Final Report for the CARMA Network-November 2009

Project Title: Hair cortisol as a non-invasive measure of long-term stress in caribou: technique development and considerations

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1. Background:

Human caused environmental change is believed to negatively affect the sustainability of many wildlife populations. Increasingly, caribou (*Rangifer tarandus*) are threatened by anthropogenic landscape change and the effects of global warming (1, 2). Long-term physiological stress in individual animals may be the predominant mechanism linking environmental change with impaired wildlife population health (3).

While the physiological response to short-term stress is adaptive, long-term stress may lead to a pathological syndrome of immunosuppression, decreased reproduction and diminished growth which may eventually manifest at the population level as reduced rates of survival and reproduction (4). Importantly, biological markers of long-term physiological stress should be measurable in individuals before health effects are apparent at the population level.

Cortisol, the primary glucocorticoid of most mammals, is a key component of the physiological stress response and has been used extensively to measure stress in the plasma, urine, feces and saliva of many wild species (5, 6, 7 and 8). Plasma collection requires capture, immobilization and invasive sampling. Urine and fecal samples are especially prone to contamination and degradation. The collection of saliva may be impractical in free ranging wildlife. More importantly, all four matrices can only assess stress in the time frame of a few hours to a few days (5, 6, 7 and 8). In contrast, hair is a relatively stable medium which can be collected non-invasively or opportunistically (e.g. hunting) and is known to incorporate blood borne hormones and xenobiotics during its active growth phase lasting weeks to months (9).

Hair cortisol concentration (HCC) has recently been validated as a biomarker of long-term stress in a variety of species (10,11 and 12) and building on techniques we have developed for use in free-ranging grizzly bears (*Ursus arctos*) and polar bears (*Ursus maritimus*), we are

investigating HCC as a sensitive, reliable and non-invasive measure of long-term stress in caribou.

2. Objectives:

1) To develop, validate and apply a technique for the determination of HCC in free-ranging caribou.

2) To examine the effects of acute stress (adrenocorticotropic hormone (ACTH) challenge) on HCC in a controlled experiment.

3) To explore HCC in free ranging caribou herds.

3. Results:

3.1 Technique development:

3.1.1 Sample quantity required:

A minimum sample quantity of 100 mg hair (approximately a 2 cm by 2cm plucked or shaved patch of hair) is required for cortisol analysis in caribou.

3.1.2 Technique summary:

- Hair samples are washed three times (3 minutes per wash with 10.0 ml methanol /100 mg hair) and dried hair is ground to a fine powder using a Retsch MM301 ball mill (3 minutes /100 mg hair at 30HZ).
- Steroids are extracted by spinning samples for 24 hours on a slow rotator with 1.0 ml methanol / 50 mg powdered hair. Extracted samples are centrifuged, supernatant is collected, and solvent evaporated under a gentle stream of Nitrogen gas at 38 ^{o}C.
- Hair extract is reconstituted with 0.4 ml of phosphate buffer for 12 hours at 4 ^{o}C in the dark and HCC is assayed with a commercial enzyme linked immunoassay kit (Oxford EA-65 Cortisol EIA kit, Oxford Biomedical, MI, U.S.A.).

3.1.3 Measures of accuracy, precision, specificity and sensitivity:

- Intra assay coefficient of variation (CV) : 6.04% and Inter assay CV: 18.39%
- Extraction Efficiency/Spike Recovery : 102.16 ± 5.20%
- Parallelism: The cortisol concentration of serially diluted *Rangifer* hair extracts and cortisol standards are highly correlated (r^2=0.998, P< 0.0001).
- The limit of detection of the assay limit is 0.04 ng/ml and corresponds to approximately 0.32 pg cortisol per mg hair.

3.1.4 Comments:

In general, assay performance in *Rangifer* is in line with our previous work in other wildlife. The high extraction efficiency and low intra assay CV suggest the technique is accurate and precise. Inter assay CV identified in caribou is greater than that observed in our work with other species but is still within acceptable limits. Parallelism between serially diluted hair extracts and cortisol standards indicates that the assay is highly specific for cortisol. The low detection limit shows the technique is also sensitive across the range of HCC determined in caribou to date (mean= 2.22 pg/mg, range= 0.60-6.90 pg/mg, n= 125).

