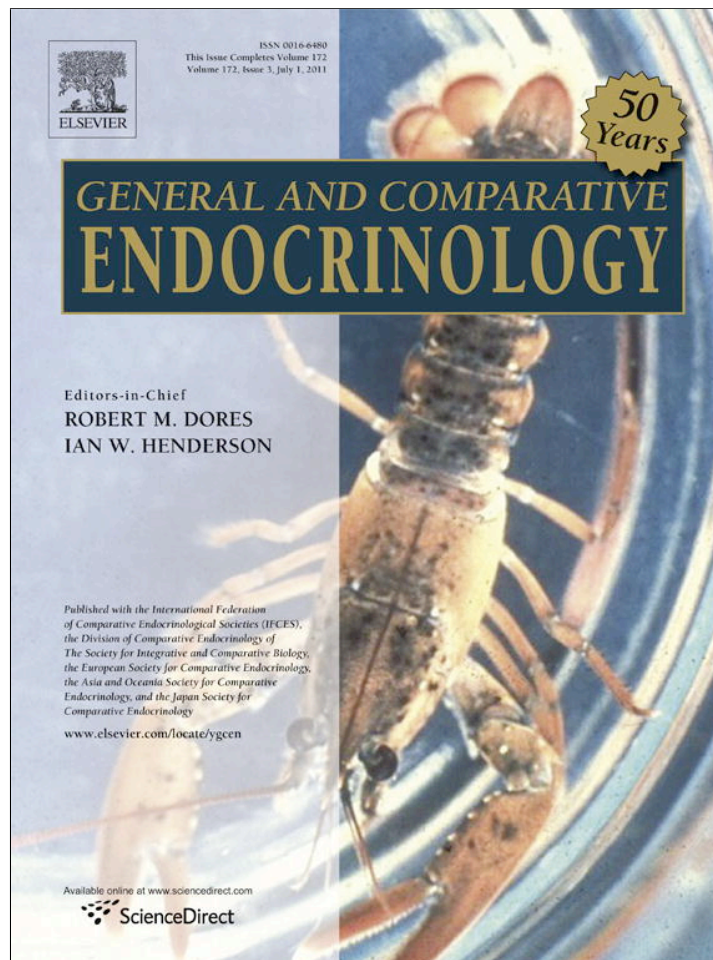


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## Glucocorticosteroid concentrations in feces and hair of captive caribou and reindeer following adrenocorticotrophic hormone challenge

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### ABSTRACT

Climate change and industrial development are contributing to synchronous declines in *Rangifer* populations across the Arctic. Chronic stress has been implicated as a proximate factor associated with decline in free-ranging populations, but its role in *Rangifer* is unspecified. Analysis of glucocorticosteroid (GC) concentration in feces, and more recently in hair, is a non-invasive method for monitoring stress in wildlife. Adrenocorticotrophic hormone (ACTH) released from the pituitary gland stimulates GC release from the adrenals and can be administered to reflect adrenal activation. In this study, we assessed concentrations of GC metabolites in feces and cortisol in hair of Alaskan caribou (*Rangifer tarandus granti*) and reindeer (*R. t. tarandus*) following ACTH treatment. We predicted that ACTH challenge would increase concentrations of fecal GCs, but not hair cortisol because steroid deposited into the hair shaft occurs over an extended period of time (months) and is likely insensitive to acute adrenal stimulation. Adult caribou ( $n = 10$ ; mean age, 6.5 years old) exhibited a peak increase in fecal GCs 8 h following a 2 IU/kg dose of ACTH compared to pre-injection concentrations. In contrast, sub-adult reindeer ( $n = 10$ , 0.8 years old) elicited a diminished response to the same dose. Quadrupling the dose (8 IU/kg) prolonged the fecal GC response in female reindeer, but male reindeer were unresponsive. Hair cortisol was unaffected by a single ACTH challenge. Further investigation is required to ascertain whether subspecific differences in adrenal sensitivity are attributed to age or sex differences, or historical selective pressures from semi-domestication and/or sedentary life cycle in reindeer.

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### 1. Introduction

Recent evidence suggests that many herds of caribou and reindeer (both *Rangifer tarandus*) in the circumpolar Arctic are declining [82]. Cumulative stressors from accelerated climate warming and increased industrial development in the Arctic have been proposed as major contributing factors [53,82]. Specifically, climate warming has increased the likelihood of extreme weather events (e.g. freezing rain and/or deep snow; [3], which has the potential of preventing access to lichens and other vegetation, leading to starvation and in some cases, mass mortality [40,50] (but see [80]). Warmer temperatures have increased the duration and intensity of insect harassment, which disrupts foraging [37,88]. Climate alterations have also affected plant phenology, which may alter quality and abundance of foods used for growth and

reproduction [21,56]. These effects are thought to contribute to reduced summer weight gain in adults and decreased survival of calves because the energetic demands of lactation are not being met [57]. Increased logging and oil and gas activities in *Rangifer* habitat have also restricted access to preferred foraging and calving areas [20,53,81,83]. Taken together, these cumulative effects have been proposed as factors that limit productivity of herds.

The study of stress has become increasingly integrated into the population assessments of wild vertebrates as a biomarker of health [51,64,89]. A number of studies have attributed population decline to the effects of chronic stress in free-living mammals [14,15,22,59] and increased anthropogenic disturbance has been shown to elicit a physiological stress response in wildlife [4, 26,85]. Activation of the hypothalamo–pituitary–adrenal (HPA) axis, a key component of the physiological response to stress in vertebrates, culminates in glucocorticosteroid (GC; cortisol in ungulates) secretion from the adrenal cortex. These steroids facilitate rapid recovery from acute stressors through increased energy mobilization, increased foraging behavior, and temporary suppression of the reproductive axis, as well as return to baseline

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conditions through negative feedback to the brain [91]. Alternatively, chronic exposure to stressors can lead to detrimental effects that include muscle wasting, suppression of growth, inhibition of reproduction, neuronal cell death, and suppression of immune function [70]. These maladaptive effects are thought to contribute to reduced fecundity and survival in chronically stressed populations [15,22,26,64,85].

