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Prey cortisol affects the usefulness of fecal glucocorticoid metabolite concentration as an indicator of stress in a carnivore

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Abstract: The non-invasive nature of sample collection makes analysis of fecal hormone concentrations useful for examining endocrine responses in free-living wild animals. Glucocorticoid hormones (i.e., cortisol and corticosterone) are frequently measured as an indicator of activation of the endocrine stress axis. However, many factors may influence glucocorticoid concentrations in feces, and the influence of prey glucocorticoids on concentrations in the feces of predators is rarely considered. We tested whether cortisol consumption influenced concentrations of glucocorticoid metabolites in feces of captive Arctic foxes (*Vulpes lagopus* L., 1758) by adding cortisol (5 mg cortisol per kg fox body mass) to the foxes' diet. Food supplemented with supraphysiological concentrations of cortisol increased fecal glucocorticoid metabolite concentrations of Arctic foxes by 97% in males and 51% in females, compared to controls. In addition, fecal metabolite concentrations in non-treatment samples were higher for females ($22.2 \pm 3.3 \text{ ng g}^{-1}$, mean $\pm$ SE) than males ($13.3 \pm 1.5 \text{ ng g}^{-1}$), suggesting female Arctic foxes may have higher baseline cortisol concentrations or females may be more sensitive to captivity or relocation. These results indicate that prey cortisol can influence measurement of glucocorticoid metabolites in carnivore feces and suggest caution may be needed when interpreting such measurements in wild carnivores.

Key words: stress, diet, Arctic fox, *Vulpes lagopus*, glucocorticoid, cortisol, fecal hormone analysis

Introduction

Organisms prepare for changes in their environment by storing energy, but unexpected events can require additional energy inputs. Such events provoke a stress response, increasing production of glucocorticoid (GC) hormones, which mobilize stored energy in order to improve the individual's chances of survival (Sapolsky 1982; Sapolsky et al. 2000; Boonstra 2004). Prolonged or repeated stress (i.e. chronic stress) can result in excess GC exposure, which can be maladaptive, reducing long-term survival or reproductive output (Sapolsky 1983; Boonstra et al. 1998; Breuner et al. 2008; Bonier et al. 2009; Boonstra 2013). Because of this relationship between the environment, GC concentrations, and an organism's fitness, hormone concentrations have become a popular metric in ecological studies.

Researchers have measured GC concentrations in a variety of tissues, including blood, saliva, hair, and urine (for review see Sheriff et al. 2011). Several studies have used measurements of glucocorticoid metabolites in feces to assess the impact of challenging events or conditions (i.e., stressors) on free-ranging individuals (Wasser et al. 1997; Millspaugh et al. 2001; Sands and Creel 2004; Cyr and Romero 2007; Dantzer et al. 2010). Fecal samples can be collected non-invasively, minimizing interactions between researchers and their study organisms that can cause stress and increase circulating levels of GCs (Kenagy and Place 2000; Fletcher and Boonstra 2006; Romero et al. 2008; Delehanty and Boonstra 2009). Fecal hormone concentrations integrate circulating concentrations over time and are less sensitive to acute fluctuations in hormone production (Palme et al. 1996; Möstl and Palme 2002; Goymann 2005; Palme et al. 2005), which may be advantageous for research on chronic stress. Nonetheless, researchers need to be cautious about the assumptions of fecal hormone techniques and confounding influences when interpreting results from these methods (Palme et al. 2005; Touma

and Palme 2005; Dantzer et al. 2011; Goymann 2012).

A fundamental assumption of fecal hormone analysis is that hormone concentrations in feces correlate to circulating levels in the blood and therefore reflect hormone synthesis and release (Miller et al. 1991; Cavigelli 1999; Mateo and Cavigelli 2005; Sheriff et al. 2010). Indeed, correlations between fecal and plasma hormone concentrations have been previously reported (Miller et al. 1991; Sheriff et al. 2010), but diet and metabolism may also influence this relationship (Goymann 2005; Dantzer et al. 2011). Metabolism can vary according to sex, age, or behaviour, and therefore the proportions of hormone metabolites excreted in urine and feces may differ among individuals or species (Taylor 1971; Palme et al. 1996; Bahr et al. 2000; Touma et al. 2003; Palme et al. 2005; Goymann 2012). A few studies have related increased levels of circulating GC hormones and excretion of GC hormones to oral administration of GC hormones, but the route of excretion (i.e. urine vs feces) varied between species (Heazelwood et al. 1984; Cooper et al. 1996; Andersson and Skakkebaek 1999; Brinkman et al. 2010).

