AN EVALUATION OF HAIR CORTISOL CONCENTRATION AS A POTENTIAL BIOMARKER OF LONG-TERM STRESS IN FREE-RANGING GRIZZLY BEARS (*Ursus arctos*) POLAR BEARS (*Ursus maritimus*) AND CARIBOU (*Rangifer tarandus* sp.)

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By

Bryan James Macbeth

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S7N 5B4
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ABSTRACT

Human-caused ecological change negatively affects the sustainability of many wildlife populations but may be especially challenging for large carnivores and ungulates. Long-term physiological stress may be an important mechanism linking ecological change with impaired health and reduced population performance in these groups. The determination of hair cortisol concentration (HCC) has recently demonstrated potential as a biomarker of long-term stress in humans and domestic animals, and may also represent a practical technique for use in free-ranging wildlife. The objectives of this research program were to: 1) develop and apply an accurate and reliable method for measuring cortisol levels in hair collected opportunistically or remotely from free-ranging grizzly bears (*Ursus arctos*), polar bears (*Ursus maritimus*), and caribou (*Rangifer tarandus* sp.), and 2) to evaluate the utility of HCC as a biomarker of long-term stress (and thus potentially useful conservation tool) in these threatened species.

An enzyme-immunoassay (EIA) based technique for measuring HCC in non-human primates was successfully modified for use with small quantities (5-100 mg) of hair representative of samples which may be obtained through opportunistic (e.g. hunting, research captures, archives) or remote (e.g. barb wire snagging) methods in each species. HCC was determined in 151 free-ranging grizzly bears from Alberta, Canada (mean 2.84 pg/mg, range 0.62-43.33 pg/mg); 185 free-ranging polar bears from southern Hudson Bay, Canada (mean 0.48 pg/mg range, 0.16-2.26 pg/mg); in 12 captive Alaskan caribou (*R. t. granti*) (mean 2.31 pg/mg, range, 1.57-3.86 pg/mg) and 12 captive reindeer (*R .t. tarandus*) (mean 2.88 pg/mg, range 2.21-3.40 pg/mg) injected either with adrenocorticotropic hormone (ACTH) or saline; and in 94 free-ranging caribou (*R. t. groenlandicus*) from West Greenland (mean 2.21 pg/mg, range 0.60-6.90 pg/mg). Factors influencing HCC in each species were then explored including: 1) technical considerations for the prudent use of HCC analysis and 2) potential relationships between HCC, biological traits, health, and prevailing environmental conditions. Evidence revealed in this study suggests that, with further research, this technique may show potential as a practical conservation tool for use in free-ranging grizzly bear, polar bear, and caribou populations.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACTH	adrenocorticotropic hormone
AICc	Akaike's Information Criterion corrected for small sample size
ΔAICc	change in Akaike's Information Criterion values
AK	Akia-Maniitsoq caribou population (West Greenland)
$AlCl_3$	aluminum chloride
ANOVA	analysis of variance
AVP	arginine vasopressin
BCI	body condition index (for ursids) as defined in Cattet et al. (2002)
BFR	body fat reserves (for West Greenland caribou) as defined in Chapter 5
ºC	degrees centigrade
$C_8H_5NaO_7S$	sodium dihydrogen 4-sulphonatophthalate
CARMA	CircumArctic *Rangifer* Monitoring and Assessment Network
CBG	cortisol binding globulin
CCWHC	Canadian Cooperative Wildlife Health Centre
cm	centimeters
cm^2	square centimeters
CO_2	carbon dioxide
CRH	corticotrophin releasing hormone
CRH-R1	corticotrophin releasing hormone receptor 1
CSF	cerebrospinal fluid
C.V.	coefficient of variation
DOCA	4-dimethylaminocinnamaldehyde
DEXA	dual energy-X-ray absorptiometry
DNA	deoxyribonucleic acid
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
ESGBP	Eastern Slopes Grizzly Bear Project
FRIGBP	Foothills Research Institute Grizzly Bear Program

FSH	follicle stimulating hormone
GC(s)	glucocorticoid(s)
GH	growth hormone
GnRH	gonadotropin releasing hormone
GR	glucocorticoid genomic receptor
HCC	hair cortisol concentration
HPA axis	hypothalamic-pituitary-adrenal axis
HPLC-MS	high performance liquid chromatography–mass spectrometry
HSD type 2	11ß-hydroxysteroid dehydrogenase type 2
Hz	hertz
IGF-1	insulin like growth factor 1
IM	intra muscular
IU	international units
IU/kg	international units per kilogram
KQ	Kangerlussuaq-Sisimiut caribou population (West Greenland)
kg	kilogram
km	kilometer
km^2	square kilometers
L	litre
LH	luteinizing hormone
MC2-R	type 2 mineralocorticoid receptor
mg	milligram
MJ	megajoule
MJ/kg	megajoule per kilogram
ml	millilitre
mm	millimeter
MR	mineralocorticoid receptor
n	number of samples
NACl	salt
$NaHCO_3$	sodium bicarbonate

ng	nanogram
ng/g	nanogram per gram
ng/ml	nanogram per millilitre
nmol	nanomole
nmol/L	nanomole per litre
PCB	polychlorinated biphenyl
pg	picogram
pg/mg	picogram per milligram
POMC	pro-opiomelanocortin
R^2	coefficient of determination; proportion of variability in the data set accounted for by the statistical model
ΔR^2	change in coefficient of determination
RIA	radio immunoassay
rpm	revolutions per minute
s	second
SBBRP	Scandinavian Brown Bear Research Project
S.D.	standard deviation
S.E.	standard error
SH	Southern Hudson Bay polar bear subpopulation
StD β	standardized coefficient
T_3	triiodothyronine
T_4	thyroxine
TSH	thyroid stimulating hormone
µl	microlitre
V1B	vasopressin receptor 1b
vs	versus
WH	Western Hudson Bay polar bear subpopulation
wi	Akaike weight; model probability

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Background

Anthropogenic landscape modification and climatic change driven by human activity are recognized to alter ecosystems around the world and are now regarded as the principal threats to global biodiversity (Lindenmayer and Fischer 2006, IPCC 2007, IUCN 2012). Although habitat loss, habitat fragmentation, changing land use patterns, and climate change affect many animals, human-caused ecological change may be particularly challenging for species that are large bodied (> 3 kg) (Cardillo et al. 2005, Collins and Kays 2011), hunted (Brashares et al. 2004, Milner et al. 2007), rare or restricted in their distribution, specialized, intolerant of disturbance, or dependent on seasonal environments (Thomas et al. 2004, Issac 2009, Lebreton 2011).

Large mammalian carnivores possess many of these characteristics and the long-term sustainability of populations inhabiting landscapes altered by human activity is often uncertain (Ray et al. 2005, Paviolo et al. 2008, Durner et al. 2009, Rajeev et al. 2011, Proctor et al. 2012). Biological traits such as low population density, low reproductive rates, extensive home range requirements, restricted foraging behaviour, and limited dispersal capability combine to make this group inherently vulnerable to the effects of human-caused ecological change (Weaver et al. 1996, Cardillo et al. 2004, Ray et al. 2005). Large carnivores are also among the species most affected by direct conflict with humans (Treves and Karanth 2003, Wilson et al. 2006, Bauer et al. 2010). Most carnivores are intolerant of human activity, and in some regions these species have been or continue to be heavily persecuted (Mattson and Merill 2002, Ray et al. 2005, Tian et al. 2011). As a result, many large carnivore populations already contain relatively few individuals and the likelihood of their persistence may be further reduced by interactions between anthropogenic ecological conditions, limiting biological traits, stochastic events (e.g. disease outbreaks; Roelke-Parker et al. 1996, Murray et al. 1999), and geographic or genetic isolation (Schultz et al. 2010, Janecka et al. 2011, Proctor et al. 2012).

Like large carnivores, many ungulates possess life history traits which make them intrinsically vulnerable to changing landscape or climatic conditions (Murray et al. 2006, Ogutu et al. 2009, Hu and Jiang 2011, Festa-Bianchet et al. 2011, Bhola et al. 2012). For example, the life cycles of most ungulates are closely tied to the acquisition of specific forage plants and trace minerals which may be seasonally or geographically restricted in their distribution (Post and Forchhammer 2008, Mcart et al. 2009, Bhola et al. 2012). As a result, these species may be

especially sensitive to anthropogenic changes in access to seasonal foraging areas or mineral licks and to changes in the phenology, distribution, and nutritional quality of important forage plants (Keller and Bender 2007, Post et al. 2008, Parker et al. 2009). Human activity (Aastrup et al. 2000, Siep et al. 2007, Nellemann et al. 2010) and human-caused ecological change may also displace ungulates from vital migration routes, calving grounds, dispersal areas, or refugia from intraspecific competition, inclement weather, disease, and predation (Racey et al. 2005,Vistnes and Nellemann 2008, Ogutu et al. 2009, Bjorneraas et al. 2011). As for large carnivores, the effects of hunting pressure (Milner et al. 2007, Zhou and Zhang 2011, Zafir et al. 2011), geographic and genetic isolation (Hebblewhite et al. 2009, Yang et al. 2011, Collins et al. 2012) along with interactions between anthropogenic ecological conditions and natural drivers of population dynamics (e.g. population density, disease) may further diminish the resilience of ungulate populations inhabiting disturbed landscapes (Morgan et al. 2006, Couturier et al. 2009a, Kutz et al. 2012).

In both large carnivores and ungulates, the consequences of human-caused ecological change are well documented and may include: altered patterns of movement, dispersal, or distribution (Vistnes and Nellemann 2008, Musiani et al. 2010, Polfus et al. 2011, Proctor et al. 2012); aberrant behaviour (Amstrup et al. 2006, Stirling et al. 2008, Hayward and Hayward 2009); an increase in the occurrence of disease (Roelke-Parker et al. 1996, Kutz et al. 2005, Cross et al. 2010; Woodroffe et al. 2012); and mortality in individual animals (Courtois et al. 2007, Schwartz et al. 2010, Durner et al. 2011, Collins and Kays 2011). Diminished reproductive output (Milner et al. 2007, French et al. 2011) and survival rates (Schwartz et al. 2006, Wittmer et al. 2007, Ciarniello et al. 2009, Pinard et al. 2012), along with the decline, extirpation, or extinction of affected populations may also occur (Mattson and Merill 2002, Ogutu et al. 2009, Rajeev et al. 2011). Although outcomes are well recognized, a greater understanding of the biological mechanisms responsible for many of these adverse effects is lacking, but is required to develop conservation strategies which ensure the continued persistence of these ecologically (Boudreau and Payette 2004, Helfield and Naiman 2006, Goheen et al. 2010), economically (Mason et al. 2007, Dowsley et al. 2009, Naidoo et al. 2011) and culturally (Anderson 2000, Van Daele et al. 2001, Goldman et al. 2010) important species.

Increasingly, long-term physiological stress is being recognized both as a consequence of human-caused ecological change, and as a potential mechanism linking habitat alteration with impaired health and population performance in free-ranging wildlife (e.g. Wikelski and Cooke 2006, Martinez-Mota et al. 2007, Van Meter et al. 2009,Wasser et al. 2011, Ellis et al. 2012). In disturbed landscapes, wildlife may be subject to stressors that may differ in type and exceed the intensity or duration of those related to normal life history processes in unaltered habitats (McEwen and Wingfield 2003, Wikelski and Cooke 2006, Ellis et al. 2012). Whereas the physiological response to short-term stress is adaptive (Sapolsky et al. 2000), long-term stress (lasting weeks to months) may lead to a pathological syndrome of distress in individual animals characterized by poor health, diminished growth, and decreased reproduction (Charmandari et al. 2005, Busch and Hayward 2009, Linklater 2010). In turn, rates of survival, reproductive output, and overall abundance may decline as the number of distressed individuals in a population grows (Wikelski and Cooke 2006, Busch and Hayward 2009, Ellis et al. 2012).

Presently, there are few tools available to assess long-term stress in free-ranging wildlife, and the development of such techniques may enhance our understanding of relationships between human-caused ecological change, health, and population dynamics in large carnivores and ungulates (Cattet et al. 2006, Ashley et al. 2011, Ellis et al. 2012). Biological markers of long-term stress should be measurable in individual animals before adverse effects are apparent at the population level and, ultimately, these techniques may also evolve into practical tools for use in conservation initiatives (Wikelski and Cooke 2006, Cattet et al. 2006, Ellis et al. 2012). Importantly, considerations and biological relationships revealed through the development and application of novel techniques in large carnivores and ungulates may be broadly applicable to other mammals, and may also lead to long-term solutions that better preserve the integrity of ecosystems as a whole (Clark et al. 2001, Caro 2003, Cluff and Paquet 2003).

The mammalian stress response is mediated by the actions of the Hypothalamic-Pituitary-Adrenal (HPA) axis and its glucocorticoid effector hormones (Sapolsky et al. 2000). Biological media commonly used to assess these parameters include blood, saliva, urine and feces (Cattet et al. 2003a, Millspaugh et al. 2002, Constable et al. 2006, Wasser et al. 2011). However, the utility of these media for the study of long-term stress in free-ranging wildlife may be limited by a variety of practical considerations (e.g. Jessup et al. 1988, Caulkett and Shury 2008, Cattet et al.

2008a, Johnstone et al. 2012). In the last decade, pioneering studies in Procavids (Koren et al. 2002), humans (Raul et al. 2004), and non-human primates (Davenport et al. 2006) observed that cortisol (the primary glucocorticoid of most mammals; Sapolsky et al. 2000) could be measured in hair. More recently, hair cortisol levels have been linked with other measures of systemic HPA axis activity (e.g. Sauvé et al. 2007, Accorsi et al. 2008, Bennett and Hayssen 2010), and elevated cortisol levels have been recorded in hair collected from humans (e.g. Yamada et al. 2007, Kirschbaum et al. 2009, Steudte et al. 2011) and animals (e.g. Davenport et al. 2006, Accorsi et al. 2008, Dettmer et al. 2009) chronically exposed to stressful stimuli or exhibiting diminished health (e.g. Thompson et al. 2010, Manenschijn et al. 2011a, Fairbanks et al. 2011). Hair is also a relatively stable medium (Quadros and Monterio-Filho 1998, Kintz 2004, Webb et al. 2010) that can be collected opportunistically or remotely from many species of free-ranging mammals (e.g. Woods et al. 1999, Belant et al. 2007, Ausband et al. 2011), and is known to incorporate blood borne hormones and xenobiotics during its active growth phase which may last many weeks to months (Maurel et al. 1986, Pragst and Balikova 2006, Kidwell and Smith 2007). All considered, hair cortisol analysis may be uniquely suited for the study of long-term stress in wild mammals threatened by human activity and environmental change (Macbeth et al. 2010, Ashley et al. 2011, Bechshøft et al. 2011). A thorough evaluation of this technique in large carnivores and ungulates is warranted.

In order for the determination of hair cortisol concentration (HCC) to be revealed as a defensible biomarker of long-term stress (and thus a potentially useful conservation tool) in these groups: 1) accurate and reliable techniques must first be developed to measure cortisol in hair collected from target species (Davenport et al. 2006), 2) factors which may influence HCC must be identified (Gow et al. 2010, Russell et al. 2012), 3) linkages must then be established with prevailing environmental conditions and markers of individual animal health, and 4) these should ideally be related to fitness characteristics such as rates of survival and fecundity at the population level (Busch and Hayward 2009, Sheriff et al. 2011).

1.2 General Research Objectives

The general objectives of this research program were to: 1) develop and apply an accurate and reliable method for measuring cortisol levels in hair collected opportunistically or remotely from three large North American mammals threatened by anthropogenic landscape modification

or human-caused climatic change: the grizzly bear (*Ursus arctos*) (AGBRP 2008, Festa-Bianchet 2010), the polar bear (*Ursus maritimus*) (Derocher et al. 2004, Peacock et al. 2011), and the caribou (*Rangifer tarandus*) (Vors and Boyce 2009, Festa-Bianchet et al. 2011), and 2) to evaluate the utility of HCC as a potential biomarker of long-term stress and practical conservation tool in these species.

1.3 Working Hypothesis

The working hypothesis of this research program is that "hair cortisol concentration is reliably quantified in free-ranging wildlife and is associated with life history variables indicative of long-term stress".

1.4 The Mammalian Stress Response

1.4.1 Stress, the Hypothalamic-Pituitary-Adrenal (HPA) axis, and cortisol

Stress is often defined as a state of threatened or perceived as threatened homeostasis which may result from exposure to noxious stimuli commonly referred to as "stressors" (Sapolsky et al. 2000, Carrasco and Van de Kar 2003, Romero 2004). The physiological response to stress is a rapidly initiated and complex process that is coordinated in higher centers of the central nervous system and mediated by a variety of peripheral endocrine and autonomic effectors that are closely associated and highly integrated (Sapolsky et al. 2000, Carrasco and Van de Kar 2003, Papadimitriou and Priftis 2009). The characteristics of a stimulus that may incite a stress response along with the strength and duration of that response may be a function of a variety of factors including: genetics (Solberg et al. 2006, Fairbanks et al. 2011), sex (Handa et al. 1994, Boonstra 2005), maternal effects (Seckel 2004, Sheriff et al. 2010), individual temperament, social status, and prior experience (McEwen and Wingfield 2003, Reeder and Kramer 2005, Edeline et al. 2010). An individual's health status and life history stage along with food availability, species specific life history traits and physiology may also be important (Boonstra and McColl 2000, Landys et al. 2006, Romero et al. 2008).

The Hypothalamic-Pituitary-Adrenal (HPA) axis is the principal endocrine pathway of the mammalian stress response (Sapolsky et al. 2000, Carrasco and Van de Kar 2003, Papadimitriou and Priftis 2009). Activation of the HPA axis occurs within seconds of an organism's identification of a stressful stimuli (Sapolsky et al. 2000) when signals from higher brain centers stimulate the synthesis and release of corticotropin releasing hormone (CRH) and

arginine vasopressin (AVP) in the paraventricular nucleus of the hypothalamus (Sapolsky et al. 2000, Papadimitriou and Priftis 2009). Upon release from parvocellular neurons, CRH and AVP enter the hypophyseal portal system and are transported to the anterior pituitary gland (Habib et al. 2001). In the pituitary, these hormones act synergistically (by means of CRH-R1 and V1B receptors respectively) to induce pituitary expression of the pro-opiomelanocortin gene (POMC) and subsequent processing of the POMC pro-hormone into adrenocorticotropic hormone (ACTH) (Carrasco and Van de Kar 2003, Papadimitriou and Priftis 2009). ACTH is then released from the anterior pituitary into the blood stream and transported to the adrenal cortex where it binds to type 2 melanocortin (MCR-2) receptors on the plasma membrane of cells in the zona fasciculata (Charmandari et al. 2005, Papadimitriou and Priftis 2009). ACTH binding triggers a signalling cascade stimulating adrenal steroidogenesis along with the release of glucocorticoid (GC) effector hormones into the systemic circulation (Sapolsky et al. 2000, Carrasco and Van de Kar 2003, Papadimitriou and Priftis 2009).

In most mammals, cortisol is the primary GC secreted by the cells of the zona fasciculata and elevated levels of this hormone are a key component of the stress response (Sapolsky et al. 2000, Carrasco and Van de Kar 2003, Papadimitriou and Priftis 2009). Within seconds to minutes following exposure to a stressful event, elevated cortisol levels act to mobilize and repartition energy thus promoting changes in physiology or behaviour that facilitate immediate survival in the face of an unexpected challenge (Sapolsky et al. 2000, Landys et al. 2006, Busch and Hayward 2009). These changes may include transient permissive effects such as an increase in cardiac output, blood pressure, cerebral blood flow, hepatic gluconeogenesis, and cerebral glucose utilization along with enhanced mobilization of fat and protein stores, improved memory consolidation, and increased cognition (Sapolsky et al. 2000, Carrasco and Van de Kar 2003, Boonstra 2005). Suppressive effects such as the inhibition of energy storage, growth, immunity, reproductive behaviour and physiology along with decreased feeding and appetite also occur (Sapolsky et al. 2000, Habib et al. 2001, Charmandari et al. 2005).

When noxious stimuli are removed, elevated levels of circulating cortisol act to terminate the stress response by means of negative feedback on the hypothalamus and pituitary and subsequent down regulation of CRH and ACTH secretion (Carrasco and Van der Kar 2003). As such, the effects of elevated cortisol levels may be modulated and the physiological response to

short-term stress is generally considered to be adaptive (Sapolsky et al. 2000, McEwen and Wingfield 2003, Charmandari et al. 2005). However, feedback inhibition of the stress response is not infallible. For example, persistent physiological stress has been associated with an increase in arginine vasopressin (AVP) secretion (Black 2002, Carrasco and Van de Kar 2003, Schmidt et al. 2008). Acting synergistically with CRH or alone, AVP is known to enhance ACTH secretion. Thus, AVP secretion may supersede negative feedback on ACTH release (caused by elevated GCs) and lead to continued responsiveness of an organism to novel stressors (Carrasco and Van de Kar 2003). When repeated or chronic activation of the HPA axis occurs over many weeks to months (long-term stress), the effects of persistently elevated cortisol may adversely impact many organ systems and physiological pathways (e.g. McEwen and Wingfield 2003, Seckl 2004, Charmandari et al. 2005). These changes may lead to the development of a pathological syndrome of distress characterized by poor health and reduced fitness in individual animals (Charmandari et al. 2005, Busch and Hayward 2009, Linklater 2010). In turn, distress occurring in many individuals may manifest as diminished rates of survival and reproduction at the population level and may ultimately culminate in the decline, extirpation, or extinction of wildlife inhabiting disturbed environments (Wikelski and Cooke 2006, Ellis et al. 2012) (Fig1.1).

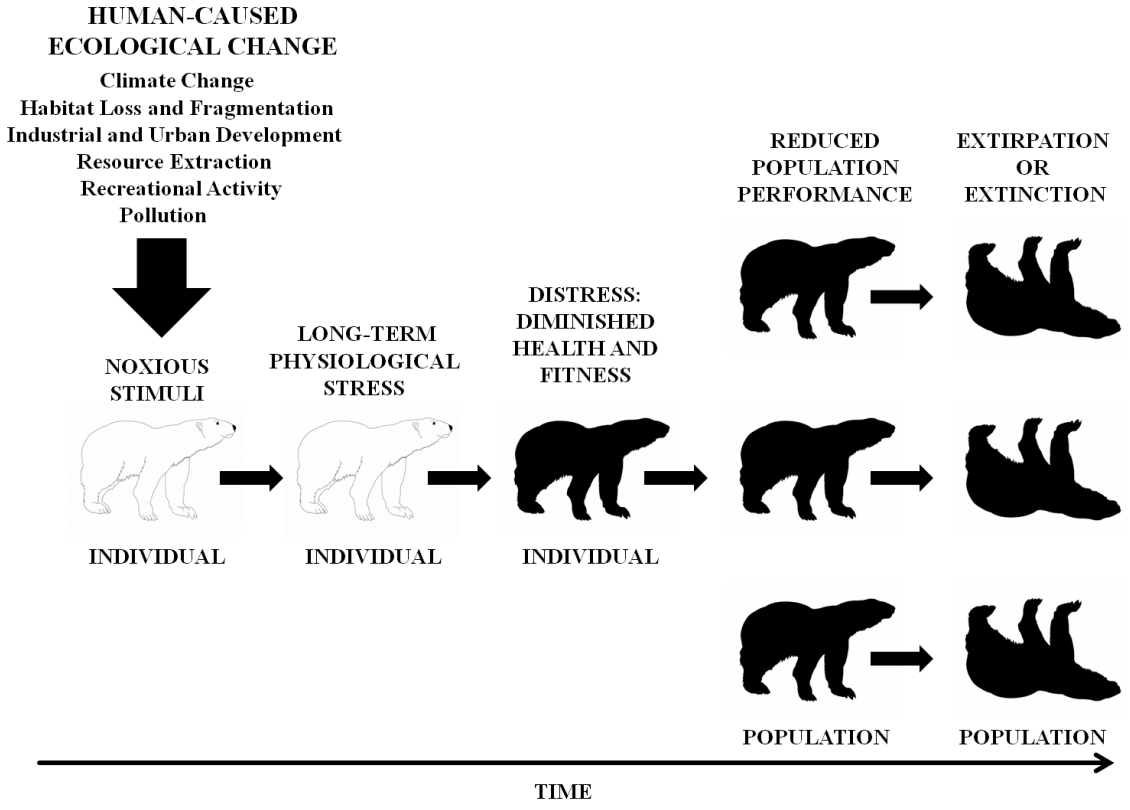

Figure 1.1 Long-term stress: a potential mechanism linking human-caused ecological change with diminished health and reduced population performance in free-ranging wildlife.