GCs are routinely measured to assess adrenocortical activity in free-ranging vertebrates and can be detected in blood, urine, feces, saliva, and hair [28,47,52,55,61,76]. GCs can increase in response to emotional and nutritional stressors, as well as increased thermoregulatory demands. Assessment of stress hormones in the circulation can be confounded by disturbance-induced stress of capture, handling, and immobilization [51,86] since GCs rise rapidly in the circulation (within minutes). In some species, rapid restraint and blood sampling before elevation of GCs is impossible or impractical. Assays that measure GC metabolites in waste products, such as feces [36,39,42,51,52,55,77] or other biomaterials that accumulate steroids, such as hair [1,28,47], permit the noninvasive monitoring of adrenocortical activity in free-living vertebrates. Importantly, these types of samples represent a cumulative measure of GC release over a longer period of time, rather than a “snapshot” or point sample that is reflected in plasma samples [36,39,51,86]. Furthermore, plasma GC concentrations are pulsatile and exhibit a circadian rhythm. In contrast, fecal concentrations represent a pooled fraction of conjugated steroid that mixes with digesta passing through the intestines over several hours or days [55]. Cortisol concentrations in hair are deposited over an extended time frame (weeks to months) and are thus distinctly suited for evaluating long-term stress in wild-life populations [11,44,47]. Steroids enter the hair shaft primarily through passive diffusion from the dermal papilla in direct proportion to unbound concentrations of circulating GCs, with deposition only occurring during the anagen (growth) phase of hair follicles [28,58].

An important prerequisite for relying upon noninvasive measures of glucocorticosteroids is validating that samples collected accurately reflect adrenal activity in captive animals [78,86]. This relationship is typically achieved by stimulating the release of endogenous GCs from the adrenal by injecting adrenocorticotrophic hormone (ACTH), a peptide hormone synthesized and released from the pituitary gland. Following an ACTH challenge, the rise in circulating glucocorticosteroids is rapid, but concentrations return to baseline within a few hours. A similar increase should be detected in feces, but the kinetics and peak concentrations are delayed due to species-level differences in the rates of conjugation, bile flow, and digesta flow that affect fecal concentrations [55,78,86]. An increase in hair cortisol concentration from a single ACTH challenge is unlikely given continuous deposition of steroid into the hair shaft [28,58] that likely masks any acute increases.

HPA axis reactivity varies within and between species [38,61–63,66,94] and can be blunted when wild animals are exposed to captive conditions [65] or selected for domestication [2,79,87]. Reindeer in Alaska are semi-domesticated and were introduced from Siberia, Russia between 1892 and 1902 from a founder population of 1280 animals [27]. Although caribou and reindeer are considered the same species, reindeer and wild caribou of Alaska are genotypically and phenotypically distinct. Besides genetic divergence of mtDNA haplotypes and microsatellite alleles [27], caribou are larger than reindeer (although this difference is most pronounced in males), have longer legs and less subcutaneous body fat, and undergo long-distance migrations every year to reach calving grounds. In contrast, reindeer remain sedentary throughout their range, although there is seasonal movement between grazing areas [33,34]. Stress responses may differ markedly between these subspecies depending on the season and stimulus. For example, fe-

male reindeer with large stores of body fat do not increase plasma cortisol during winter fasting when activity is low whereas male reindeer exhibit large increases in plasma cortisol when food intakes are low and activity is high during rut in autumn [6,8]. Differences in migratory behavior between subspecies could also alter HPA axis responsiveness, as documented in seasonally-breeding birds [90]. Furthermore, Siberian reindeer and barren ground caribou may perceive stressors differently because of differences in traits selected for tolerance to external threats, body condition, activity patterns, timing of breeding, and interactions with conspecifics, insects, predators, and people [7,9,31,67,73].

The main objective of this study was to validate the measurement of GCs in feces and hair of caribou and reindeer. We investigated whether acute activation of the HPA axis induced by ACTH administration results in a corresponding increase in GC metabolite excretion in fecal material of Alaskan barren ground caribou (*Rangifer tarandus granti*) and reindeer (*R. t. tarandus*) in captivity. If an effect was documented, then the temporal dynamics of GC excretion in relation to time of ACTH challenge and sex were evaluated. Because selective pressures from semi-domestication or sedentariness in reindeer could dampen the HPA axis response to stress, we also tested the hypothesis that reindeer exhibit reduced adrenal sensitivity to ACTH compared to caribou. In addition, we measured cortisol concentrations in hair of both subspecies. Because steroids are continuously incorporated into actively growing hair [28,58], we predicted that a single ACTH challenge would not lead to a detectable increase in hair cortisol.

## 2. Materials and methods

### 2.1. Animals

Experiments were approved by the Institutional Animal Care and Use Committee, University of Alaska, Fairbanks (protocol #08–01). This study was conducted at the Robert G. White Large Animal Research Facility at the University of Alaska, Fairbanks (65°N, 146°W), where captive populations of caribou and reindeer are maintained for research purposes and have been prevented from interbreeding. Caribou used in the study were descended from Delta and Porcupine herds of barren ground caribou in Alaska. Reindeer were founded from Siberian herds that were introduced into Western Alaska during the early twentieth century. Adult males ( $n=6$ ) and females ( $n=6$ ) of each subspecies were separated into outdoor pens of similar size (0.5–1.0 ha) on February 19, 2008. Male caribou were divided between two pens whereas male reindeer, female reindeer and female caribou were held together in their respective groups. Male caribou were separated to avoid strong dominance hierarchies between young (1.8 years) and mature males (>3 years). All reindeer were born in 2007 and were 0.8 years old at the start of the study. Female reindeer were separated from males during the rut and were therefore not pregnant. Female caribou were between 4 and 14 years old ( $8.95 \pm 4.12$  years) at the start of the study. All caribou females had been held with males in the previous breeding season and five of those females gave birth to calves in the following spring (2008). Male caribou were between 1.8 and 7.8 years old ( $3.94 \pm 2.55$  years) at the start of the study. Animals were fed *ad libitum* on a pelleted formulation (16% crude protein, 34% plant cell walls; Alaska Pet and Garden, Anchorage, AK) that has been used for growth and maintenance of both caribou and reindeer for 10 years [8]. The feeding regime simulated high quality range with abundant forages that are similar in composition to willow (*Salix* sp.) in summer. Water was available *ad libitum* as snow in winter and in water troughs through the spring and summer.

## 2.2. ACTH challenge

Caribou and reindeer were trained to routinely move from their pens to a smaller handling area, which led to a chute with a load scale for measures of body mass ( $\pm 0.1$  kg). In addition, reindeer were trained as calves with halters and lead ropes to walk with handlers and to be tethered for sampling blood. ACTH challenges were performed on March 4 and 5, 2008, for reindeer and caribou, respectively. A second trial was conducted using the same reindeer on June 17, 2008. Approximately 10–20 min prior to challenge, reindeer and caribou were moved to the handling area. Reindeer were tethered and manually restrained for ACTH administration whereas caribou were held in a hydraulic chute.

