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Faecal metabolites and hair cortisol as biological markers of HPA-axis activity in the Rocky mountain goat

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ABSTRACT

Monitoring glucocorticoids in faeces and hair is increasingly used in ecological studies and provides a powerful and minimally intrusive mean to identify physiological challenges faced by wild animals. Using a cortisol and a corticosterone immunoassays, we conducted an adrenocorticotropic (ACTH) challenge with five weekly repeated injections to validate the use of faecal glucocorticoid metabolites and hair cortisol concentration as biological markers of the HPA-axis activity in captive mountain goats (*Oreamnos americanus*). We also investigated the effect of endogenous (age, sex, reproductive status) and methodological (faecal sample collection date, freezing delay and hair type) variables on cortisol values using faecal and hair samples collected from marked wild mountain goats during a long-term study. The cortisol enzyme immunoassay was reliable for mountain goat faeces and hair, and was sensitive enough to detect a clear rise in glucocorticoid concentration following ACTH injections for both matrices. Age and sex had no detectable effect on faecal glucocorticoid metabolites, but hair cortisol concentration was higher in kids and yearlings than in older goats, and lower in adult males compared to adult females. Reproductive status had no detectable effect on both faecal and hair measurements. Faecal metabolite concentrations increased with sample collection date in late spring until mid-summer and decreased afterward until early fall. Guard hair had nearly twice as much cortisol per gram as undercoat hair. Prolonged delay to freezing reduced the concentration of faecal glucocorticoid metabolites, but degradation seemed limited when samples were exposed to wind and sun or when ambient temperature was low. We conclude that faeces and hair can be used as valid biomarkers of the HPA-axis activity in mountain goat provided that confounding variables are taken into account when interpreting measurements.

1. Introduction

One of the primary physiological processes helping vertebrates cope with challenges, such as seasonality and various forms of physiological disturbances, is the stimulation of the hypothalamic-pituitary-axis (HPA) leading to the release of glucocorticoid hormones (Landys et al., 2006; Reeder and Kramer, 2005). Glucocorticoids (GCs; corticosterone/cortisol) are metabolic hormones involved in the maintenance of energetic balance that also act as key components of the acute stress response (Landys et al., 2006; Sapolsky et al., 2000). At baseline, GCs are drivers of the circadian rhythm and vary within the year to help animals meet seasonal needs (Dallman et al., 1993; Landys et al., 2006; Romero, 2002). Peak concentrations of GCs are reached as part of acute stress responses and redirect energy from long term functions towards functions required to rapidly cope with the stressor (Hawlena and Schmitz, 2010). While useful to avoid short term threats, prolonged periods of high glucocorticoid concentration may alter immune function, reproduction, and reduce survival (Wingfield and Sapolsky, 2003). Both baseline and stress-induced stimulation of the HPA axis are therefore informative of the life-history and environmental challenges faced by individuals (Landys et al., 2006; Reeder and Kramer, 2005).

Glucocorticoid concentrations have traditionally been measured in blood serum or plasma, but pulsatile fluctuations (Windle et al., 1998) and capture/restraint increase of GC concentration in the blood (Müller et al., 2006; Widmaier and Kunz, 1993) have prompted the development of techniques using alternative biological matrices such as faeces and hair. Glucocorticoid metabolite levels in faeces provide a short-term integrated measure of the HPA axis activity during gut passage time (Palme, 2005; Palme et al., 1996). The types of metabolites formed, as well as the routes and time course of excretion can, however,

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differ greatly among species, sex or age-classes (Palme et al., 2005; Touma et al., 2003). Variation according to species, sex, and age must therefore be investigated before using faecal metabolites as an indicator of an animal's physiology. Hair, on the other hand, has the potential to record several months of glucocorticoid response. While not fully understood, the incorporation of hormones into hair is thought to occur during the period of active growth (i.e. anagen) when the hair bulb is closely associated with the capillary system surrounding the follicles (Harkey, 1993; Pragst and Balikova, 2006), albeit follicular cells have been shown to locally produce cortisol that does not necessarily reflect systemic concentrations (Ito et al., 2005). Several studies comparing traditionally used substrates (e.g. blood, faeces, saliva) to hair or examining the impact of stress-related conditions on hair cortisol concentration in both animal and human models provided evidence that hair is a suitable matrix for glucocorticoid analysis (Accorsi et al., 2008; Carlitz et al., 2014; González-de-la-Vara et al., 2011; Short et al., 2016; Tallo-Parra et al., 2015; Van Uum et al., 2008). Nevertheless, very low recovery of radio-labelled cortisol was reported in the hair of guinea pigs *(Cavia aperea f. porcellus)* (Keckeis et al., 2012) and non-systemic factors partly explained hair cortisol concentrations in brown bear *(Ursus arctos)* (Cattet et al., 2014). Further studies improving our understanding of hair as an indicator of HPA axis activity are thus needed (Cattet et al., 2017; Russell et al., 2012).

Glucocorticoid measurements using alternative biological matrices are increasingly used in ecological studies and can be especially useful for wild animal populations that are sensitive to invasive sampling (Buchanan and Goldsmith, 2004; Kersey and Dehnhard, 2014; Sheriff et al., 2011). These alternative methods are, however, only suitable if native or metabolized glucocorticoid concentrations within the biological matrix reflect the physiological state of the animal (Sheriff et al., 2011). This assumption must be validated for each new matrix and species using procedures such as an adrenocorticotropic hormone (ACTH) challenge (Buchanan and Goldsmith, 2004; Sheriff et al., 2011; Touma and Palme, 2005). With appropriate sample collection, faecal validations in captivity are usually successful and the expected peak in glucocorticoid metabolites is often detected several hours after the injection. Hair validation is especially difficult because the extensive integration time of this matrix makes it unlikely to reflect a single injection (Ashley et al., 2011). To our knowledge, a successful hair validation has not been published for non-domestic ungulates (but, see González-de-la-Vara et al. (2011) and Ashley et al. (2011) for attempts on cattle and caribou, respectively).

The mountain goat (*Oreamnos americanus*) is an alpine ungulate especially sensitive to anthropogenic disturbance (Côté, 1996; Côté et al., 2013; Richard and Côté, 2016; White and Gregovich, 2017). Adverse effects of capture and chemical immobilization have been reported for this species, which exhibited life-history consequences following chemical immobilization such as increased risk of kid abandonment and decreased kid production in young females (Côté et al., 1998). As with most mammals, cortisol is the dominant glucocorticoid in this species (Koren et al., 2012). Our objective was to validate the use of faecal glucocorticoid metabolite (FGM) and hair cortisol (HCC) concentrations as biomarkers of the HPA activity of the Rocky Mountain goat. As such, we performed an ACTH challenge on captive animals using five weekly repeated injections. The effects of age, sex and reproductive status on glucocorticoid measurements were also assessed using faecal and hair samples collected from marked wild individuals during a long-term study. The influence of sample collection date (for faeces) and hair type (guard or undercoat) were also evaluated. Finally, we conducted a faecal degradation experiment to evaluate the impact of the time-lag between excretion and sample freezing on FGM measurements in alpine habitats.