3.2 Hair cortisol among sex classes and body regions in captive caribou:

3.2.1 Sex classes:

- In captive caribou there is no difference in HCC among adult male and adult female caribou (Independent samples t- test, $t_{(10)} = 0.287$, P= 0.780). These findings are in line with our previous work in free-ranging grizzly bears (B. Macbeth, unpublished data).

3.2.2 Body regions in captive caribou:

- Hair cortisol concentration varies among different body regions in captive caribou (Fig 1).

- Within body regions, variation in HCC is greatest among samples taken from the neck (mean CV 75.54%, SD 21.90 %, n=12) and least in those collected from the shoulder (mean CV 11.52%, SD 9.13%, n=12). Samples collected from the rump exhibit an intermediate level of variation (mean CV 17.04%, SD 13.78%, n=12).

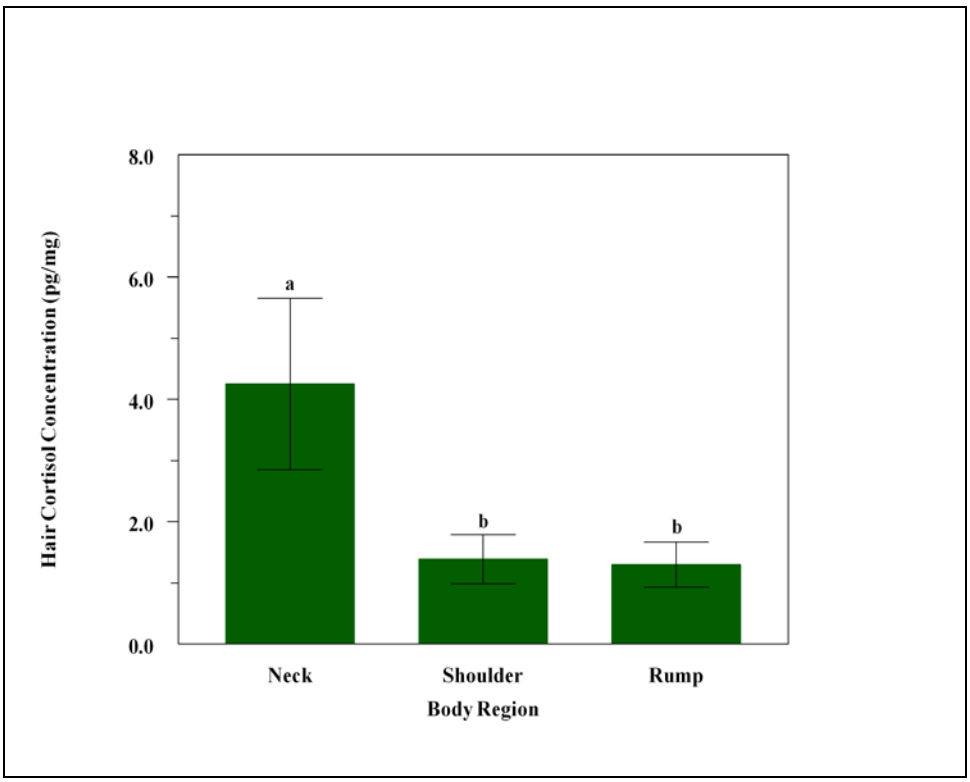

Figure 1. Hair cortisol concentration among body regions (neck, shoulder and rump) in captive caribou. Data are presented as mean ± SD of hair cortisol determinations in n=12 animals. Hair cortisol concentration varies among body regions (One-way repeated measures ANOVA, F= 52.813, P< 0.0001). Data marked with different letters are significantly different from each other (Tukey-Kramer, P< 0.05).

3.2.3 Body regions in free-ranging caribou:

- The pattern of HCC among body regions in a free-ranging caribou bull from Southampton Island, Canada was different from that in captive animals (Fig 2).