In the first trial, five of the six animals of both subspecies were randomly assigned to receive an intramuscular ACTH challenge at a dose of 2 IU/kg of body mass (stock concentration of 40 IU/ml; gel form; Meds for Vets, Inc., Sandy, Utah). Dosage was similar to previous studies in other ungulate species [86]. In a second trial in reindeer, the ACTH dose was increased fourfold (8 IU/kg) because reindeer were relatively insensitive to the lower dose (see Section 3). In both trials, the remaining animal in each group received a control injection of physiological saline (Butler Animal Health, Dublin, Ohio). Total volume of ACTH or saline injected varied between 4.6 and 8.7 ml in caribou (92–173 kg body mass) and 2.8–4.6 ml in reindeer (79 and 92 kg body mass). For both subspecies, injections occurred sequentially, instead of simultaneously because caribou could only be immobilized one animal at a time. Individual doses were administered in equal amounts into the right and left shoulder, respectively, with an 18-gauge needle. Time of challenge ranged from 09:06 to 09:33 Alaska Standard Time (AKST) for reindeer and 09:07 to 09:41 AKST for caribou, after which animals were released back into their normal pens.

## 2.3. Fecal sampling

Spontaneously voided feces were collected from caribou and reindeer ca. 4, 8, 24, 48, 72, and 168 h after injection, and pre-injection (baseline) samples were collected 7 days prior to ACTH challenge at ca. 0900 AKST (Alaska Standard Time). For each collection period, several observers entered pens and watched from a distance to record when each animal defecated. Reindeer were distinguished from each other by halter color and number, while caribou were identified by body size, pelage color and antler morphology differences. Once voided, the entire fecal sample was immediately collected with gloves from the ground and stored at  $-20^{\circ}\text{C}$  until analysis. Actual fecal voidance times (relative to time of challenge) were not exact and varied among individuals (time after challenge, mean  $\pm$  SEM: 4 h sampling:  $4.05 \pm 0.08$  h (caribou),  $4.50 \pm 0.20$  h (reindeer); 8 h sampling:  $8.02 \pm 0.17$ ,  $8.20 \pm 0.12$  h; 24 h sampling:  $24.02 \pm 0.20$ ,  $24.42 \pm 0.23$ ; 48 h sampling:  $47.85 \pm 0.13$ ,  $48.6 \pm 0.08$ ; 72 h sampling:  $71.78 \pm 0.10$ ,  $72.40 \pm 0.10$ ; 168 h sampling, times not reported because of recording oversight).

## 2.4. Hair sampling

Hair was shaved from the neck (along the jugular vein equidistant from the shoulder to the mandible), shoulder (at the center of the scapula), and rump (at the greatest curvature of the buttock) 1 week before and 2 weeks after ACTH challenge. Reindeer were also sampled 19 weeks after the second administration of ACTH. Hair was removed without breaking the skin by using an electric shaver fitted with a close blade for surgical preparations (1/10 mm; size 40; Oster A5). We shaved  $40 \times 40$  mm of skin at each site to collect all the hair into a plastic bag with clean gloves and forceps. We sampled from the left side before dosing and from

the right side after the ACTH dose. Hair follicles were collected after dosing to evaluate the stage of growth by using forceps to pluck hair from each body region in each animal. Growth stage was assessed in subsamples of 100 randomly selected hair shafts from each site using trichograms [30] and special staining techniques [5].

## 2.5. Blood sampling

Blood was sampled from the jugular vein (1.5"  $\times$  18 gauge needle) when animals were handled for sampling hair. Blood samples were drawn immediately before sampling hair within 10 min of entering the handling area and again 30 min later to evaluate the effect of handling on blood cortisol. Blood was drawn into 10 ml evacuated tubes without additive (Vacutainer Systems; Becton-Dickinson Co., Franklin Lakes, New Jersey). Serum was separated at 3000 g for 10 min in a centrifuge and stored at  $-20^{\circ}\text{C}$  for analysis.

## 2.6. Fecal GC analysis

Fecal samples were freeze-dried with a lyophilizer for 24 h and then mixed uniformly. Approximately 0.1 g of dry fecal material was extracted twice with 15 ml of 70% ethanol (30% distilled water), vortexed (30 min), and then centrifuged (2200 rpm for 20 min). The supernatant was collected and combined from each extraction and stored at  $-20^{\circ}\text{C}$ . Prior to analysis a portion of the extract was evaporated under forced air and reconstituted with assay buffer. To measure fecal GCs, a corticosterone double-antibody  $^{125}\text{I}$  radioimmunoassay kit was used (MP Biomedicals, Solon, OH; catalog #07120102) according to the manufacturer's protocol. The primary antibody used in the kit exhibits a low cross-reactivity with cortisol (0.05%), but has been shown to effectively bind to fecal metabolites of cortisol and corticosterone in a number of vertebrate species, including ungulates [23,86]. Although the major circulating GC of *Rangifer* is cortisol [6,19,68], circulating concentrations are rapidly metabolized to cortisone-type metabolites in fecal material that more readily bind to antibodies raised against corticosterone than cortisol [86]. Cross reactivities with other steroids were negligible (<0.34%) and listed in the manufacturer's protocol. Thus, instead of specifically reporting fecal cortisol concentrations here, we rely upon fecal GCs as the metric for assessing adrenal sensitivity. Serial dilution curves derived from pooled *Rangifer* fecal extracts were not significantly different from the slope of the standard curve ( $t_7 = 2.53$ ,  $p = 0.15$ ), indicating adequate parallelism. The slope of standards spiked with diluted fecal extract was also highly accurate ( $r^2 = 0.99$ ). Intra- and interassay variations were 2.9% and 7.5%, respectively.

## 2.7. Hair cortisol analysis

For hair, cortisol concentrations were specifically measured. Techniques used for hair processing, cortisol extraction, and analysis were modified after those reported in [28] and [47]. Hair samples were washed three times (3 min per wash) with 10.0 ml of methanol per 100 mg of hair. This method is effective for removing visible contamination and measurable cortisol from the surface of clean and moderately blood-contaminated hair in *Rangifer* (Macbeth, unpublished data). Although this wash protocol may be safely extended to six washes without removing intrinsic cortisol from the hair shaft, it may not be effective in removing surface contamination from samples severely contaminated with blood (Macbeth, unpublished data). Importantly, all samples in the present study were free of visible blood contamination (Macbeth, personal communication). Presently, we recommend that analysis of hair cortisol in *Rangifer* be restricted to samples that are clean or