1.4.2 Consequences of chronic HPA axis activation in individual animals

1.4.2.1 Metabolism, tissues, and organ systems

Carbohydrate, protein and lipid metabolism along with most tissues and organ systems are responsive to cortisol and other GCs (Sapolsky et al. 2000). In general, these hormones have an anti-insulin effect and increase blood glucose at the expense of protein and lipid catabolism (Sapolsky et al. 2000, McEwen and Wingfield 2003, Charmandari et al. 2005).Thus, cortisol acts to mobilize and redirect energy away from protein and adipose stores in response to energetic challenges (Landys et al. 2006, Busch and Hayward 2009). Specifically, elevated cortisol levels enhance protein breakdown and lipolysis to provide substrates (e.g. amino acids and glycerol) for the process of hepatic-gluconeogenesis which is also up regulated in response to stressful stimuli (Sapolsky et al. 2000, Desborough 2000, Landys et al. 2006). Elevated GCs also increase available glucose by stimulating glycogen deposition and glycogenolysis in the liver while

simultaneously inhibiting glucose uptake and use in peripheral tissues such as muscle, skin and bone (Sapolsky et al. 2000, Landys et al. 2006). Accordingly, muscle atrophy, collagen break down, delayed wound healing, and skin disease or dysfunction may be observed in chronically stressed animals (Habib et al. 2001, Chiu et al. 2003, Ramos-e-Silva and Jacques 2012). Osteoblast activity is also suppressed by GCs, and prolonged stimulation of the HPA axis may also lead to osteoporosis and delayed bone healing (Charmandari et al. 2005). In addition to direct actions, GC effects on bone may be potentiated by simultaneous decreases in growth hormone (GH) and sex steroids caused by elevated CRH levels (Charmandari et al. 2005). Ultimately, increased energy mobilization from protein and lipid stores in combination with a decrease in the maintenance of structural tissue (i.e. muscle, collagen, and bone) may lead to diminished growth (body size, body mass, or body condition) in chronically stressed animals (Landys et al. 2006, Edeline et al. 2010, Macbeth et al. 2012).

Persistently elevated levels of circulating cortisol may also affect the gastrointestinal and cardiovascular systems (Habib et al. 2001, Charmandari et al 2005). Decreased motility, mast cell degranulation, and oxidative injury along with increased susceptibility to inflammation and ulceration characterize the gastrointestinal tract's response to long-term stress (Salim 1988, Charmandari et al. 2005, Bulbul et al. 2012). These changes may ultimately compromise the integrity of the intestinal epithelium and increase the potential for uptake of disease causing antigens, toxins, and microorganisms from the gut lumen (Söderholm and Perdue 2001). Long-term stress may also induce hypertension and increase the risk of other cardiovascular disorders (Sapolsky et al. 2000, Esler et al. 2008, Dimsdale 2008).

1.4.2.2 Immune function and endocrine pathways

The relationship between the immune system and GCs is complex and characterized by both stimulatory and suppressive actions depending on the type and duration of stressor encountered (McEwen and Seeman 1999, Sapolsky et al. 2000). However, virtually all components of the immune system are inhibited by persistent HPA axis activity (McEwen et al. 1997, Sapolsky et al. 2000, Charmandari et al. 2005). For example, GCs are lympholytic and promote T cell and B cell apoptosis which, in turn, may be associated with atrophy of the thymus and other lymphoid tissue (McEwen et al. 1997, Sapolsky et al. 2000). Immunoglobulin production along with cytokine synthesis, release, or efficacy, monocyte differentiation into

macrophages, and macrophage phagocytic activity are also diminished (McEwen et al. 1997, Sapolsky et al. 2000, Charmandari et al. 2005). Elevated cortisol levels may also reduce the inflammatory response which is a vital component of efficient immune function (Sapolsky et al. 2000, O'Conner et al. 2000, Black 2002). Through these mechanisms long-term stress may decrease an individual's resistance to infectious, autoimmune, allergic, or neoplastic diseases (McEwen et al. 1997, O'Conner et al. 2000, Charmandari et al. 2005).

Persistently elevated cortisol may also influence a variety of endocrine pathways (Sapolsky et al. 2000, Tsigos and Chrousos 2002, Charmandari et al. 2005). Through the direct actions of cortisol (and indirectly via increased CRH) long-term stress may reduce secretion of growth hormone (GH) and other growth factors (e.g. insulin-like growth factor, IGF-1) (Charmandari et al. 2005) which may diminish long-term growth potential (Tsigos and Chrousos 2002). Similarly, GCs may decrease production of thyroid stimulating hormone (TSH) along with the conversion of thyroxine (T4) to biologically active triiodothyronine (T3) (Charmandari et al. 2005). T3 is an important regulator of carbohydrate and protein metabolism and diminished T3 levels may adversely impact growth, development, and reproduction in affected animals (Tsigos and Chrousos 2002, Choksi et al. 2003). Prolonged HPA axis activity may also inhibit the secretion or release of essential sex hormones such as gonadotropin releasing hormone (GnRH), luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Sapolsky et al. 2000, Tsigos and Chrousos 2002, Charmandari et al. 2005). Furthermore, long-term stress may render gonad tissue resistant to sex steroids which may lead to gonadal atrophy (Charmandari et al. 2005). In aggregate, these changes may ultimately reduce proceptive and receptive reproductive behaviours (Sapolsky et al. 2000), conception rates, and pregnancy success in chronically stressed individuals (Ebbesen et al. 2009, Latendresse 2009, Sheriff et al. 2009a).

1.4.2.3 HPA axis programming

In humans and a variety of domestic or laboratory species, long-term maternal stress during gestation has also been associated with lasting alterations in the development and function of the HPA axis in offspring (HPA axis programming; Seckel 2004). The consequences of HPA axis programming may include persistent increases in HPA activity (or reactivity) and elevated levels of circulating GCs (Seckel 2004, Kapoor et al. 2006, Meany et al. 2007). In turn, these changes may negatively impact the growth and development of fetuses along with their

subsequent health, long-term viability, and lifetime fitness (Kapoor et al. 2006, Meaney et al. 2007, Sheriff et al. 2010). Maternal stress may also have multigenerational impacts as reduced fecundity may also occur in the offspring of chronically stressed females (Dunn et al. 2010, Sheriff et al. 2010). Alternately, HPA axis programming may be an adaptive response which prepares offspring to cope in environments where stressors are frequently encountered or particularly intense (Love et al. 2013, Dantzer et al. 2013). The impacts of maternal stress may be further enhanced by the transfer of cortisol in milk during the early neonatal period during which HPA axis development and maturation continue (Catalani et al. 2000, Sullivan et al. 2010, Macri et al. 2011).

1.5 Allostasis

The concept of allostasis provides a useful theoretical framework to investigate mechanisms through which long-term stress may link human-caused ecological change to diminished health and fitness in wildlife populations (McEwen and Wingfield 2003, Landys et al. 2006, Busch and Hayward 2009).

In any environment, animals are faced with a variety of challenges that may be related to relatively predictable life history processes (e.g. growth, migration, reproduction, feeding behaviour, moult, seasonal environments) or unpredictable challenges (e.g. extreme weather events, predation, disease) (Wingfield et al. 2005, Landys et al. 2006, McEwen and Wingfield 2010). In order to survive and successfully reproduce, animals must maintain the stability of essential physiological systems (homeostasis) within a range optimal for their current life history stage (McEwen and Wingfield 2003). These goals may be achieved by altering behaviour and physiology in order to acquire or re-allocate energy (McEwen and Wingfield 2003, Landys et al. 2006, Busch and Hayward 2009).

Allostasis refers to this process of achieving stability in internal systems through physiological or behavioural change (McEwen and Wingfield 2003). Allostatic load may therefore be described as the cost to an individual of being forced to adapt to changing environments (McEwen 1998, McEwen and Wingfield 2003). It is the result of daily and seasonal routines to obtain food and survive plus additional energy needed for critical life cycle events or to deal with unexpected challenges (McEwen 1998, McEwen and Wingfield 2003). More simply, allostatic load may be considered as the difference between an organism's

available energy (from food or intrinsic energy stores) and that required to deal with energetic challenges related to periodic life processes and unpredictable events (Landys et al. 2006, Busch and Hayward 2009) (Fig 1.2). In addition to their role as effectors of the stress response, GCs play an important part in energy metabolism and in an organism's response to changing energetic demands (Landys et al. 2006, Busch and Hayward 2009). As such, these hormones are considered to be primary mediators of allostasis and as allostatic load increases baseline levels of GCs tend to rise (Sapolsky et al. 2000, McEwen and Wingfield 2003, Landys et al. 2006).

As allostatic load changes, the effects of GCs are mediated at the molecular level in part through differential binding to a high affinity nuclear mineralocorticoid receptor (MR) and a low affinity nuclear GC receptor (GR) (Sapolsky et al. 2000, Landys et al. 2006). These receptors are present in most organs and organ systems (Sapolsky et al. 2000, Landys et al. 2006). In an undisturbed animal at rest relatively low (basal) levels of circulating GCs act through high affinity MR receptors to maintain glucose, salt, and water balance within the minimum operating levels necessary to sustain life (Sapolsky et al. 2000, Landys et al. 2006). Low to moderate increases in GC levels (compared to basal) are associated with an organism's response to the added demands of daily (e.g. feeding behaviour, general activity patterns) or seasonal (e.g. migration, reproduction, moult) life history events (McEwen and Wingfield 2003, Landys et al. 2006, Busch and Hayward 2009). At these levels, GCs begin to saturate high affinity MR receptors which enhances catabolic processes and behavioural changes (e.g. increased feeding or locomotion) (Sapolsky et al 2000, Landys et al. 2006) that provide the extra energy necessary to counter increased allostatic load and maintain physiological systems within an elevated (but modulated) operating range (Landys et al. 2006). These changes may be considered to be within the reaction norm of a particular species and may be observed as circadian, seasonal, or life history stage associated alterations in levels of circulating GCs (e.g. Boonstra 2005, Landys et al. 2006, Romero et al. 2008).

As allostatic load continues to grow (and GC levels increase) GCs begin to bind low affinity GR receptors (Landys et al. 2006, Busch and Hayward 2009). Increased binding of GR receptors is associated with the suppressive effects of GCs which act to reduce allostatic load by mobilizing or repartitioning available energy through the temporary inhibition of feeding behaviour, energy storage, growth, immunity, and reproduction (Sapolsky et al. 2000). If energy

demands exceed energy available, a physiological state of allostatic overload may occur which is characterized by dysregulation of allostatic mediators, a reduction in an animal's ability to cope with additional challenges, and an increase in the potential for adverse health effects (McEwen and Wingfield 2003, Busch and Hayward 2009). High levels of circulating GCs are associated with allostatic overload which may be considered an emergency life history stage (Wingfield 2005, Landys et al. 2006, McEwen and Wingfield 2010) (Fig 1.2).

Unpredictable challenges may require extra energy compared to demands that fall within the reaction norm for a particular species and life history stage (McEwen and Wingfield 2003, Wingfield 2005, Landys et al. 2006, Wingfield et al. 2011). As such, exposure to unpredictable events superimposed on the demands of regular life history processes may increase allostatic load in affected organisms (Wingfied 2005, McEwen and Wingfield 2010, Wingfield et al. 2011) (Fig 1.2). For many species, anthropogenic ecological change may function in this capacity and may therefore contribute to allostatic load and overload in organisms inhabiting perturbed environments (Wingfied 2005, Busch and Hayward 2009, Wingfield et al. 2011). Increases in allostatic load associated with anthropogenic factors may also render affected animals less able to cope with challenges related to normal life history and may therefore make affected individuals more prone to allostatic overload (Busch and Hayward 2009). Persistently elevated GCs associated with repeated or long-lasting periods of allostatic overload may result in the variety of adverse health effects, pathological syndrome of distress, and population effects previously described (section 1.4.2, Fig 1.1).

The adverse effects of human-caused ecological change may follow a gradient of disturbance with the greatest altered HPA axis activity and compromised health apparent where home ranges are located in highly modified areas (Wingfield 2005, Ellis et al. 2012). Since GCs are important mediators of allostasis, the study of long-term stress may serve to increase our understanding of the importance of both natural and human-caused ecological conditions as drivers of health and population performance in free-ranging wildlife.

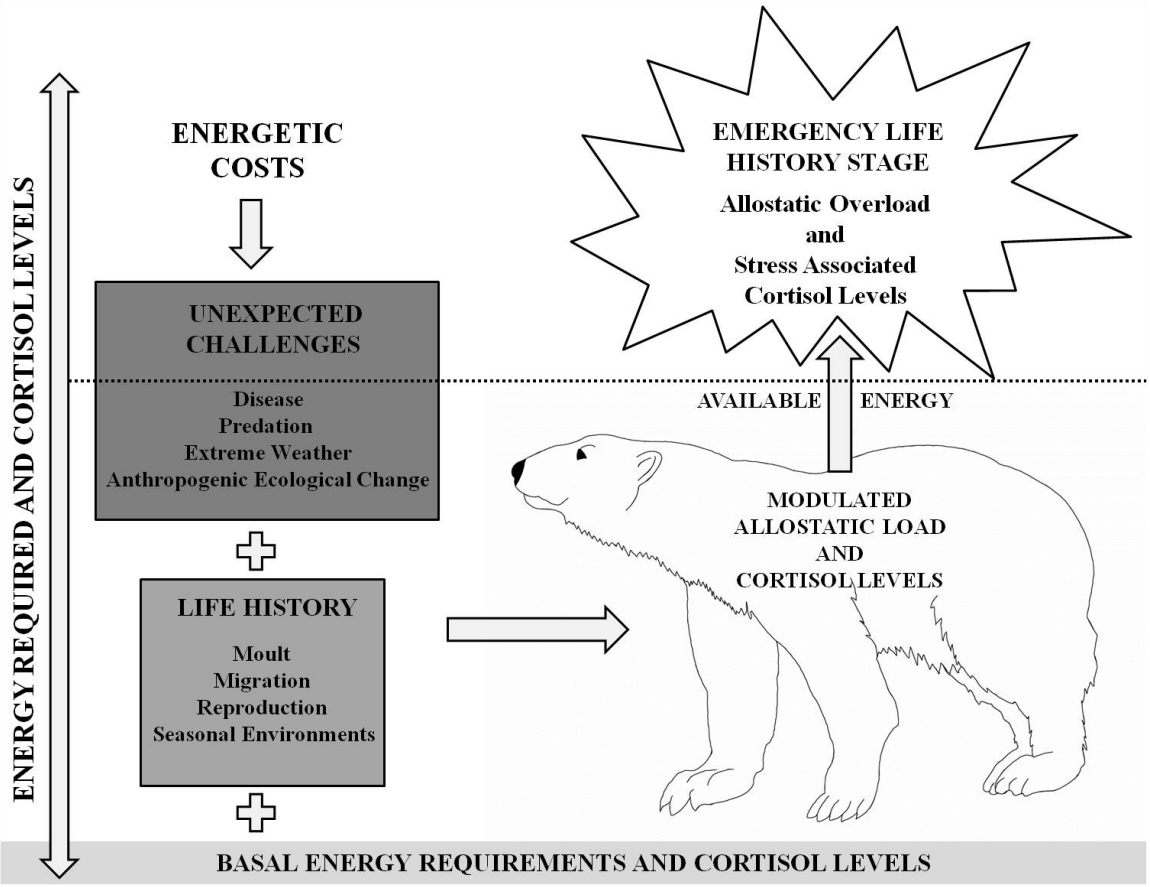

Figure 1.2 Allostasis and the relationship between allostatic load, allostatic overload and cortisol levels. Allostasis refers to the process of achieving stability in internal systems through physiological or behavioural change. Allostatic load may be considered as the difference between an organism's available energy and the costs of dealing with challenges related to life history processes and unpredictable events. Glucocorticoids are primary mediators of allostasis, and as allostatic load increases cortisol levels rise within a modulated range. When energy required to meet demands exceeds that available an organism may enter a state of allostatic overload. Allostatic overload is an emergency life history stage characterized by dysregulation of allostatic mediators and high (stress associated) levels of circulating cortisol. If allostatic load remains uncorrected, these changes may adversely impact the health and fitness of affected animals.

1.6 Species Accounts: Background, Conservation Status, and Potential Anthropogenic Sources of Long-Term Stress

1.6.1 Grizzly bear

In the Rocky Mountains and foothills of Alberta, Canada landscape change is occurring at an alarming rate and threatens the survival of the grizzly bear (Stelfox et al. 2005, AGBRP

2008, Festa-Bianchet 2010). Grizzly bears in these ecosystems live at the southern edge of their Canadian distribution and inhabit one of the most densely populated and developed landscapes in which the species survives (Gibeau and Herrero 1998, AGBRP 2008, Festa-Bianchet 2010). The number of grizzly bears in different regions of the Canadian Rocky Mountains may be highly variable owing to a combination of habitat characteristics and patterns of human land use (Mowat et al. 2005, AGBRP 2008, Proctor et al. 2012). In Alberta, grizzly bears occur at lower densities (5-18 grizzly bears/1000 km^2; AGBRP 2008, Festa-Bianchet 2010) relative to other North American populations which inhabit more productive interior or coastal regions (80-90 grizzly bears /1000km^2; McLellan 1989, MacHutchon et al. 1993). Recent DNA and habitat based population estimates also suggest that there are only 691 grizzly bears on provincial lands in Alberta, and that as few as 379 may be reproductively mature adults (Festa-Bianchet 2010). Furthermore, these investigations and radio-telemetry based studies also indicate that Alberta grizzly bears occur in seven genetically distinct and predominantly female-isolated subpopulations which are separated by the major East-West oriented provincial highways (Proctor et al. 2012). The conservation status of grizzly bears along with the sources and importance of human-caused ecological change are highly variable across these population units (AGBRP 2008, Festa-Bianchet 2010).

Critically, some subpopulations already contain < 50 grizzly bears (Festa-Bianchet 2010), and recent demographic analysis suggests that grizzly bear abundance in Alberta is likely slowly declining (Boulanger and Stenhouse 2009, Festa-Bianchet 2010). In addition, human-caused mortality of grizzly bears in some areas has recently been (Stenhouse et al. 2005a) and may continue to exceed (ASRD 2010a, ASRD 2011, ASRD 2012) sustainable levels (i.e. human caused mortality rate of 2.8% - 4.8%; AGBRP 2008, Festa-Bianchet 2010) while the reproductive rates of Alberta grizzly bears are low compared to other North American populations (Garshelis et al. 2005, Boulanger and Stenhouse 2009). Body size and body condition (traits closely tied to fitness in grizzly bears; e.g. Zedrosser et al. 2007, Dahle et al. 2006, Zedrosser et al. 2011, Steyaert et al. 2012, Robbins et al. 2012) may also vary among grizzly bears inhabiting different areas of the province (M. Cattet, personal communication).

In recognition of these threats, the spring grizzly bear hunt in Alberta was suspended in 2006, a species recovery plan has been developed (AGBRP 2008), and the conservation status of

grizzly bears in the province has been downgraded to "Threatened" (ASRD 2010b). Despite increasing legal protection and conservation efforts, grizzly bear habitat throughout Alberta continues to be compromised by a rapidly expanding human population and escalating industrial, residential, and recreational development (AGBRP 2008, Festa-Bianchet 2010). Grizzly bears are extremely vulnerable to anthropogenic disturbance and human-caused ecological change may have both direct and indirect effects on health and population performance in this species (Weaver et al. 1996, Cattet et al. 2006, AGBRP 2008, Schwartz et al. 2010, Proctor et al. 2012).

Grizzly bear mortality is directly related to the rate of human-bear encounters and the probability of a bear being killed in an encounter with humans escalates as development and human use of bear habitat increases (Mattson et al. 1996, AGBRP 2008, Schwartz et al. 2010). Transportation infrastructure built to support industry, residential developments, and recreation is of particular concern as roads and trails allow easier access for humans into grizzly bear habitat (Gibeau and Herrero 1998, Benn and Herrero 2002, Graham et al. 2010). Although grizzly bears are known to avoid roads (Gibeau et al. 2002, Waller and Servheen 2005, Proctor et al. 2012), roadside ditches (along with similar disturbances such as: forestry cut-blocks, oil and gas pipelines, and oil rigs; Stenhouse and Graham 2011) may attract grizzly bears owing to the seasonal availability of important forage plants (Gibeau et al. 2002, Nielson et al. 2004a, Nielson et al. 2004b, Roever, et al. 2008). Even in protected areas, humans are responsible for the majority of grizzly bear deaths in Alberta and most of these deaths occur within 500 meters of a road (Benn and Herrero 2002, Stenhouse et al. 2003, AGBRP 2008). Thus, despite potential nutritional benefits, developed areas may act as grizzly bear population sinks with an increased risk of mortality directly related to human access (Nielson et al. 2004b, Nielson et al. 2008, Stenhouse and Graham 2011). Significantly, this risk may be elevated in female and juvenile grizzly bears whose home ranges often contain more roads and developments compared to adult males and which may also use habitat in these areas more frequently (Gibeau et al. 2002, Mueller et al. 2004, Graham et al. 2010, Stenhouse and Graham 2011). The conservation status of some grizzly bear subpopulations in Alberta is precarious and the loss of any adult females or juveniles may threaten the species' persistence in some areas of the province (Festa-Bianchet 2010).

Anthropogenic factors may also function as a source of long-term stress for grizzly bears by altering normal behaviour or life history strategies and forcing individuals to deal with

conditions dissimilar to those encountered in pristine habitat (Wikelski and Cooke 2006, Cattet et al. 2006, Roever et al. 2010). Most grizzly bears flee from humans (McLellan and Shackleton 1989), and when grizzly bears use human dominated landscapes they tend to maintain temporal or spatial separation from human activity by using cover or visiting habitats outside periods of peak human use (Gibeau et al. 2002, Graham et al. 2010, Martin et al. 2010, Stenhouse and Graham 2011). Avoidance behaviour, fear, and anticipatory anxiety may all elevate levels of circulating GCs (e.g. Hydbring-Sandberg et al. 2004, Roelofs et al. 2009, Marechal et al. 2011) and suggest that repeated or prolonged contact with humans may directly increase long-term stress levels in grizzly bears. Although some grizzly bears may become habituated to human activity (Bader 1989, Gibeau et al. 2002, Mueller et al. 2004), sensitization may also occur with repeated exposure to humans resulting in heightened HPA axis reactivity (Harlow et al. 1992). Importantly, the energetic costs of fear, flight, or acclimation may also increase allostatic load in individuals to the point where the demands of other anthropogenic stressors or natural life cycle events may be more likely to elicit allostatic overload (Schulkin et al. 1998, Busch and Hayward 2009).

Human activity may also indirectly increase HPA axis activity in grizzly bears by altering intraspecific interactions or reducing foraging efficiency (Stenhouse et al. 2005b, Landys et al. 2006, Martin et al. 2010). For instance, dominant adult male grizzly bears generally select for high quality habitat and an absence of humans and may exclude adult females and sub adults from these localities (McLoughlin et al. 2002, Rode et al. 2006a, Libal et al. 2011). Female grizzly bears with dependent offspring and juveniles also actively avoid habitat occupied by adult males which may kill younger bears (Gibeau et al. 2002, Rode et al. 2006a, Libal et al. 2011). In developed areas, displaced cohorts may therefore have no alternative but to establish home ranges in sub-optimal habitat which may be close to human influence and may lead to a disproportionate increase in long-term stress levels in affected individuals (Gibeau et al. 2002, Mueller et al. 2004, Rode et al. 2006a).

Roads and human settlements may also prevent grizzly bears from accessing high quality habitat containing important seasonal foods (e.g. berries during hyperphagia) or shelter (Gibeau et al. 2002, Waller and Servheen 2005, Proctor et al. 2012). Even recreational activities that are often considered to be unobtrusive (e.g. bear-viewing) have been linked with decreased foraging

efficiency or success in this species (Rode et al. 2006b, French 2007). Reduced access to seasonal forage may increase energetic demands or deficits in displaced bears and both allostatic load and levels of circulating GCs may increase as a result (Busch and Hayward 2009). Like mortality, this effect may be most pronounced in female and juvenile grizzly bears (Gibeau et al. 2002, Mueller et al. 2004, Rode et al. 2006a).

Other anthropogenic factors may have similar consequences. For example, climatic warming (IPCC 2007) may alter grizzly bear denning behaviour along with the phenology, distribution, and availability of important forage plants (Pigeon et al. 2011, Stenhouse and Graham 2011, Proctor et al. 2012). The impacts of changes in forest management practices and habitat loss due to recent mountain pine beetle (*Dendroctonus ponderosae*) infestations in Alberta grizzly bear habitat may also be relevant (Nielsen et al. 2004a, Nielsen et al. 2004b, Stenhouse and Graham 2011). Likewise, climate change may facilitate an increase in the number of humans inhabiting or recreating in grizzly bear range (Proctor et al. 2012).

Although the causes of grizzly bear mortality and mitigation strategies to prevent it are well recognized (Herrero 2005, AGBRP 2008, Festa Bianchet 2010), the importance of long-term stress in this species is not yet fully understood. Nonetheless, the potential relationship between long-term stress, diminished health, and reduced fitness is increasingly recognized as an issue relevant to grizzly bear conservation (Stenhouse and Graham 2005, Cattet et al. 2006, Macbeth et al. 2010). Specifically, long-term stress associated with human-caused ecological change may negatively affect the health of individual grizzly bears and, as more animals become affected, may lead to reduced population performance (Cattet et al. 2006). These changes may have significant implications for the long-term sustainability of grizzly bears in Alberta which face increasing conflict with human interests into the foreseeable future and are already threatened by reduced abundance, slow reproductive rates, and high levels of human-caused mortality (AGBRP 2008, Festa-Bianchet 2010, ASRD 2012). Long-term stress may also be relevant in many other *U. arctos* populations across the species Holarctic distributional range which may be subject to a similar array of human-caused conservation challenges (e.g. Johnsingh 2003, McCarthy et al. 2009, Schwartz et al. 2010, Proctor et al. 2012, Straka et al. 2012).

