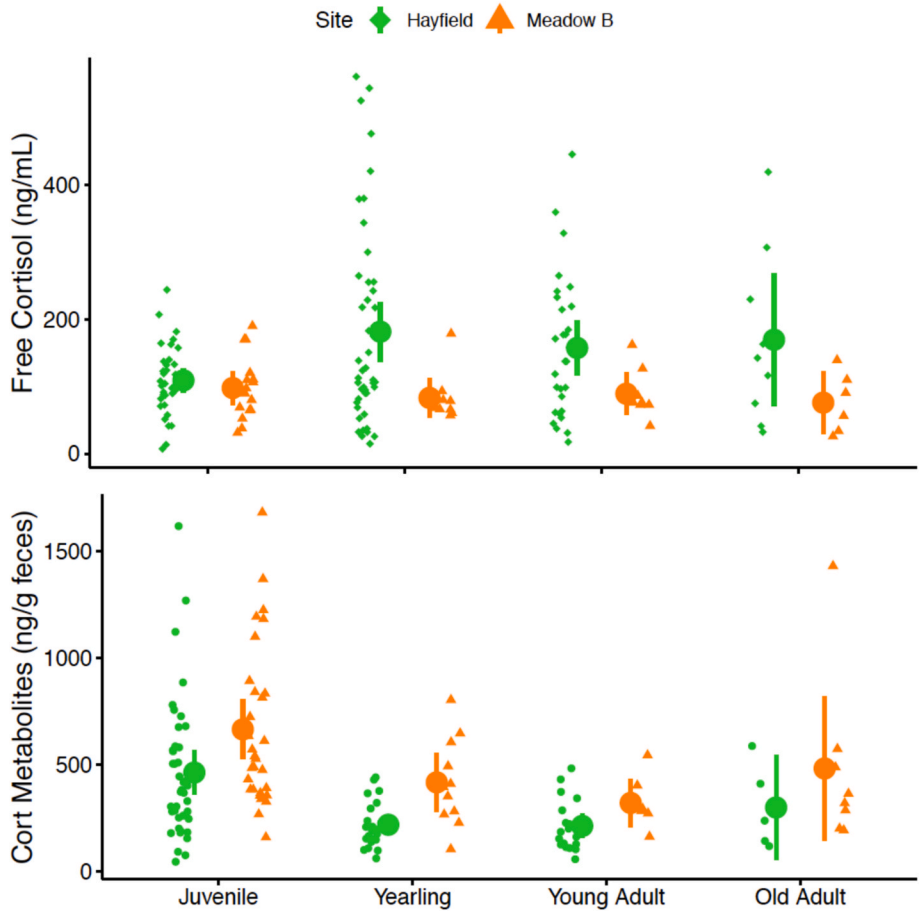

Fig. 1. The mean concentration, individual values and the 95% confidence intervals of two measures of endogenous cortisol levels – free cortisol from plasma and corticosteroid metabolites from feces – in Columbian ground squirrels from two meadows. Meadow B was a natural one that had an abundance of forbs and Hayfield was an unnatural one that had been seeded as a horse pasture and had an abundance of grasses. The sexes were pooled and ages separated into 4 classes: juveniles – born in the current year; yearlings – born previous year; young breeding adults – ages 2–5; and old breeding adults – ages 6–10. Sample sizes for Hayfield and Meadow B, respectively are: juveniles (26, 16), yearlings (43, 9), young adults (25, 8), and old adults (8, 6). Statistical differences are given in the text.

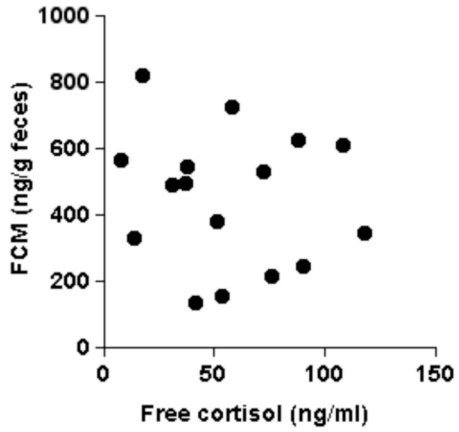

Fig. 2. Concentrations of free cortisol in plasma and of fecal corticosteroid metabolites (FCM) in feces obtained on the same Columbian ground squirrels captured on 27 July. Plasma samples were obtained within 3 min of capture and fecal boli within 15–30 min after capture. Sexes and age groups were pooled.

unit time from a larger volume of poorer quality ingested forage than did those on MB. Hence, if we had collected all the feces produced over a longer period of time (e.g. say 1 h) from both populations, weighed them, and calculated the total FCM produced, values on Hayfield would indeed have been higher and reflect higher plasma free cortisol levels.