Our study focuses on Arctic foxes (*Vulpes lagopus* L., 1758), which are known to consume Arctic rodents (e.g., lemmings and voles), which can have uncommonly high GC concentrations (Fletcher and Boonstra 2006; Romero et al. 2008). We aimed to determine whether GC measurements from Arctic fox feces are sensitive to ingested hormones. To examine whether GC concentrations in prey may affect the fecal glucocorticoid metabolite (FGM) concentrations of predators, we added cortisol (the primary GC in most mammals; Boonstra 2005) to the food of captive Arctic foxes and measured FGM concentrations before and after the treatment. We predicted that FGM concentrations would increase due to the cortisol consumption and that we would be able to detect increased FGM concentrations due to the effect of cortisol consumption.

Methods

Between 11-Nov-2010 and 1-Jan-2011, we collected fecal samples from subadult Arctic foxes that had been born in captivity during the spring of 2010 at the Assiniboine Park Zoo in Winnipeg, Manitoba, Canada. All procedures were approved by the University of Manitoba Animal Care Committee (protocol F10-06). The 12 foxes (seven female and five male) were transferred from their natal exhibits to a temporary holding facility two weeks before we began collecting samples. Siblings were housed together in two units (one per family group) that had a semi-sheltered outdoor enclosure (3.4 m x 2.4 m) and a small heated indoor enclosure (2.4 m x 0.9 m). Individuals were marked on the shoulder or back with unique dye patterns so that we could attribute fecal samples to individuals (Table 1). We collected fecal samples between 8:00 am and 4:00 pm, immediately following defecation, and stored them in a -20°C freezer to prevent degradation. Fecal samples found outside this collection window were not used because they could not be linked to specific individuals.

Zoo staff provided the foxes with daily meals of rodents, chickens, and eggs. We injected 18.0 mg of cortisol dissolved in butter into frozen baby chicks. The amount of cortisol was chosen based on the upper limit of veterinary recommendations for canines (5 mg cortisol per kg fox body mass), which we chose so we would be better able to illustrate the concept, with the intention that more realistic dosages could be considered in future studies. We calculated the treatment amount based on a 3.6 kg fox (Table 1). Treated food was given to individual foxes on four occasions, separated by at least one week. The small chicks were easily consumed, allowing us to control portions for meals that included cortisol treatments. Individual portions were given to each fox and the foxes were observed until the treated food was consumed in full. We

assumed that the entire dose was delivered once the chick was fully consumed.

The gut passage time for Arctic foxes is highly variable. The mean passage time for seeds in Arctic foxes has been estimated to be between 16.2 and 25.5 hours (Graae et al. 2004). Caged Arctic foxes have been shown to exhibit higher variability in defecation patterns, with passage times typically ranging between 9 and 27 hours (Szuman and Skrzydlewski 1962). In our study, cortisol-treated food was given to the foxes in mid-afternoon (~3:00 pm) and we expected to see higher FGM concentrations on the following day. We compared treated samples with baseline samples collected before the cortisol treatment or multiple days after the treatment. Some individuals did not defecate during our sample collection times and therefore we were only able to measure treatment effects in four male and four female Arctic foxes for which we had both treatment and control samples.

We used radioimmunoassay (RIA) to measure FGM concentrations, based on previously described methods and validation of that assay on measurement of FGMs in Richardson's ground squirrels, *Urocitellus richardsonii* (Sabine, 1822) (Ryan et al. 2012; Hare et al. 2014). Briefly, 0.2g of dried fecal matter was extracted for measurement of FGMs in each sample using ethanol (Ryan et al., 2012). The ethanol was evaporated in a vacuum centrifuge and the extracted hormone was reconstituted in RIA buffer (10 ml phosphate buffer, 90 ml Milli-Q, 0.9 g NaCl, and 0.5 g bovine serum albumin). Extraction efficiency was measured by extracting 10 samples from a pool of fecal matter with 0.2 ng of radioactively-labeled cortisol (1,777 dpm). Samples provided good parallelism with the standard curve (data not shown), extraction efficiency of cortisol from fecal samples was $73.7\% \pm 3.7\%$ (mean $\pm$ SEM), intra-assay variation was 5.8% and inter-assay variation was 17.6%.

To measure FGM concentrations, we combined 100 μ l of reconstituted cortisol with 100

μl of tritiated cortisol (PerkinElmer, Waltham, Massachusetts; 5000 disintegrations minute^{-1}) and 100 μl of cortisol antibody (Fitzgerald Industries, Acton, Massachusetts; 1:3200 dilution). The antibody used was identical to that previously validated for the measurement of FGMs in Richardson's ground squirrels (Hare et al. 2014). It was not possible to conduct similar validation techniques in the present study because the animals used were still considered part of an exhibit in the zoo. Samples were incubated for 1 hour at room temperature and then overnight at 4 °C. We then added 100 μl of dextran-coated charcoal (0.25 g dextran, 2.5 g charcoal, and 50 ml RIA buffer) to terminate the assay. Samples were centrifuged (30 minutes, 2500 x g, 4 °C) and the resulting supernatant was decanted into a scintillation vial and 4ml of scintillation cocktail was added (Ultima Gold, PerkinElmer, Waltham, Massachusetts). Radioactivity was measured on a liquid scintillation counter (LS6500; Beckman Coulter, Brea, CA). FGM concentrations were interpolated from known standards measured with each assay. All chemicals were purchased from Sigma Aldrich (Mississauga, ON, Canada) unless otherwise stated.