1.6.2 Polar bear

A large body of scientific evidence suggests that the global climate is warming at an unprecedented rate primarily resulting from an anthropogenic increase in emissions of greenhouse gases (e.g. carbon dioxide, CO_2) and human land use practices (e.g. forest clearing) (ACIA 2005, IPCC 2007, Rosenzweig et al. 2008). Although climate change has impacted many taxa in ecosystems around the globe (Walther et al. 2002, Parmesan and Yohe 2003, Root et al. 2003), the effects of this phenomenon are magnified and accelerated in Arctic and subarctic regions (ACIA 2005, Hansen et al. 2006, Walsh 2008). In these areas, a complex suite of changes have been attributed to recent warming trends and include: sea-ice, glacier, and permafrost melting; changing patterns of human land use, precipitation, water flow, and salinity; and a northward shift of species ranges (ACIA 2005, Richter-Menge et al. 2006, Laidre et al. 2008) The relative importance of these changes as conservation threats or potential sources of long-term stress in northern mammals may vary with species specific life history traits (Laidre et al. 2008, Ragen et al. 2008, Prowse et al. 2009).

Polar bears are top-level predators in Arctic marine ecosystems whose highly specialized life history, behaviour, and feeding patterns make them especially vulnerable to the effects of climate change (Derocher et al. 2004, Laidre et al. 2008, Durner et al. 2009). Polar bears depend on sea-ice as a platform for hunting their primary prey, [ringed (*Pusa hispida*) and bearded (*Erignathus barbatus*) seals] (Derocher et al. 2004), seasonal movements (Ferguson et al. 2000, Ferguson et al. 2001, Parks et al. 2006), and breeding (Ramsay and Stirling 1986, Ramsay and Stirling 1988, Zeyl et al. 2009). In some regions, sea-ice also provides critical denning habitat (Fischbach et al. 2007, Laidre et al. 2008, Durner et al. 2009). As such, the distribution and composition of Arctic sea-ice is closely related to heath, survival, and population performance of polar bears (e.g. Derocher et al. 2004, Regehr et al. 2007, Durner et al. 2009, Rode et al. 2010a, Regehr et al. 2010).

A weight of evidence indicates that human-caused climatic warming is linked with recent declines in the extent of annual sea-ice, decreases in the age, thickness and overall extent of multiyear ice and an increase in the length of the summer ice free period across the Arctic (ACIA 2005, Richter-Menge et al. 2006, Kinnard et al. 2011). With further reductions in the extent and duration of sea-ice predicted to continue indefinitely (ACIA 2005, Richter-Menge et al. 2006,

Walsh 2008), the health and fitness of individual polar bears along with the long-term sustainability of many polar bear populations is now at risk (e.g. Derocher et al. 2004, O' Neill et al. 2008, Wiig et al. 2008, Hunter et al. 2010, Molnár et al. 2010).

In different areas of the Arctic polar bears belong to ecosystems that are fundamentally different in their structure and function and where the rates and consequences of climate change are heterogeneous (Thiemann et al. 2008, IUCN PBSG 2010).The polar bear populations presently considered most at risk are those inhabiting the southern edge of the species distributional range in Hudson Bay and James Bay, Canada where a natural cycle of sea-ice melting forces bears onshore for several months during summer and fall (Stirling et al. 2004, Stirling and Parkinson 2006, Obbard et al. 2006). Over the last three decades, a significant reduction in the extent and duration of annual sea-ice has occurred in this region (Gough et al. 2004, Gagnon and Gough 2005), and polar bears now have less time to hunt during the spring and early summer when seals are most accessible and bears amass most of the adipose reserves drawn on during the onshore period (Stirling and Parkinson 2006, Obbard et al. 2006, Obbard et al. 2007). As a result, shore-bound bears are forced to fast for longer periods with less stored fat and evidence of a related decline in the health and survival of individual animals along with deteriorating population performance is mounting.

In the Western Hudson Bay (WH) subpopulation, earlier ice break-up and an extended ice free season may be associated with changes in the distribution of polar bears during the onshore period (Stirling et al. 2004, Towns et al. 2009, Towns et al. 2010) along with a general decrease in body size and body condition (Stirling et al. 1999, Derocher and Stirling 1995, Atkinson et al. 1996) reproductive output (Stirling et al. 1999, Derocher and Stirling 1995), survival (Derocher and Stirling 1996, Regehr et al. 2007), and overall population size (Regehr et al. 2007). The average body condition of polar bears inhabiting the Southern Hudson Bay (SH) subpopulation has also decreased since the mid-1980s (Obbard et al. 2006, Obbard et al. 2007). This trend is most pronounced in females and juvenile bears, and there is now concern that if the duration of the annual ice-free period continues to increase the SH subpopulation may respond similarly to the WH subpopulation (Obbard et al. 2006, Obbard et al. 2007). In recognition of these threats, both the WH and SH polar bear subpopulations are currently designated as "Threatened" (MESA 2008, OESA 2008).

Importantly, evidence of nutritional stress (Amstrup et al. 2006, Stirling et al. 2008, Cherry et al. 2009), changing patterns of habitat use (Fischbach et al. 2007, Schliebe et al. 2008, Gleason and Rode 2009), unusual mortality events (Durner et al. 2011), and declining health or population performance (Regehr et al. 2010, Sonne 2010, Rode et al. 2010a, Rode et al. 2012) are also being recorded in more northerly polar bear populations. These patterns agree with those predicted under climate change scenarios (Derocher et al. 2004, Durner et al. 2009) and may suggest that the impact of global warming on polar bears is now spreading north-ward. Such changes have lead to the recent federal classification of polar bears as "Threatened" (USFWS 2008) or of "Special Concern" (SARA 2011) in the United States and Canada respectively.

The occurrence of long-term stress in polar bears may be directly linked to climatic warming as habitat loss, declining access to prey, and nutritional stress may increase seasonal energetic deficits and thus levels of circulating GCs in affected animals (Hamilton 2007, Busch and Hayward 2009, Chow et al. 2011). Habitat loss may also lead to additional increases in HPA axis activity by forcing polar bears to change their seasonal distribution or movement patterns (Stirling et al. 2004, Towns et al. 2009, Towns et al. 2010). In turn, this may alter intraspecific interactions (Blanchard et al. 2005, Bartolomucci et al. 2005, McEwen and Wingfield 2010) or press already nutritionally stressed bears to expend extra energy travelling in open water, on fragmented ice, or on land in search of suitable habitat and food (Hurst et al. 1982, Gleason and Rode 2009, Pagano et al. 2012).

As climatic warming makes Arctic travel routes more accessible, industrial development and tourism intensify, and the regional human population grows habitat loss may also force polar bears into greater proximity to human settlements or industrial activity (Derocher et al. 2004, Hovelsrud et al. 2008, Towns et al. 2009, Dawson et al. 2010). Like habitat loss, human activity has been shown to directly influence the movement and behaviour of polar bears and, in a manner similar to grizzly bears (see section 1.5.1), increasing interactions with humans could represent an emerging source of long-term stress in this species (Amstrup 1993, Dyck and Baydack 2004, Andersen and Aars 2008). In some regions, nutritionally stressed polar bears may also be forced to search for food on land (Derocher et al. 1993a, Rockwell and Gormezano 2009, Dyck and Kebreab 2009). The importance of terrestrial foraging in this species is not fully understood. However, this strategy is generally considered to be outside normal life history

parameters for polar bears and, at the population level, may be unlikely to compensate for energetic deficits associated with reduced foraging success on sea-ice (Derocher et al. 2004, Rode et al. 2010b). Terrestrial foraging in polar bears may even be associated with an increase in energetic expenditures, allostatic load, and cortisol levels in some animals (Hurst et al. 1982, Brook and Richardson 2002, Busch and Hayward 2009). Further support for both hypotheses comes from increasing reports of emaciated polar bears killed or sighted near human settlements far inland from their typical coastal habitat (Goodyear 2003, CBC 2008, CBC 2010, SKIU 2008).

Though the effects of climatic warming may be of distinct significance, other anthropogenic influences in the Arctic may function as sources of long-term stress for polar bears. The long range transport, bioaccumulation, and biomagnification of chemical contaminants may be especially significant in this regard (Derocher et al. 2003, Sonne 2010, Holmstrup et al. 2010). Among others, polychlorinated biphenyls (PCBs) and other organochlorine pesticides (Bentzen et al. 2008), brominated flame retardants (Muir et al. 2006), and heavy metals (e.g. mercury; Dietz et al. 2009) have been identified in polar bear tissue. Many of these contaminants are endocrine disruptors that alter metabolic pathways, enzymes, hormones, and tissue homeostasis and, in polar bears, have been associated with a wide range of physical or physiological disturbances including altered HPA axis activity (Haave et al. 2003, Øskam et al. 2004, Fisk et al. 2005, Sonne 2010). Like climatic change, the relative importance of environmental toxins may vary among polar bear subpopulations (Verreault et al. 2005, Sonne 2010, McKinney et al. 2011). Other less understood phenomena such as disease (Cattet et al. 2004, Burek et al. 2008, Kirk et al. 2010a, Kirk et al. 2010b), or increasing interactions with grizzly bears (Doupe et al. 2007, Rockwell et al. 2008) may also have implications for long-term stress levels in polar bears in some regions. As for grizzly bears, increasing long-term stress may be anticipated to occur first in female and juvenile polar bears with potentially important consequences for the sustainability of affected populations (Derocher et al. 2004, Aars and Anderson 2008, Kirk et al. 2010a, Molnár et al. 2011, Bytingsvik et al. 2012).

1.6.3 Caribou

Caribou (*Rangifer tarandus* sp.) are a keystone species in Arctic and subarctic terrestrial ecosystems throughout the circumpolar region (Festa-Bianchet et al. 2011, Gunn 2011, Forde et

al. 2012). *Rangifer* population dynamics are driven by a complex, interacting array of biotic and abiotic factors (Vors and Boyce 2009, Joly and Klein 2011, Festa-Bianchet et al. 2011), and although caribou numbers may oscillate naturally over decades (Gunn 2003), a weight of evidence suggests that human-caused ecological change may be associated with the declines now being recorded in many herds (Vors and Boyce 2009, Joly et al. 2011, CARMA 2012a). Long-term physiological stress may be a consequence of these changes, and also a potential mechanism linking human-caused ecological change with impaired health and population performance in this species (Reimers 1983, Whitfield and Russell 2005, Ashley et al. 2011, Wasser et al. 2011, Renaud 2012).

Although five subspecies of caribou are currently recognized [*Rangifer tarandus groenlandicus* (Canadian barren ground caribou), *R.t. granti* (Alaskan barren ground caribou), *R.t. caribou* (woodland caribou; also described as boreal forest or mountain caribou), *R.t. pearyi* (Peary/ high arctic caribou), and Eurasian reindeer *(R.t. tarandus*); Miller 2003, Vistnes and Nellemann 2008, Festa-Bianchet et al. 2011)], the sources and relative importance of anthropogenic stressors may be highly variable across the species' vast distributional range (Joly et al. 2011, Festa-Bianchet et al. 2011, CARMA 2012a). In North America, these factors may align more closely with the habitat preferences and life history traits of caribou populations than with their taxonomic designation (Vors and Boyce 2009, Festa-Bianchet et al. 2011). As such, an alternate system of classification that separates caribou into four ecotypes [Peary/high arctic, migratory tundra, boreal forest, and mountain] based on these characteristics may be more relevant (Festa-Bianchet et al. 2011).

Peary caribou are found in the high Arctic Archipelago; a region characterized by a short growing season, limited plant growth, extreme cold, and little precipitation (Miller 2003, Tews et al. 2007, Festa-Bianchet et al. 2011). This *Rangifer* subspecies travels in small groups (tens of individuals), calves over a dispersed area at low density, and may migrate between seasonal ranges on different Arctic islands or occupy relatively small home ranges year round (Miller 2003, Festa-Bianchet et al. 2011). In contrast, migratory tundra caribou inhabit more southerly Arctic and subarctic regions from Labrador west to Alaska (Miller 2003). This subspecies is gregarious at calving, may travel in herds of many thousands, and migrates hundreds of kilometers between seasonal ranges (which may include both tundra and boreal forests) (Festa-

Bianchet et al. 2011). Boreal caribou are found in the taiga from Newfoundland to British Columbia. Although this subspecies is relatively sedentary and generally travels as individuals or in small groups, some herds may be large and have seasonal home ranges of many hundreds of square kilometers (Festa-Bianchet et al. 2011). Mountain caribou are found in the mountainous regions of Western Canada and as a relic population in the western United States (Festa-Bianchet et al. 2011, USFWS 1983). This subspecies calves at high elevations, travels in smaller herds, and may exhibit migratory or sedentary behaviour. Mountain caribou are further divided into Northern and Southern ecotypes based on differences in seasonal migration patterns and forage preferences (Festa Bianchet et al. 2011). In all caribou ecotypes, the causes, rates, and consequences of anthropogenic ecological change may be heterogeneous among regional populations (Vors and Boyce 2009, Festa-Bianchet et al. 2011, CARMA 2012a). As a result, the conservation status of *Rangifer* varies widely across the species distributional range (Vors and Boyce 2009, Fiesta-Bianchet et al. 2011, CARMA 2012a).

In Canada, the High Arctic/Peary *Rangifer* ecotype is presently listed as "Endangered" (SARA 2011b), and the Dolphin and Union populations of the migratory barren ground ecotype are considered of "Special Concern" (SARA 2011b). The conservation status of boreal forest and mountain caribou is more variable with some populations currently recognized as being "Not at Risk" (Newfoundland and Labrador population) (SARA 2011b), while others are of "Special Concern" (northern mountain population) (SARA 2011b), "Threatened" (boreal and southern mountain populations) (SARA 2011b), or "Endangered" (Atlantic-Gaspesie population) (SARA 2011b). In the United States, the only population of caribou (mountain ecotype) now found south of the Canadian border is listed as "Endangered" (USFWS 1983) while migratory tundra caribou in Alaska are not protected at the federal level.

The impacts of climatic warming may be among the most important sources of human-caused long-term stress for all *Rangifer* ecotypes (Vors and Boyce 2009, Festa-Bianchet et al. 2011, CARMA 2012a). Wherever they occur, the life history strategies of caribou are closely tied to foraging success in the spring through fall (Cameron et al. 1993, Rönnegård et al 2002, Couturier et al. 2009a) and to the availability of specific types of forage (e.g. lichens) in winter (Heggberget et al. 2002, Joly et al. 2010, Waterhouse et al. 2011). Current warming trends in *Rangifer* habitat (ACIA 2005) may alter the quantity and type (Weladji and Holand 2006, Joly et

al. 2007, Joly et al. 2009), nutritional quality (Joly et al. 2009, Cebrian et al. 2008), phenology (Post and Forchhammer 2008, Post et al. 2008), or distribution (Heggberget et al. 2002, Joly et al. 2009, Waterhouse et al. 2011) of important forage species. As a result, energetic deficits associated with reduced foraging efficiency or long-distance travel to locate alternate food sources (Dyer et al. 2001, McNeil et al. 2005, Sharma et al. 2009) may increase long-term stress levels in caribou (Landys et al. 2006, Busch and Hayward 2009). These factors may also enhance allostatic load making affected animals less able to deal with other natural or anthropogenic challenges (Busch and Hayward 2009). Overall, changes in the quality, distribution, and phenology of important forage species may even negate any benefit of warming related to a general increase plant biomass in caribou habitat (Post et al. 2008, Tyler et al. 2008, Gunn et al. 2009). Moreover, warmer temperatures may also be associated with an increase in the intensity and duration of insect harassment which may further reduce foraging efficiency and increase HPA axis activity during the summer period (Hagemoen and Reimers 2002, Weladji et al. 2003, Witter et al. 2011).

Climatic warming may also be connected to an increase in the occurrence of extreme weather such as rain on snow (ground icing) events, deep snow, and fire (IPCC 2007, ACIA 2005). Ground icing and deep snow may prevent *Rangifer* from accessing winter forage (Tews et al. 2007, Jandt et al. 2008, Joly et al. 2011) and have been associated with large scale die-offs in a number of populations (Miller and Gunn 2003, Tveraa et al. 2007, Hansen et al. 2011). Deep snow may also increase the risk of predation (Brotton and Wall 1997, Bergerud and Luttich 2003, Ferguson and Elkie 2005) while forest and tundra fires may reduce access to and use of core habitat for extended periods (Racey 2005, Jandt et al. 2008. Joly et al. 2010). In addition, secondary growth in burned areas may enhance interspecific competition along with the threat of mortality due to predation (Racey 2005, Robinson et al. 2012). Climatic warming may also facilitate the range expansion of disease bearing southern species into caribou habitat [e.g. meningeal worm (*Parelaphostrongylus tenuis)* in white-tailed deer (*Odocoileus virgineanus*); Trainer et al. 1993, Lantham and Boutin 2008, Boutin et al. 2012] or may lead to changes in the annual life cycles of endemic internal parasites resulting in greater burdens in infected caribou (Kutz et al. 2005, Hughes et al. 2009, Kutz et al. 2012). Energetic expenditures (or deficits) related to foraging in or migrating from compromised habitat along with the effects of disease

and increased predation risk may further enhance long-term stress levels in caribou (Busch and Hayward 2009, McEwen and Wingfield 2010, Sheriff et al. 2010). It should also be recognized that the effects of climate change may interact with other anthropogenic landscape features and natural drivers of *Rangifer* population performance (e.g. population density and disease) to further influence HPA axis activity in this species (Vors and Boyce 2009, Tyler 2010, Festa-Bianchet et al. 2011).

Like the grizzly bear, caribou are considered to be relatively intolerant of human disturbance (e.g. Aastrup 2000, Nellemann et al. 2000, Vistnes and Nellemann 2008, Nellemanm et al. 2010), and, in many regions, *Rangifer* habitat is threatened by increasing resource extraction (oil, gas, timber, and mineral) activities along with escalating residential, and recreational development (Schindler et al. 2007, Vistnes and Nellemann 2008, Whittington et al. 2011). Transportation infrastructure and industrial or recreational developments are of particular concern for caribou conservation (Dyer et al. 2001, Gunn et al. 2009, Whittington et al. 2011). Roads and trails increase human access into caribou habitat and may enhance the risk of contact with humans and human caused mortality (hunting or poaching) (Apps and McLellan 2006, Gunn et al. 2009, Festa-Bianchet et al. 2011). Roads and trails may also increase the probability of contact with predators (James and Stuart-Smith 2000, Whittington et al. 2011, Boutin et al. 2012). In addition, these and other anthropogenic landscape features may act as barriers to caribou movements and reduce habitat availability (Dyer et al. 2001, Reimers and Colman 2006, Gunn et al. 2009). For example, boreal and mountain caribou have been found to avoid habitat near roads, other anthropogenic linear landscape features (Dyer et al. 2002, Schindler et al. 2007, Fortin et al. 2008), and petroleum extraction or forestry related disturbances (Sorensen et al. 2007, Schaefer and Mahoney 2007, Courtois et al. 2007, Hins et al. 2008). Although the effects of industrial development on migratory tundra caribou are more controversial (Cronin et al. 2000, Haskell, et al. 2006, Haskell and Ballard 2008), a review of studies on a regional scale suggests that avoidance of roads and oil pipelines may occur in this subspecies in the vast majority of cases (Vistnes and Nellemann 2008). Caribou have also been found to avoid recreational development (e.g. resorts, cabins) even in the presence of high quality habitat in otherwise poor quality landscapes (Nellemann et al. 2000, Vistnes and Nelleman 2008, Nellemann et al. 2010). Likewise, backcountry recreational activity (e.g. snowmobiling, skiing,

road traffic) may displace caribou from preferred winter habitat, calving areas, or refugia from predators (Reimers and Colman 2006, Seip et al. 2007, Helle et al. 2012). As for grizzly bears, avoidance of human activity may have the potential to directly increase HPA axis activity in caribou (Cook 1996, Bristow and Holmes 2007, Busch and Hayward 2009).

This behaviour may also lead to the crowding of caribou in remaining areas of undisturbed habitat (Vistnes and Nellemann 2008). In turn, crowding may indirectly increase levels of circulating cortisol in caribou through the density dependent effects of overgrazing (Reimers 1983, Couturier et al. 2009b, Mahoney et al. 2011) and social stress (Holand et al. 2004a, Bartolomucci et al. 2005, Fauchald et al. 2007). Avoidance may also prevent caribou from migrating to circumvent the localized effects of stochastic weather events or may force caribou into sub optimal habitat in which diminished foraging efficiency, intra and interspecific competition, or an increasing risk of predation further enhance long-term stress levels (Vistnes and Nellemann 2008, Vors and Boyce 2009, Sheriff et al. 2010). As for grizzly and polar bears, increases in long-term stress levels and associated health effects related to anthropogenic factors may occur first in female and juvenile caribou which may have important consequences for the long-term sustainability of herds inhabiting disturbed areas (Whitfield and Russell 2005, Vors and Boyce 2009, Festa-Bianchet et al. 2011).

1.7 Biological Media Used for the Study of HPA Axis Activity in Wildlife and the Need for an Alternate Approach to Evaluate Long-term Stress

A variety of biological media including blood plasma or serum, saliva, urine, and feces have been used to assess GC levels, HPA axis activity, and the stress response in wild species. Quantitative measurements of physiological stress have traditionally relied on the determination of cortisol (or other GC) concentrations in blood (Mormède et al. 2007, Amaral 2010, Sheriff et al. 2011). However, plasma or serum cortisol levels represent a point in time measure of HPA axis activity (Mormède et al. 2007, Sheriff et al. 2011), and the utility of these media for the study of long-term stress may be limited by natural fluctuations in cortisol levels related to circadian and seasonal effects (Bubenik et al. 1998, Boonstra et al. 2001, McKenzie and Deane 2003). In most species, the majority of circulating cortisol is tightly bound to the plasma protein corticosteroid binding globulin (CBG) and only unbound (free) cortisol (5-10% of the total) is considered to be biologically active (Henley and Lightman 2011). Cortisol measured in blood

reflects total cortisol and to determine free levels it is necessary to know the equilibrium dissociation constant of CBG and the maximum corticosteroid binding capacity of the blood sample (Sheriff et al. 2011). Furthermore, blood collection itself may be stressful and may bring about an artificial increase in cortisol levels if samples are not obtained shortly (approximately ≤ 3 minutes) after the initiation of handling (Romero and Reed 2005, Mormède et al. 2007, Sheriff et al. 2011). This may be especially important in large species of free-ranging wildlife where blood collection most often requires capture (involving physical restraint or pursuit) and chemical immobilization (Morton et al. 1995, Cattet et al. 2003a, Omsjoe et al. 2009). In addition to handling stress (Dickens et al. 2010), some sedative and immobilization drugs (e.g. alpha-2 adrenergic agonists, opioids) may also directly increase blood cortisol levels (Bubenik and Brown 1989, Arnemo and Ranheim 1999, Pascoe et al. 2008). Indeed, capture and immobilization have been associated with elevated blood cortisol and other stress related physiological changes in grizzly bears (Cattet et al. 2003a, Kusak et al. 2005, Cattet et al. 2008a), polar bears (Cattet et al. 1999, Tryland et al. 2002, Øskam et al. 2004), and caribou (Rehbinder 1990, Säkkinen et al. 2004, Omsjoe et al. 2009). Importantly, blood to be used in cortisol analyses must also be centrifuged and derived plasma or serum kept cool (or frozen) to minimize steroid metabolism (Sheriff et al. 2011). In many field-based studies these requirements may be difficult to achieve.

The measurement of cortisol in saliva has been explored as an alternate method with which to estimate HPA axis activity in wildlife (Millspaugh et al. 2002, Heintz et al. 2011, Marcilla et al. 2012). Cortisol measured in saliva represents a point in time measure of circulating free cortisol levels (Mormède et al. 2007, Sheriff et al. 2011). Like blood, salivary cortisol may be influenced by circadian rhythms, season (Ekkel et al. 1996, Heintz et al. 2011, Marcilla et al. 2012), and sample collection methods (Kobelt et al. 2003, Poll et al. 2007, Dreschel and Granger 2009). Unlike blood, cortisol levels in saliva may be somewhat less affected by handling procedures (Kobelt et al. 2003, Sheriff et al. 2011), and may remain stable at room temperature for a few days to weeks so long as samples remain wet (Kirschbaum and Hellhammer 1989, Chen et al. 1992, Sheriff et al. 2011). Saliva may also be collected relatively non-invasively from captive wildlife (Millspaugh et al. 2002, Rehbinder and Hau 2006, Heintz et

al. 2011, Menargues et al. 2008). Despite these potential advantages, the collection of saliva from free-ranging animals is usually impractical (Sheriff et al. 2011).