moderately contaminated with blood. Each dried sample was then pulverized into a fine powder using a ball mill [(Retsch MM301; Retsch Inc., Newtown, PA, USA) (3 min/100 mg hair at 30 Hz; 10 ml stainless steel grinding jar, single 5 mm stainless steel grinding ball)], and steroids were extracted with 1 ml of methanol (EMD Chemicals, Gibbstown, NJ, USA) per 50 mg powdered hair for 24 h on a slow rotator. Samples were then centrifuged (15 min/4500 rpm/20 °C), the supernatant collected, and transferred to a glass 12 mm test tube. To ensure all extracted steroids were recovered, the ground hair sample was rinsed two times by adding 1.0 ml of fresh methanol, gently vortexing (40 s), centrifuging (4500 rpm/15 min/20 °C) and pooling supernatants. The pooled supernatant was dried at 38 °C under a gentle stream of nitrogen gas and reconstituted with 0.4 ml of phosphate buffer. Cortisol was determined using an enzyme-linked immunosorbent assay (ELISA; EA-65 Cortisol EIA kit, Oxford Biomedical, Lansing, MI, USA). According to the manufacturer, cross reactivity of the antibody used for the cortisol kit is: cortisol (100%), prednisolone (47.72%), cortisone (15.77%), 11-deoxycortisol (15%) prednisone (7.83%), corticosterone (4.81%), 6-beta-hydroxycortisol (1.37%), 17-hydroxyprogesterone (1.36%) and deoxycorticosterone (0.94%). All other hormones and intermediates tested by the manufacturer exhibited cross reactivity of  $\leq 0.06\%$ . The approximate limit of detection of the EIA kit (0.04 ng/ml) was determined by calculating the absorbance (mean + 2SD) of the zero standard provided with the EIA kit based on 12 runs. This corresponds to approximately 0.32 pg cortisol per mg hair in a processed 50 mg hair sample. Extraction efficiency was  $94.53 \pm 3.52\%$  based on five determinations of recovery from a 4.0 ng/ml spike of a hair extract solution containing ground hair (Fluka Hydrocortisone Analytical Standard 31719; Sigma-Aldrich, Munich, Germany). Cortisol measured in serially-diluted *Rangifer* hair extract was parallel ( $r^2 = 0.998$ ,  $p < 0.001$ ) with serially-diluted cortisol standards provided with the EIA kit. Intra-assay coefficient of variation (CV) was 6.04% ( $n = 6$ ) while inter-assay CV was 18.39%. ( $n = 6$ ).

### 2.8. Serum cortisol analysis

Serum cortisol was determined following procedures described previously [48]. Briefly, cortisol was extracted into diethyl ether, evaporated under nitrogen, and reconstituted in 0.2 ml of phosphate buffer. Samples were run in triplicate using ELISA (EA-65 Cortisol EIA kit, Oxford Biomedical, Lansing, MI, USA). Intra-assay CV was  $<10\%$  and the detection limit was 20 pg/ml.

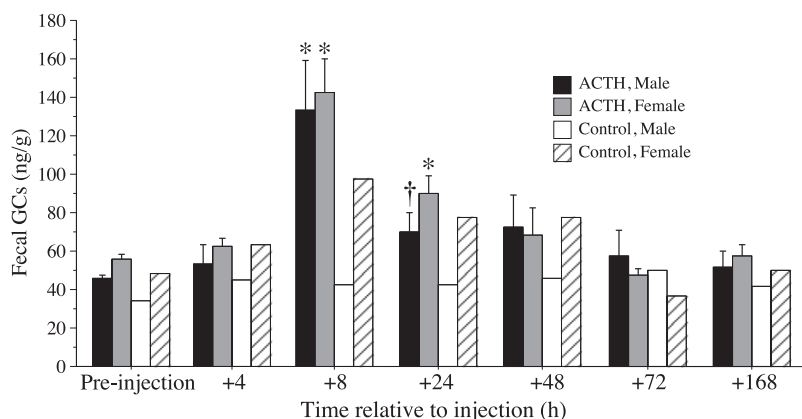
### 2.9. Statistical analysis

Because it was predicted *a priori* that ACTH challenge would elevate GC concentration, one-tailed paired *t*-tests were used to examine whether concentrations of fecal GCs after challenge were higher than concentrations before challenge. For these tests, Type I error probabilities were adjusted for the number of comparisons made using the sequential Bonferonni method [74]. Data were logarithmically transformed to satisfy assumptions of normal distribution and homogeneity of variances in these parametric tests. Statistical evaluation of GC levels in saline-injected controls was not possible given low sample size, thus animals served as their own controls. However, concentrations from saline-injected controls provided a relative baseline for comparisons with ACTH animals (see Figs. 1–3). Sexual and subspecific differences in GC concentration (log-transformed) of ACTH-injected animals and serum cortisol levels were evaluated using two-way repeated measures analysis of variance (ANOVA). Sex and subspecies were the main factors and time was the repeated measure. A two-way repeated measures ANOVA was used to test for differences in hair cortisol concentrations across regions in pre-injected caribou and reindeer with sex and subspecies as main factors and body region as the repeated measure. Fischer's protected least significance difference (PLSD) test was employed for multiple comparisons between groups.