2. Materials and methods

2.1. Animals

The ACTH challenge was conducted on captive mountain goats housed at the Calgary Zoo (Alberta). One adult male and 2 adult females were available for the faecal ACTH challenge. The same animals and a yearling female were used for the hair ACTH challenge. Both adult females had kids (~6 months old). Goats were fed a high-quality diet composed of a supplemented pelleted formula and mixed hay with *ad libidum* access to water. The habitat was divided into a large enclosure for exhibit, 2 pens where animals could be isolated, and 1 metal and polyethylene panelled restraint chute allowing handling without sedation. Goats were habituated to keepers entering the habitat on a daily basis and had been previously handled through the restraint chute.

2.2. Faecal ACTH challenge

The faecal ACTH challenge was conducted in late November 2017. Animals were used as their own control because of the low number of goats available. Females were isolated and challenged 1 day apart so that kids would not be left alone. The experiment was conducted as follows: 1) One day before injection, we collected a baseline sample from every adult by entering the pen for less than half an hour and collecting spontaneously voided samples. The goats did not show signs of arousal such as quick movements or adopting a threatening position. 2) On the day of injection, goats were isolated into the restraint chute and we collected a second baseline sample either via spontaneous defecation or directly from the rectum. 3) Goats were administered 50 IU Synacthen Depot by intramuscular injection and kept isolated for a minimum of 20 h. Based on studies in other ungulates, we expected faecal glucocorticoid metabolites to peak 12 to 20 h post-injection of ACTH (Ganswindt et al., 2012; Gross et al., 1996; Kleinsasser et al., 2010; Miller et al., 1991; Spaan et al., 2017). We did not have access to the zoo facilities overnight. Therefore, we scheduled injections at 17:30 in order to produce a peak in glucocorticoid metabolites during the day, when we could precisely record excretion time. 4) We sampled all faeces voided after injection by collecting 10–20 pellets without visible urine contamination. First post-injection sample collection occurred at 07:30 (14 h post-injection). We then visited the enclosures every 2 h until the animals were released. For overnight samples collected in the morning or when more than one sample was excreted between two visits, we determined relative time of excretion based on the faeces physical characteristics (i.e. hot and moist samples considered more recent than dry and cold ones). Females were released 20 h and 24 h after injection according to the availability of the keepers. As a result of the unexpected low defecation rate in the adult male (only 1 post-injection sample after 20 h), we maintained isolation for an additional 19 h. Number of collected samples and post-injection isolation time were respectively 8 samples in 20 h for the first female, 14 samples in 24 h for the second female and 6 samples in 39 h for the male. 5) We obtained group composite samples 5 and 6 days after the last injection by collecting overnight faeces in the pen where all animals were kept. Following the injection of the first female, we also opportunistically collected overnight samples from the pen where the other female (pre-injection) and 3 kids were kept. These group composite samples and all pre-injection samples were used to establish a baseline range of FGM concentration. All samples were stored at −20 °C immediately after collection. The timetable of the experiment is in Appendix 1.

2.3. Hair ACTH challenge

The hair ACTH challenge was conducted from August to September 2016 during the period of active hair growth (Côté and Festa-bianchet, 2003; Holroyd, 1967). Goats were guided and restrained in the chute. A

small patch (3 cm × 3 cm) of rump hair was completely shaved using electric clippers and stored for analysis. Goats were administered 25 IU Synacthen Depot by intramuscular injection. Animals were then released in the enclosure and injections were repeated every 7 days for a total of 5 injections over 35 days. One week after the last injection, regrown hair was collected via shaving from the same patch initially shaved. Hair samples were stored in envelopes at room temperature until analysis.

2.4. Age, sex, reproductive status, date and hair type differences

We used 840 faecal samples and 580 hair samples (511 guard; 79 undercoat) collected on known individuals from 1990 to 2016 from a wild mountain goat population studied at Caw Ridge (54° 03′ N, 119° 23′ W), west-central Alberta, Canada. All goats included in the study were marked as juveniles and closely monitored every summer. Exact age, sex and reproductive status of each individual were therefore known for all samples. Details on the Caw Ridge study area and population are published elsewhere (Côté and Festa-Bianchet, 2001a, 2001b; Hamel et al., 2011).

All hair samples and most faecal samples (n = 537) were collected upon capture. Hair samples were plucked from the rump and faeces were collected directly from the rectum of the goat. Both were frozen immediately after handling. Some faecal samples (n = 303) were also collected opportunistically in the field from 2013 to 2016. We used a 50× camera mounted on a tripod to record the exact location and identity of individuals defecating and retrieved samples once the group had left (usually less than 30 min after excretion). Opportunistic samples were frozen within 2–6 h post-excretion (mean freezing delay ± 95%CI of 175 samples for which defecation time was recorded in 2016 was 3.2 ± 0.3 h). All faecal samples were stored in sterile individual bags and kept frozen in the dark until analysis to prevent any effect of UV light or changes in storage environment (e.g. humidity and temperature) on GC concentration. While other studies have shown that measurements in hair are stable even after several months or years at room temperature (Macbeth et al., 2010, 2012; Yamanashi et al 2016), hair samples were kept in the same storage condition as the faecal samples as an added precaution.

2.5. FGM degradation experiment

To assess the stability of FGM in alpine environments, we collected 5 faecal samples immediately after excretion. Each fresh sample was divided into 7 subsamples containing 6 pellets and treated as follows: one was immediately frozen (control), 3 were left outside on the ground exposed to wind and sun (sun group), and 3 were left outside placed in individual paper bags within a backpack in the shade (bag group). One subsample from each treatment group was later frozen after 4, 8 and 21 h of exposure. The percent change in FGM concentration was calculated as the ratio between experimental subsamples and the associated control subsample. The experiment was conducted from 29 July 2017 at 11 h 19 to 1 August 2017 at 8 h 19. Shade temperature ranged from 14.6 °C to 6.1 °C and the sky was clear. We also measured temperature at 0, 4, 8 and 21 h and report it along with the results.