It should also be recognized that cortisol levels have been evaluated in cerebrospinal fluid (CSF) (Luna and Taylor 1998, Kaufman et al. 2007), milk (Fukasawa et al. 2008, Sullivan et al. 2010), and adipose tissue (e.g. whale blubber; Amaral 2010). However, these media are not widely employed in wildlife studies (Sheriff et al. 2011).

In contrast, the measurement of GC metabolites in urine and feces is regularly used to study HPA axis activity in wild species (e.g. urine: Owen et al. 2005, Constable et al. 2006, Jaimez et al. 2011; e.g. feces: von der Ohe et al. 2002, Sheriff et al. 2010, Hulsman et al. 2011). Glucocorticoids in blood are metabolized by the liver and excreted in the urine or feces (Touma and Palme 2005, Mormède et al. 2007, Sheriff et al. 2011), and both media may offer potential advantages over blood and saliva in that they may represent the accumulation of GC metabolites over several hours to several days (Hunt and Wasser 2003, Lepschy et al. 2007, Ashley et al. 2011, Sheriff et al. 2011). Cortisol metabolites measured in urine and feces reflect circulating free cortisol levels in this time period (Sheriff et al. 2011). These media can also be collected opportunistically or remotely from free-ranging species (e.g. Wasser et al. 2004, Constable et al. 2006, Wasser et al. 2011, Jaimez et al. 2011). Furthermore, the study of fecal GCs in particular has demonstrated clear potential as a non-invasive technique with which to evaluate the effects of environmental stressors on wildlife health and population performance (Sheriff 2009a, Sheriff et al. 2009b, Sheriff et al. 2010). The use of urinary or fecal GCs to study stress in grizzly bears, polar bears (von der Ohe et al. 2004, Wasser et al. 2004, Shepherdson et al. 2004) and caribou (Rehbinder and Hau 2006, Ashley et al. 2011, Wasser et al. 2011) has been investigated. However, a number of confounding factors may diminish the utility of these techniques in field-based studies of these species.

Under most field conditions the collection of fresh, uncontaminated urine samples is difficult and may limit the practical use of this technique to captive wildlife or species that can be closely monitored in their natural habitat (Muller and Wrangham 2004, Constable et al. 2006, Jaimez et al. 2011). Although fecal samples may be more easily collected (e.g. through the use of scat detection dogs; Wasser et al. 2004), levels of fecal GC metabolites may be influenced by environmental exposure (Washburn and Millspaugh 2002, Rhenus et al. 2009). Furthermore,

naturally occurring bacterial enzymes may degrade steroid metabolites in as little as a few hours and while the age of fecal samples is important it is often unknown in wildlife studies (Millspaugh and Washburn 2002, Touma and Palme 2005, Sheriff et al. 2011). Accordingly, urine and fecal samples require immediate preservation once collected (Brown et al. 1995, Millspaugh et al. 2003, Sheriff et al. 2011). However, GC levels in both media may also be influenced by preservation, storage, and sample preparation protocols (Brown et al. 1995, Wasser et al. 2000, Millspaugh et al. 2003, Sheriff et al. 2011).

The time delay in the excretion of cortisol metabolites corresponds to the time between urination events or gut transit time (Hulsmann et al. 2011, Sheriff et al. 2011). Most species urinate several times per day, and circadian variations in levels of urinary GCs are commonly recorded (Muller and Wrangham 2004, Owen et al. 2004, Bennett et al. 2008). Seasonal variations in urinary GC levels have also been observed (Saltz and White 1991, Hay et al. 1994, Owen et al. 2005). While the detection of circadian changes in fecal GC levels may be limited to small-bodied species with relatively rapid gut transit (Sheriff et al. 2011), seasonal changes in fecal GC levels are known from many mammals (Foley et al. 2001, von der Ohe et al. 2004, Konjević et al. 2011). GC levels in both media may also vary by species and with an animal's life history stage (Owen et al. 2005, Mooring et al. 2006, Kersey et al. 2011). Where samples are collected remotely, and if additional analyses are not performed (e.g. DNA-based species and sex determination), these factors may introduce bias into the interpretation of subsequent GC results generated from wildlife (Miller and Washburn 2004, Sheriff et al. 2011).

An animal's diet may also influence fecal GC levels (von der Ohe and Servheen 2004, Keay et al. 2006, Ganswindt et al. 2012). This may be especially important for omnivorous or carnivorous species (such as grizzly and polar bears) where GCs derived from the tissue of prey may be found in feces or where the fiber content of an animals' diet may influence gut transit time (von der Ohe and Servheen 2002, von der Ohe et al. 2004). Likewise, seasonal changes in the fiber content of plant forage may be relevant in herbivores (Drucker et al. 2010) while the consumption of inorganic material during feeding (or mineral acquisition) may be important in both groups (Klein and Thing 1989, Sato et al. 2005, Ganswindt et al. 2012). It is also probable that dietary components or nutritional status may influence GC metabolites measured in urine by altering urinary pH or other factors which could influence the GC stability (Gevaert et al. 1991,

Delgiudice et al. 1994). Most importantly, neither media can assess HPA axis activity occurring over weeks to months without the repeated sampling of individuals (Sheriff et al. 2011). Clearly, alternate approaches are needed for the evaluation of long-term stress in free-ranging wildlife.

1.8 The Importance of a Non-invasive Technique

The handling of wildlife is an invaluable component of many ecological studies and is often the only practical means through which biological samples or some types of information can be obtained (e.g. Cattet et al. 2002, Laske et al. 2011, Peterson et al. 2012). Nonetheless, the capture and immobilization of wildlife does not occur under controlled conditions and always involves the risk of injury or mortality even in healthy animals (Arnemo et al. 2006, Cattet et al. 2008a, Dickens et al. 2010). Remote drug delivery systems have the potential to cause serious injury (Valkenburg et al. 1983, Valkenburg et al. 1999, Cattet et al. 2003b). Misplaced darts may be directly fatal. More insidiously, contamination of dart wounds may lead to secondary infection or more generalized illness that may debilitate animals for extended periods (Cattet et al. 2003b, Cattet et al. 2008a). Morbidity or mortality may also occur as a direct result of anesthesia (Larsen and Gauthier 1989, Caulkett and Shury 2008, Mentaberre et al. 2012) or may occur as a result of other events secondary to capture or recovery from immobilization drugs [e.g. capture myopathy (Montane et al. 2002, Cattet et al. 2003b); trauma (Arnemo et al. 2006, Cattet et al. 2008a, Jacques et al. 2009); predation (Larsen and Gauthier 1989, Swenson et al. 1999, Dickens et al. 2010)]. The benefits of wildlife capture may be further limited by practical or financial considerations (e.g. Jessup et al. 1988, Goodrich et al. 2001, Reed et al. 2011) along with an intrinsic risk to the health and safety of researchers (Caulkett and Shury 2008). Many of these concerns are especially relevant in the study of large mammals, and the development and application of non-invasive sampling strategies to evaluate wildlife stress should be encouraged.

1.9 The Utility of Hair

In free-ranging wildlife, hair has been used successfully to identify exposure to blood borne environmental contaminants (e.g. Christensen et al. 2007, Dietz et al. 2009, Lokken et al. 2009) and to assess the importance of dietary components (e.g. Mowat and Heard 2006, Gray et al. 2008, Horton et al. 2009, Drucker et al. 2010). Capture-mark-recapture based estimates of wildlife abundance have also been undertaken using DNA collected from hair bulbs to identify individual animals, their gender, and patterns of movement (e.g. Mowat et al. 2005, Lindsay and

Belant 2008, Proctor et al. 2012). As a result, protocols for the remote collection of hair from free-ranging wildlife are well established in a wide range of species [e.g. Woods et al. 1999 (Ursids); Garcia-Alaniz et al. 2010, Ausband et al. 2011 (Felids and Canids); Williams et al. 2007 (Mustelids); Belant et al. 2007, Fickel et al. 2012 (Cervids); non-human primates (Bradley et al. 2008)]. Remote hair collection (i.e. through the use of barb wire or other snagging apparatus) is considered to be among the least invasive sampling methodologies currently available. Furthermore, hair is commonly collected as part of ongoing research programs and may also be obtained from archives or wildlife killed accidently, as a result of human-animal conflict, or hunting (Macbeth et al. 2010, Dietz et al. 2009, Bechshøft et al. 2012).

In addition to potential for non-invasive or opportunistic sampling, hair is known to incorporate blood borne hormones and xenobiotics during its active growth phase which may last many weeks to months (Maurel et al. 1986, Pragst and Balikova 2006, Kidwell and Smith 2007). Compared to blood, saliva, urine or feces, hair is a relatively stable medium that can be transported and stored at room temperature (Quadros and Monterio-Filho 1998, Felicetti et al. 2003, Jaspers et al. 2010) and in which steroid hormones (Davenport et al. 2006, Koren and Geffen 2009, Macbeth et al. 2010) or toxins (Pragst and Balikova 2006, Dietz et al. 2009, Bechshøft et al. 2012) can often be measured years to centuries after their deposition (Kintz 2004, Macbeth et al. 2010, Webb et al. 2010). In the last decade, the measurement of cortisol in hair has emerged as a promising indicator of long-term stress in humans and domestic animals where elevated HCC has been associated with chronic exposure to a variety of stressful stimuli and adverse health effects in individuals (Gow et al. 2010, Sheriff et al. 2011, Russell et al. 2012). All considered, hair may also represent an ideal medium with which to study long-term stress in free-ranging wildlife (Koren et al. 2002, Macbeth et al. 2010, Ashley et al. 2011, Bechshøft et al. 2011).

1.10 Hair Structure and Biology

1.10.1 An overview of hair structure and hair growth

The potential advantages of hair as a medium in which to assess long-term stress in free-ranging wildlife are closely tied to its structural and biological characteristics. In general terms, hair is a flexible keratinized fibril produced by small sac like organs called hair follicles which are embedded in the epidermis and are closely associated with sebaceous glands, apocrine sweat

glands, and eccrine sweat glands (Harkey 1993, Stenn and Paus 2001, Pragst and Balikova 2006) (Fig. 1.3). Hair visible above the skin surface is termed the shaft while hair within the follicle is known as the root which has a terminal knob referred to as the bulb (Harkey 1993, Pragst and Balikova 2006) (Fig. 1.3). The hair shaft is composed of three layers and contains approximately 65-95% protein, 1-9% lipid along with relatively small quantities of trace elements, polysaccharides, and water (Kidwell and Blank 1996). An outer cuticle of flat keratinized cells surrounds a central cortex of densely packed keratinized cells, melanin, and nuclear remnants (Harkey et al. 1993, Kidwell and Blank 1996, Pragst and Balikova 2006) (Fig 1.3). The cuticle may be further divided into three layers: the outermost A-layer, the exocuticle, and the endocuticle (Kidwell and Blank 1996). Depending on the type of hair, the cortex may surround a medulla consisting of loosely packed cuboidal cells, irregular fibrous proteins, and air filled spaces (Harkey 1993, Kidwell and Blank 1996, Pragst and Balikova 2006) (Fig. 1.3).

Within the hair follicle, the innermost zone around the base of hair bulb is the site of hair synthesis (Stenn and Paus 2001, Pragst and Balikova 2006). This region is referred to as the germination center and is composed of a germinal epithelium containing keratinocytes and melanocytes (also referred to as matrix cells) (Harkey 1993, Stenn and Paus 2001, Pragst and Balikova 2006) (Fig 1.3). Cells from the germination zone differentiate into the protective outer and inner root sheaths and the three layers of the hair shaft as rapid mitosis in the zone of germination forces matrix cells towards the distal aspect of the hair follicle (Stenn and Paus 2001, Pragst and Balikova 2006) (Fig 1.3). Upon leaving the zone of germination, matrix cells first enter a keratogenous zone where melanin is synthesized and the process of keratinization begins (Harkey 1993, Pragst and Balikova 2006) (Fig 1.3). As cells progress further towards the hair follicle opening at the epidermal surface, they die, dehydrate, and decompose forming dense protein fibrils in the zone of mature hair (Harkey 1993, Pragst and Balikova 2006) (Fig 1.3). Cellular residue is further bound by proteins and a protein-lipid complex (cell membrane complex) originating from the plasma membranes of dead matrix cells (Pragst and Balikova 2006). The hair shaft itself is a metabolically inactive tissue (Harkey 1993, Kidwell and Blank 1996, Pragst and Balikova 2006).

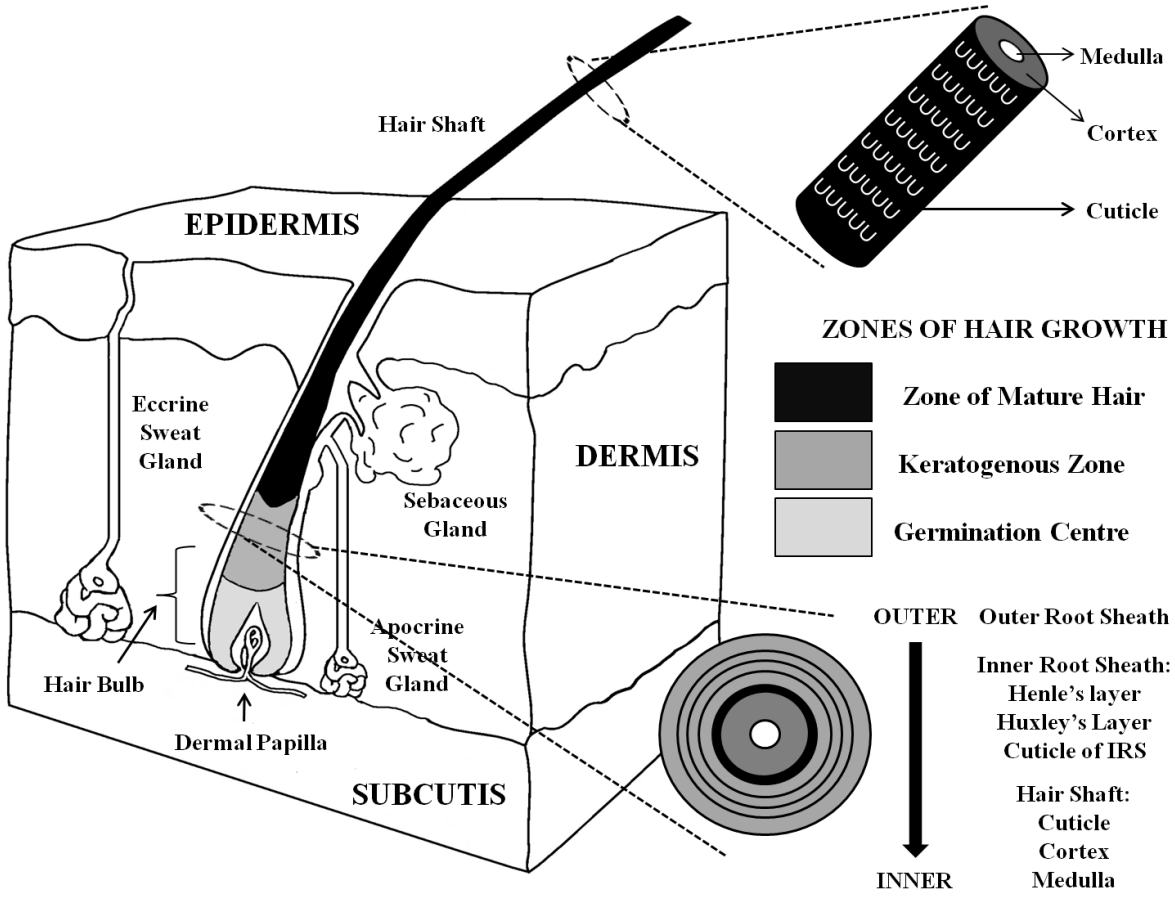

Figure 1.3 Structure of the hair follicle and hair shaft.

1.10.2 The hair cycle and mechanisms for incorporation and retention of substances in hair

1.10.2.1 The hair cycle

Hair does not grow continually but instead follows a cyclical pattern of alternating periods of active growth and quiescence (Stenn and Paus 2001, Pilkus and Chuong 2008, Müntener et al. 2011). A hair follicle which is actively producing hair is said to be in anagen (Harkey 1993, Stenn and Paus 2001, Pragst and Balikova 2006). In anagen, new hair is formed as matrix cells in the zone of germination multiply and differentiate into the hair shaft's three layers. During this period, the hair bulb is closely associated with the blood supply of the cutaneous plexus and the dermal papilla (Harkey 1993, Stenn and Paus 2001, Pragst and Balikova 2006) (Fig. 1.3). Accordingly, drugs, metabolites, and hormones circulating in the blood are believed to be incorporated into hair primarily during this active growth phase (Davenport et al. 2006, Pragst and Balikova 2006, Kidwell and Smith 2007). Although the

duration of anagen is a species specific trait, this growth phase typically lasts for many weeks to many months (e.g. Samuel et al. 1986, Maurel et al. 1986, Cuyler and Ørtisland 2002). Cortisol levels in hair should therefore represent an integrated measure of HPA axis activity occurring during this time and may provide the longest record of cortisol levels currently available (Macbeth et al. 2010, Sheriff et al. 2011). Importantly, the longest hair types (typically guard hairs in wildlife) may also have the longest period of anagen and thus the greatest time frame in which to incorporate stress hormones (Harkey et al. 1993, Jones et al. 2006, Macbeth et al. 2010).

After anagen, hair enters a relatively short transitional phase (catagen) which may last days to weeks and in which follicular regression occurs, cell division stops, and hair shaft elongation ends (Stenn and Paus 2001, Pragst and Balikova 2006, Müntener et al. 2011). In catagen the hair bulb becomes fully keratinized, club shaped, and migrates distally in the regressing hair follicle. Following catagen, the hair follicle enters a quiescent stage (telogen) in which relatively little follicular activity occurs and which, like anagen, may last for many months (Stenn and Paus 2001, Pragst and Balikova 2006, Müntener et al. 2011). In telogen, the hair bulb is held loosely in the distal portion of the regressed follicle, and blood borne substances are unable to enter the hair shaft (Stenn and Paus 2001, Pragst and Balikova 2006, Müntener et al. 2011). With the onset of a new anagen phase old hair is forced from the follicle by growth of the new hair and shed (sometimes referred to as exogen; Müntener et al. 2011).

1.10.2.2 Mechanism for incorporation

In the forensic drug field, the passive diffusion of substances from blood in capillaries of the cutaneous plexus to cells in the zone of germination is considered to be the most important mechanism of incorporation into hair (Harkey 1993, Pragst and Balikova 2006, Kidwell and Smith 2007). This process is currently believed to be restricted to the period of active hair growth (anagen), and incorporation may also occur in direct proportion to levels of bioavailable (free) xenobiotics in the circulation (Baumgartner et al. 1992, Pragst and Balikova 2006, Kidwell and Smith 2007). However, substances may also enter the growing hair root or hair shaft (with some temporal delay; Kidwell and Smith 2007) via diffusion from the external environment, tissues surrounding actively growing hair, or as the results of glandular apocrine, sebaceous, and sweat secretions in and around the follicle (Henderson 1993, Pragst and Balikova 2006, Kidwell

and Smith 2007). Among these alternate pathways, glandular secretions may be particularly important (Pragst and Balikova 2006, Kidwell and Smith 2007). Like drugs and other xenobiotics, blood borne diffusion during anagen is currently believed to be the primary pathway for the incorporation of free cortisol into hair (Gow et al. 2010, Russell et al. 2012). The study of hair cortisol is a rapidly emerging field and new evidence suggests that alternate mechanisms of cortisol incorporation into hair [e.g. cortisol containing biological contaminants, glandular secretions, and local (hair follicle) cortisol production] are nearly certain, however, both their overall and relative importance in different species are currently unknown (Sharpley et al. 2009, Macbeth et al. 2010, Keckeis et al. 2012, Xie et al. 2012).

In humans, the incorporation of substances from the blood stream into the growing hair shaft is believed to occur over a length of 1.2-1.5 mm between the level of the matrix cells and the distal end of the zone of keratinisation in the hair root (Pragst and Balikova 2006) (Fig 1.1). This process takes approximately three days in humans (Pragst and Balikova 2006) with an additional week or more required for that hair segment to emerge as the hair shaft visible at the skin surface (Kidwell and Smith 2007, Xie et al. 2012). A delay in incorporation has recently been recorded for cortisol entering the hair shaft in both humans and animals and suggests that even actively growing hair may be relatively resistant to the effects of short-term increases in circulating levels of GCs (Ashley et al. 2011, Gonzalez de la Vera et al. 2011, Xie et al. 2012). This feature may represent an additional benefit of using hair in wildlife studies where capture and immobilization protocols are well known to cause a rapid increase in cortisol levels (Romero and Reed 2005, Mormède et al. 2007, Sheriff et al. 2011).

1.10.2.3 Binding and retention

Regardless of the mechanism of incorporation, structural proteins, melanin, and sulf-hydryl containing amino acids are considered to be the primary binding sites which entrap substances in hair (Cone 1996, Kintz 2004, Kidwell and Blank 1996). As such, the cortex is most often considered to be the primary region for the binding and retention of blood borne substances within the hair shaft (Kidwell and Blank 1996). However, lipophilic molecules such as cortisol may also be bound in the cell membrane complex (Pragst and Balikova 2006).

Once bound with the hair shaft, the structural characteristics of hair may protect even unstable molecules from degradation (Kintz 2004, Webb et al. 2010, Gonzalez de la Vera et al.

2011). For example, the keratin in the exocuticle contains large quantities of the amino acid cysteine which, through the formation of disulfide bonds, attaches this layer firmly to A-layer and makes the cuticle both resilient and resistant to damage (Kidwell and Blank 1996). This characteristic along with the tiled arrangement of the cuticle, and the presence of sulfhydryl containing amino acids in other hair layers may explain why hair is relatively resistant to the effects of environmental or chemical exposure (Kidwell and Blank 1996, Quadros and Monterio-Filho 1998, Kintz 2004), and why substances incorporated into hair often remain detectable for many years to centuries (Kintz et al. 2004, Dietz et al. 2009, Bechshøft et al. 2012). Recent studies suggest that cortisol levels in hair may also be resistant to environmental conditions (Macbeth et al. 2010) and may remain stable for many months to hundreds of years (Webb et al. 2010, Macbeth et al. 2010, Gonzalez de la Vera et al. 2011, Bechshøft et al. 2012).

1.10.2.4 Control of the hair cycle

In individual hair follicles, the hair cycle is controlled through a variety of complex interactions between endogenous and exogenous stimuli (reviewed in Stenn and Paus 2001, Randall 2007). Endogenous factors include a wide range of hormones (most notably melatonin and prolactin), cytokines, and growth regulators (Stenn and Paus 2001, Randall 2007), while photoperiod and to a lesser extent temperature exert exogenous influences (e.g. Pinter 1968, Ryder 1978, Maurel et al. 1986, Heydon et al. 1995, Celi et al. 2003). In humans and some laboratory species (e.g. guinea pig, *Cavia porcellus*), each hair follicle has its own inherent rhythm, and the growth cycles of adjacent hair follicles may be asynchronous (Stenn and Paus 2001). A similar mosaic pattern of hair growth and replacement has also been recorded in some breeds of domestic dog (Al Bagdadi et al. 1979, Diaz et al. 2004a, Diaz et al. 2004b, Müntener et al. 2011) and in the domestic cat (Baker et al. 1974). In contrast, hair in most wild mammals grows in waves in which the activity of many follicles is synchronized (e.g. Pearson 1975, Ryder 1978, Maurel et al. 1986, Samuel et al. 1986, Cuyler and Ørtisland 2002). When many follicles enter anagen simultaneously the result is visible as seasonal shedding (moult) while old hairs are forced from the follicle by new growth (Maurel et al. 1986, Samuel et al. 1986, Stenn and Paus 2001). Importantly, active hair growth is initiated before and continues beyond the cessation of visible moult (Stenn and Paus 2001).

In general, the onset and duration of moult along with the number of seasonal moults experienced by a species is a unique life history trait (Maurel et al. 1986, Samuel et al. 1986, Cuyler and Ørtisland 2002). Hair growth is also an energetically costly process (Randall 2007) and may be further influenced by an individual's nutritional or health status (Pinter 1968, Mathur and Deo 1976, Forrester et al. 1993, Heydon et al. 1995), sex, or life history stage (Pearson 1975, Ryder 1978, Stewart and Macdonald 1997, Neuhaus 2000). In addition, trauma to the skin is known to stimulate active hair growth (Stenn and Plaus 2001) while the timing and duration of hair growth may also vary among body regions and even among adjacent hair shafts (or groups of hair shafts) within body regions (Maurel et al. 1986, Stenn and Paus 2001, Müntener et al. 2011). Nevertheless, it is likely that the period of active hair growth in a particular species closely parallels that of visible moult (Maurel et al. 1986, Macbeth et al. 2010). This feature may allow for an estimate to be made of the time frame over which cortisol is deposited in hair samples obtained from wild species at different times of the year (Macbeth et al. 2010).