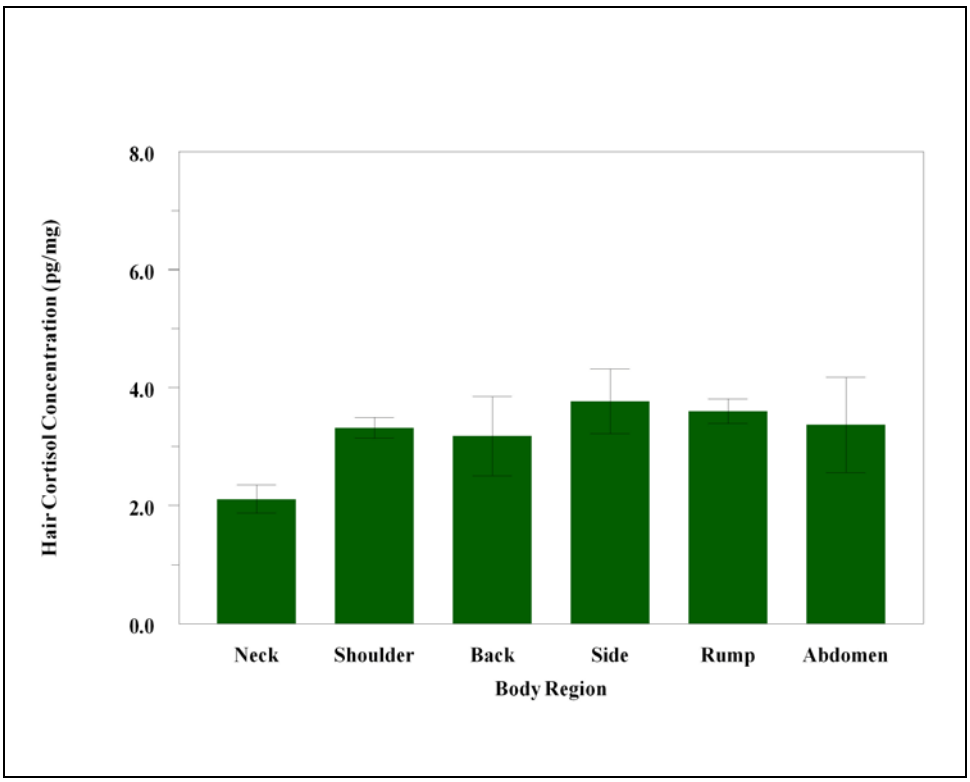

Figure 2. Hair cortisol concentration among body regions in a mature free-ranging caribou bull from Southampton Island, Canada. Data are presented as the mean ± SD of five independent cortisol determinations in each body region. No statistical analysis was performed in this preliminary analysis.

3.2.4 Comments:

Significant differences among body regions suggest that sampling should be standardized to one body region in caribou. Owing to its high variability, we recommend that the neck is discarded as a potential site for HCC analysis and that all future work in this species use shoulder or rump hair.

4. ACTH challenges:

ACTH trials were performed by P. Barboza et al. at the University of Alaska Fairbanks in February, 2008. Five adult male caribou and 5 adult female caribou were injected with 2 IU/kg ACTH, IM. One male and 1 female caribou were injected with saline and served as controls. ACTH has been used successfully in *Rangifer* to artificially elevate plasma cortisol concentration and effectively mimics an episode of acute stress (13).

Hair was collected from three body regions (neck, shoulder and rump) before and one week after injection by shaving as close to the skin as possible with electric clippers. Post ACTH administration, 100 hairs were plucked from each body region in each animal for growth stage analysis using trichograms and special staining techniques (14, 15).