2.6. Glucocorticoid extraction

Faecal samples collected in the wild were freeze-dried and crushed into powder to control for potential variability in water content associated with changing environmental conditions. The ACTH samples were collected from animals housed in a zoo under controlled conditions and were therefore extracted immediately post-thaw. Measurements from 'wet' and 'dry' samples are both valid and usually highly correlated, but absolute values vary because dried samples are more concentrated (Palme et al., 2013). To extract steroid hormones, we homogenised the samples and added 5 ml of 80% methanol to 0.20 ± 0.01 g of dry faeces or 0.50 ± 0.01 g of wet faeces. Immersed samples were left on a rotator plate overnight. After 22 h, we centrifuged the samples to precipitate faecal material, transferred the supernatant into clean vials and stored them at −20 °C until analysis. Prior to quantification by enzyme immunoassay (EIA), extracts were warmed to room temperature and diluted 1:20 in assay buffer (0.1 mM sodium phosphate buffer, pH 7.0, containing 9 g of NaCl and 1 g of bovine serum albumin per litre).

Hair extractions were conducted in a laboratory and with tools that were never used for faecal or blood analyses. Hair samples were thoroughly examined for visible traces or smell of urine, faeces or blood and discarded if any signs of contamination were detected. Unstained samples were then sorted according to hair type and cut at their base to remove follicles. We cut hair in 5 mm pieces into pre-weighed 7 ml scintillation vials to obtain a sample mass (Mastromonaco et al., 2014). To avoid non-visible contamination with biological fluids, we washed all samples by vortexing them for 10 s in 100% methanol and immediately removing all methanol with a pipettor (Mastromonaco et al., 2014). This wash procedure was designed to prevent any surface contamination without starting the extraction of GCs from the hair shaft. Immediately thereafter, we extracted steroid hormones by adding 80% methanol at a ratio of 0.01 g of hair/ml of methanol, vortexing the vials for 5 s and leaving them on a plate shaker for 24 h (MBI Orbital Shaker; Montreal Biotechnologies Inc., Montreal, QC, Canada). We then centrifuged the samples at 2,400g during 10 min and pipetted the supernatant into new vials. Finally, extracts were air dried in a fume hood and stored at −20 °C until analysis. Prior to quantification by EIA, dried extracts were warmed to room temperature and reconstituted in assay buffer for one hour with vortexing at the beginning and end of reconstitution. Due to the low GC concentration present in hair, dried extracts were reconstituted as a 10-fold concentration of the original extracts.

2.7. HPLC analysis

Prior to immunoassay, characterization of steroid metabolites in the faecal extracts was done using reverse phase high performance liquid chromatography (HPLC). Preparation of the samples for HPLC was done by evaporating 25 ml of faecal extracts from a pool of 5 samples. The preparation was then purified by solid-phase extraction using Sep-Pak C_{18} cartridges (Waters Scientific, Mississauga, ON, Canada) and analysed using a Waters Alliance 2695 HPLC separation system as previously described by Kummrow et al. (2011). Cortisol and corticosterone (Steraloids Inc.) were run as reference standards. Absorption was recorded at 254 nm. Eluent fractions were collected every minute for 20 min and then assayed with both cortisol and corticosterone EIA to determine immunoreactivity. Based on immunoreactivity peak, we selected the cortisol EIA for all further analyses (see results).

2.8. Immunoassay

FGM and HCC were assayed by EIA using the cortisol antiserum R4866 (raised against cortisol-3CMO-BSA; C. Munro, University of California, Davis, CA, USA) and cortisol-HRP conjugate (C. Munro, University of California, Davis, CA, USA) following the method previously described by Majchrzak et al. (2015). In brief, microtitre plates (Nunc maxisorp, VWR, Mississauga, ON, Canada) were coated with cortisol antiserum diluted 1:12,000 in coating buffer overnight at 4 °C. Faecal extracts were diluted 1:20 in EIA buffer and evaporated hair extracts were reconstituted in EIA buffer at a 10X concentration. Samples, cortisol standards (78–20,000 pg/ml; Sigma H-0135), and controls were loaded along with horseradish peroxidase conjugate (diluted 1:34,000 in EIA buffer) onto the plate and incubated for 2 h at room temperature. The substrate solution (ABTS) was then added and absorbance was measured at 405 nm using a spectrophotometer (MRX microplate reader, Dynex Technologies, Chantilly, VA, USA). The cross-

reactivities of the cortisol antiserum were: cortisol, 100%; prednisolone, 9.9%; prednisone, 6.3%; cortisone, 5%; corticosterone, 0.7%; 21-deoxycortisone, 0.5%; deoxycorticosterone, 0.3%; other, <0.3% (Young et al., 2001). Assay sensitivity was 41.8 pg/mL. Biochemical validation (parallelism and recovery) of the cortisol EIA was done using pooled faecal and hair extracts. Inter-assay CV's were <15% and intra-assay CV's were <6% for both assays and sample matrices.

2.9. Statistical analysis

We defined the baseline range for the faecal ACTH challenge as the 95% confidence interval (hereafter CI) of average FGM concentration using all samples collected prior to and over 5 days after injection. FGM concentrations outside this baseline range were considered elevated. We compared the highest FGM concentration of each injected animal with the mean baseline concentration using a one-sided *t*-test. We also report the relative increase between pre-injection and maximum concentrations. For the hair ACTH challenge, we conducted a one-sided paired *t*-test to compare pre- to post- injection HCC while accounting for individual variation. Relative increase in HCC are also reported.

We assessed the impact of collection date on FGM and hair type on HCC using samples collected from the wild population of Caw Ridge. For faecal samples, we compared a set of candidate linear mixed models using Akaike information criterion (AIC_c) (Burnham and Anderson, 2002). The base model included age class (kid, yearling, 2-years-old or adult) and sex as fixed covariates, and year and goat identity as random factors, while other candidate models also included Julian date of collection fitted either as 1st, 2nd, 3rd or 4th polynomial order.

For hair, we used 50 samples containing both guard and undercoat hair and compared cortisol concentration of hair types with a paired *t*-test using the sample ID as grouping factor. We also conducted an analysis of variance to compare different hair samples (n = 598) from the Caw Ridge population that were sorted as guard or undercoat before extraction. Finally, we tested the correlation between guard and undercoat HCC with a linear model. One highly influential value (Cook's distance: 9.17) was excluded when fitting the model, but the outcome of the test was the same with or without this value.

We built a set of candidate linear mixed models to test the effects of age class, sex, reproductive status (lactating/non-lactating) and the interaction between age class and sex on FGM and HCC, and selected the most parsimonious model using AIC_c. The base model included goat identity and year fitted as random factors. Other candidate models also included age class, sex or both age class and sex. We conducted a second model selection based exclusively on adult females and using the same random factors (identity and year) to evaluate the influence of reproductive status. Date of sample collection was included as a fixed covariate (second order polynomial term) in all FGM models to account for its impact on FGM concentration (see results, Subsection 3.3). Only guard hair samples were used in the hair analyses because of the effect of hair type on HCC (see results, Subsection 3.3). Because mountain goat hair grow between May and early December (Côté and Festa-bianchet, 2003), fully grown hair collected during summer were used to evaluate the physiological status of the previous year.