Data were analysed in Program R (R Development Core Team, 2009). FGM concentrations were log-transformed to achieve normality; normality and homogeneity of variance were confirmed using visual inspection of histograms and residual plots. We tested whether FGM concentrations were related to time of defecation using linear regression. We tested whether FGM concentrations were affected by sex using a linear mixed-model, considering individual and Julian date covariates, which we compared to a null model using a likelihood ratio test. We used a linear mixed-model to test for an increase in FGM the day after cortisol treatment, considering both treatment and sex as fixed effects and date and individual as random effects (no interaction was found between treatment and sex). We used Markov-Chain Monte Carlo (MCMC) sampling (10,000 simulations) to estimate *P*-values of fixed effects (Rosa

et al. 2003; Lele et al. 2007). We also used a likelihood ratio test to compare the mixed-model to a null model that included only random effects.

Results

FGM concentrations were not related to the time between feeding (3:00 pm) and defecation ($F_{1,94} = 0.564$; $P = 0.45$). FGM concentrations were higher for females ($22.2 \pm 3.3 \text{ ng g}^{-1}$, mean $\pm$ SE) than for males ($13.0 \pm 1.5 \text{ ng g}^{-1}$; Fig. 1), excluding samples collected the day following a cortisol treatment. The mixed-effect model using sex as an explanatory variable performed better than the null model (Likelihood ratio test; $t_1 = 6.060$; $P = 0.0045$). FGM concentrations were higher in samples collected the day after cortisol consumption (Fig. 1; $t_{85} = 2.797$, $P = 0.013$) by an average of 97% for males and 51% for females. The linear mixed-effect model that included treatment effects performed better than the null model (Likelihood ratio test; $t_2 = 12.600$; $P = 0.0002$).

Discussion

The use of fecal hormone analysis to examine the physiological status of captive and free-living organisms has increased dramatically in recent years, reflecting the widespread and growing interest in this technique for monitoring the response of both captive and free-living populations to potential environmental stressors. Fecal hormone analysis is ideal for some studies because fecal samples can be collected non-invasively and relatively inexpensively. Furthermore, fecal hormone concentrations reflect the average circulating plasma hormone concentration over a longer period of time compared to markers such as saliva or plasma, making fecal concentrations an indicator of stress that is less sensitive to acute fluctuations in circulating

concentrations (Sheriff et al. 2011).

Several confounding influences on hormone concentrations may affect how an individual metabolizes and excretes glucocorticoid hormones, including age, sex, and diet (Goymann 2012). Our results indicate that dietary factors, not directly related to metabolism, can influence FGM concentrations in Arctic foxes. Such dietary sources of hormones might confound studies using fecal hormone concentrations to reflect stress physiology of organisms, particularly those examining hormone concentrations in carnivores that consume prey with high hormone concentrations. Dietary sources of hormones may also transfer across the gut and enter circulation (Heazelwood et al 1984; Cooper et al. 1996; von der Ohe et al. 2004; Brinkman et al. 2010), thus it is possible that consumed glucocorticoids may play an integral role in the regulation of endogenous production of glucocorticoids. To answer these questions would require repeated blood sampling following ingestion of a meal, which itself would likely influence GC release in the consumer. Nonetheless, use of labelled GCs in the diet of a carnivore could be used in clearance studies to examine partitioning of consumed GCs.

Some prey species can produce high concentrations of GCs. Plasma cortisol concentrations in snowshoe hare (*Lepus americanus* (Erxleben, 1777)) can increase from 38.6 ng ml⁻¹ (on average) to over 200 ng ml⁻¹ in response to stress (Boonstra et al. 1998). Plasma corticosterone concentrations can exceed 700 ng ml⁻¹ in meadow voles (*Microtus pennsylvanicus* (Ord, 1815); Fletcher and Boonstra 2006) and reach 3000-4000 ng ml⁻¹ in female brown lemmings (*Lemmus trimucronatus* (Richardson, 1825); Romero et al. 2008). Caribou (*Rangifer tarandus* L., 1758) have lower circulating cortisol concentrations (8-25 ng ml⁻¹) but a larger volume of blood and therefore a high total quantity of circulating cortisol (Ashley et al. 2011). Predators that consume these prey types in the wild may be exposed to high doses of ingested

GC hormones. If these consumed hormones enter the circulation, the increased concentration of GC hormones could affect the consumer by diverting energy, as if the consumer was experiencing a stress response (Heazelwood et al. 1984; Cooper et al. 1996; Andersson and Skakkebaek 1999; Brinkman et al. 2010).