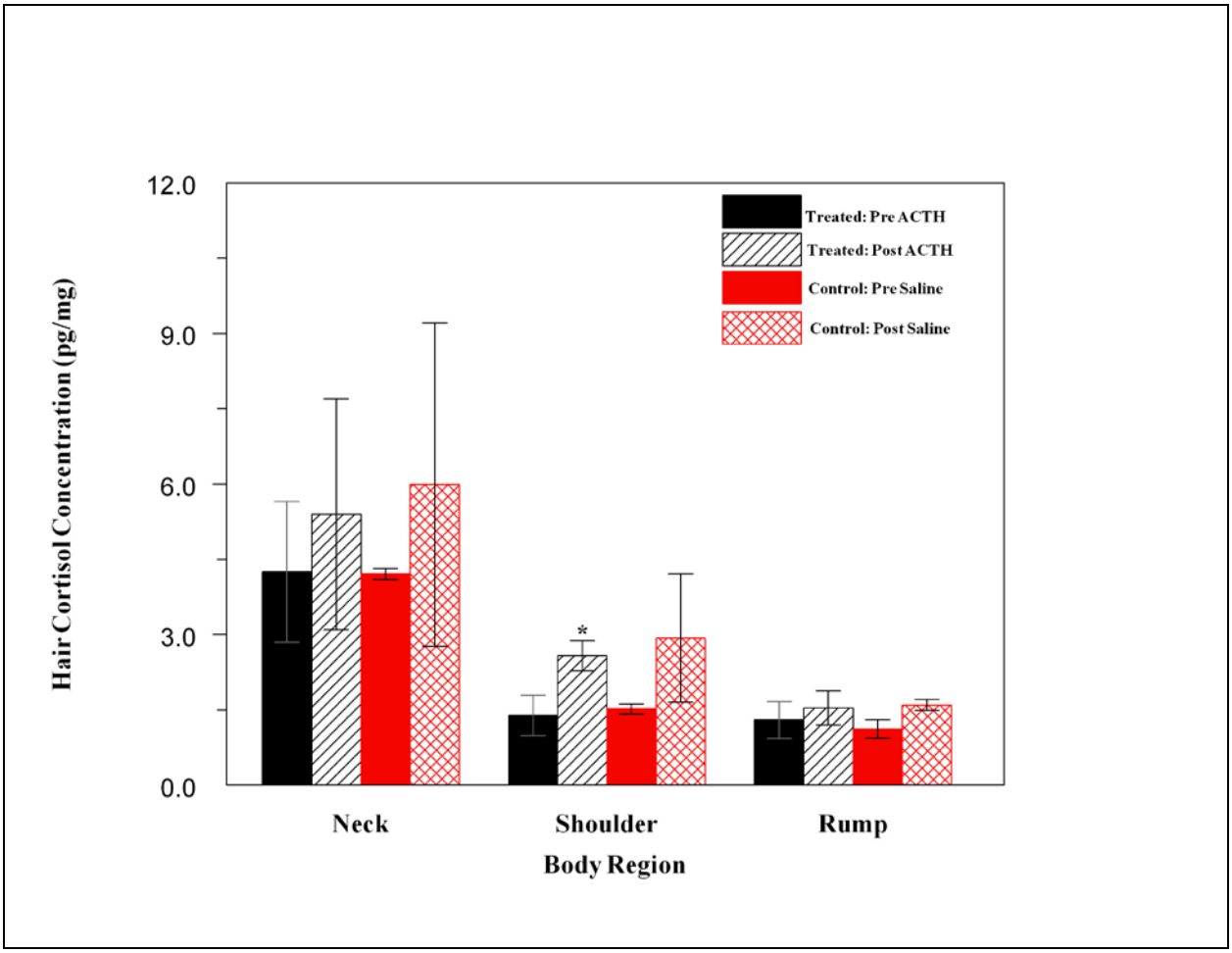

Figure 3. Hair cortisol concentration pre and post ACTH (or saline) administration in neck, shoulder and rump hair of caribou. Data are presented as mean ± SD of hair cortisol determinations in n=10 caribou. There is no significant difference in the cortisol concentration of caribou neck hair (Paired samples t-test, $t_{(9)} = 2.213$, P= 0.054) or rump hair (Paired samples t-test, $t_{(9)} = 2.195$, P= 0.056) pre and post ACTH administration. *There is a significant increase in hair cortisol concentration in shoulder hair (Paired Samples t-test, $t_{(9)} = 10.581$, P< 0.0001) post ACTH administration. In all body regions the pattern of HCC pre and post ACTH administration follows that of n=2 control animals (red) injected with saline.