1.11 Patterns of Hair Biology in Study Species

1.11.1 Grizzly bear

In the grizzly bear, pelage varies from nearly white (light blond) to black (Harting 1987, Schwartz et al. 2003, B. Macbeth personal observation). Grizzly bear hair is often lightest at the distal aspect of the hair shaft (tips) and may also be characterized by alternating bands of light and dark hair in some hairs of some individuals. Alternately, hair shafts may be a uniformly dark or light in colour (Harting 1987, Schwartz et al. 2003, B. Macbeth personal observation). All variations of grizzly bear hair colour may be found within the same body region with the exception of the lower legs which are most often uniformly dark (Harting 1987, B. Goski personal communication, B. Macbeth personal observation). Newly grown grizzly bear hair is dark brown to black and may lighten distally with time due to a combination of genetic and environmental factors (i.e. weathering/bleaching) (Rogers 1980, Harting 1987, Schwartz et al. 2003, B. Macbeth personal observation). Based on gross examination of live captured animals and skin biopsies collected from dead grizzly bears, it is likely that this species has a compound hair follicle characterized by a single large guard type hair and several, smaller secondary hairs emerging from the same follicular opening (B. Macbeth, personal observation, Müntener et al. 2011). The colour, length, width and medullary size of grizzly bear hair may also vary among

different body regions and different grizzly bear populations (Harting 1987, Elgmørk and Riiser 1990).

Grizzly bears replace their hair coat once annually and active hair growth is believed to begin soon after emergence from dens in the spring (Pearson 1975, Harting 1987, Schwartz et al. 2003). However, latitude, sex, and age may influence hair growth in this species and its onset and duration may be highly variable in individual bears (Pearson 1975, Harting 1987, Schwartz 2003). In free-ranging grizzly bears, visible moult is often observed first in adult males while the process may be delayed in immature males, solitary females and females with dependent offspring (Pearson 1975, Harting 1987, Schwartz et al. 2003). The onset of moult may also occur earlier in more northerly grizzly bear populations (Pearson 1975). Accordingly, moult may be complete by as early as late July or as late as early September depending on latitude and individual (Pearson 1975). However, active hair growth is not believed to stop until the onset of hibernation (October-November) in this species (Christensen et al. 2007).

Within individual bears, the pattern of hair growth may also be influenced by hair type. Specifically, the growth of guard hair may begin in early spring and last until late fall while the growth of undercoat may not be initiated until late summer (Jones et al. 2006, C. Robbins personal communication, B. Macbeth personal observation). Furthermore, the growth of undercoat may be delayed in captive male grizzly bears compared to females (C. Robbins, personal communication). In captive grizzly bears, the onset of hair growth has also been observed to be directly related to nutrient intake (C. Robbins, personal communication). Precisely when hair growth begins in each body region may also vary even among bears inhabiting the same general geographic area (B. Goski, personal communication, B. Macbeth personal observation).

Since grizzly bears exhibit a single annual moulting period it is likely that there is a seasonal concentration of follicular activity which (for guard hair) occurs from spring to fall (a period of approximately 5 months; Felicetti et al. 2003a, Jones et al. 2006, Christensen et al. 2007). In this time it is likely that the majority of hair follicles are in anagen, actively growing and incorporating blood borne substances like cortisol (Stenn and Paus 2001, Davenport et al. 2006, Pragst and Balikova 2006). Hair growth in the grizzly bear has been estimated to occur at a rate of approximately 500 µm per day (Christensen et al. 2007). Consequently, each 1 cm

segment of hair from the root to tip may reflect approximately 20 days of exposure to blood borne substances (Christensen et al. 2007).

Although the duration of catagen in grizzly bears is unknown it is likely hair follicles begin to actively regress sometime in the late summer or early fall and are in telogen by early hibernation. Since no hair growth is believed to occur during hibernation (Jones et al. 2006, Christensen et al. 2007) telogen may last from late fall until the initiation of moult in the following spring. If telogen guard hairs are collected on emergence from dens the longest possible growth period (and thus exposure to potential stressors) should be represented. In such samples, the tip and middle of the hair should reflect HPA axis activity occurring in the spring and summer of the previous year while the area near the hair root should reflect the most recent exposure prior to the cessation of hair growth near hibernation (Jones et al. 2006, Christensen et al. 2007). By sampling the entire length of the hair shaft an integrated estimate of annual stress levels may be possible in this species (Macbeth et al. 2010).

1.11.2 Polar bear

Polar bear hair is unpigmented but may appear white, yellow, orange or blue depending on ambient lighting and season (Ørtisland and Ronald 1978, Amstrup 2003). Polar bears appear whitest after moulting in autumn but may be off white to yellow in late winter and spring as the result of pelage contamination with oils from prey and oxidation by the sun (Amstrup 2003). In captivity, polar bears may also appear green owing to the incorporation of algae into the hollow medulla of guard hair (Lewin and Robinson 1979).

Compared to grizzly bears, less has been reported about factors influencing moulting patterns in this species. Moult in polar bears is known to take place once a year and active hair growth is believed to occur from late April/early May until September or October (Born et al. 1991, Amstrup 2003, Jaspers et al. 2010). As such, it is probable that the pattern of follicular activity (and subsequent duration of cortisol incorporation) may be comparable in grizzly and polar bears. Both species also have grossly similar hair types (guard hair and undercoat) however there are minor structural differences in the hair of grizzly and polar bears (Grojean et al. 1980, Elgmørk and Riiser 1990).

1.11.3 Caribou

In caribou, hair colour varies with age and ecotype (Miller 2003). The general pattern of adult colouration is a relatively dark body with lighter neck, ventral surface and sides while calves are a uniform light brown or red brown with lighter undersides (Cuyler and Ørtisland 2002, Miller 2003). In general woodland caribou have the darkest pelage (dark brown) while Peary caribou have the lightest (brown grey to white). Barren ground caribou have hair of many shades from light to dark (Miller et al. 2003). Coat colour in all ecotypes may also vary with body region, within and among hair shafts, and with season (Miller et al. 2003). Like grizzly bears, individual hair shafts from caribou may be uniformly dark or light or may contain both light and dark segments (Cuyler and Ørtisland 2002, B. Macbeth, personal observation). All hair shaft colour patterns may also be found within a single body region (B. Macbeth, personal observation). In caribou, guard hairs are relatively thick and large and contain a well developed medullary space while the under coat is very fine and relatively short (Cuyler and Ørtisland 2002, B. Macbeth personal observation).

As in grizzly and polar bears, caribou exhibit a single annual moult which begins in the late spring or early summer and continues until late fall (Cuyler and Ørtisland 2002, Miller 2003, Drucker et al. 2010). Moult is often completed fastest in mature bulls and may be delayed in juveniles and yearlings, solitary cows, and cows with dependent offspring (Cuyler and Ørtisland 2002, Miller et al. 2003). By late summer all but cows with calves or animals in poor condition may be in new coats (Cuyler and Ørtisland 2002, Miller 2003), and by the onset of rut (September to early October) hair coats are fully developed and exhibit maximal colour intensity (Miller et al. 2003). As time after growth increases, caribou hair tends to become bleached and damaged resulting in a general lightening and loss of colour contrast (Cuyler and Ørtisland 2002, Miller 2003). Like bears, the HCC measured in an intact hair shaft collected during the spring moult may represent an integrated measure of HPA axis activity occurring in the summer and fall of the preceding year (Drucker et al. 2010).

1.12 Hair Cortisol Concentration as a Potential Biomarker of Long-Term Stress

1.12.1 The emerging field of hair cortisol analysis and hair cortisol in humans and non-human primates

The study of hair cortisol concentration (HCC) is a rapidly emerging field of research that has increased from only four peer-reviewed reports [in humans (Cirimele et al. 2000, Raul et al.

2004); in rock hyrax, *Procavia capensis* (Koren et al. 2002); in captive non-human primates (Davenport et al. 2006)] at the start of this research program (June, 2007) to more than sixty published accounts at the time of writing (reviews in Gow et al. 2010, Russell et al. 2012, Meyer and Novak 2012). The earliest HCC studies were primarily limited to the detection of cortisol (Cirimele et al. 2000) or the determination of cortisol levels in hair from target species (Koren et al. 2002, Raul et al. 2004). The first intervention study to demonstrate a potential linkage between long-term stress and HCC was performed by Davenport et al. (2006) who found that cortisol measured in the hair of captive rhesus macaques (*Macaca mulatta*) increased as the result of exposure to a prolonged stressor (movement between housing units). Davenport et al. (2006) also determined that HCC and salivary cortisol levels in study animals were directly associated and that HCC levels returned to normal (pre relocation) levels approximately 1 year after translocation. Salivary cortisol levels are known to be closely associated with the amount of bioavailable (free) cortisol in the blood (Morméde et al. 2007), and together these finding provided the first evidence that HCC may: 1) reflect systemic HPA axis activity, and 2) respond dynamically to changes in circulating cortisol levels occurring over extended periods.

Building on the results of early studies a range of analytical techniques have now been applied in HCC studies (reviewed in Gow et al. 2010, Russell et al. 2012, Meyer and Novak 2012). Most protocols used for HCC analysis involve the solvent based (usually methanol) extraction of cortisol from cut or pulverized hair followed by the determination of cortisol levels in samples of dried and then reconstituted (with phosphate buffer) hair extract. Techniques that have been applied in the determination of HCC include enzyme-linked immunosorbent assays (ELISA) (most studies), radio-immuno assays (RIA), and high performance liquid chromatography-mass spectrometry (HPLC-MS) (Gow et al. 2010, Russell et al. 2012, Meyer and Novak 2012).

Since Davenport et al. (2006), most HCC studies have been performed in humans or non-human primates (Gow et al. 2010, Russell et al. 2012, Meyer and Novak 2012). In addition to revealing a variety of biological (e.g. sex, age, species) or hair sample related factors (e.g. effects of UV radiation, water, hair treatment, hair colour) that may influence HCC, research findings in both groups have provided encouraging evidence that cortisol in hair may indeed be a biologically relevant marker of long-term stress (Gow et al. 2010, Russell et al. 2012, Meyer and

Novak 2012). For example, as in non-human primates (Davenport et al 2006), hair cortisol levels in humans have been shown to be directly associated with cortisol levels recorded in saliva (D'Anna-Hernandez et al. 2011, Xie et al. 2012) and also urine (Sauvé et al. 2007). In humans, hair cortisol analysis has also been used successfully to track the clinical course of physiological states (pregnancy), diseases, or medical treatments resulting in hypercortisolism (Kirschbaum et al. 2009, Thomson et al. 2010, Gow et al. 2011, D'Anna-Hernandez et al. 2011, Manenschijn et al. 2011a).

The majority of HCC studies in humans and non-human primates also support a direct relationship between hair cortisol levels and chronic exposure to stressful stimuli (Gow et al. 2010, Russell et al. 2012, Meyer and Novak 2012). For instance, compared to unaffected controls, HCC levels in humans have been found to be higher in those individuals that have experienced prolonged physical or psychosocial stress in the months preceding hair sampling [e.g. hospitalization (Yamada et al. 2007), chronic pain (Van Uum et al. 2008), unemployment (Dettenborn et al. 2010), alcohol withdrawal (Stadler et al. 2010), perceived stress (Kalara et al. 2007, Karlén et al. 2011, Stalder et al. 2012), clinical depression (Dettenborn et al. 2012), shift work (Manenschijn et al. 2011b), post traumatic stress disorder (Steudte et al. 2011), and athletic training (Skoluda et al. 2011, Stalder et al. 2011)]. Direct associations between HCC levels and the intensity or duration of physical (Yamada et al. 2007, Skoluda et al. 2011) or psychological stress (Steudte et al. 2011) have also been identified. In humans, HCC has also been found to remain relatively stable within individuals in the absence of major stressors which may further highlight the potential utility of this technique for evaluating stress levels in populations exposed to divergent stressors (Stalder et al. 2011).

Among non-human primates, recent studies have replicated the results of Davenport et al. (2006) through the identification of elevated HCC in animals subjected to relocation stress (Fairbanks et al. 2011, Dettmer et al. 2012) as well as in individuals chronically exposed to other forms of adverse stimuli [e.g. removal from mothers (Dettmer et al. 2009, Dettmer et al. 2012)]. Recent studies in non-human primates also add support to the belief that hair cortisol levels are a biologically relevant indicator of long-term HPA axis activity. For example, direct associations have now been identified for both pre relocation (unstressed) and post relocation (stressed) hair cortisol levels among non-human primates with similar genetic makeup which may provide the

first evidence that genetic influences may influence HCC as well as environmental factors (Fairbanks et al. 2011). HCC levels have also been found to reflect known patterns of HPA axis activity related to age and taxonomic designation in a wide range of non-human primate species (Fourie et al. 2011).

Other compelling evidence in support of HCC as a valid biomarker of long-term stress comes from the increasing recognition of associations between elevated HCC and diminished health in humans and captive non-human primates. In humans, HCC levels have been found to be directly associated with diminished mental health [e.g. post traumatic stress disorder (Steudte et al. 2011); clinical depression (Dettenborn et al. 2012); bipolar disorder (Van Rossum et al. 2011)]. Elevated HCC has also been identified in human patients with chronic pain disorders (Van Uum et al. 2008) as well as in individuals with cardiovascular disease (Pereg et al. 2010), or having metabolic ailments characterized by hypercortisolism [e.g. Cushing's Disease, (Thomson et al. 2010, Manenschijn et al. 2011a). More recently, direct associations between HCC levels and indices of obesity (a known risk factor for many cardiovascular and metabolic diseases in humans) have been identified [e.g. waist circumference and waist to hip ratio (Manenschijn et al. 2011a); body mass index; (Manenschijn et al. 2011b, Stadler et al. 2012)].

In non-human primates, elevated HCC levels caused by chronic stressors (removal from mothers or relocation) have been found to be associated with diminished cognitive performance (Dettmer et al. 2009) and increased anxiety behaviour (Dettmer et al. 2012) in juvenile animals.

1.12.2. Hair cortisol in domestic mammals

To date, there are relatively few accounts of HCC analysis in domestic animal species [e.g. dogs and cats (Accorsi et al. 2008, Bennett and Hayssen 2010), reindeer (Ashley et al. 2011), cattle (Maiero et al. 2005, Comin et al. 2008, Comin et al. 2011, Gonzalez de la Vera et al. 2011). However, where applied these studies also offer support for the validity of HCC as a biomarker of long-term stress. As in primates, studies in domestic carnivores have demonstrated that HCC may be directly associated with other measures of systemic GC levels including feces (Accorsi et al. 2008) and saliva (Bennett and Hayssen 2010). More recently, investigations in domestic ungulates employing ACTH stimulation tests have found evidence to support the hypothesis that cortisol from the blood stream may not be incorporated into non-growing hair (Ashley et al. 2011) while incorporation into actively growing hair may be delayed by many days

(Gonzalez de la Vera et al. 2011). Investigations in domestic cattle and reindeer have also identified elevated HCC in neonatal vs older juvenile or in juvenile vs adult animals (Maiero et al. 2005, Comin et al. 2008, Ashley et al. 2011, Gonzalez de la Vera et al. 2011, Comin et al. 2011). These findings agree with known patterns of HPA axis activity in these species (Jacob et al. 2001, Omsjoe et al. 2009) and also with HCC findings in non-human primates (Fourie et al. 2011). Studies in domestic cattle have also identified elevated HCC levels in response to a relocation event (movement between valley bottom and high mountain pastures) (Comin et al. 2011).

1.12.3 Hair cortisol in free-ranging wildlife

When this research program was initiated only one published account of hair cortisol analysis in wildlife was available (Koren et al. 2002). Since this time, the potential utility of this technique has garnered significant interest among wildlife researchers. However, evidence in support of HCC as a valid and practical indicator of long-term stress in wild species is only now beginning to emerge. Koren et al. (2002) measured cortisol in hair collected from free-ranging rock hyrax and were the first to investigate HCC in wildlife. Later studies by the same researchers recorded higher HCC levels in dominant members of this communally living species (Koren et al. 2008). These findings were attributed to the stress of social domination that has been observed in many group living mammals (e.g. Sands and Creel 2002, Mooring et al. 2006, Berg et al. 2008), and were suggested to provide evidence that HCC may accurately reflect long-term stress related to social behaviour in this species (Koren et al. 2008). Martin and Réale (2008) measured HCC in free-ranging eastern chipmunks (*Tamis striatus*) and observed that HCC levels were directly associated with fecal glucocorticoid levels and also that cortisol levels measured in both media changed seasonally. As in other species, these results may support the commonly held belief that HCC provides an estimate of systemic HPA axis activity occurring over extended periods (Gow et al. 2010, Russell et al. 2012, Meyer and Novak 2012).

This research program has been integral in the development of HCC as a potential tool for use in large carnivores and ungulates, and was also the first to evaluate HCC in a large free-ranging mammal of any kind (grizzly bears from Alberta, Canada). Research findings describing this study and aspects of HCC analysis in polar bears from Southern Hudson Bay, Canada, and

caribou from Alaska and Greenland are presented in detail in the following chapters and in (Macbeth et al. 2010, Ashley et al. 2011, Macbeth et al. 2012).

Outside of this research program, HCC has recently been assessed in polar bears from East Greenland (Bechshøft et al. 2011, Bechshøft et al. 2012) and caribou from Quebec, Canada (Renaud 2012). As in Macbeth et al. (2010), life history traits and procedural effects that may influence HCC in polar bears were explored in Bechshøft et al. (2011) and Bechshøft et al. (2012). Although Bechshøft et al. (2012) found that HCC was a weak predictor of contaminant burden in East Greenland polar bears, HCC could be measured in polar bear hair many years old. Renaud (2012) also identified biological and procedural considerations relevant to HCC analysis in caribou. However, Renaud (2012) also observed that HCC levels were highest among free-ranging caribou inhabiting areas disturbed by human activity. This finding was the first account of a potential relationship between elevated HCC levels and anthropogenic landscape disturbance in wildlife.

1.13 Hair Cortisol Concentration as a Potential Conservation Tool

If physiological stress levels in wildlife are adversely affected by human-caused ecological change, biological markers of long-term stress should be measurable in individual animals before adverse health effects are apparent at the population level (Wikelski and Cooke 2006, Cattet et al. 2006, Ellis et al. 2012). Once validated as a defensible biomarker of long-term stress (Busch and Hayward 2011, Macbeth et al. 2012), the use of HCC analysis may therefore increase the ability of wildlife managers to monitor the response of wildlife to changing environments and may also provide opportunities to alleviate human-caused stressors before population performance is compromised (Cattet et al. 2006, Cook and O'Conner 2010, Ellis et al. 2012). Furthermore, HCC may be valuable to establish baseline data in unaffected but at risk wildlife populations (Carey 2005, Cooke and Suski 2008, Cook and O'Conner 2010). Where habitats or wildlife have already been compromised by human activities, this technique may also serve to direct or monitor the effectiveness of restoration efforts (Cook and O'Conner 2010, Ellis et al. 2012). Moreover, the remote collection of hair from wildlife requires little training, is relatively cost effective, and may also be useful for the simultaneous evaluation of other parameters that may be of interest to wildlife managers [e.g. DNA-based species identification and population inventories (e.g. Mowat et al. 2005, Garcia-Alaniz et al. 2010, Fickel et al. 2012),

assimilated diet (Felicetti et al. 2003a, Gray et al. 2008, Drucker et al. 2010) and toxins (Duffy et al. 2005, Christensen et al. 2007, Jaspers et al. 2010)]. As such, HCC analysis may also represent a practical tool with which to foster scientific collaboration, engage local communities, and enhance the dissemination of scientific knowledge along with its application towards conservation initiatives in wildlife at risk (Brook et al. 2009, Larter 2009, Aryal 2012).

CHAPTER 2[a]

HAIR CORTISOL CONCENTRATION AS A NON-INVASIVE BIOMARKER OF LONG-TERM STRESS IN FREE-RANGING GRIZZLY BEARS (*Ursus arctos*) FROM ALBERTA, CANADA: CONSIDERATIONS WITH IMPLICATIONS FOR OTHER WILDLIFE

This investigation was performed as the first of four studies included in this thesis. Hair collected from grizzly bears in Alberta, Canada was targeted for study owing to the "Threatened" conservation status of this species in Alberta and to the findings of ongoing research programs which have identified regional differences in the health status and population performance of grizzly bears across the species' provincial range. Genetic information obtained from remotely collected (barb-wire snagging) hair samples has also been used as the cornerstone of grizzly bear population surveys in Alberta and elsewhere. Together, these features suggested that hair cortisol analysis may be a particularly useful tool with which to assess long-term stress in this species. In this study, extensive laboratory validation was performed to determine if cortisol could be accurately and reliably measured in hair collected from free-ranging grizzly bears. The effects of a variety of procedural and biological factors which may influence hair cortisol concentration were evaluated. Through this work, important guidelines for the prudent use of hair cortisol analysis were developed for grizzly bears. The findings of this investigation subsequently guided efforts to modify, apply, and evaluate the utility of hair cortisol analysis in the other threatened species [polar bears (*Ursus maritimus*) and caribou *Rangifer tarandus* sp.] examined in this thesis (Chapters 3, 4, and 5). Importantly, this work also served as the foundation for ongoing collaborative studies in *U. arctos* from Alberta, Scandinavia, Nunavut, and Mongolia which are evaluating potential relationships between long-term stress, health, and anthropogenic landscape conditions in this species.

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2.1 Introduction

In many areas of the world human-caused landscape change is occurring at an alarming rate and negatively affects the sustainability of wildlife populations (Lindenmayer and Fisher 2006, Chown and Gaston 2008). While landscape change has been implicated in the decline of many species (e.g. van der Zee et al. 1992, Ecke et al. 2006, Wittmer et al. 2007), the underlying biological mechanisms responsible for these declines are poorly understood. Increasingly, the physiological response of wildlife to ecological perturbations is being recognized as a potential cause of poor population performance (Wikelski and Cooke 2006, Cattet et al. 2006, Chown and Gaston 2008). The consequences of chronic stress may be of particular importance as species inhabiting disturbed landscapes are forced to deal with stressors differing in type, intensity or duration from those typically encountered in pristine habitat (McEwen and Wingfield 2003, Reeder and Kramer 2005, Wikelski and Cooke 2006).

In vertebrates the stress response is an evolutionarily conserved process mediated by the hypothalamic-pituitary-adrenal (HPA) axis which allows organisms to respond rapidly to unpredictable changes in their environment (McEwen and Wingfield 2003, Reeder and Kramer 2005). It is initiated when higher brain centers perceive endogenous or exogenous noxious stimuli that threaten homeostasis and culminates in the release of glucocorticoids from the adrenal cortex (Habib et al. 2001). Elevated levels of circulating glucocorticoids exert a variety of catabolic, antireproductive, antigrowth and immunosuppressive effects which mobilize and repartition energy to help organisms restore homeostatic balance (Habib et al. 2001, Charmandari et al. 2005). While the physiological response to short-term stress is adaptive ("fight or flight"), long-term stress (occurring over weeks to months) may lead to a pathological syndrome of distress characterized by immunosuppression, decreased reproduction and diminished growth (Moberg 2000, Habib et al. 2001, Charmandari et al. 2005, Boonstra 2005). Accordingly, measures of population performance such as survival, reproductive output and abundance may be reduced as the proportion of distressed individuals in a population increases.

Cortisol, the primary glucocorticoid of most mammals, has been used to assess the stress response in the plasma, urine, feces and saliva of many species (e.g. Cattet et al. 2003a, Constable et al. 2005, Keay et al. 2006, Millspaugh et al. 2002). However, intrinsic diurnal and seasonal variations along with sampling protocols, life history stage, diet and environmental

exposure may influence glucocorticoids measured in these media (Ekkel et al. 1996, Beerda et al. 1996, Cattet et al. 2003a, von der Ohe et al. 2004, Owen et al. 2005, Constable et al. 2006, Keay et al. 2006, Mormède et al. 2007). Most importantly, these media reflect short-term stress occurring over hours to days and cannot assess HPA activity occurring over weeks to months without the repeated sampling of individuals (Owen et al. 2005, Keay et al. 2006).

In contrast, hair is a relatively stable medium known to incorporate blood-borne hormones and xenobiotics during its active growth phase lasting weeks to months (Pragst and Balikova 2006). It can be transported and stored at ambient temperature and under optimal conditions cortisol and other molecules incorporated into hair may remain detectable for years to centuries (Kintz 2004, Webb et al. 2010). The measurement of hair cortisol concentration (HCC) as an index of long-term stress has recently been explored in Procaviids (Koren et al. 2002, 2008), captive non-human primates (Davenport et al. 2006), domestic dogs and cats (Accorsi et al. 2008), humans (Raul et al. 2004, Kalra et al. 2007, Sauvé et al. 2007, Yamada et al. 2007; Van Uum et al. 2008, Kirschbaum et al. 2009, Thomson et al. 2010, Webb et al. 2010, Gow et al. 2010), and rodents (Martin and Réale 2008). Like drugs and other xenobiotics, cortisol is believed to enter the hair shaft in direct proportion to its free concentration in the blood, a process restricted to the phase of active hair growth in individual hair follicles (Pragst and Balikova 2006, Davenport et al. 2006). Accordingly, HCC determined in an intact hair shaft should represent an integrated measure of systemic HPA axis activity during the active growth phase of the hair being evaluated. Encouragingly, HCC has been linked with other measures of systemic HPA axis activity [e.g. salivary cortisol by Davenport et al. (2006), fecal glucocorticoids by Accorsi et al. (2008)] and elevated HCC has been identified in humans and animals chronically exposed to stressful stimuli (e.g. Davenport et al. 2006, Yamada et al. 2007, Van Uum et al. 2008, Kirschbaum et al. 2009). Importantly, protocols for the non-invasive collection of hair from free-ranging wildlife are well established [e.g. Woods et al. 1999 (Ursids), McDaniel et al. 2000 (Felids), Williams et al. 2009 (Mustelids), Belant et al. 2007 (Cervids)]. Hair may also be obtained opportunistically, from archives and museums or from wildlife killed as a result of human/animal conflict or hunting. All considered, hair may be the best available medium to study long-term stress in free-ranging wildlife.