Second, squirrels from the two meadows differ in their intestinal microbiome because of the different forage available. The microbiome is shaped by both the host and by environmental factors (Kolodny and Schulenburg 2020). The dynamic nature of the microbiome suggests that it could be a critical enabler of rapid acclimation to changes in the environment (Alberdi et al., 2016). Greater microbial digestion on higher quality forage on MB than on Hayfield could thus reduce both the volume and composition of the feces produced on MB, and hence lead to greater FCM levels on MB. Even in a natural system (the boreal forest and the snowshoe hare 9–11 year cycle), with regular seasonal changes (summer to winter) and long-term yearly fluctuations in population density and forage, the microbiome changed markedly (Stothart et al. 2025). Thus, it is critical to link the species' biology and demography to variation in habitat and vegetation quality, both of which will vary markedly within a year (e.g. peak of the growing season versus senescent vegetation at the end of it; and summer versus winter diets for mammals active year-around). In Columbian ground squirrels, it would give insight into their demography to assess microbiome differences among age groups and among populations living in different environments and if these are adaptive or non-adaptive.

Third, the plasma GC levels did not accurately reflect the internal state and condition of the squirrels. Thus, the problem was not with the FCM measurements, but with the GC measurements. However, we think this is unlikely as blood samples (in all but those obtained on 27 July) were collected over 1 h after capture on squirrels from both populations and levels tend to stabilize because of negative feedback to the pituitary

in many sciurids (e.g. Boonstra and McColl, 2000; Place and Kenagy, 2000), but not all (Delehanty and Boonstra 2012).

Our attempt to determine if a squirrel's true baseline level of free plasma cortisol at capture was concordant with their baseline level of FCM failed (Fig. 2). Part of this may be a sample size issue (ours was small), but it is likely more fundamental than that. The free cortisol signature would reflect the levels actually produced as the squirrels emerged from their burrows the first thing in the morning. However, given the circadian rhythm of the sleep-wake cycle in mammals (Oster et al., 2017), the FCM signature would reflect free cortisol broken down by the liver ~ 7 h previous (Bosson et al. 2009) – i.e. in the middle of the night (~1–3 am) when the squirrels were asleep in their burrows. Thus, in terms of interpreting either of these matrices relative to the environment of the study species, timing of collection is critical.

Would it have been better to conduct our study in a laboratory setting where all environmental aspects that might influence plasma GC and FCM levels could be controlled (i.e. diet, weather, social structure, age, etc.)? Possibly, but we do not think so. It would be extremely difficult to replicate key aspects of their natural environment during this critical time in their annual cycle – the short pre-hibernation period and, in females, eliminating the philopatry which is the basis of their social structure (Arnaud et al., 2012; Hare and Murie, 2007). It would also increase their lab-induced stress (Bosson et al. 2009). In addition, providing variation in forage quality (forbs vs grasses), with and without high amounts of polyunsaturated fats, would be a challenge. The alternative would be an intensive field study of these squirrels, documenting the forage available within each territory and what was actually eaten using microhistology, stable isotopes (Hobbie et al. 2017) and/or DNA metabarcoding techniques in their feces and/or hair (Ando et al. 2020), and simultaneously collecting plasma and feces for GC and FCM analysis.

The evidence in sciurids indicate that diet is critical to FCM levels (e.g. different types of seeds in the granivorous red squirrels – Dantzer et al., 2011). Because of their simple digestive tract, FCM levels in herbivorous sciurids may give counterintuitive results when diet is not optimal and thus their foraging environment must be considered in the interpretation of their stress levels (e.g. Mateo, 2007; Sheriff et al. 2012; Wey et al., 2015). In addition, foraging selectivity may be partially age dependent. Juveniles are highly philopatric and are forced to forage near the maternal burrow, independent of forage quality whereas older squirrels may forage further from their burrow and be selective to obtain the diet they need to build up fat reserves to survive hibernation.

5. Conclusions

In our study three factors came together in these squirrels to account for the differences we observed: the simplicity of their digestive system, the differences in the forage available on the two meadows, and the critical time in their life cycle – preparing for hibernation. Though these factors may play less of a role in the stress measures in other mammal species, they should be considered along with other ecological life history factors.

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CRediT authorship contribution statement.

RB and COB designed the study; RB funded it through his NSERC grant and carried out the data analysis; RB, JC, and COB carried out field work, data curation and lab analysis; all coauthors contributed to writing, reviewing and editing, and approved the final version of the manuscript.

CRediT authorship contribution statement

Rudy Boonstra: Writing – review & editing, Writing – original draft, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Joanne Castillo:** Writing – review & editing, Validation, Project administration, Methodology, Investigation, Data curation. **Tim J. Karels:** Writing – review & editing, Writing – original draft, Investigation, Data curation, Conceptualization. **F.Stephen Dobson:** Writing – review & editing, Writing – original draft, Validation, Investigation, Conceptualization. **Rupert Palme:** Writing – review & editing, Writing – original draft, Resources, Methodology. **Curtis O. Bosson:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Data curation, 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.

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