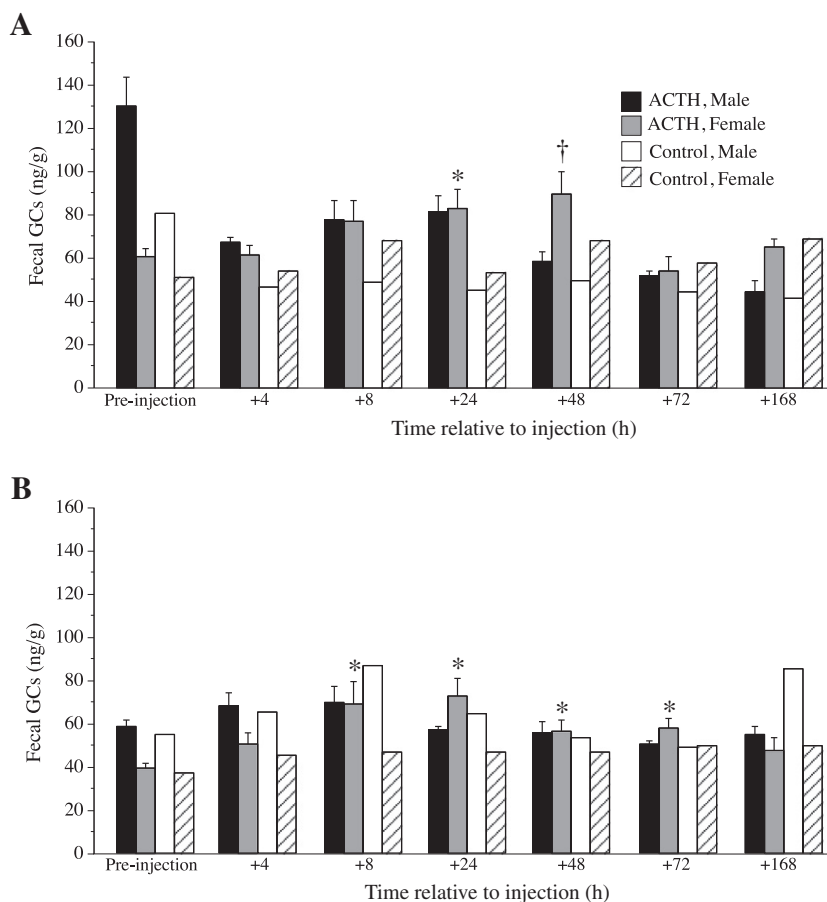
## 3. Results

### 3.1. Fecal GC levels

A 2 IU/kg dose of ACTH was sufficient to increase fecal GCs in male and female caribou (Fig. 1). Fecal GCs were highest 8 h after ACTH challenge in both male (mean, 133.5 ng/g) and female (142.1 ng/g) caribou, representing a 190% and 154% increase, respectively, from pre-injection concentrations (Fig. 1; paired *t*-tests: males,  $t = -4.27$ ,  $p = 0.0065$ , corrected  $\alpha = 0.0083$ ; females,  $t = -10.13$ ,  $p = 0.0005$ , corrected  $\alpha = 0.0083$ ). Mean fecal GC levels in saline-injected male and female caribou 8 h after challenge were 42.4 and 97.4 ng/g, respectively, compared to pre-injection levels of 34.1 and 48.5 ng/g, representing a 24% and 101% increase from baseline (Fig. 1). Approximately 24 h following ACTH administration, fecal GC concentration remained significantly elevated in females, but not in males (although there was a non-significant trend) (Fig. 1; paired *t*-tests: males,  $t = -2.74$ ,  $p = 0.029$ , corrected



**Fig. 1.** Fecal GC concentrations (ng/g) of male and female caribou before and after ACTH (2 IU/kg) or saline (control) injection. An asterisk indicates post-injection concentrations are significantly greater than pre-injection levels following sequential Bonferonni comparison of one-tailed paired *t*-tests. A dagger indicates a significant trend ( $p < 0.05$  but lacking significance following sequential Bonferonni correction) for concentrations to be higher in post-injected caribou compared to pre-injection values. Note that control animals are only used as a reference point and not statistically compared because of low sample size ( $n = 1$ ). Error bars indicate 1 SEM.



**Fig. 2.** Fecal GC concentrations (ng/g) of male and female reindeer before and after ACTH or saline (control) injection. (A) In March, a 2 IU/kg dose of ACTH was administered. (B) In May, reindeer received a second dose of ACTH (8 IU/kg) and fecal GC levels were measured. An asterisk indicates post-injection concentrations are significantly higher than pre-injection concentrations following sequential Bonferroni correction using one-tailed paired *t*-tests. A dagger indicates a trend ( $p < 0.05$  but lacking significance following sequential Bonferroni correction) for higher concentrations in post-injected reindeer compared to pre-injection levels. Note that control animals are used as a reference point and not statistically compared because of low sample size ( $n = 1$ ). Error bars indicate 1 SEM.

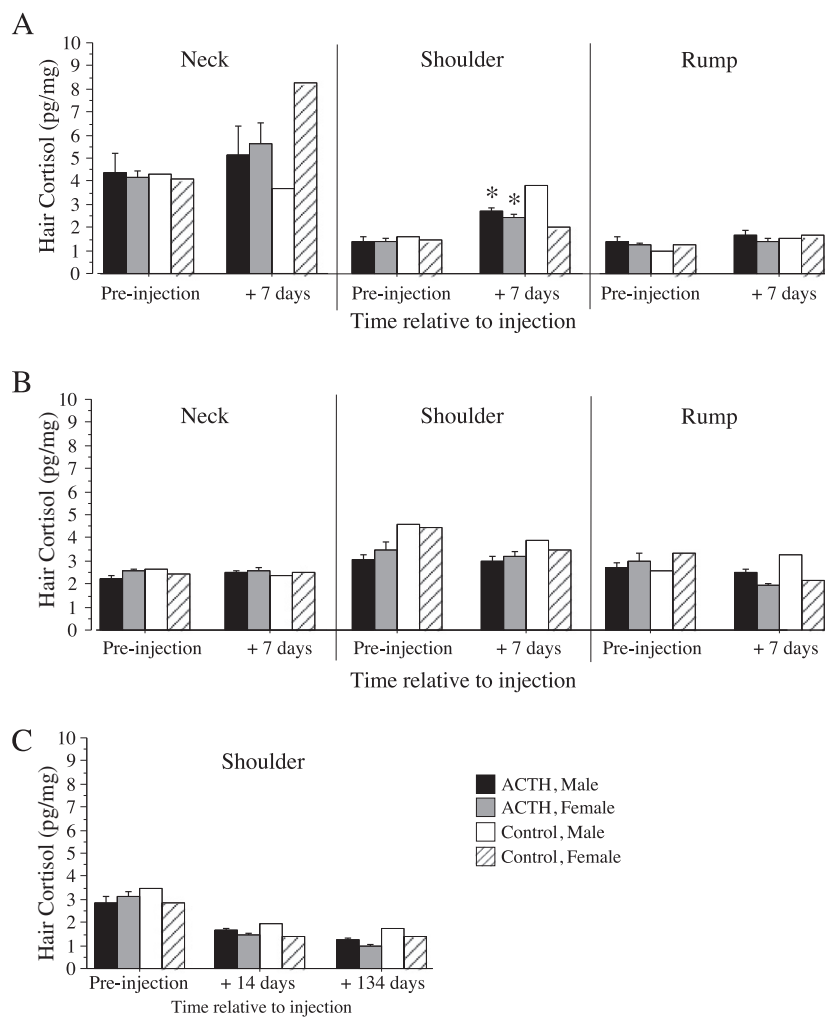
$\alpha = 0.01$ ; females,  $t = -4.34$ ,  $p = 0.0061$ , corrected  $\alpha = 0.01$ ). Thereafter, fecal GCs measured at 48, 72, and 168 h following injection were statistically indistinguishable from concentrations measured before challenge (Fig. 1; paired *t*-tests, all  $p > 0.07$ ).

Male and female reindeer responded differently than caribou for the same dose of 2 IU/kg ACTH dose as indicated by a significant interaction between subspecies, sex, and time for fecal GC concentrations (repeated measures ANOVA,  $F(6, 96) = 5.731$ ,  $p < 0.0001$ ). Specifically, in female reindeer, fecal GCs measured 24 h after challenge (mean, 82.6 ng/g) were significantly higher compared to pre-injection levels (60.66 ng/g; Fig. 2A; paired *t*-test,  $t = -4.434$ ,  $p = 0.0057$ , corrected  $\alpha = 0.0083$ ). There was a non-significant trend for concentrations to remain elevated 48 h after ACTH administration (89.6 ng/g; paired *t*-test,  $t = -3.70$ ,  $p = 0.0101$ , corrected  $\alpha = 0.01$ ). These changes represented a 36% and 47% increase, respectively, in fecal GCs relative to baseline concentrations (prior to ACTH injection). For the same time periods (24 and 48 h post-injection), the female control exhibited a 3.9% and 31% increase, respectively, in fecal GC concentration compared to baseline levels. In male reindeer, fecal GCs measured prior to challenge (mean, 130.2 ng/g) were atypically elevated compared to all post-injection concentrations (Fig. 2A). This may have blunted their fecal GC response to ACTH challenge compared to baseline concentrations (paired *t*-tests, all  $p > 0.99$ ). Barring this confound, post-injection concentrations in males were similar to those found in females, except for the 48 h post-sampling period (Fig. 2A).