Whereas limited work has been undertaken in humans (e.g. Raul et al. 2004, Sauvé et al. 2007), factors affecting the measurement and interpretation of HCC have not been thoroughly investigated in animals. In wildlife, the use of non-invasive or opportunistic collection techniques presents additional challenges as these methods may be subject to a suite of confounding factors associated with sampling protocols and the quantity or quality of sample available for analysis (e.g. von der Ohe and Servheen 2002, Touma and Palme 2005).

Grizzly bears (*Ursus arctos*) in Alberta, Canada occupy one of the most heavily populated and exploited landscapes across the species distributional range (Gibeau and Herrero 1998, AGBRP 2008). Increasingly, grizzly bear habitat is threatened by a rapidly expanding human population and escalating industrial, residential and recreational development (Stelfox et al. 2005). Grizzly bears are extremely vulnerable to anthropogenic disturbance and the ability of the species to persist in compromised ecosystems is uncertain (Mattson et al. 1996, Noss et al. 1996, Weaver et al. 1996, McLellan et al. 1999, AGBRP 2008). Recent population estimates suggest there may be as few as 691 grizzly bears in Alberta (Festa-Bianchet 2010) and the species has been designated as "Threatened" under Alberta's Wildlife Act (ASRD 2010b). Furthermore, some grizzly bear populations within the province are among the slowest reproducing documented for this species (Garshelis et al. 2005). The development of tools to enhance understanding of the effects of landscape change and to assess the impact of conservation actions on grizzly bear health and population performance is of the utmost importance.

2.2 Objectives

The purpose of this investigation was 1) to develop an accurate and reliable method to measure HCC in samples collected non-invasively (using barb wire snagging; Woods et al. 1999) or opportunistically from free-ranging grizzly bears and 2) to examine HCC in relation to procedural, structural and biological characteristics which may have bearing on its measurement or interpretation in this species. Cortisol concentration was determined in hair from 151 grizzly bears collected during ongoing research programs in Alberta. The effects of decontamination protocols, hair type, body region, distribution along the hair shaft, colour, sample quantity, weathering, laboratory storage, age, sex class and capture method were investigated. Hair cortisol concentration in individual grizzly bears captured multiple times during different seasons

or different years was also examined. Important considerations broadly applicable to the use of HCC as a monitoring tool for long-term stress in grizzly bears and other wildlife were developed.

2.3 Materials and Methods

2.3.1 Sample collection, storage, and handling

Hair was obtained from 146 grizzly bears captured as part of the Foothills Research Institute Grizzly Bear Program (FRIGBP) 1999-Present (research goals summarized in Stenhouse and Graham 2006) or the Eastern Slopes Grizzly Bear Project (ESGBP) 1994-2005 (research goals summarized in Herrero 2005), 4 grizzly bear hides and 1 barb wire snag. Hides were collected from free roaming bears killed as a result of human-animal conflict and were removed and frozen immediately following death. Research bears were captured via remote drug delivery (from helicopter [*n*=19], from ground [*n*=4], leg-hold snare [*n*=94] or culvert trap [*n*=29]) (see Stevens and Gibeau 2005 and Cattet et al. 2008a, respectively for detailed summaries of capture and handling protocols used in the ESGBP and FRIGBP). Hair was collected from the neck, shoulder, rump and abdomen of 15 individuals to facilitate a comparative analysis of HCC among different body regions. In most other samples the body region of origin was unknown.

Hair was collected at a single capture or sampling event in 133 bears, at two different captures or sampling events in 16 bears and at three different captures or sampling events in 2 bears for a total of 171 sampling events from 151 animals. In bears sampled multiple times, hair was obtained from 12 live captured animals, 2 dropped radio collars, 2 hides and from 2 carcasses. Carcasses were located by radio telemetry approximately 1 and 2 weeks after death, respectively. Of the 171 hair samples examined 131 were obtained in spring (defined as emergence from hibernation to June 20) while 25 were recovered in summer (June 21-September 20) and 15 were recovered in fall (September 21 or later). Hair was collected by shaving as close to the skin as possible with electric clippers or by manual plucking with haemostats. Samples were stored in dry paper envelopes at room temperature in the dark (Felicetti et al. 2003). Gross contaminants (mud, stones and vegetation) and hair shafts with visible damage were removed. Guard hair and undercoat were identified based on length, thickness and degree of curl (Elgmørk and Riiser 1991, E. Jones and D. Heard, personal communication) (Fig 2.1), isolated and

grouped according to a subjective classification scheme representing the range of colouration observed in all available samples (Table 2.1A, Fig 2.2).

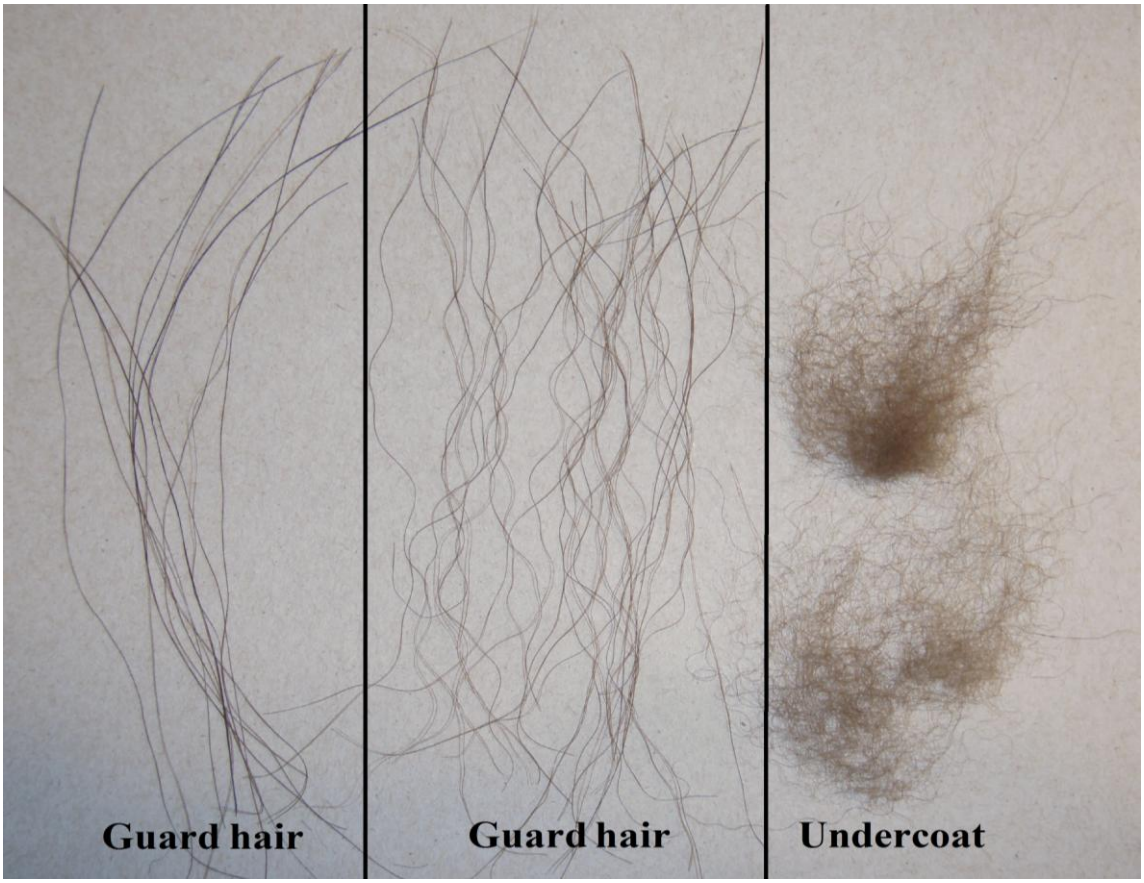

Figure 2.1 Classification of hair types for hair cortisol analysis in grizzly bears (*Ursus arctos*).

Table 2.1 Classification of grizzly bear (*Ursus arctos*) guard hair by (A) colour and (B) amount of biological surface contamination.

(A) Colour Classification	Description
Class 1	Uniformly white to light blond
Class 2	Dirty blond to light brown or red brown ± relatively light tips
Class 3	Moderately dark brown with relatively light tips
Class 4	Dark brown to black with relatively light tips
Class 5	Uniformly dark brown to black
(B) Contamination Category	**Description**
Category 1	< 25% of hair sample surface contaminated with biological (e.g. blood, lipid, urine, feces, or trap bait)
Category 2	25 to75% of hair sample surface covered with biologicals
Category 3	> 75% of the hair sample surface covered with biologicals

Figure 2.2 Examples of colour classification scheme (Table 2.1A) for grizzly bear (*Ursus arctos*) guard hair.

Hair cortisol concentration in guard hair was the focus of this investigation. HCC was only explored in undercoat to facilitate a comparative analysis among hair types. Hair from 15 grizzly bears was used to assess HCC along the length of the hair shaft. Hair for segmental

analysis was obtained from a 5 cm^2 patch of hair on the rump (n=12) or shoulder (n=3). The length of hair shafts within samples varied by up to 3 cm and among different individuals by up to 10 cm. As such, samples were divided into proximal, middle and distal segments by aligning all hairs from an individual bear against a straight edge and cutting each hair in the sample into equal thirds as measured with a ruler.

Multiple hair samples collected from the shoulder region of a single grizzly bear were exposed to ambient environmental conditions in Alberta grizzly bear habitat (latitude, longitude: 52°22′22" N, 119°45′31"W) for up to 18 days from May 7 to May 25, 2008. Sub samples weighing approximately 500 mg were flattened then placed within 10 cm by 10 cm screen pouches (Appendix 1, Fig A1.1) and exposed to the elements in two mock barb-wire snag sets following the methods of Woods et al. (1999). One mock snag was set in an open meadow and the second in a forested site relatively protected from the elements (Appendix 1, Fig A1.2). Weather during the trial ranged from -1ºC with rain and snow to +15°C with sun. A severe spring blizzard deposited > 50 cm of wet snow on May 7, 2008 and all open meadow site samples were snow covered for one week or more (Appendix 1, Fig A1.2).

2.3.2 Removal of surface contamination

Methods used in this investigation were developed upon those reported by Davenport et al. (2006). The surface of hair obtained from free-ranging animals may be covered with a variety of contaminants containing cortisol (e.g. blood, saliva, feces and urine). As such, the development of effective decontamination protocols is vital for the accurate measurement and interpretation of HCC (Davenport et al. 2006). In this study, many grizzly bear hair samples were covered with dirt and debris while mild to severe contamination with trap bait (beaver [*Castor canadensis* Kuhl, 1820] or moose [*Alces alces* L., 1758] carcasses), feces, urine, blood or lipid was observed in some. The amount of surface contamination could not be explicitly quantified in this investigation and a subjective classification scheme containing three categories based on the approximate amount of biological contamination on the hair surface was developed (Table 2.1B). Approximately 80% of samples fit into contamination Category 1, 18% into Category 2, and 2% into Category 3.

Methanol is commonly used in forensic decontamination protocols for hair and is known to be effective in removing neutral and lipophilic contaminants such as cortisol from the surface

of hair (Pragst and Balikova 2006, Kidwell and Smith 2007). A wash procedure using five short methanol washes was developed for use in this study. Hair was washed five times with 0.04 ml methanol/ mg hair for three minutes per wash on a slow rotator (Fisher Scientific Haematology and Chemistry Mixer 346, Fisher Scientific, Ottawa, Ontario, Canada). Methanol was changed and hair samples gently blotted between washes for maximum cleaning efficiency. Washed hair was placed in plastic dishes and air dried on a laboratory bench top for three days at room temperature.

To assess procedural efficacy wash kinetics were examined in contamination Category 1-3 guard hair collected from six grizzly bears. Two samples of each category were randomly selected from those available and exposed to an extended wash protocol (9 washes/0.04 ml methanol per mg hair/3-minutes per wash). The amount of cortisol in each wash was quantified and wash kinetics in each category were assessed. No decontamination techniques have gained widespread acceptance in the forensic drug field or the emerging field of hair cortisol analysis (Baumgartner and Hill 1992, Davenport et al. 2006, Kidwell and Smith 2007). In this investigation, external contamination was considered removed when wash cortisol concentration fell below the limit of detection of the assay (0.04 ng/ml) and visible contamination was no longer apparent (Kidwell and Smith 2007)**.** For endogenously produced substances such as cortisol, rigid cut-off values and other criteria used in the forensic drug field to distinguish external contamination from intrinsic concentration (e.g. Baumgartner and Hill 1992) are not applicable. As such, evidence of penetration of the hair shaft was deemed to have occurred if the cortisol concentration in successive washes rose above the reliable limit of detection after previously having been below it (Kidwell and Smith 2007)**.**

To confirm the wash protocol did not extract intrinsic cortisol from the hair shaft (Davenport et al. 2006), cortisol concentration was compared in Category 1 and Category 2 guard hair from 12 grizzly bears. Six samples each were randomly selected from those available and within categories paired samples from individual bears were washed five or nine times. Owing to their low prevalence and to findings of the wash kinetics investigation, Category 3 samples were not included in this experiment. Heavily contaminated undercoat was not explicitly examined in this investigation. However, the wash protocol was assessed in and applied to

Category 1 undercoat to facilitate a comparative analysis of HCC between the two hair types in 15 randomly selected bears.

2.3.3 Hair preparation

Washed and dried hair was ground to a fine powder with a Retsch MM 301 Mixer Mill (Retsch Inc, Newtown, Pennsylvania, USA). The quantity of hair available for analysis varied from 10 mg to greater than 1000 mg. Grinding time was determined based on the quantity of hair available and when possible, larger samples (100-200 mg) were ground preferentially to ensure the best representative sample was used and to guard against loss due to static.

Samples between 100 mg and 200 mg were ground for 0.03 minutes/mg hair at 30 Hz (10 ml stainless steel grinding jars with a single 12 mm stainless steel grinding ball), samples between 40 mg and 100 mg were ground for 0.15 minutes/mg hair at 30Hz (5 ml stainless steel grinding jar with a single 7 mm stainless steel grinding ball) and those < 40 mg were ground for 1.0 minute/mg hair at 30 Hz (1.5 ml stainless steel grinding jar with a single 5 mm stainless steel grinding ball). All grinding times resulted in a fine powder that was grossly similar across sample quantities of the same hair type. For the occasional hair sample that did not grind sufficiently, processing was continued at 30Hz in 30 s intervals until a fine powder was achieved. Whenever possible, extra ground hair was stored in 1.5 ml plastic vials at room temperature in the dark.

The quantity of hair collected using barb wire snagging methods for grizzly bears is commonly ≤ 30 mg (B. Macbeth, personal observation) and to account for loss during processing a target sample size of 25 mg powdered hair was set for subsequent steroid extraction and analysis in all bears. However, in 14 bears sample quantity was < 25 mg after grinding and HCC was determined in quantities ranging from 5- 25 mg.

2.3.4 Steroid extraction

Ground hair samples were immersed in 0.5 ml of high resolution gas chromatography grade methanol (MX 0480-1, EMD Chemicals, Gibbstown, New Jersey, USA), gently vortexed (10 s) and placed on a slowly spinning rotator to extract for 24 hours. Twenty-four hours was effective in extracting all measurable cortisol from the hair shaft of grizzly bear hair as no additional cortisol could be recovered when the same hair samples were immersed in fresh methanol for another 24 to 48 hours (n = 10 samples guard hair, n = 10 samples undercoat).

After 24 hours samples were centrifuged (15 minutes/4500 rpm/ 20 ˚C), the supernatant collected, and transferred to a glass 12 mm test tube. Preliminary investigation found that all measurable cortisol could be recovered if a hair sample was rinsed by adding 0.5 ml of fresh methanol, gently vortexing (40 s), centrifuging (4500 rpm/15 minutes/ 20 ˚C) and collecting a second supernatant (Table 2.2). However, to ensure all extracted steroids were recovered the rinse-recovery protocol was repeated twice for a total of three collections. Pooled supernatant was dried at 38 ˚C under a gentle stream of nitrogen gas. Steroids were concentrated at the bottom of the test tube by rinsing the sides with five consecutive methanol washes (0.4 ml, 0.3 ml, 0.2 ml, 0.18 ml and 0.15 ml) then reconstituted with 0.2 ml of phosphate buffer (12 hours/4 ˚C) in the dark. Reconstituted samples were gently vortexed (40 s), collected and transferred to 1.5 ml plastic vials. Samples were centrifuged (4500 rpm/5 minutes /20 ˚C) to remove any remaining particulates and duplicate 50 µl aliquots of extract were analyzed with a commercially available enzyme linked immunoassay kit (Oxford EA-65 Cortisol EIA kit, Oxford Biomedical, Lansing, Michigan, USA). This EIA kit was chosen based on an established record of performance for cortisol analysis in our laboratory. 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 ≤0.06%.

Table 2.2 Cortisol recovered from four successive rinses (0.5 ml methanol per rinse) of grizzly bear (*Ursus arctos*) hair previously extracted for 24 hours with 0.5 ml methanol per 25 mg powdered hair. Data for each rinse is presented as the percentage of the total cortisol recovered from all rinses.

Hair Type	Rinse 1	Rinse 2	Rinse 3	Rinse 4
Guard hair 1	80.4	19.6	-	-
Guard hair 2	81.5	18.5	-	-
Guard hair 3	78.5	21.5	-	-
Undercoat 1	72.9	27.1	-	-
Undercoat 2	78.7	21.3	-	-
Undercoat 3	78.2	21.8	-	-

2.3.5 Data analysis

Data were analyzed using SPSS 17.0 (SPSS Statistics Version 17.0.0, August 23, 2008) with the level of significance set at $P \leq 0.05$. Where data failed tests of normality, values were log transformed for subsequent analysis. Simple linear regression was used to assess parallelism between cortisol standards provided in the EIA kit and serially diluted hair extracts run in the same assay. Cortisol distribution along the length of the hair shaft, among different body regions and sample quantity were assessed using one-way repeated measures analysis of variance (ANOVA). The effects of laboratory storage and hair type were examined using paired *t*-tests while sex class was assessed using an independent samples *t*-test. The effects of capture method and hair colour were analyzed with one-way ANOVA while Pearson's product moment correlation was used to explore the association of HCC and age. For bears captured more than once, only samples from the earliest capture at which hair was collected were used in comparative HCC analyses. When hair from more than one body region was available from an individual animal the average HCC value obtained from all regions was used in analyses. Owing to an apparent animal capture method effect, data from 29 bears captured by culvert trap were excluded from analysis of HCC among sex classes, age groups and hair colour. Contamination Category 3 hair samples were not used in comparative analyses.

2.4 Results

2.4.1 Hair processing and storage

2.4.1.1 Assay performance

Intra-assay coefficient of variation (C.V.) was 3.58% ($n = 6$) while inter-assay C.V. was 5.75%. ($n = 6$) The approximate limit of detection of the EIA kit (0.04 ng/ml) was determined by calculating the (mean + 2 S.D.) absorbance of the zero standard provided with the EIA kit based on 12 runs which corresponds to approximately 0.32 pg cortisol per mg hair in a processed 25 mg hair sample. Results from serially-diluted grizzly bear hair extract were parallel ($r^2 = 0.997$, $P \leq 0.001$) with results from serially-diluted cortisol standards provided with the EIA kit. Extraction efficiency was 94.16 ± 5.12% based on four 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). In addition, no additional cortisol could be measured when a previously extracted hair sample was re-extracted with fresh methanol for an

additional 24 or 48 hours (i.e. 48 hrs or 72 hours total extraction time, n = 10 guard hair, n = 10 undercoat). Likewise, no further cortisol could be recovered when an additional 0.2 ml of reconstitution buffer was added to leftover residue in the glass test tube (i.e. after 12 hours reconstitution) and reconstituted for a further 12 hours (n = 10 guard hair, n = 10 undercoat).

2.4.1.2 Decontamination protocols

Five, 3-minute washes with 0.04 ml methanol per mg hair were effective in removing all visible contamination (i.e. visible dirt, debris or biologicals) from all categories of grizzly bear guard hair. Five washes also removed all measurable cortisol from the surface of Category 1 and 2 hair but did not remove all measurable cortisol from the surface of Category 3 hair (Fig 2.3). Regardless of degree of contamination, a single wash was effective at removing most (> 80%) of the measurable cortisol associated with the hair surface (Fig 2.3). An additional 3 to 4 washes were required to remove hair surface cortisol from Category 2 hair (Fig 2.3). Following five washes, cortisol was not detected in additional washes of Category 1 and Category 2 hair while hair surface cortisol was measurable in all nine washes in Category 3 hair (Fig 2.3). Further, a comparison of hair shaft cortisol concentration in Category 1 and Category 2 hair washed five times or nine times showed no difference (Paired samples t-test, $t_{(11)} = 0.451$, $P = 0.661$, $n = 12$). Five, 3-minute washes with 0.04 ml methanol per mg hair also removed all visible contamination and measurable cortisol from the surface of Contamination Category 1 grizzly bear undercoat (Table 2.3). As in guard hair, a single wash was effective at removing most of the measurable cortisol associated with the hair surface (Table 2.3). Likewise, a comparison of hair shaft cortisol concentration in Category 1 undercoat washed five times or nine times showed no difference (Paired samples t-test, $t_{(11)} = 0.685$, $P = 0.507$, $n = 12$).

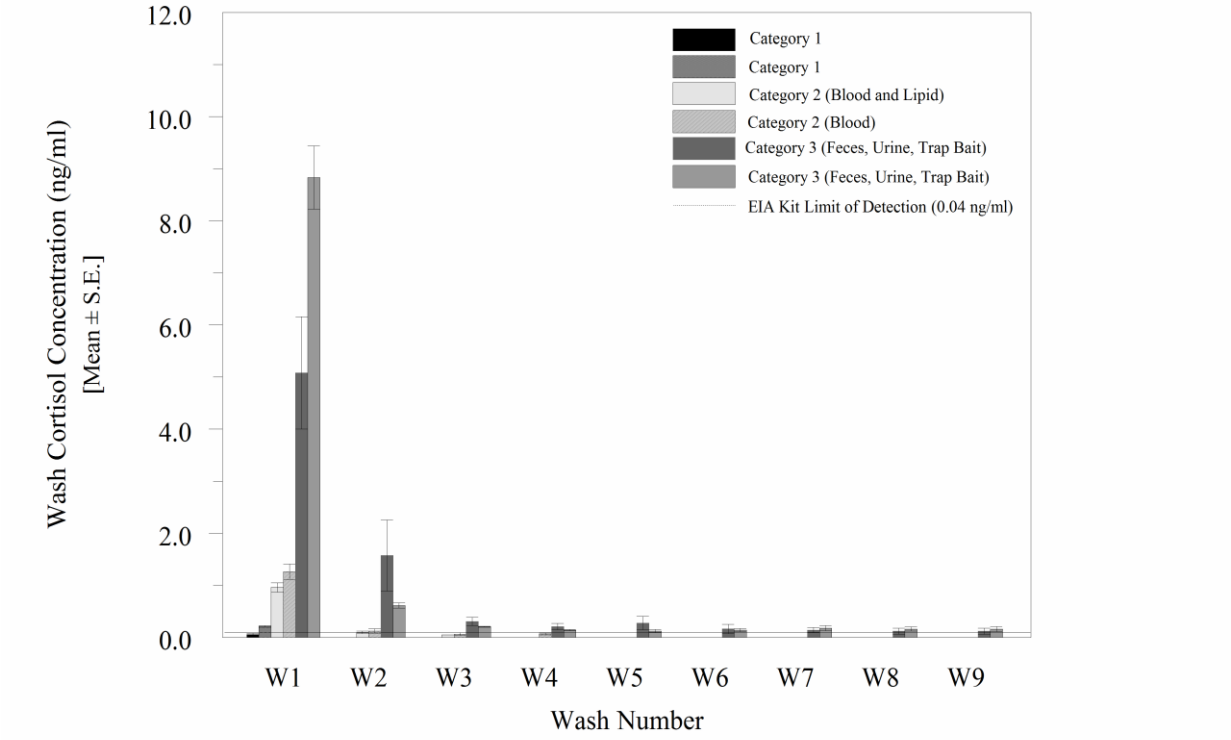

Figure 2.3 Dynamics of a wash procedure in contamination Category 1-3 grizzly bear (*Ursus arctos*) guard hair exposed to nine 3-minute washes with 0.04 ml methanol per mg hair. Each wash specific cortisol concentration is the mean ± S.E. of 3 independent determinations in each category.