We built a linear mixed model with freezing delay (0, 4, 8 and 21 h) and treatments (sun/bag/none) as fixed effects and sample identity as random factor to assess the impact of congelation delay on FGM concentration in alpine environments. We report parameter estimates and 95% confidence interval.

All statistical analyses were conducted with R 3.4.3 (R Core Team, 2017). We used the package "lme4" (Bates et al., 2015) to conduct linear mixed models and "AICcmodavg" (Mazerolle, 2017) for model selection. Normality and homoscedasticity were inspected visually through the distribution of residuals and residuals-fitted plots. To meet these assumptions, FGM data were ln-transformed for all analyses except the ACTH challenge and the degradation experiment whose residuals were normally distributed. For simple linear regression, analysis of variance and *t*-test, we report p-values with a significance threshold of $\alpha = 0.05$. For AIC_c selection, we present 95% CI for all parameters included in plausible models ($\Delta AIC_c < 2$) but considered the most parsimonious model as the best one (Burnham and Anderson, 2002). For all mixed models, we calculated r^2 using MuMIn package (Barton, 2016) and present the marginal and conditional r^2 as defined by Nakagawa and Schielzeth (2013). For linear mixed models with categorical variables, we conducted multiple comparisons under a model selection framework using *multComp* function of the package "AICcmodavg" (Mazerolle, 2017).

3. Results

3.1. Validity of immunoassay

Biochemical validations showed that the cortisol assay was suitable for faeces and hair. The recoveries of known concentrations of exogenous cortisol from faecal and hair extracts were 111 ± 4% and 104 ± 5%, respectively. The measured hormone concentrations in the spiked samples correlated with the expected concentrations (faecal cortisol $r = 0.999$, $p < 0.001$; hair cortisol $r = 0.996$, $p < 0.001$; Appendix 4). Serial dilutions of pooled faecal and hair extracts showed parallel displacement with the cortisol standard curve (faecal cortisol $r = 0.994$, $p < 0.001$; hair cortisol $r = 0.987$, $p < 0.001$; Appendix 5).

Chromatographic separation of pooled faecal extracts resulted in several peaks at fractions near the cortisol reference standard and no major peak thereafter (Fig. 1A). Immunoreactivity of the eluted fractions was evaluated using both cortisol and corticosterone EIAs. The cortisol EIA produced one major peak, which corresponded to the fraction that eluted at the same time as the cortisol standard on the chromatogram (Fig. 1). The corticosterone EIA did not produce any significant peaks, but smaller peaks that did not correspond to the elution time of either cortisol or corticosterone standards on the chromatogram (Fig. 1). These results determined the selection of the cortisol EIA for all further analyses.

3.2. ACTH challenge

The faecal ACTH challenge revealed a clear rise in FGM concentrations for two of the three injected animals and a moderate rise for the third one (Fig. 2). The adult male and one adult female reached maximum FGM concentrations 32 h (192% of baseline concentration) and 21 h (223%) post-injection, respectively. Overnight samples from the other adult female collected the morning after injection were elevated (127%), but maximum concentration (131%) occurred 20 h after injection in the last sample. Peak FGM concentrations were significantly higher than the baseline average for all animals (Adult female 1: $t_{12} = -18.5$, Adult female 2: $t_{12} = -6.0$, Adult male: $t_{12} = -7.6$; All p's < 0.0001).

For the hair ACTH challenge, there was a clear rise in HCC following repeated ACTH injections ($t_3 = -5.4$, $p = 0.006$; Fig. 3). Relative change in cortisol concentrations for the two adult females, the yearling female and the adult male were respectively 264%, 147%, 240% and 233%.

3.3. Faecal sample collection date and hair type

The best model assessing the effect of collection date on FGM concentrations included Julian date as a second order polynomial term ($R^2_m = 0.07$, $R^2_c = 0.38$; AIC table in Appendix 2). The model with Julian date as a fourth order polynomial term had equivalent support but was less parsimonious ($R^2_m = 0.07$, $R^2_c = 0.39$, AIC table in Appendix 2). Average FGM concentrations increased until mid-July and decreased afterwards (ln-scaled estimate [95% CI]: Day −0.14 [−1.21, 0.94], Day^2 −3.64 [−4.51, −2.77]; Fig. 4). Effect size was large with average cortisol concentration in June and July being 1.5 times higher than in May and 2 times higher than in September (FGM[95%CI] average: May

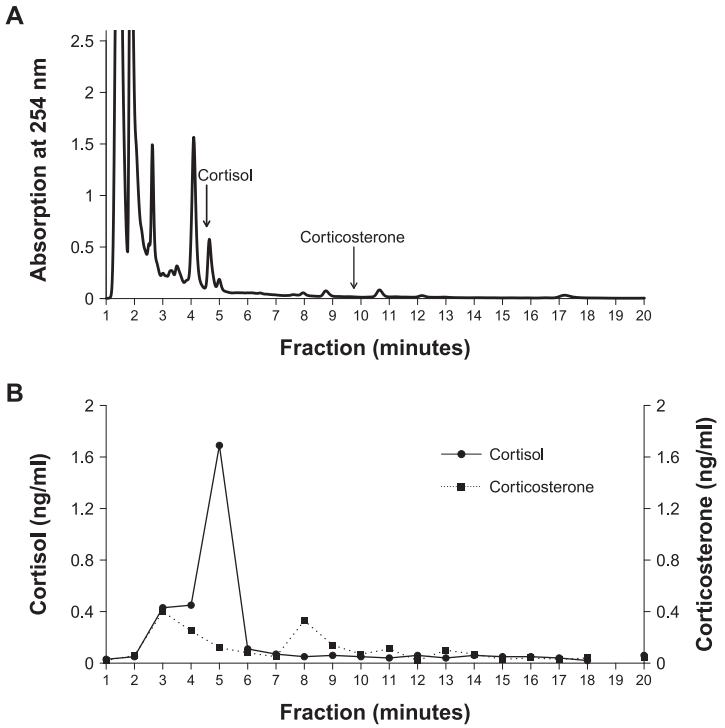

Fig. 1. Reverse phase high performance liquid chromatography (HPLC) separation of pooled faecal extracts from captive mountain goats: (A) chromatogram and (B) immunoreactivity profile as determined by cortisol and corticosterone enzyme immunoassay.