Because the increase in fecal GCs of female reindeer after a 2 IU/kg dose of ACTH was small compared to increases measured in caribou and because concentrations prior to challenge in male reindeer were atypically elevated, a second trial was conducted in reindeer to assess if a higher dose of ACTH (8 IU/kg) was necessary to elevate fecal GCs in both sexes. For this trial, pre-injection concentrations of males were lower (58.16 ng/g) than the first trial (Fig. 2B). Nonetheless, fecal cortisol after ACTH administration was not significantly elevated compared to baseline concentrations, albeit there was a non-significant trend for higher cortisol concentrations 4 h after challenge (Fig. 2B; paired *t*-tests: 4 h post-injection,  $t = 0.034$ ,  $p = 0.034$ , adjusted  $\alpha = 0.0083$ ; other post-injection times,  $t > -1.08$ ,  $p > 0.17$ ). In contrast, female reindeer exhibited significantly elevated concentrations of fecal cortisol 8, 24, 48, and 72 h following a 8 IU/kg dose of ACTH compared to concentrations before challenge (Fig. 2B; paired *t*-tests: 8 h post-injection). The percentage increase from baseline was 75%, 85%, 43%, and 47%, respectively, compared to smaller percentage increases from baseline (22% to 26%) in the control. Furthermore, these increases were small compared to those reported in female caribou that received one-quarter the ACTH dose.

### 3.2. Hair cortisol concentrations

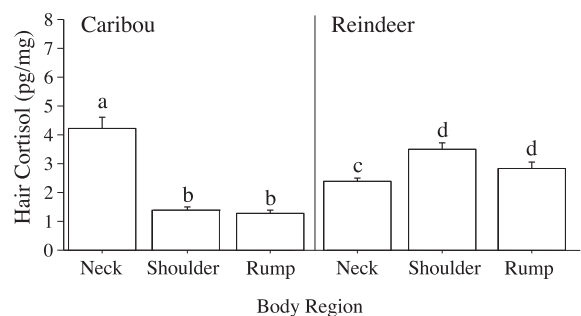
ACTH administration (2 IU/kg) did not increase cortisol concentrations of hair sampled 7 days following challenge from the neck or rump of caribou or from the neck, shoulder, or rump of reindeer



**Fig. 3.** Hair cortisol concentrations (pg/mg) of male and female (A) caribou and (B) reindeer before dosing and 1 week following an injection of ACTH (2 IU/kg) or saline (control) injection. Hair was sampled from three body regions: neck, shoulder, and rump. (C) In a second trial, hair cortisol was measured from the shoulder region of male and female reindeer before and 2 and 19 weeks following an 8 IU/kg dose of ACTH or saline (control) injection. An asterisk indicates significantly higher cortisol concentrations compared to pre-injection levels. Note that control animals are used as a reference point and not statistically compared because of low sample size ( $n = 1$ ). Error bars indicate 1 SEM.

(Fig. 3A and B; paired  $t$ -tests, all  $t > -1.465$ ,  $p > .0885$ ). However, a significant elevation of cortisol was detected in the shoulder hair of both male and female caribou compared to concentrations prior to challenge (paired  $t$ -test, male;  $t = -5.82$ ,  $p < 0.002$ ; female,  $t = -10.50$ ,  $p = 0.0002$ ), although saline-injected controls exhibited comparable increases (Fig. 3A). A higher ACTH dose (8 IU/kg) was insufficient to elevate cortisol in the shoulder hair of male or female reindeer 2 or 16 weeks post-injection (Fig. 3C), even though the hair sampled in October (16 weeks post-injection) was actively growing. Hair collected before and 2 weeks after the second ACTH injection was not growing, indicating growth from the previous year (2007).

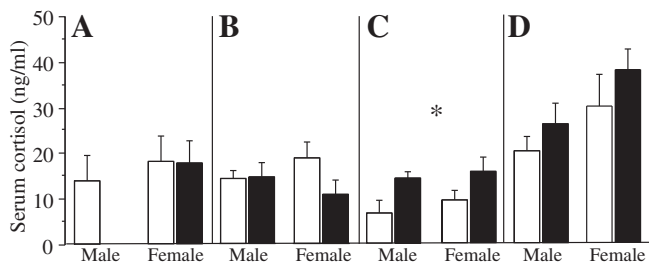
Pre-treatment concentrations of hair cortisol were differently affected by an interaction between body region and subspecies (Fig. 4; two-way repeated measures ANOVA; subspecies  $\times$  body region,  $F(2, 38) = 94.1$ ,  $p < 0.0001$ ). Specifically, in reindeer, cortisol concentrations of shoulder and rump hair were significantly higher than those for neck hair (Fig. 4). These concentrations were bounded by statistically higher cortisol concentrations in the shoulder hair of caribou and statistically lower concentrations in the neck and rump regions of caribou (Fig. 4). No sexual differences in hair cortisol were detected for either subspecies ( $F(1, 19) = 0.48$ ,  $p = 0.40$ ).



**Fig. 4.** Prior to ACTH injection, hair cortisol concentrations varied significantly across body region (neck, shoulder, rump) in caribou and reindeer. Shared letters indicate no significant differences among groups. Error bars indicate 1 SEM.

### 3.3. Serum cortisol concentrations

Circulating cortisol was more variable in caribou ( $16.0 \pm 13.3$  ng/ml) than in reindeer ( $16.6 \pm 6.6$  ng/ml) before animals were handled to sample hair in March (Fig. 5A). Male caribou were not re-sampled in March, but handling for 30 min did not significantly affect cortisol among females of either subspecies in late winter



**Fig. 5.** Serum cortisol concentrations (ng/ml) before hair sampling and after 30 min of additional restraint in male and female (A) caribou in March and (B) reindeer in March, (C) June, and (D) October. An asterisk indicates a significant effect of time in the overall two-way repeated measures ANOVA. Open bars correspond to pre-hair sampling and closed bars correspond to 30 min of restraint stress. Error bars indicate 1 SEM.

(Fig. 5A). Handling increased circulating cortisol in reindeer during summer (June,  $F(1, 10) = 6.52$ ,  $p = 0.029$ ; Fig. 5C) when initial concentrations were lower ( $8.1 \pm 5.9$  ng/ml) than those in late winter (March; Fig. 5B) or at the end of the breeding period (October  $25.0 \pm 13.5$  ng/ml; Fig. 5D).