Based on our results, predators that consume prey with high GC concentrations could exhibit elevated FGM concentrations, regardless of the stress levels experienced by individual predators or the health status of the predator population. Arctic foxes eat lemmings in high quantities when they are abundant (Angerbjörn et al. 1994; Roth 2002; McDonald et al. 2017) and presumably ingest hormones present in the lemming tissues. Arctic foxes likely experience elevated corticosterone concentrations related to lemming consumption, which could increase the overall concentration of GC hormones in the bloodstream and may affect Arctic fox reproduction or survival. The influence of prey GC levels on predator FGM concentrations would also depend on the type of GC being consumed. Arctic foxes may ingest substantial quantities of corticosterone in lemmings and voles, but these ingested hormones may not affect fecal measurements of cortisol metabolites. Perhaps a better real-world scenario would include avian predators that consume lemmings and voles, since both the predator and the prey produce the same type of GC hormone.

We found differences in FGM concentrations between captive male and female Arctic foxes, caused potentially by sex-related difference in baseline production of cortisol, metabolism, or excretion (Palme et al. 1996; Touma et al. 2003; Palme et al. 2005). Males and females may also respond differently to environmental stressors such as relocation or confined space, which have been shown to cause stress (Morton et al. 1995; Watson et al. 2005; Davenport et al. 2006). Although we previously found no differences in cortisol concentrations between free-living male

and female Arctic foxes (McDonald 2014), additional stressors in the natural environment may mask sex differences in baseline hormone concentrations.

In summary, cortisol additions to the food of both male and female Arctic foxes caused increased concentrations of glucocorticoid metabolites in feces, suggesting dietary hormone concentrations should be considered when evaluating the response of wildlife to potential stressors. We hope that future studies will consider these influences when studying predators in their natural environment, especially when examining FGM in predators that consume prey with substantial GC concentrations. We have shown that estimating predator fecal GC concentrations without consideration of prey GC levels could potentially lead to erroneous conclusions and inaccurate discussion of the stress status of wild carnivores, particularly those whose prey are known to vary in GC concentrations between and within species.

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Figure Caption

Fig. 1 Fecal glucocorticoid metabolite (FGM) concentrations (untransformed mean $\pm$ SE) of captive Arctic foxes (4 male and 4 female) before (control) and after cortisol consumption (treatment). This figure represents the eight foxes with cortisol treatment measurements; multiple measurements were made for every individual. These results are from 45 control and 9 treatment samples for males, and 22 control and 11 treatment samples for females (sometimes several samples were collected from a single individual on a single day). Day and individual were included as random effects to control for repeated measurements.

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Table 1. Individual profiles and the number of samples collected for each of the 14 captive juvenile Arctic foxes (*Vulpes lagopus*).

Dye Pattern	Sex	Mass (kg)	# Control	# Treatment	# Total
Green Back	M	3.9	15	2	17
Yellow Shoulder	M	4.1	14	1	15
Blue Back	M	3.9	8	4	12
Red Shoulder/Red Back	M	3.9	8	2	10
Blue Shoulder	M	3.9	3	0	3
Black Shoulder/Black Back	F	4.0	8	5	13
Red Shoulder/Green Back	F	3.5	7	2	9
Green Shoulder	F	3.6	9	0	9
Red Back	F	3.7	9	0	9
Black Shoulder	F	3.7	8	0	8
Red Shoulder	F	3.7	4	2	6
Green Shoulder/Green Back	F	3.7	3	2	5
Blue Shoulder/Blue Back	F	3.4	0	0	0
Yellow Shoulder/Yellow Back	F	3.3	0	0	0

Note: Only individuals that provided treatment samples, allowing for within-individual comparison, were included in the analyses of treatment effects.

Fig. 1 Fecal glucocorticoid metabolite (FGM) concentrations (untransformed mean $\pm$ SE) of captive Arctic foxes (4 male and 4 female) before (control) and after cortisol consumption (treatment). This figure represents the eight foxes with cortisol treatment measurements; multiple measurements were made for every individual. These results are from 45 control and 9 treatment samples for males, and 22 control and 11 treatment samples for females (sometimes several samples were collected from a single individual on a single day). Day and individual were included as random effects to control for repeated measurements.

60x42mm (300 x 300 DPI)