355[294,417], June 563[534,593], July 545[500,591], Aug. 459[434,485], Sept. 248[204,292]).

Hair cortisol concentration was highly related to hair type for guard and undercoat hair belonging to the same sample ($t_{51} = 11.7$, $p < 0.001$). Guard hair had on average 2.35 times more cortisol than undercoat hair. The same pattern was observed when comparing all available hair samples taken on different individuals at Caw Ridge (mean ± SE: Guard 10.4 ± 0.2, Undercoat 7.2 ± 0.1; $F_{1,596} = 51.9$, $p < 0.001$). Samples for which both guard and undercoat hair were analysed revealed a highly significant but weak correlation of cortisol concentration among hair types (estimate ± SE: 0.98 ± 0.23, $F_{1,49} = 18.2$, $p < 0.001$, $R^2 = 0.27$; Fig. 5).

3.4. Age, sex and reproductive status

Age class and sex do not appear to have a strong influence on mountain goat FGM as these variables were not included in the best model (Table 1). The model that included sex alone had equivalent support as the base model, but was less parsimonious and the estimate's confidence interval overlapped 0 (ln-scaled estimate [95%CI]: 0.02[−0.04,0.09]). The model with the lowest ΔAICc for hair included the interaction between age class and sex (Table 1, $R^2_m = 0.10$, $R^2_c = 0.36$). According to this model, males had lower HCC than females, but only when adults (estimate[95%CI]: Yearling:Male −0.68[−2.02,0.65], 2-years-old:Male 0.24[−2.33,2.82], Adult:Male −1.83[−3.41,−0.26]). However, the model including age class alone had equivalent support (Table 1) and was more parsimonious (Table 1; $R^2_m = 0.09$, $R^2_c = 0.34$; estimate[95%CI]: Yearling 0.03[−0.65,0.70], 2-years-old −2.06[−3.08,−1.04], Adult −2.47[−3.22,−1.71]). Multiple comparisons revealed that kids and yearlings had higher HCC than 2-years-olds and adults (Fig. 6; Appendix 3). Reproductive status of females did not appear to influence FGM or HCC as the base models had equivalent support and were more parsimonious (Table 1). Both FGM and HCC seemed marginally lower in barren females, but the confidence intervals of these estimates included 0 (ln-FGM: −0.1[−0.24,0.04]; HCC: −0.54[−1.98,0.90]).

3.5. Faecal metabolites degradation

The degradation experiment did not reveal clear directional variation of FGM concentration for samples exposed to sun (4 h: 0.2[−11.2,11.6], 8 h: −3.3[−14.7,8.0], 21 h: 4.9[−6.5,16.2]; Fig. 7). Average concentrations of samples kept in a bag were consistently lower than concentrations of controls, but effect size were similar regardless of the duration of the freezing delay (4 h: −8.3[−18.6,2.0], 8 h: −11.1[−21.4,−0.9], 21 h: −7.6[−17.9,2.7]; Fig. 7). FGM concentration varied widely between controls and treatment subsamples with a total range of −19.9% to 19.4% for sun exposed samples and −28.2% to 4.8% for samples kept in a bag.

4. Discussion

We validated a method to measure HPA axis activity in mountain goats using faecal and hair samples. We showed that a cortisol EIA can be used to reliably monitor FGM and HCC in this species. To our knowledge, this is also the first demonstration that systemic HPA axis activity is reflected in the hair of a non-domestic ungulate.

4.1. Validity of immunoassay

EIA performance was validated with parallelism, accuracy and precision tests, which showed that the assay could reliably measure the hormones present in the faecal and hair extracts without confounding effects of factors within the extract. Characterization of the faecal hormone metabolites by HPLC revealed that the immunoreactive substances present in our extracts were similar to the cortisol standard in terms of polarity and that the cortisol-EIA was best suited to measure them. Previous studies have shown that native cortisol is often virtually absent in the faeces of mammals (reviewed in Palme et al., 2005, but see Goncalves et al., 2016; Heistermann et al., 2006). Therefore, it is unclear if the assay measured authentic cortisol or cross reacted with metabolized forms of similar polarity (Möstl et al., 2005). Interestingly, no significant peak was detected on the chromatogram or the

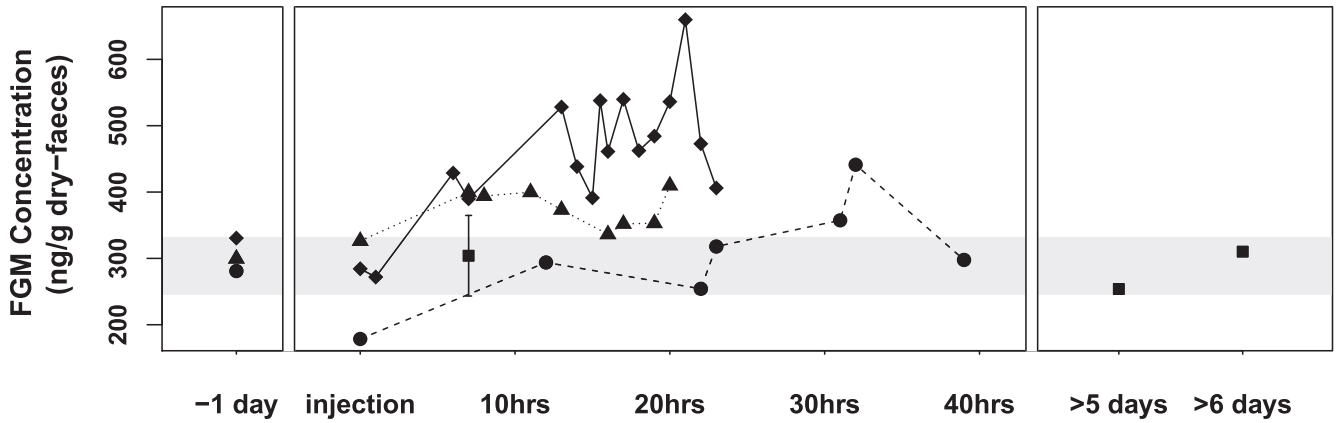

Fig. 2. Faecal glucocorticoid metabolite (FGM) concentrations of two adult females (diamond and triangle) and one adult male (circle) mountain goat following one adrenocorticotropic hormone (ACTH) injection. Black squares are baseline group composite samples. Shaded grey area represents 95% confidence interval of the average baseline concentration.

immunogram over 7 min, suggesting that less polar metabolites typically observed in ruminant feces (11,17-dioxoandrostanes; Palme and Mostl, 1997) were not present in our extracts. Since the HPLC fractions were collected every minute, we do not know if the major immunoreactive peak observed in fraction 5 was caused by the compounds identified by the chromatogram at approximately 4.1 min, 4.6 min, or by both. However, irrespective of the specific identity of the substance being measured, the detection of the ACTH injection by the cortisol-EIA demonstrates that this assay quantifies faecal metabolites in a biologically-relevant manner.