## 4. Discussion

### 4.1. Effect of ACTH upon fecal GCs

This study demonstrates that measurement of fecal GCs in barren ground caribou is sensitive to short-term increases in adrenal activity. This type of validation is essential for assessing stress responses in free-ranging populations as fecal concentrations should reflect endogenous levels within the circulation, even though there is a species-specific latency period between adrenal release of steroids and the final step of defecation [78,86]. Male and female caribou exhibited peak concentrations of GC metabolites 8 h following ACTH administration that were still elevated up to 24 h later. Because samples were collected at discrete periods, it is likely that the actual peak falls between these two time points. This excretion lag time is within the range of those found in roe deer (*Capreolus capreolus*; 8–23 h; [29]) but shorter than those reported in other cervid species, such as Roosevelt elk (*Cervus elaphus roosevelti*; 22 h; [86]), red deer (*Cervus elaphus elaphus*; 18 h; [41]), and white-tailed deer (*Odocoileus virginianus*) during summer (20–24 h; [52]). However, in the latter species, lag times were reduced by half in winter (10–13 h) possibly due to seasonal changes in metabolism, which correspondingly affect rates of liver conjugation of steroids and gut passage [52]. A similar effect of season was likely for caribou and reindeer in this study because food intakes decline to the annual minimum at the end of winter in March and April [8].

We provide evidence to support the hypothesis that reindeer exhibit reduced adrenocortical sensitivity to ACTH challenge compared to caribou. Female reindeer elevated fecal GCs 24 h after ACTH administration, but this increase only represented a 31% rise from baseline compared to a nearly twofold increase in male and female caribou, respectively. Male reindeer failed to exhibit any discernable increase in fecal GCs following ACTH challenge. Furthermore, a fourfold increase in ACTH dose prolonged the fecal GC response in female reindeer, but not in males, which were still unresponsive.

It is unclear why subspecific differences in adrenal sensitivity to ACTH occur in *Rangifer*, but there are several possibilities. First, the most parsimonious explanation is that reindeer were younger than caribou in this study and their HPA axis had not fully matured, resulting in hyposensitivity. In developing vertebrates, there is a

distinct period of hyporesponsiveness to stress [10,25,46,49, 69,72,84] and this suppression is hypothesized to minimize the deleterious effects of elevated GCs upon growth and development (for review see [69]). Additional study is warranted to disentangle age effects from other factors discussed below. Second and inextricably connected with age is the potential to reproduce, which could also affect responsiveness to stress [13,75,93]. The majority of female caribou in this study were pregnant whereas female reindeer were virgins. However, stress responsiveness is typically reduced during pregnancy [18,54], which would produce an opposite effect whereby female caribou exhibit less sensitivity to stress and ACTH stimulation than reindeer. Nonetheless, aforementioned age effects could mask such an effect. Third, a history of semi-domestication in Alaskan reindeer could have selected for diminished adrenocortical response to stress, including adrenal insensitivity. Reduced responsiveness of the HPA axis has been demonstrated in a number of domesticated vertebrate species [2,45,79,87], as well as for species that exhibit tameness from inhabiting islands that are predator-free [60]. Fourth and more specifically, differences between caribou and reindeer in social and migratory behavior may directly influence stress reactivity. Barren ground caribou are nomadic and migrate long distances on a biannual basis, which likely alters their sensitivity to unpredictable environmental perturbations that are encountered during migration, such as increased predation and storms. Reindeer, on the other hand, are largely sedentary and more reliant on large stores of body fat to contend with austere winter conditions. These alterations in behavior are likely reflected by underlying reactions to stressful perturbations in the environment. Considerable research on migratory birds indicates modulation of HPA axis during migration (for review see [90]). Generally speaking, baseline corticosterone in plasma is elevated during migration compared to breeding, although stress-induced concentrations are typically blunted; variability is also high, especially between spring and autumn migration and among species [90]. Examining sensitivity to ACTH challenge in non-migratory woodland caribou (*R. t. caribou*) or Peary caribou (*R. t. pearyi*) could help differentiate between semi-domestication and sedentariness as the major factor influencing adrenal responsiveness.

Another important finding of the study was the sexual difference in fecal GCs profiles in response to ACTH in reindeer, although this effect may be an artifact due to high concentrations in males before ACTH challenge. During the first trial, fecal GCs in males were unexpectedly elevated prior to challenge. It is unclear why such an effect occurred, but it is possible that a dominance hierarchy among male reindeer could have been formed after completion of rut in the fall (Barboza, personal communication). This hierarchy could have led to an elevation of circulating GCs. For the second trial, baseline concentrations of fecal GCs were slightly higher in males than females, and this difference was less pronounced compared to the first trial. As discussed above, the increased dose predictably enhanced adrenal sensitivity in females but not in males. This dimorphism is supported by another study in reindeer where a sexual difference was observed in fecal GC concentrations using a smaller dose of ACTH (1 IU/kg; [35]). A sexual dimorphism in HPA axis responsiveness is well described in rodents, with males exhibiting lower adrenocortical responses than females [32].

Several possibilities could explain the lack of responsiveness to ACTH in male reindeer. First, if males were previously exhibiting a stress response before ACTH challenge, then ACTH receptors in the adrenal cortex may have been saturated and unresponsive to further stimulation. This explanation is a possibility in this study because GC concentrations prior to challenge were elevated in males relative to females in both ACTH trials. However, in a previous study, male reindeer did not elevate fecal GCs concentrations in response to a lower dose of ACTH (1 IU/kg) and pre-injection concen-

trations were not abnormally elevated [35]. These findings suggest that insensitivity to ACTH in male reindeer is consistent with previous studies and not necessarily confounded by high pre-injection concentrations. Second, males may require even a higher dose of ACTH to activate the adrenals. This hypothesis could be examined further if plasma cortisol was measured shortly after ACTH challenge to assess whether the adrenal gland was activated. However, the only study measuring circulating cortisol in reindeer following ACTH challenge was conducted in females [68]. If circulating concentrations rise, then downstream effects are likely responsible for the lack of a fecal steroid response. Such effects could involve altered catabolism of steroids in the liver [17] or buffering of excess steroid by corticosteroid-binding globulin [16]. Both scenarios could lead to decreased excretion of specific GC metabolites measured in feces. Further experiments are necessary to distinguish between these mechanisms.