4.1 Hair growth stage analysis:

4.1.1 Hair bulb morphology:

Hair growth follows a cyclical pattern of alternating periods of active growth (anagen), transition (catagen) and quiescence (telogen) and the morphology of plucked hair is useful in

determining the stage of hair growth (9, 14). Trichograms use hair bulb morphology to identify stages of the hair cycle and club shaped bulbs are indicative of non-growing (telogen) hairs (14).

To interpret pre and post ACTH patterns of HCC trichograms were performed using 100 plucked hairs from each body region of each animal. In this investigation most hairs from all body regions in all animals were club shaped (Table 1, Fig 4). A small percentage of hairs were broken and bulb morphology could not be assessed (Table 1). Hair from the neck of one animal was not available in sufficient quantity for growth stage analysis. Our findings are in line with known patterns of hair biology in caribou as samples were collected in late winter when all animals should have mature (non-growing) hair coats (16).

Cortisol is believed to enter the hair shaft primarily from the systemic circulation and only during periods of active growth (10). This, together with morphological findings suggests that the differences in shoulder HCC observed pre and post challenge were not due to ACTH administration. This assertion is further supported by the fact that the patterns of HCC pre and post injection in treated and control animals were the same (Fig 2).

Table 1: Hair bulb morphology in hair collected post ACTH (or saline) administration from 12 caribou.

Body Region	Mean % Club Shaped Hair Bulbs	Mean % Broken Hairs	Total Number Hairs Examined
Neck	**97**	**3**	**n=1100**
Shoulder	**99**	**1**	**n=1200**
Rump	**95**	**6**	**n=1200**

4.1.2 Staining with 4-dimethylaminocinnamaldehyde:

To confirm hair was not growing a sub sample of 25 plucked hairs from each body region in each animal was stained with 4-dimethylaminocinnamaldehyde (DOCA). DOCA distinguishes growing hair (which contains the root sheath protein citrulline) from non-growing hair which does not. Growing hair stains bright red while non-growing hair stains pale orange or not at all (15).

The results of this experiment were inconclusive. All hairs from all animals stained light pink to orange (Fig 4). Importantly, DOCA turns pink on contact with air (B. Macbeth, personal observation). The width of caribou guard hairs prevented proper convergence of the cover slip and slide in staining preparations. As a result, the consistent submersion of hair in stain was difficult to control. It is likely that the pale pink observed in all samples was a result of this phenomenon and was not due to the presence of anagen hairs. This assertion is supported by the preceding morphological analysis and known patterns of hair biology in caribou (16).