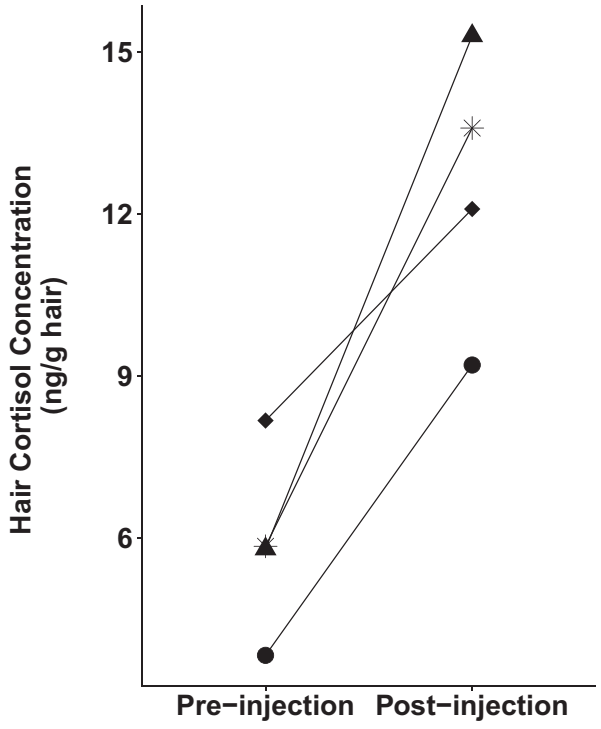

Fig. 3. Hair cortisol concentrations (HCC) of two adult females (diamond and triangle), one yearling female (asterisk) and one adult male (circle) mountain goat before and after a trial of 5 repeated adrenocorticotropic hormone (ACTH) injections.

4.2. ACTH challenge

Administration of ACTH induced a clear response in FGM for the male and one female, but the excretion pattern for the second female was not as obvious. Because we expected a shorter delay between injection and excretion of immunoreactive substances, this animal was released 20 h post-injection whereas peak concentration was observed after 21 h in the other female. It is therefore unclear whether FGM concentration continued to rise or decreased after it was released in the exhibit. Nevertheless, post-injection samples were elevated compared to baseline samples indicating that this female reacted to ACTH but might not have been kept long enough to measure the decrease in FGM concentration. Highest FGM concentrations were detected 20 to 32 h post-injection. This is comparable but slightly later than what was reported for other ungulates (dairy goat (*Capra aegagrus*) 13 h (Kleinsasser et al., 2010), red deer (*Cervus elaphus*) 18.75 h (Huber et al., 2003b), African buffalo (*Syncerus caffer*) 10–20 h (Spaan et al., 2017), sheep (*Ovis aries*) 12 h and horse (*Equus caballus*) 24 h (Palme et al., 1996)). Comparing horses, sheep and pigs (*Sus domesticus*), Palme et al. (1996) showed that the time course of steroid excretion in the faeces was highly related to gut passage time. Gut passage time decreases when an animal is active (Touma et al., 2003). Delay between injection of ACTH and peak FGM concentration should therefore be longer when injection occurs at a time of low activity (Touma et al., 2003). Mountain goats are diurnal and animals in our study were injected at sunset which could explain the slightly longer delay we observed. While it is unclear why the male had such a low defecation rate (only 1 faecal output after 20 h compared to 8 and 11 for the females), this is likely the cause of the much later peak observed for this individual. Variability in the time course of excretion should be kept in mind when interpreting FGM measurements from wild mountain goats especially when the impact of short-term stressors is assessed.

The hair ACTH challenge resulted in an unequivocal rise of HCC for all animals after injection. This clearly demonstrates that systemic fluctuations of cortisol can be reliably monitored in this substrate. However, while relative elevation was similar across individuals (mean [95%CI]: 221 ± 26%), there was substantial variability in absolute CORT concentration. Indeed, after a 233% rise in cortisol concentration, the male peak HCC was similar to the baseline HCC of one female. Using single samples without knowing an animal baseline level could therefore be misleading. It should also be kept in mind that the goats in our study were in ideal conditions avoiding excessive abrasion on the skin or other sources of local stress that could artificially elevate HCC (Salaberger et al., 2016).

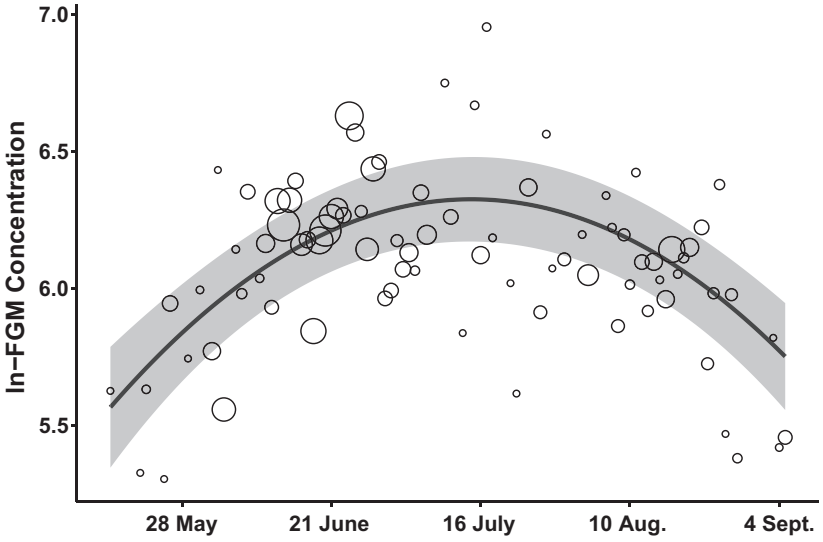

Fig. 4. Faecal glucocorticoid metabolite (FGM) concentration during summer in mountain goats from the Caw Ridge population (Alberta) sampled between 2000 and 2016. Dot size is proportional to sample size. The fitted quadratic regression is presented with its 95% confidence interval and includes age class and sex as fixed covariates and goat identity and year as random factors.