Table 2.3 Wash cortisol concentration determined in Contamination Category 1 undercoat collected from $n = 3$ grizzly bears (*Ursus arctos*) and exposed to nine 3-minute washes with 0.04 ml methanol per mg hair. Wash specific cortisol concentration is presented as the mean ± S.E. of $n = 3$ independent determinations in each animal.

Grizzly Bear	Sample Quality	Wash Cortisol (ng/ml)								
		W1	W2	W3	W4	W5*	W6	W7	W8	W9
1	blood feces	0.324 ±0.048	0.079 ±0.007	0.061 ±0.004	0.057 ±0.004	-	-	-	-	-
2	feces	-	-	-	-	-	-	-	-	-
3	no visible biologicals	-	-	-	-	-	-	-	-	-

* As for grizzly bear guard hair, 5 washes were required to remove visible contamination (i.e. dirt and biologicals) from the hair surface of most undercoat samples.

2.4.1.3 Minimum sample quantity required and laboratory storage

Hair cortisol concentration was similar in subsamples of ground hair weighing 25 mg, 15 mg and 5 mg (one-way repeated measures ANOVA, $F_{(2, 14)} = 1.162$, $P = 0.328$, $n = 15$). Hair cortisol concentration in grizzly bear hair stored in powdered form in plastic vials at room temperature in the dark decreased after 9 months storage (Paired samples t-test, $t_{(14)} = 6.513$, $P \leq 0.001$, $n = 15$). In contrast, HCC in hair stored intact in dry paper envelopes under similar conditions for 8 to 17 months (mean storage time 12.5 months) remained similar (Paired samples t-test, $t_{(14)} = 1.245$, $P = 0.234$, $n = 15$).

2.4.1.4 Environmental exposure

HCC measured in hair exposed to ambient environmental conditions for ≤ 18 days was within 95% confidence intervals (1.62-2.43 pg/mg, n=15) calculated for subsamples of hair collected from the same bear (and body region), but stored instead in dry paper envelopes in the laboratory (Fig 2.4).

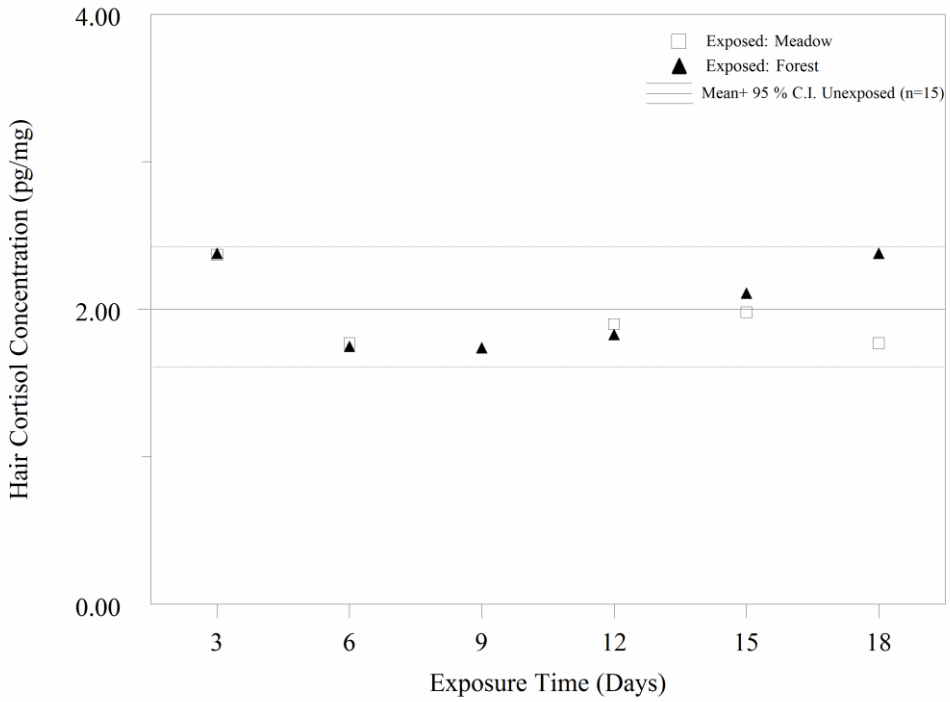

Figure 2.4 Comparison of hair cortisol concentration between subsamples of grizzly bear (*Ursus arctos*) guard hair stored under laboratory conditions and hair exposed to weathering for ≤ 18 days in mock barb wire snag set-ups located in (□) open meadow and (▲) forest. All subsamples were collected from the same body region of a single bear.

2.4.2 Features of hair

2.4.2.1 Hair type and body regions

Hair cortisol concentration was compared in Category 1 guard hair and Category 1 undercoat collected from 15 grizzly bears randomly selected from available samples containing both hair types. Guard hair contained significantly more cortisol than undercoat (mean ± S.D. $_{\text{guard hair:}}$ 2.78 ± 1.51 pg/mg vs. mean ± S.D. $_{\text{undercoat}}$: 2.11 ± 1.58 pg/mg; paired samples t-test, $t_{(14)} = 4.254$, $P \leq 0.001$). Hair cortisol concentration varied significantly among guard hair samples collected from different body regions of 15 grizzly bears (one-way repeated measures ANOVA, $F_{(3,14)} = 6.626$, $P \leq 0.001$) with highest values in the neck (Tukey-Kramer, $P \leq 0.05$) and lower values in the shoulder, rump and abdomen (Fig 2.5). A similar regional pattern was also detected

in undercoat (one-way repeated measures ANOVA, $F_{(3, 7)} = 7.636$, $P \leq 0.001$). With both hair types, mean variation in HCC between body regions was greater than mean variation in HCC measured within body regions (mean C.V. ± S.D. $_{\text{guard hair}}$: 33.6 ± 13.8%, $n = 15$ vs. 11.7 ± 6.3%, $n = 14$; mean C.V. ± S.D. $_{\text{undercoat}}$: 63.3 ± 22.3%, $n = 8$ vs. 17.2 ± 5.2%, $n = 7$).

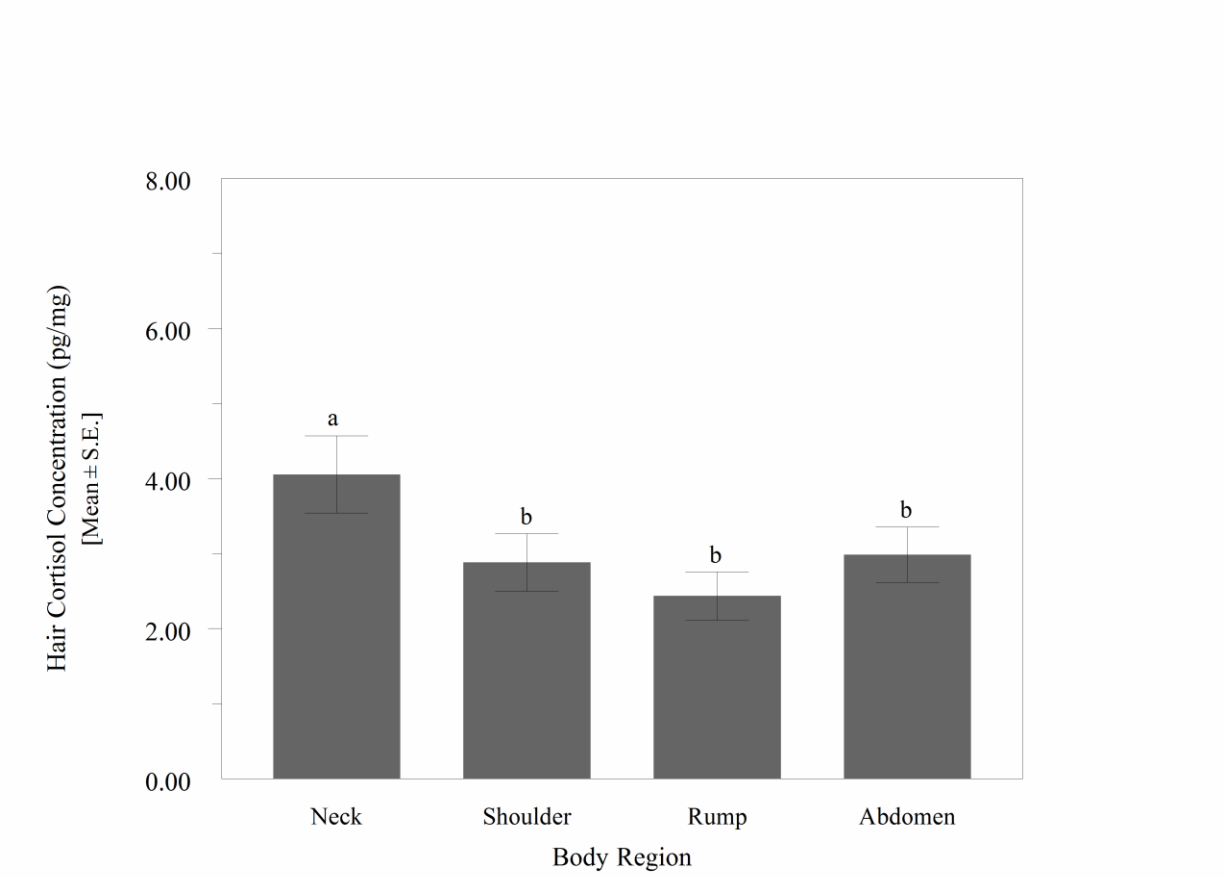

Figure 2.5 Comparison of hair cortisol concentration between neck, shoulder, rump and abdomen in the guard hair of $n = 15$ grizzly bears (*Ursus arctos*). Mean values are represented by bars and S.E. represented by error bars. Significant differences (Tukey-Kramer, $P \leq 0.05$) in hair cortisol concentration between body regions are indicated by different letters.

2.4.2.2 Hair colour and cortisol distribution along the length of the hair shaft

No differences in HCC were detected between proximal, middle and distal segments of the hair shaft in samples collected from 15 grizzly bears (one-way repeated measures ANOVA, $F_{(2,14)} = 1.129$, $P = 0.338$). Variation in HCC in 120 individual grizzly was not explained by differences in hair colour of bears (one-way ANOVA, $F_{(4,115)} = 1.104$, $P = 0.358$) (Hair colour

categories: Class 1 n = 15, Class 2 n = 27, Class 3 n = 53, Class 4 n = 16, Class 5 n = 9). We did, however, find in several bears differences in HCC between light and dark hair samples, collected from the same animal (and body region), that suggest HCC tends to be higher in dark than in light hair.

2.4.3 Features of grizzly bears

2.4.3.1 Range of hair cortisol values determined in Alberta grizzly bears

The median HCC recorded in 151 grizzly bears was 2.84 pg/mg (range 0.62-43.33 pg/mg).

2.4.3.2 Age and sex

There was no association between age (mean 7 years, range 1- 22 years, n = 111) and HCC (Pearson product moment correlation r = 0.023, P = 0.814). There was no difference in HCC between male (median 2.62 pg/mg, range 0.77-32.39 pg/mg, n = 63) and female (median 2.74 pg/mg, range 0.62-27.9 pg/mg, n = 58) grizzly bears (Independent samples t-test, $t_{(119)}$ = 0.918, P = 0.360).

2.4.3.3 Capture method

Hair cortisol concentration was highest in grizzly bears captured in culvert traps but similar between bears captured either by leg-hold snare or by remote drug delivery from helicopter (helicopter darting) or ground (free-roaming) (one-way ANOVA, $F_{(3,147)}$ = 6.730, $P \leq$ 0.001, Tukey-Kramer: $P \leq 0.05$) (n = 29 culvert trap, n = 94 leg-hold snare, n = 19 helicopter darting and n = 9 free-roaming) (Fig 2.6).

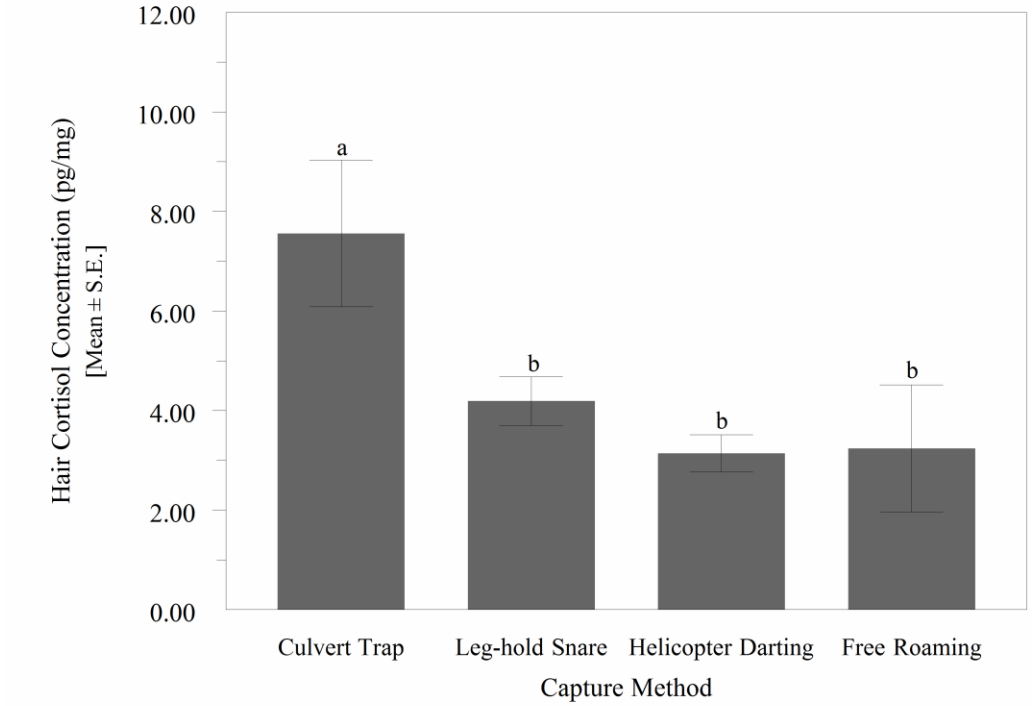

Figure 2.6 Comparison of hair cortisol concentration in guard hair from grizzly bears (*Ursus arctos*) either captured by different methods (culvert traps [*n* = 29], leg-hold snares [*n* = 94], remote drug delivery from helicopter [*n* = 19]) or sampled in other ways (free-roaming includes barb wire hair snag [*n* = 1], hunting [*n* = 4], and remote drug delivery from ground [*n* = 4]). Mean values are represented by bars and S.E. represented by error bars. Significant differences (Tukey-Kramer, $P \leq 0.05$) in hair cortisol concentration by capture and sampling methods are indicated by different letters.

2.4.3.4 Variation in different years and different seasons

Hair cortisol concentration measured in individual animals captured multiple times, sometimes in different seasons or in different years, was highly variable (Fig 2.7). In some bears HCC was similar in different years (e.g. Bears 1, 2, 3 and 6) or in different seasons of the same year (e.g. Bears 4 and 5 [spring 2008 and fall 2008]). In other bears HCC was highly variable across different years (e.g. Bears 15, 16, 17 and 18).

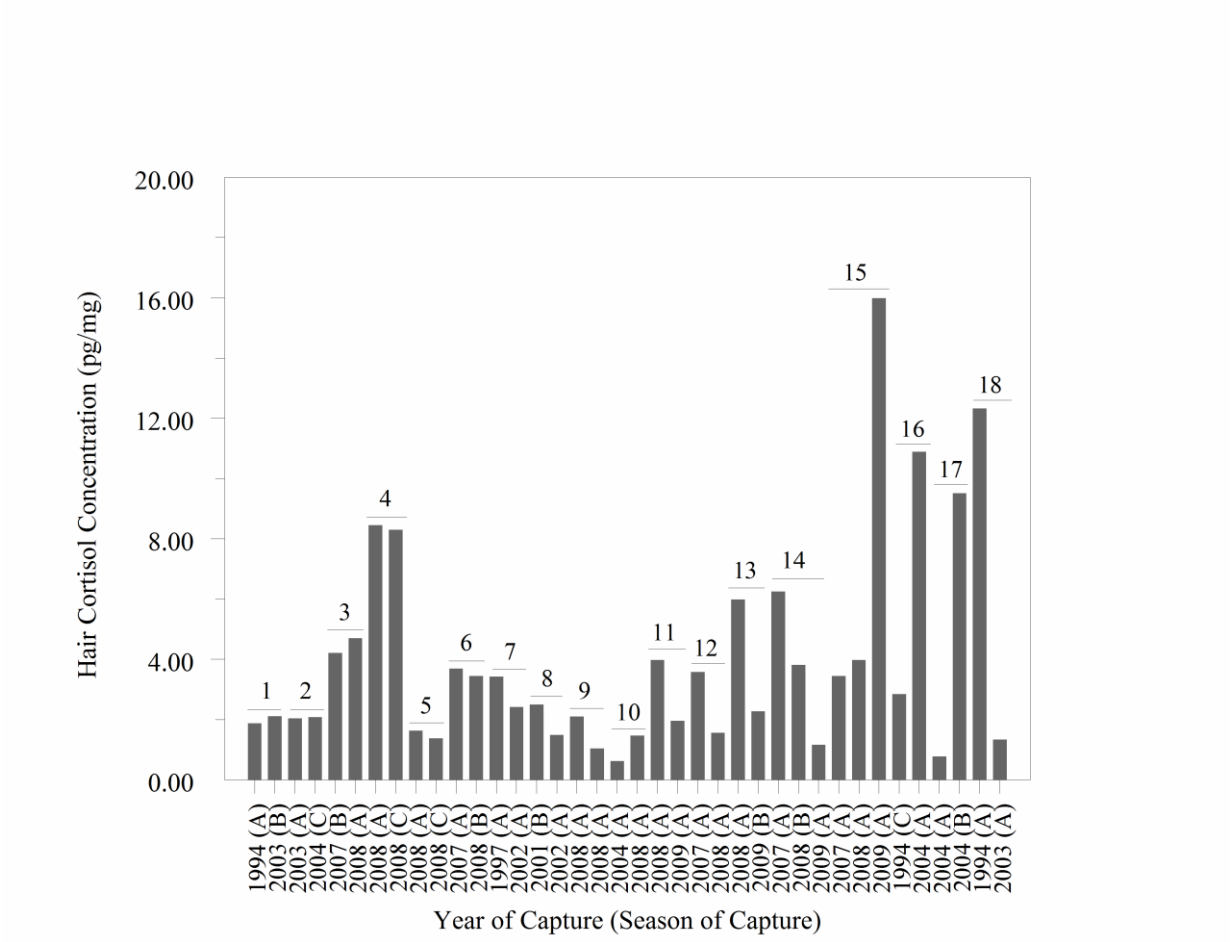

Figure 2.7. Hair cortisol concentration measured in *n* = 18 grizzly bears (*Ursus arctos*) captured multiple times in different years or in different seasons ([A] spring, [B] summer and [C] fall). Underscored numbers represent hair cortisol data from individual grizzly bears.

2.5 Discussion

To our knowledge, this is the first investigation of HCC in a free-ranging large mammal and the first study to explore in detail factors which may influence HCC in animals. In the grizzly bear, HCC can be measured precisely and accurately in as little as 5 mg of powdered guard hair. This amount represents approximately 5-10 unprocessed guard hairs which can be readily obtained through established non-invasive or opportunistic sampling techniques in ursids (e.g. Woods et al. 1999). These findings are important as the development and application of non-invasive techniques should be encouraged where possible as negative consequences associated with the capture and handling of wildlife are well recognized (e.g. Cattet et al. 2003, 2008a, 2008b, Arnemo et al. 2006).

Highly significant parallelism with cortisol standards and the low limit of detection suggest that this technique is also highly specific for cortisol and sensitive across the range of concentrations determined in Alberta grizzly bear hair to date (median 2.84 pg/mg range 0.62-43.33 pg/mg, *n* =151). The median HCC determined in Alberta grizzly bears is similar to mean values reported in domestic carnivores (dogs 2.10 ± 0.22 pg/mg [*n* = 29] and cats 3.32 ± 0.27 pg/mg [*n* = 27], Accorsi et al. 2008). The observed range indicates an approximately 100-fold variation in HCC among individual grizzly bears; nearly 10 times greater than reported in humans (e.g. Raul et al. 2004, Sauvé et al. 2007, Van Uum et al. 2008) or non-human primates (Davenport et al. 2006).

To date, factors influencing HCC have not been thoroughly investigated in animals. Sampling and processing protocols along with the intrinsic characteristics of hair are known to influence the measurement and interpretation of drug, xenobiotic or hormone concentrations in this medium (Cone and Joseph 1996, Wennig 2000, Kidwell and Smith 2007, Davenport et al. 2006, Sauvé et al. 2007). Potential confounding factors may be of particular importance when cortisol, an endogenously produced hormone, is being measured and where hair is obtained non-invasively or opportunistically from free-ranging wildlife.

Only limited attention has been given to external contaminants and decontamination protocols in the emerging field of HCC analysis (e.g. Davenport et al. 2006). Special consideration should be directed to these topics in wildlife whose hair may be exposed to cortisol-containing blood, urine, feces or saliva prior to collection (e.g. Gosling and McKay 1990, Whittle et al. 2000, Dawson et al. 2000). In the grizzly bear, contamination of hair with blood, feces and urine are of particular concern. Blood contamination may occur when hair samples are collected opportunistically from carcasses. It may also compromise samples from animals having recently fed/scavenged or hair covered in attractants associated with barb wire snagging (blood lures) or live capture (animal carcass baits). Urinary and fecal contamination may be significant in some grizzly bears captured and held in culvert traps (B. Macbeth, personal observation).

Five-3-minute washes with 0.04 ml methanol per mg hair removed all visible contamination (i.e. dirt, debris and biologicals) from the surface of all contamination categories (Table 2.1B) of grizzly bear guard hair. However, this protocol was not effective in removing all

measurable surface contamination from Category 3 samples even though they appeared visibly clean after five washes (Fig 2.3). We observed no difference in hair shaft cortisol concentration measured in samples of Category 1 and 2 guard hair washed five or nine times with methanol suggesting that cortisol within the hair shaft is not lost when guard hair from these categories is washed up to nine times with methanol. Together, we interpret these results as evidence a liberal wash protocol using five-3-minute washes with 0.04 ml methanol/mg hair is effective in removing visible contamination and measurable cortisol from the surface of Category 1 and 2 grizzly bear guard hair. Although this wash protocol may be safely extended to nine washes without removing intrinsic cortisol from the hair shaft, it may not be effective in removing surface contamination from samples severely compromised by biological materials (Category 3). Accordingly, HCC analysis in the grizzly bear should be restricted to samples from contamination Categories 1 and 2.

Our findings disagree with those reported by Davenport et al. (2006) where a wash protocol using three-3-minute methanol washes was found to remove cortisol from the hair shaft of non-human primates while a similar protocol using isopropanol was interpreted as safe and effective. These discrepancies suggest that requirements for an effective and safe wash protocol may be species-specific. This may relate to sample quality (i.e. probable type and amount of contamination or hair shaft damage) or to the inherent resilience of hair among different species or different hair types within species (Harkey 1993, Cone and Joseph 1996, Kidwell and Smith 2007). Results reported here and in Davenport et al. (2006) suggest that multiple short washes with methanol or isopropanol are effective in removing visible contamination and measurable cortisol from animal hair and may provide a starting point for work in other species.

In any species, the effects of hair type, body region and hair colour on HCC may be of consequence in the small quantity of hair typically obtained with non-invasive or opportunistic collection techniques (e.g. Woods et al. 1999, Bradley et al. 2008). Different types of hair have distinct chemical and physical properties which may influence the uptake and retention of xenobiotics (e.g. Elgmørk and Riiser 1991, Cone and Joseph 1996, Kidwell and Blank 1996, Jones et al. 2006). Furthermore, hair types may also have disparate periods of active growth which translates into type-specific time frames in which substances in the systemic circulation

may be incorporated into the hair shaft (Al Bagdadi et al. 1979, Maurel et al. 1986, Felicetti et al. 2003a, Jones et al. 2006).

In the grizzly bear both guard hair and undercoat can be collected in sufficient quantities for HCC analysis using non-invasive (barb wire snagging) or opportunistic sampling (carcasses or rub trees) (B. Macbeth, personal observation). However, we identified that guard hair contains more cortisol than undercoat in this species indicating that comparative analysis should be restricted to one hair type. We suggest that for grizzly bears, the use of guard hair is preferable to undercoat for three reasons. Foremost is the distinct probability that guard hair represents a superior measure of long-term stress than undercoat in this species. In the grizzly bear, guard hair is longer than undercoat (Elgmørk and Riiser 1991) and longer hair shafts are known to originate from hair follicles with relatively long periods of active growth (Harkey 1993). Consequently, in an individual, cortisol should be incorporated into guard hair over a longer period of time relative to undercoat (Harkey 1993, Pragst and Balikova 2006). This pattern of hair growth is supported by observations of live captured grizzly bears, carcasses and hair samples obtained at different times of the year in Alberta which suggest that the growth of undercoat occurs primarily in late summer and fall (B. Goski, personal communication). This assertion is also supported by observations in captive grizzly bears which have demonstrated that undercoat growth begins later in the season than guard hair in shaved patches of hair (C. Robbins, personal communication). Secondly, HCC was the least variable in guard hair across different body regions and within body regions. Given that the body region of origin is often unknown when hair is collected non-invasively, this strengthens our contention that guard hair represents a superior medium to assess HCC in this species. Finally, during the course of technique development, we found that the quality of guard hair samples was easier to gauge and control (e.g. visible hair shaft damage). Guard hair was also more reliably cleansed and ground to a uniform powder than undercoat. These recommendations do not preclude the use of undercoat cortisol concentration as a measure of long-term stress in the grizzly bear as long as its limitations are considered in study design. In fact, if the goal of HCC analysis is to identify seasonal patterns of stress, analyzing both hair types in conjunction may be useful.