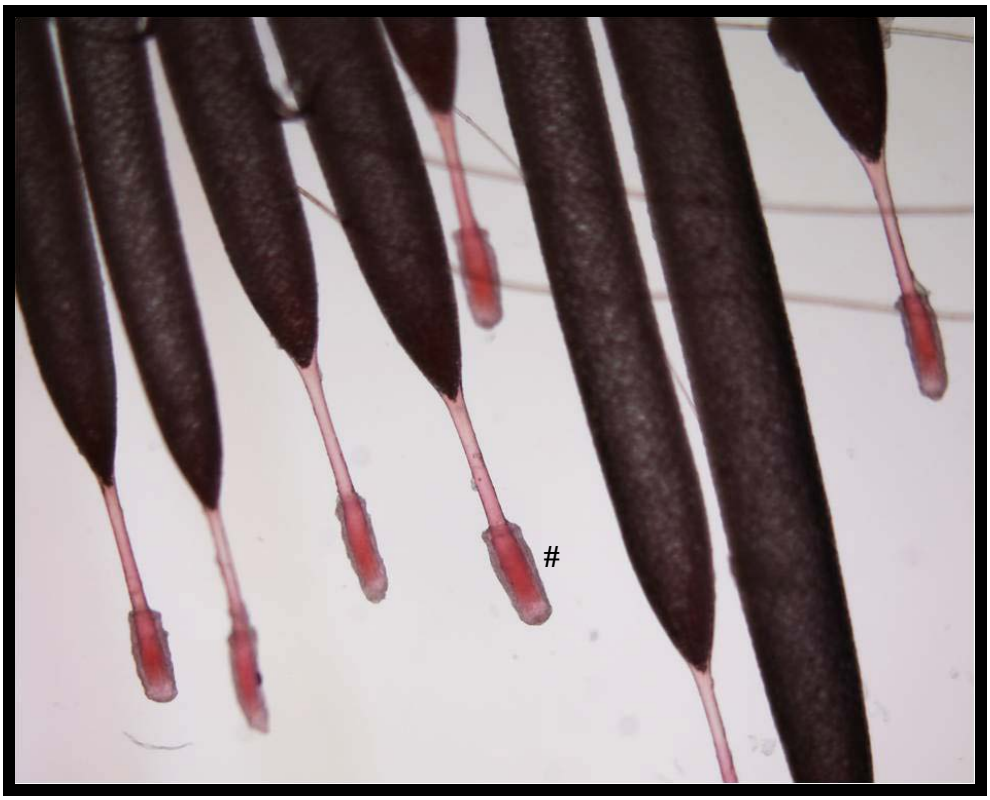

Figure 4. Plucked caribou hair stained with 4-dimethylaminocinnamaldehyde at 100X magnification showing club shaped hair bulbs (e.g. #).

4.1.3 Comments:

Studies of HCC in humans have identified significant variation in HCC in different areas of the scalp (17) and we have observed large variation in HCC measured within body regions in some grizzly and polar bears (B. Macbeth, unpublished data). Hair collected post ACTH came from different locations than had been sampled pre challenge. As such, it is probable that variability within body regions accounts for the increase in shoulder hair observed in this investigation.

HCC recorded in each hair shaft within a body region reflects an average measure of hypothalamic-pituitary-adrenal axis activity during its period of active growth. Factors affecting the onset and duration of moult among different body regions are highly variable as are patterns of follicular activity within body regions (e.g. 14, 16 and 18). Disparate periods of active growth among sites sampled pre and post challenge is the most likely cause of the differences in shoulder HCC observed in this study. However, hair colour and the distribution of cortisol along the length of the hair shaft may also be important (12, 19 and 20).

The relationship between HCC and hair colour is unclear. When these parameters are measured across many different individuals there appears to be no effect of hair colour on HCC

(17, B. Macbeth, unpublished data). However, this relationship has not been thoroughly investigated in individuals of any species. Our preliminary work in grizzly bears suggests that within individuals dark hair may contain more cortisol than light hair (B. Macbeth, unpublished data). These findings are in line with studies in the forensic drug field where dark hair has been found to integrate and retain significantly more drug than light hair (both within individuals and among different individuals) (e.g. 19, 20). Melanin is considered to be an important binding site for xenobiotics and different quantities and types of melanin in coloured hair are believed to be responsible for observed patterns (19).

Hair collected in this trial was shaved as close to the skin as possible. Caribou used in this study were not tractable and the uniformity of hair samples was somewhat difficult to control (P. Barboza, personal communication). In humans, significant differences in HCC have been identified along the length of the hair shaft (12). In contrast, no differences have been observed in other species (e.g. non-human primates: 10, grizzly bears: B. Macbeth, unpublished data). The pattern of cortisol distribution along the length of the hair shaft is unknown in caribou. If cortisol distribution is highly variable, sample heterogeneity may have influenced pre and post ACTH HCC determinations. Unfortunately, an accurate assessment of HCC along the length of the hair shaft could not be performed using the shaved hair samples available for this experiment.

Further work to identify factors which may influence HCC in caribou is warranted.

5. Hair cortisol concentration in free-ranging caribou herds:

In collaboration with investigators from the Ontario Ministry of Natural Resources and the Greenland Institute of Natural Resources cortisol concentration was determined in hair collected from 15 woodland caribou captured as part of ongoing research in Ontario, Canada and 97 caribou killed as part of two research hunts in south west Greenland. HCC was also examined in a single mature bull killed as part of a research collection on Southampton Island, Canada (B. Elkin, Environment and Natural Resources, Government of the Northwest Territories).