#### 4.2. Effect of ACTH upon hair cortisol

Cortisol levels deposited in hair of caribou and reindeer were unaffected by ACTH treatment, except in one case. Cortisol concentrations were elevated in the shoulder hair of caribou 7 days following injection compared to baseline levels. However, this effect is most likely a sampling artifact, because controls administered saline exhibited a similar increase. Growth of hair is cyclical and undergoes alternating periods of active growth (anagen), transition (catagen), and inactivity (telogen), and steroids only passively diffuse from the circulation during periods of active growth [28,58]. In the first trial (March), ACTH treatment was not expected to increase cortisol levels in hair because hair growth was arrested. In the second trial (July and August), reindeer were actively shedding their winter coat and simultaneously growing new hair (anagen). Nevertheless, ACTH challenge failed to increase hair cortisol in female reindeer during this period despite a significant elevation in fecal GCs. Because steroids are incorporated into hair follicles during anagen, these results suggest that a single dosing of ACTH does not elevate hair cortisol concentrations in *Rangifer*. Perhaps a higher dose of ACTH or repeated stimulation is required to detect increased cortisol concentrations in hair. We had intended to test the hypothesis that hair cortisol concentration would increase between serial hair samples collected from the same animal following sequential ACTH challenges at 4- and 8-week intervals. However, lack of funds and logistical challenges limited us to trials based on single injections only. In rhesus macaques (*Macaca mulatta*), a prolonged stressor that involved translocation and exposure to new conspecifics was sufficient to increase hair cortisol [28]. For our study, the other possibility is that increased steroid deposition occurred but was exceedingly small relative to the total amount of cortisol in the entire hair shaft, which creates a dilution effect that prevented detection of any discernable increase.

Hair cortisol concentrations prior to ACTH administration varied between caribou and reindeer and among body region sampled. Similar variation has been reported in humans [71] and grizzly bears [47]. Hair samples collected from five different areas of the human scalp were variable compared to repeated sampling within a single region [71]. Similar to caribou, cortisol in neck hair of grizzly bears was higher than concentrations measured in other body regions (shoulder, rump, and abdomen), and these differences were attributed to variability in the onset and timing of the annual body molt [47]. Because new hair growth commences in advance of any discernable body molt in grizzly bears, it is possible that neck hair was growing before other regions or that increased glandular secretions containing cortisol were more abundant in the neck region compared to other areas, which, in turn, could elevate hair cortisol [58]. Reindeer exhibited an opposite pattern where cortisol concentrations in shoulder and rump

hair were significantly elevated compared to those in neck hair. Given that a detailed analysis of the annual cycle of hair growth in *Rangifer* is lacking, it is unclear why caribou and reindeer exhibit different patterns of cortisol deposition across body regions sampled. Hair was sampled in late winter when growth was not occurring, thus these concentrations likely reflected growth from the previous year that could have been influenced by a number of factors, such as age and social interactions. Generally speaking, reindeer fur is longer and coarser than fur of this particular caribou herd, which could affect steroid deposition [24,43]. Timing of molts also varies between subspecies in addition to the timing of other productive demands such as birth and lactation (reindeer are typically earlier than caribou; [7]), which in turn could affect the temporal dynamics of cortisol accumulation in hair. Lastly, hair color (melanin type and quantity) influences hair cortisol concentration in domestic dogs (*Canis lupus familiaris* [12]) and mule deer (*Odocoileus hemionus*; Jeffrey, personal communication), but not in humans [71], and this effect could potentially mediate differences in hair cortisol concentrations between reindeer and caribou. Among grizzly bears, hair color failed to explain any variation in hair cortisol [47]. However, there was a tendency for darker hair to contain more cortisol than lighter hair within individual bears [47]. In the current study, hair color was generally similar between caribou and reindeer, among body regions, and within individuals (pre- and post-sampling; Macbeth, personal communication). Some of the samples originating from the neck in both caribou and reindeer were predominantly white and in one individual some of the body region samples were red brown (Macbeth, personal communication). If hair color affects hair cortisol deposition in *Rangifer*, then these effects were probably minor in this study. Nevertheless, more research is warranted to accurately assess melanin content and its effect upon hair cortisol concentration in *Rangifer* and other species.

#### 4.3. Effect of restraint stress upon serum cortisol

Serum cortisol concentrations from blood collected within 10 min of initial immobilization were more variable in caribou than reindeer. Since GCs rise rapidly in the circulation within 3–4 min of initial capture and restraint [92], it is likely that serum cortisol concentrations were influenced by different immobilization procedures and the time that blood samples were taken. Although animals had been routinely exposed and trained to immobilization procedures prior to the experiment, it is possible that caribou may have responded differently to the hydraulic chute compared to tethering in reindeer, and that this difference was reflected by greater variability in serum cortisol concentrations. Additional studies are required to test this hypothesis.

Both sexes of reindeer did not elevate serum cortisol following 30 min of restraint stress in late winter, suggesting reduced sensitivity of the HPA axis during this time. This reduced sensitivity was seasonal, as reindeer responded to restraint stress by elevating plasma cortisol concentrations in summer, but not in autumn. Seasonal modulation of the adrenocortical response to restraint stress is widespread in seasonally-breeding vertebrates (for review see [92]). If sensitivity to stress is enhanced in summer, then we would expect reindeer to exhibit increased responsiveness to ACTH, especially since the dosage was quadrupled. However, this effect was demonstrated in female reindeer, but not in male reindeer.

## 5. Conclusion

This study verifies that analysis of fecal GC metabolites can serve as an effective, non-invasive method for assessing physiological stress in barren ground caribou. Stimulation of the adrenals by

ACTH led to a predicted rise in fecal GC metabolites that is typical of other studies. Taking into account the excretion lag period, an increase in fecal concentrations would correspond to release of GCs from the adrenal at least 8 h earlier. The next step is to measure GC concentrations in free-ranging caribou to establish effects of season, sex, and age in the wild and among populations that differ in exposure to environmental stressors. This non-invasive technique could also complement demographic trends to evaluate impacts of climate change and industrial activity upon alterations in fecundity and mortality that are hypothesized to contribute to population declines.

For reindeer, more research is needed to understand fecal hormone profiles before extrapolation to the field. Reindeer exhibited diminished adrenal sensitivity to ACTH compared to caribou possibly as a result of selective pressures from semi-domestication and/or sedentariness, but effects from age and sex differences in this experiment cannot be ruled out. Additional studies that assess brain and pituitary sensitivity to stress would increase our understanding of the mechanistic pathways that alter HPA responses to stress. Adrenal insensitivity of male reindeer to ACTH was not expected and should be further investigated to examine whether higher doses of ACTH are needed to stimulate the adrenal gland and whether ACTH increases plasma cortisol. Lastly, more research is necessary to understand the dynamics of hair cortisol deposition in relation to acute and chronic stress. Validating the rate of steroid deposition into the hair follicle relative to amount of circulating steroid (bound and unbound) as well as identifying the minimum threshold required for acute stress/adrenal activation to increase hair cortisol levels would prove useful. Taken together, these non-invasive measures of stress hormones have great potential as monitoring tools to evaluate the role of stress in regulating the physiological health of *Rangifer* populations in the Northern hemisphere.

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