4.3. Faecal sample collection date and hair type

The hormonal pattern observed for the effect of collection date, with highest FGM levels occurring in mid-summer, is consistent with seasonal FGM variation in elk (*Cervus canadensis*; Millspaugh et al., 2001) and bighorn sheep (*Ovis canadensis*; Goldstein et al., 2005), but opposite to Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*: Dalmau and Manteca, 2007) and red deer (Huber et al., 2003a,b). Annual variation of GC concentration can be the result of various factors including changes in anthropogenic activity (Millspaugh et al., 2001), altered gut passage time due to changes in forage quality (Hofmann, 1989) and changes in life-history stages such as lactation (Landys et al., 2006; Romero, 2002). All-terrain vehicles (ATVs) have access to Caw Ridge and their presence usually increases after mid-June, but a previous study suggested they were not perceived as a major threat by the goats (St-Louis et al., 2013) and large groups of ATVs can be seen in early September when average FGM concentration was at the lowest. Increased GC concentration due to longer gut passage time is also unlikely since forage quality peaks in mid-summer and should therefore shorten gut passage time. Finally, elevation of GC concentration could help females sustain lactation (Landys et al., 2006), but this does not explain a similar pattern in males and juveniles. Our sample size in early and late summer was low compared to mid summer. Further analyses with standardized sample collection across summer would help understand the drivers of the observed variation in FGM. Nonetheless, we advise to take seasonal patterns into account as not doing so could bias the interpretation of FGM results.

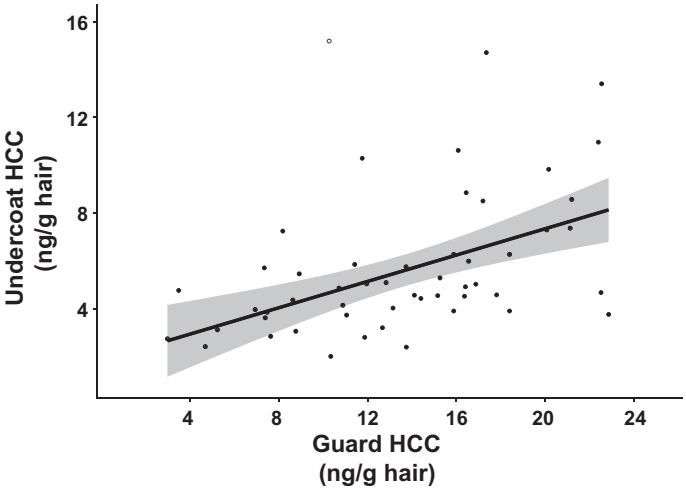

Fig. 5. Correlation between hair cortisol concentrations (HCC) of guard and undercoat hair in mountain goats, at Caw Ridge, Alberta (2000–2016) from 50 samples that contained both hair types. Linear regression is presented with 95% confidence interval and was fitted without the influential point represented by an empty circle (Cook's distance 9.17).

Hair cortisol concentration was much lower in undercoat than guard hair. Hair type was a strong predictor of HCC even when pooling all samples taken between years 2000 and 2016 from individuals likely exposed to different stressors. This result is consistent with a previous study by Macbeth et al. (2010) which showed that guard hair contains more cortisol than undercoat hair in brown bear. Hormone incorporation can vary according to chemical and physical characteristics of hair types (Harkey, 1993; Pragst and Balikova, 2006). Another possibility is that guard and undercoat hair growth period do not perfectly overlap as seen in other ungulates (Cowan and Raddi, 1972; Nixon et al., 1991). Both hair types would therefore provide distinct but equally valid information on cortisol exposure. The systematic differences we observed could for instance be explained by seasonal variation in cortisol levels. Cortisol concentrations were significantly correlated when analysing both hair types from the same sample. However, the predictive power of the correlation was low and would likely yield imprecise estimates if it was used to account for the effect of hair type in mixed data sets. It is important to note that all samples in our study were collected from the rump to standardize for any differences in HCC due to body location (Ashley et al., 2011; Macbeth et al., 2010; Terwissen et al., 2013). Our results support those of Macbeth et al. (2010) and strengthen the idea that comparative analyses should be restricted to one hair type.

4.4. Age, sex and reproductive status

Age class and sex had no detectable impact on mountain goat FGM concentration in our study. While sex-specific differences in FGM excretion have been found in various taxa including rodents and birds (Goymann, 2012; Palme et al., 2005), no effect of sex on FGM measurements were detected in other wild ungulates such as red deer (Huber et al., 2003a), chamois (Hadinger et al., 2015) and elk (Millspaugh et al., 2001). Aging has been shown to influence systemic glucocorticoid concentration through alteration of the HPA-axis

Table 1
Model selection for the influence of age class, sex and reproductive status on faecal glucocorticoid metabolite concentration and hair cortisol concentration in mountain goats, at Caw Ridge, Alberta (2000–2016). First set of candidate models (a) is based on the full data set (nfaeces = 761; nhair = 511), second set (b) is based on a subset of adult females for which reproductive status was known (nfaeces = 271; nhair = 115). Individual identity and year were included in all models as random factors. K is the number of parameters. Selected models are highlighted in bold.

	Faecal Metabolite Concentration				Hair Cortisol Concentration			
Models	K	AICc	ΔAICc	AICc weight	K	AICc	ΔAICc	AICc weight
a)								
Base	**6**	**793.0**	**0**	**0.59**	4	2732.8	50.7	0
Age	8	795.9	3.0	0.13	**7**	**2682.6**	**0.5**	**0.44**
Sex	7	795.6	1.6	0.27	5	2724.3	43.2	0
Age + Sex + Age:Sex	11	801.5	8.6	0.01	11	2681.1	0	0.56
b)								
Base	**6**	**262.6**	**0**	**0.54**	**4**	**623.8**	**0**	**0.70**
Reproductive status	7	262.9	0.3	0.46	5	624.5	1.7	0.30

responsiveness in rodents (e.g. Sapolsky, 1991) and primates (e.g. Goncharova and Lapin, 2002). Pavitt et al. (2015,2016) showed that red deer also have higher FGM concentration as they age and attributed this to a decline in body condition. Our results suggest that age and sex have limited influence on mountain goat FGM concentration or that other environmental factors are more influential under natural conditions.

Age class and, to a lesser extent, sex influenced hair cortisol concentrations. Kids and yearlings had higher HCC than older individuals, and females had higher HCC than males but only when adult. Plasma cortisol concentration can vary according to age and sex (Touma and Palme, 2005), but molt speed and hair morphology may also influence cortisol incorporation into hair (Henderson, 1993). The age- and sex-specific differences observed in our study match the differences in molt timing observed by Déry et al. (2019) on the same population. Indeed, according to this study, yearlings and adult females complete the shedding of their winter coat later than older individuals and males, likely because of variation in energetic constraints and access to nutritional resources (Déry et al., 2019). The age- and sex- specific differences we observed could therefore either reflect real changes in systemic HPA-axis activity potentially also linked to energy management or represent an artefact of molting patterns. Nevertheless, the clear change in HCC after 2 years and the potential sex difference for adults underline the importance of individual identification when using hair samples to assess cortisol concentration. The use of non-invasive sampling methods such as barbed-wire hair traps should be coupled to other methods allowing age- and sex- identification (e.g. DNA analysis, camera).