5.1 Ontario:

- Free-ranging woodland caribou from Ontario were sampled by M. Gauthier et al. (Ontario Ministry of Natural Resources) between February 20 and March 10, 2009.

- Samples were collected from the shoulder by cutting the hair as close to the skin as possible with scissors. Animals sampled included 12 adult females and 1 adult male along with 1 calf and 1 sub adult.

Table 2: Hair cortisol concentration determined in 15 free-ranging woodland caribou from Ontario, Canada.

Caribou Herd Designation	Mean Hair Cortisol (pg/mg)	Hair Cortisol Range (pg/mg)	Sample Size
Fraserdale	**2.08**	**NA**	**n=1**
Nagagami	**2.94**	**1.20-4.67**	**n=2**
Onakawana	**1.91**	**0.82-2.61**	**n=5**
Detour	**1.84**	**0.89-3.87**	**n=7**

- Animals from the Fraserdale and Nagagami regions inhabit heavily disturbed habitat as a result of forestry activities. There is also significant transportation infrastructure development (roads, a railway, transmission lines) in these areas. In contrast, Onakawana animals are only impacted by a railway line on the eastern aspect of their range and have access across large rivers to pristine habitat to the north and west. Detour animals are located along the Ontario/Quebec border and have been exposed to mines, mining exploration, forestry operations, paved highway and numerous winter roads (M. Gauthier, personal communication).

5.2 Greenland**:**

- Free ranging caribou from Greenland were sampled by C. Cuyler et al. (Greenland Institute of Natural Resources). Hair was collected from the Akia-Maniitsoq herd between March 29 and April 13, 2008 and from the Kangerlussuaq-Sisimiut herd between March 3 and March 17, 2009.

- Samples collected in 2008 were taken from the neck while those from 2009 were taken from the shoulder. In both years, hair was obtained by cutting as close to the skin as possible with a sharp knife.

- In both herds the harvests were selective for females. A range of ages, body conditions and reproductive classes were taken. Most cows were adults (> 3-years old) while some were sub adults (1½ to 2½-years old). Calves of the year between 9 and 10-months old were also harvested (C. Cuyler, personal communication).

Table 3: Hair cortisol concentration determined in 97 free-ranging caribou from Greenland.

Caribou Herd Designation	Mean Hair Cortisol (pg/mg)	Hair Cortisol Range (pg/mg)	Sample Size
Akia-Manittisoq	2.15	0.60-6.90	n=48
Kangerlussuaq-Sisimut	2.30	1.21-3.98	n=49