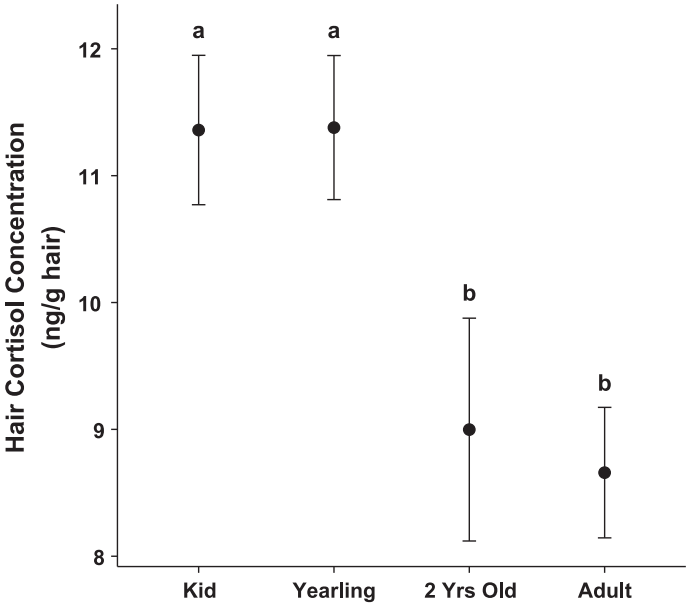

Fig. 6. Average hair cortisol concentrations according to age-classes in mountain goats, at Caw Ridge, Alberta (2000–2016). Error bars represent 95% confidence intervals, letters indicate grouping structure determined by multiple comparisons.

We did not detect a difference in FGM and HCC between lactating and non-lactating females. Female glucocorticoid concentration is elevated during lactation in most mammalian species (Edwards and Boonstra, 2018). However, even toed ungulates such as sheep, cows (*Bos taurus*) and red deer do not show this pattern (Edwards and Boonstra, 2018). Our results in mountain goats are therefore consistent with other similar species.

4.5. Faecal metabolites degradation

Prolonged exposure to ambient temperature reduced the immunoreactive substances measured by our assay for subsamples kept in a bag but not for subsamples exposed to sun. Glucocorticoid metabolites can be further metabolized by bacterial enzymes following defecation (Mostl et al., 2002). FGM concentrations measured by an immunoassay will then either increase, decrease or remain stable depending upon the assay sensitivity to the altered metabolites (Lexen et al., 2008; Ludwig et al., 2013; Mostl et al., 2002). Bacterial activity might have been facilitated by the relative humidity of the bag but limited in faeces dried after sun exposure. Interestingly, subsamples exposed for 4, 8 and 21 h had similar FGM concentrations suggesting that bacterial degradation occurred in the first hours of exposure. Ambient temperature decreased during the experiment and might have limited further bacterial activity. The relative FGM concentration varied widely between subsamples. Because we wanted our experiment to reflect real exposure conditions, we avoided breaking structural integrity of the pellets and did not homogenize the samples prior to separating them. Some of the variability we observed is thus likely attributable to intra-sample variation (Millspaugh and Washburn, 2003). Our results suggest that glucocorticoid metabolite degradation can occur in mountain goat faeces exposed to environmental conditions. However, degradation seemed limited by exposure to wind and sun or by low ambient temperature.

This study demonstrates that glucocorticoid concentrations can be successfully measured in both faeces and hair of mountain goats using a cortisol enzyme immunoassay. Metabolites in the faeces are representative of GC concentrations one or two days prior to sample collection and hair can reflect multiple weeks of glucocorticoid exposure. To avoid biased measurements, faecal samples should be kept at low temperature and frozen as soon as possible. Age class, sex and reproductive status had no detectable effect on FGM measurements, but

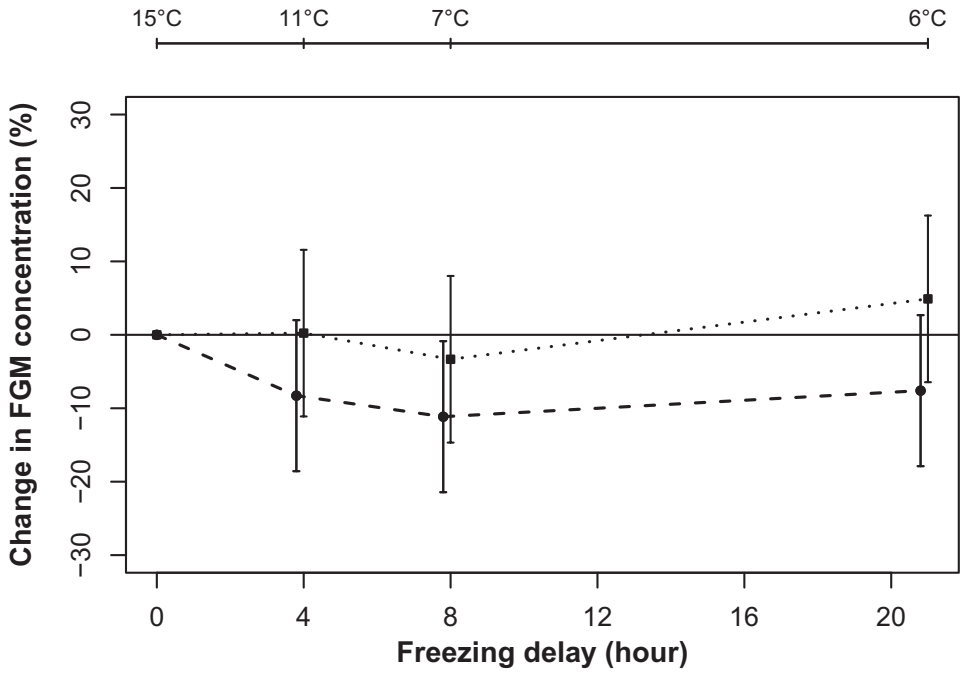

Fig. 7. Variation of FGM concentration in mountain goats, at Caw Ridge, Alberta (2000–2016), after 0, 4, 8 and 21 h of exposure to environmental conditions either in the sun (square) or in a bag (circle). Error bars represent 95% confidence intervals. Ambient shade temperature recorded at 0, 4, 8 and 21 h is shown above graph.

date of sample collection must be considered as it could impact interpretation. On the other hand, hair cortisol concentration of different age- and sex- classes should be analysed separately and comparative analyses should be restricted to one hair type.

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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.2019.04.022.

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