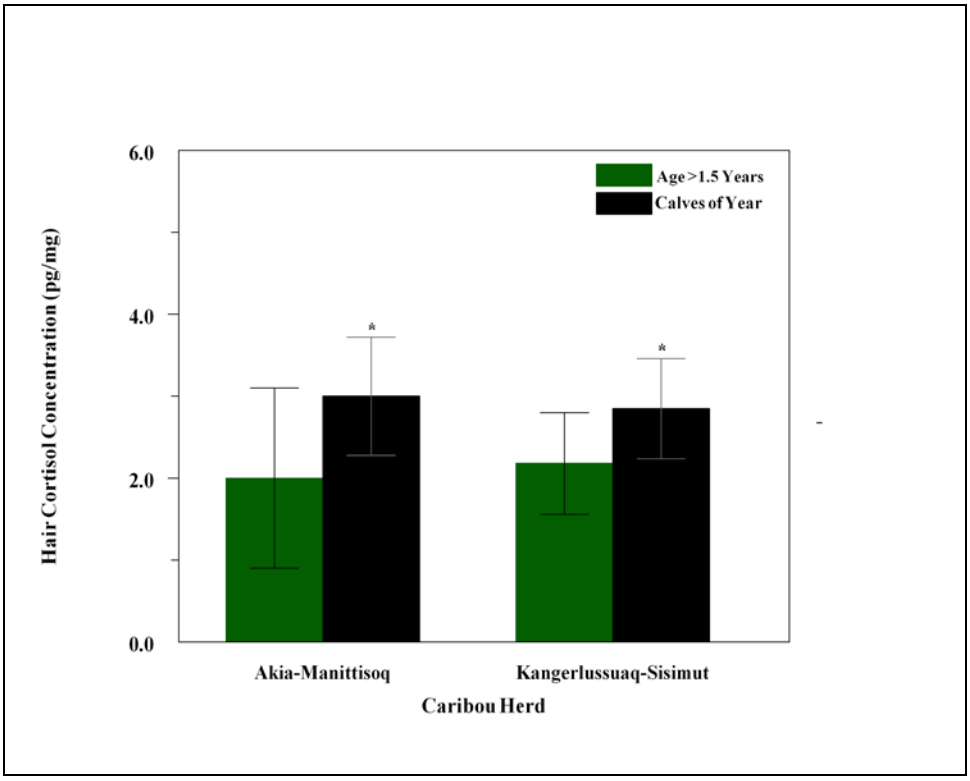

Figure 5. Hair cortisol concentration in free-ranging female caribou > 1.5 years of age and claves of the year from the Akia-Manittisoq and Kangerlussuaq-Sisimut herds in Greenland. In both herds, hair cortisol concentration was highest in calves of the year (Akia-Manittisoq: Paired samples t-test, $t_{(46)} = 2.300$, P= 0.024, n=41 >1.5 years and n=7 calves) and (Kangerlussuaq-Sisimut: Paired samples t-test, $t_{(47)} = 2.941$, P= 0.005, n=40 >1.5 years and n=9 calves).

6.0 Summary:

Table 4. Summary (mean ± range) of hair cortisol concentration determined in seven free-ranging and one captive caribou herds.

Caribou Herd Designation	Mean Hair Cortisol (pg/mg)	Hair Cortisol Range (pg/mg)	Sample Size
Captive	2.31	1.56-3.86	n=12
Fraserdale	2.08	NA	n=1
Nagagami	2.94	1.20-4.67	n=2
Onakawana	1.91	0.82-2.61	n=5
Detour	1.84	0.89-3.87	n=7
Southampton Island	3.23	NA	n=1
Akia-Manittisoq	2.15	0.60-6.90	n=48
Kangerlussuaq-Sisimut	2.30	1.21-3.98	n=49

7.0 Conclusions and recommendations:

- We have developed an enzyme-linked immunoassay-based technique to measure hair cortisol concentration (HCC) in caribou.
- A minimum sample quantity of 100 mg hair (approximately a 2 cm by 2cm plucked or shaved patch of hair) is required for cortisol analysis.
- In general, assay performance in *Rangifer* is in line with our previous work in other wildlife.
- The high extraction efficiency (102.16 ± 5.20%) and low intra-assay coefficient of variation (CV=6.04%) indicate that the technique is accurate and precise.
- Inter-assay CV identified in caribou is greater than that observed in our work with other species but is still within acceptable limits.
- Parallelism between serially-diluted hair extracts and cortisol standards (r^2=0.998, P< 0.0001) suggest that the assay is highly specific for cortisol.
- A low detection limit (0.32 pg cortisol per mg hair) indicates the technique is sensitive across the range of HCC determined in caribou to date (mean = 2.22 pg/mg, range = 0.60-6.90 pg/mg, n=125).
- HCC varies among body regions in caribou. Owing to its low intra-region variability, we recommend collecting only shoulder or rump hair for HCC analysis.
- HCC does not vary with sex class in captive animals.
- HCC varies with age class in some free-ranging herds.
- HCC in quiescent hair is not influenced by adrenocorticotropic hormone (ACTH) challenge, a procedure that mimics short-term stress.
- Hair cortisol concentration is a promising measure of long-term stress in *Rangifer*.
- Further work to identify factors affecting HCC in caribou is warranted.
- HCC analysis should be expanded to include all age, sex and reproductive classes of free-ranging caribou.
- Future studies should also explore the association of HCC with other measures of stress and health in free-ranging caribou (e.g. body condition, *Brucella suis* type 4)

6. References:

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