We found that inter-body region variability in HCC was greater than intra-body region variability in both hair types. Our findings are somewhat similar to those reported in humans.

Sauvé et al. (2007) identified large variation (C.V. 30.5 %) in HCC determined in five sections of the human scalp, while variation in a single region of the scalp was less (C.V. 15.6%). In the grizzly bear, the most plausible explanation for the variation observed in this study is differences in the timing and pattern of hair growth in individuals (Al Bagdadi et al. 1979, Maurel et al. 1986, Diaz et al. 2004a). Grizzly bears replace their hair coat once annually and moult is believed to begin soon after emergence from dens in spring (Pearson 1975, Schwartz et al. 2003, Felicetti et al. 2003, Jones et al. 2006). Factors influencing moulting patterns vary considerably. Latitude, sex, age and reproductive class are known to influence the onset and duration of moult (Pearson 1975, Schwartz et al. 2003). Observations in captive grizzly bears suggest that moult may also be strongly influenced by food availability (C. Robbins, personal communication). Importantly, patterns of moult among body regions may be highly variable among different bears inhabiting the same general geographic area (Pearson 1975, B. Goski, personal communication). The pattern of moulting within body regions may also be irregular (B. Macbeth, personal observation). Hair cortisol concentration recorded in different body regions (and each shaft within that region) should reflect an average measure of HPA activity in an individual during that period of active hair growth. When the variety of factors affecting the onset and duration of moult are taken into consideration, variation in the pattern of HCC among different body regions in individual animals is not surprising.

The elevated HCC recorded in neck hair and the similar patterns of HCC among body regions in both hair types suggest that the pattern of moult in grizzly bears is not as random as observational studies indicate. This is likely given the fact that new hair growth is initiated well in advance of obvious moult and the precise timing of active hair growth cannot be ascertained through observation alone (Maurel et al. 1986, Diaz et al. 2004). Where moulting patterns have been thoroughly investigated in other species they have been found to be remarkably predictable (Maurel et al. 1986). Elevated serum cortisol has been recorded in ursids in the spring and in years of poor food availability (Harlow et al. 1990, Hellgren et al. 1993). Elevated neck HCC may be the result of such elevations overlapping with periods of neck hair growth in grizzly bears. Alternately, some structural feature of neck hair (Elgmørk and Riiser 1991) or functional characteristics of the neck's integument (e.g. increased glandular secretions containing cortisol) may be important (Pragst and Balikova 2006). Currently, the seasonal hair cycle, rates of hair

growth and histological characteristics of the grizzly bear integument are poorly understood. Studies examining these factors in captive and free-ranging animals using simple, portable and relatively non-invasive dermatological techniques (e.g. trichograms; Diaz et al. 2004a and growth stage staining; Baden et al. 1979) would be useful in this regard.

Presently, we suggest that sampling be standardized to a single body region for HCC analysis in grizzly bears. Cortisol is elevated in neck hair relative to the shoulder, rump or abdomen (Fig 2.5), and we recommend that neck hair should not be not routinely collected for HCC analysis in this species. In bears captured by leg-hold snare or by culvert trap hair from the rump and abdomen may be contaminated with significant quantities of dirt or biologicals making decontamination and sample processing more labour intensive (B. Macbeth, personal observation). We currently recommend collecting hair from the top of the shoulder (hump) for HCC analysis in grizzly bears. This anatomic location is easily identified and may remain relatively clean in snared or culvert trapped bears (B. Macbeth, personal observation). In addition, cortisol measured in this body region is similar to that from the rump and abdomen (Fig. 2.5) which may be considered as alternate sampling sites should shoulder hair be unavailable or of poor quality.

In the forensic drug field, hair colour has been identified as an important factor which influences the incorporation and retention of drugs and other xenobiotics in hair (Kidwell and Smith 2007). Dark hair has been found to integrate and retain significantly more drug than light hair in individuals and among different individuals (e.g. Rothe et al. 1997, Kidwell and Smith 2007). 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 (Rollins et al. 2003). In the grizzly bear, hair colour is influenced by a combination of genetics and weathering (bleaching) (Pearson 1975, Schwartz et al. 2003). Pelage varies from nearly white (light blond) to black and often varies in colour along the length of the hair shaft (Pearson 1975, Harting 1987, Schwartz et al. 2003). Predominant colour patterns may vary with age, geographic location, sex and season, making hair colour a potentially important confounding factor in HCC analysis (Pearson 1975, Harting 1987, Schwartz et al. 2003).

When hair colour and HCC were compared in guard hair from 120 individual grizzly bears, no significant difference in HCC could be identified among five different colour

categories ranging from nearly white to black. These findings agree with studies in humans which report no difference in HCC among blond, brown or black hair (Sauvé et al. 2007, Raul et al. 2004) and suggest that hair colour may be of limited importance when comparing HCC among different grizzly bears. However, in several bears differences in HCC were observed between light and dark hair samples collected from the same animal (and body region), that suggest HCC tends to be higher in dark than in light hair. Variation in HCC among different colours of hair could be an important confounding factor in longitudinal studies of HCC in individual animals. Currently, this relationship is poorly understood and we are opportunistically pursuing the effects of hair colour on HCC in individual grizzly bears captured as part of ongoing research in Alberta. Importantly, hair from the legs of grizzly bears is most often uniformly dark; a finding consistent across age-sex classes, geographic regions and seasons (Pearson 1975, Harting 1987, Schwartz et al. 2003, B. Goski, personal communication). Should ongoing work identify hair colour as an important source of variation in HCC within individual grizzly bears, leg hair may provide an optimal site for hair sampling in future studies. Commonly used leg-hold snare sets could also easily be modified to facilitate non-invasive hair sampling from limbs.

In this study, no difference in HCC was identified among proximal, middle and distal segments of grizzly bear hair. These findings agree with those of Davenport et al. (2006) who found no difference in HCC measured in proximal and distal segments of hair from non-human primates. In contrast, studies in humans have identified elevated HCC in segments of hair corresponding to the third trimester of pregnancy (Kirschbaum et al. 2009) and measured segmental variation in HCC over the clinical course of Cushing's syndrome (Thomson et al. 2010). The lack of segmental differences observed in this study could be due to the magnitude of these differences being too small to identify or may suggest that the scale of analysis was not fine enough. Variation in the length of hair obtained from different individuals or in the length of hair shafts within samples could also have concealed segmental differences in HCC. These findings emphasize the importance of using intact hair samples to obtain the best possible estimate of annual HPA activity in the grizzly bear. While samples collected non-invasively are generally intact, plucked hair samples should be collected preferentially in live-captured bears to ensure the entire length of the hair shaft is available. If shaved, hair should be cut as close to the

skin and as evenly as possible. Further work examining the incorporation and distribution of cortisol along the length of the hair shaft in grizzly bear hair is warranted. In this regard, controlled studies in captive animals [e.g. adrenocorticotropic hormone (ACTH) stimulation tests] during periods of active hair growth would be beneficial.

The highly significant elevation in HCC in culvert trapped bears observed in this investigation was unexpected. Three possible explanations for these findings should be considered: 1) a direct effect of short-term stress (capture), 2) an indirect effect of acute stress secondary to contamination of hair with feces and urine in some animals, and 3) the presence of landscape features (e.g. road density, human activity) in the home ranges of bears captured in culvert traps which may act as sources of long-term stress.

Short-term stress is unlikely as the direct cause of elevated HCC in culvert trapped bears. Most hair samples used in this investigation were collected in the spring prior to the onset of moult. As such, samples represented non-growing hair from the previous summer (Felicetti et al. 2003; 2004); hair incapable of incorporating cortisol directly from the systemic circulation (Pragst and Balikova 2006). Furthermore, the pattern of HCC and serum cortisol concentration among different capture methods is not what would be expected if short-term stress directly influenced HCC. Serum cortisol concentration (a measure of short-term stress) in bears captured by leg-hold snare (85.30 ± 68.50 nmol/L, $n = 49$) was higher than that in bears captured by culvert traps (55.45 ± 57.81 nmol/L, $n = 8$) or by remote drug delivery (42.90 ± 36.78 nmol/L, $n = 11$) (FRIGBP ongoing work). This difference among capture methods was statistically significant (one-way ANOVA, $F_{(3.74)} = 3.757$, $P = 0.028$) with bears captured by leg-hold snare having significantly higher serum cortisol (Tukey-Kramer, $P \leq 0.05$) than those captured by helicopter but not different than those captured in culvert traps (Tukey-Kramer, $P > 0.05$). If short-term stress was the direct cause of elevated HCC in culvert trapped bears, a similar elevation in HCC in snared bears would be expected. This was not observed, instead snared bears had significantly lower HCC than culvert trapped bears and there was no difference in HCC among bears captured by snare, helicopter darting or free roaming.

An alternate hypothesis is that elevations in fecal and or urinary cortisol (due to the stress of capture) may influence HCC when grizzly bear hair is wet or damaged and bears are held in traps for extended periods (≥ 12 hours). ACTH stimulation tests performed by Hunt and Wasser

(2003) in two captive grizzly bears suggest that the effects of an acute stressor may lead to significant elevations in fecal glucocorticoids within 15 hours. Grizzly bears captured in culvert traps may be held up to 24 hours prior to processing and on removal some bears may be contaminated with a significant amount of feces or urine over all areas of the body (B. Macbeth, personal observation; Appendix 2, Fig A2.1, Fig A2.2). It is plausible that the hair of bears captured and held in culvert traps could be contaminated with feces or urine containing elevated cortisol related to the capture event. The severity, body regions affected and ultimate importance of this contamination may be a function of: 1) the magnitude of the individual stress response, 2) the amount of waste deposited, 3) how long the bear is held, 4) how it behaves while trapped (e.g. struggling to escape or sitting still) and critically 5) the amount of moisture (water or urine) present in the trap or on the hair coat (Appendix 2, Fig A2.1, Fig A2.2).

Experimental studies in the forensic drug field indicate that external contaminants may penetrate the hair shaft deeply when hair is wet (Kidwell and Blank 1996, Kidwell and Smith 2007). Water expands the hair cuticle facilitating permeability and providing a medium for diffusion. Once the hair shaft has been breached contaminants may become tightly bound in the hair matrix and impossible to remove with decontamination protocols (Kidwell and Blank 1996). Importantly, this process is enhanced by damage to the hair shaft and prolonged exposure (Kidwell and Smith 2007). In contrast, substances placed on hair when dry are relatively easily removed with decontamination protocols (Kidwell and Smith 2007).

Evidence in support of this hypothesis may come from observations in an adult male grizzly bear captured in three different years and with all three capture methods (Bear 15, Fig 2.7). When captured in 2007 (spring capture/snare) and 2008 (spring capture/helicopter darting) this bear exhibited similar HCC (3.47 pg/mg [2007] and 3.97 pg/mg [2008]). In spring 2009, this animal was captured in a culvert trap and held for approximately 12 hours prior to processing. On removal from the trap the bear was noted to be uniformly soaking wet and covered in a copious mixture of feces, urine and trap bait (Appendix 2, Fig A2.2). HCC was subsequently recorded as 14.99 pg/mg, nearly five times that recorded in previous years. The bear was in good physical condition with no evidence of disease or injury (B. Macbeth, personal observation) and hair appeared grossly clean after our standardized wash protocol had been implemented.

It should be noted that this effect is unlikely to have occurred in all culvert trapped bears. Most bears are contained in culvert traps for periods < 12 hours and often no more than a few hours. Further, many bears come out of culvert traps visibly clean and there is a general attempt by field researchers to collect samples from non-contaminated body regions. However, in some bears and under some conditions the collection of clean and dry samples is not possible. If the preceding hypothesis is correct it is probable that large elevations in HCC occurring in a few bears increased mean HCC recorded among all culvert trapped bears and artificially skewed results. This hypothesis was explored in a controlled laboratory study using grizzly bear guard hair of known HCC exposed to water, urine, and feces spiked with known concentrations of cortisol (Appendix 2).

We recognize that in the grizzly bear, fecal glucocorticoids are usually measured with a corticosterone RIA which exhibits limited cross reactivity with cortisol (0.05%) (Wasser et al. 2000, Hunt and Wasser 2003, von der Ohe et al. 2004). However, our work (wash validation; Fig 2.3, Table 2.2) with an EIA kit specific for cortisol (100%) and exhibiting limited cross reactivity with corticosterone (4.81%) suggests that this species may also excrete intact cortisol (or cross reactive metabolites) in feces. This hypothesis is supported by ACTH stimulation tests performed in the Himalayan black bear (*Ursus thibetanus* Cuvier, 1823) which suggest some ursids may excrete native cortisol in feces (Young et al. 2004). The types of urinary glucocorticoids excreted by grizzly bears are unknown. However, cortisol has been measured in the urine of other bear species (Giant Panda [*Ailuropoda melanoleuca* David, 1869], Owen et al. 2005). Presently, we recommend that if severely contaminated or wet hair samples are recovered from grizzly bears held for extended periods in culvert traps they should not be used in HCC analysis.

A final explanation for observed increases in HCC in culvert trapped bears may relate to landscape features in the home ranges of bears captured using this method. We are currently exploring the linkage between HCC and landscape variables (e.g. proportion of protected area, road density and anthropogenic influence within a bear's home range) in collaboration with ongoing research at the FRIGBP.

When hair is collected non-invasively, potentially confounding life history traits such as age and sex class are usually unknown or costly to determine. Differences in basal levels of

circulating glucocorticoids along with the strength and activity of the stress response have been attributed to sex-and age-class variation in a variety of mammals (Reeder and Kramer 2005, Boonstra 2005). We found no evidence of sex-or age-class effects on HCC in the grizzly bear. These findings agree with those reported by von der Ohe et al. (2004) who identified no differences in fecal glucocorticoid concentrations measured in different age-sex classes of free-ranging grizzly bears in Alaska. Knowledge that sex and age-class do not influence HCC is advantageous where non-invasive techniques are employed and may be important in studies of long-term stress among populations of grizzly bears, which may be small and have skewed sex ratios or age ranges.

Moisture and ultraviolet radiation are known to enhance the removal of xenobiotics from hair (Kidwell and Blank 1996, Kidwell and Smith 2007). In free-ranging wildlife natural weathering cannot be controlled and may ultimately represent the most important confounding factor in HCC analysis. The effects of weathering may be especially important when hair is obtained non-invasively or opportunistically (e.g. carcasses or rub trees) as samples may be exposed to the elements for days to weeks prior to collection. Our results suggest that HCC does not change in grizzly bear guard hair exposed to ambient spring environmental conditions in Alberta grizzly bear habitat over an 18-day period. This is an important finding as barb wire sets used in DNA census studies of Canadian grizzly bear populations are typically left for 10-14 days prior to hair sample collection (e.g. Mowat and Strobeck 2000). *Ursus arctos* is widely distributed in North America and around the globe and bears may inhabit regions with vastly different climatic conditions (Schwartz et al. 2003). Further work examining the effects of temperature, moisture and UV is warranted to define the longest possible time frame in which grizzly bear hair may be left in the field prior to collection.

Our work indicates that HCC does not change for > 1 year when samples are stored as intact hair in dry paper envelopes in the dark. In contrast, hair stored in ground form exhibits a significant decrease in HCC over a shorter time period (9 months). Webb et al. (2010) successfully measured dynamic patterns of cortisol deposition in centuries-old human hair. In the grizzly bear, studies of HCC in museum specimens, mounts and archived hides could provide an important historical record of long-term stress in and among populations. As such, we are continuing longitudinal studies to better understand the implications of long-term storage on

HCC in this species. We have also explored the effects of chemical-based taxidermy protocols on HCC in grizzly bear hair (Appendix 3).

Our preliminary findings indicate that the temporal pattern of HCC in individual grizzly bears may be very stable or highly variable. As such, studies of HCC may provide an important tool with which to better understand the linkages between long-term stress, grizzly bear health and population performance (Cattet et al. 2006). In this investigation we have identified a number of important factors which may influence HCC in the grizzly bear and have made recommendations to reduce their potential effects. While our present study focused on one species, factors identified here should be considered in the development, application and interpretation of HCC analysis in other species. Moreover, techniques developed here may facilitate the exploration of other steroid hormones in wildlife hair. For example, we have also quantified corticosterone, progesterone, estradiol, testosterone, and thyroid hormones in hair from Alberta grizzly bears (D. Janz, unpublished data).

In addition, we have recently initiated a collaborative research program between the FRIGBP, the Scandinavian Brown Bear Research Project (SBBRP), and the Canadian Cooperative Wildlife Health Centre (CCWHC) examining HCC in hair collected from three additional populations of grizzly bears (also known as brown bears, *U. arctos*). In this regard, we have measured cortisol in hair from an additional $n = 634$ brown bears from four populations in Canada (Alberta and Nunavut), Scandinavia (Sweden), and Mongolia. Ongoing data analysis [(M. Cattet (CCWHC) and G. Stenhouse (FRIGBP)] seeks to more vigorously assess the utility of HCC as a biomarker of long-term stress in *U. arctos* by evaluating the relationships between HCC, grizzly bear biology, health, and anthropogenic landscape characteristics in these populations. Like our current study, this work will have important implications for the development and application of HCC as a conservation tool in this species and in other wildlife.

CHAPTER 3[a]

AN EVALUATION OF HAIR CORTISOL CONCENTRATION AS A BIOMARKER OF LONG-TERM STRESS IN FREE-RANGING POLAR BEARS (*Ursus maritimus*) FROM SOUTHERN HUDSON BAY AND JAMES BAY, CANADA

This investigation was performed as the second of four studies included in this thesis and was undertaken to evaluate the utility of hair cortisol analysis in polar bears. Hair was collected from the "Threatened" Southern Hudson Bay subpopulation of polar bears in Ontario, Canada. In this region, ongoing research programs have identified a decline in the body condition of polar bears which may be related to long-term stress associated with anthropogenic climatic warming. Hair cortisol techniques developed for use in grizzly bears (*Ursus arctos*) (Chapter 2) were modified for use in free-ranging polar bears and laboratory validation was performed to determine if cortisol could be accurately and reliably measured in hair collected from this species. The effects of procedural considerations and an expanded array of biological factors which may influence hair cortisol concentration were also explored and considerations for the prudent use of hair cortisol analysis were developed for polar bears. In addition, a combination of hypothesis testing and information theory was used to develop and assess models to evaluate potential relationships between hair cortisol concentration and indices of growth (mass, length, and body condition index) closely related to survival and fecundity in this species. Hair cortisol levels identified in this study may also serve as a baseline for the continued monitoring of long-term stress levels in polar bears from the Southern Hudson Bay subpopulation.

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3.1 Introduction

In the Arctic, factors related to climatic warming are considered among the most important drivers of ecological change and threaten the survival of wild species throughout the circumpolar region (ACIA 2005, Laidre et al. 2008). For polar bears (*Ursus maritimus*), the annual Arctic sea-ice serves as core habitat which allows this top-level carnivore access to its primary prey: ringed (*Pusa hispida*) and bearded (*Erignathus barbatus*) seals, facilitates seasonal movement and mating, and in some areas provides critical den sites (Derocher et al. 2004, Laidre et al. 2008). A weight of evidence indicates that climatic warming is linked with recent declines in the extent of annual sea-ice, decreases in the age, thickness and overall extent of multiyear ice, and an increase in the length of the summer ice-free period across the Arctic (ACIA 2005, Walsh 2008). With this trend forecast to continue indefinitely (IPCC 2007), the long-term persistence of many polar bear populations is now in question (e.g. Stirling and Parkinson 2006, Durner et al. 2009, Molnár et al. 2010).

The polar bear populations considered most at risk are those at the southern edge of the species distributional range in Hudson Bay and James Bay, Canada where a natural cycle of sea-ice melting forces bears onshore for several months during summer and fall (Stirling et al. 2004, Stirling and Parkinson 2006). In this region, a significant reduction in the extent and duration of annual sea-ice has occurred over the last three decades (Gough et al. 2004, Gagnon and Gough 2005), and polar bears now have less time to hunt seals during spring and early summer, accumulate less fat prior to ice break-up, and fast over a longer ice-free period (Stirling and Parkinson 2006, Obbard et al. 2006). In western Hudson Bay, long-term monitoring programs have demonstrated that earlier ice break-up and an extended ice free season may be associated with changes in the distribution of the Western Hudson Bay (WH) polar bear subpopulation (Stirling et al. 2004, Towns et al. 2010), diminished body size and body condition in individuals (Stirling et al. 1999, Derocher and Stirling 1995, Atkinson et al. 1996) along with decreased reproductive output (Stirling et al. 1999, Derocher and Stirling 1995), survival (Derocher and Stirling 1996, Regehr et al. 2007), and overall population size (Regehr et al. 2007). Although the Southern Hudson Bay (SH) subpopulation has not been monitored continuously throughout this period, the average body condition of SH polar bears handled since 2000 is significantly lower than that recorded for SH bears from 1984-1986 (Obbard et al. 2006, Obbard et al. 2007).

However, the size of the SH polar bear subpopulation appears to be unchanged from the mid-1980s, and annual variation in mean body size and condition since 2000 (2000-2009) show no clear trends (Obbard et al. 2007, M. Cattet, unpublished data). Nevertheless, there is concern that SH polar bears may be under increasing stress and that, if the trend of earlier sea-ice melting and later freeze-up continues in eastern and southern Hudson Bay, the SH subpopulation may respond similarly to the WH subpopulation (Obbard et al. 2007). As a result, the conservation status of the SH polar bear subpopulation is currently designated as "Threatened" (OESA 2008). With the growing recognition that long-term stress is an important factor linking ecological change with impaired health and population performance in wildlife (e.g. Wikelski and Cook 2006, Sheriff et al. 2010, Ellis et al. 2012), the ability to monitor long-term stress in polar bears may prove invaluable for conservation efforts in southern Hudson Bay and elsewhere.

In most mammals, cortisol is the principal effector hormone of the stress response (Busch and Hayward 2009) and over the past decade the measurement of cortisol in hair has shown promise as a reliable biomarker of long-term stress in a variety of species including: Procavids (Koren et al. 2002), non-human primates (Davenport et al. 2006), humans (Gow et al. 2010), domestic dogs and cats (Accorsi et al. 2008, Bennett and Hayssen 2010), and ungulates (Ashley et al. 2011, Gonzalez de la Vara et al. 2011). Like other blood-borne hormones and xenobiotics, cortisol is thought to enter the hair shaft in direct proportion to its biologically available concentration in the blood; a process believed to be restricted to the phase of active hair growth in individual hair follicles (Davenport et al. 2006, Pragst and Balikova 2006). As such, the measurement of cortisol in a full length of hair should represent an integrated measure of hypothalamic-pituitary-adrenal (HPA) axis (the primary endocrine pathway of the physiological stress response; Sapolsky et al. 2000) activity during the period of hair growth which may reflect a time frame of weeks to months (Macbeth et al. 2010). In addition, hair cortisol concentration (HCC) may not be as sensitive to short-term stress (Ashley et al. 2011, Gonzalez de la Vara et al. 2011) as are other measures of HPA activity, such as serum cortisol (Cattet et al. 2003a), salivary cortisol (Millspaugh et al. 2002) or glucocorticoids measured in urine and feces (Owen et al. 2004, Hunt and Wasser 2003). Furthermore, hair itself is a relatively stable medium which can be collected non-invasively or opportunistically from free-ranging animals (Macbeth et al. 2010, Bechshøft et al. 2012), transported and stored at ambient temperature, (Macbeth et al. 2010,

Jaspers et al. 2010), and, under optimal conditions, cortisol incorporated into hair may remain detectable for years to centuries (Webb et al. 2010, Macbeth et al. 2010, Gonzalez de la Vara et al. 2011). Recently, techniques for measuring HCC have been refined for use with hair collected from free-ranging grizzly (*U. arctos*) (Macbeth et al. 2010) and polar bears (Bechshøft et al. 2011, Bechshøft et al. 2012). However, in order to serve as a useful conservation tool, factors influencing HCC in these species must first be identified (Macbeth et al. 2010, Bechshøft et al. 2011, 2012) and then revealed to establish unequivocal linkages between prevailing environmental conditions and the fitness of individual animals (Busch and Hayward 2009).

3.2 Objectives

The purpose of this investigation was to: 1) modify HCC methods developed in grizzly bears for use in polar bears, 2) examine the effect of decontamination protocols, sample quantity, laboratory storage time, body region, hair type, sex, age, family group status, and capture period on HCC in free-ranging polar bears of the SH subpopulation, and 3) evaluate the association of HCC (as a potential biomarker of long-term stress) and measures of growth [length, mass, and body condition index (BCI; Cattet et al. 2002)] in SH polar bears. Growth is a natural history trait for which much evidence has been amassed for its direct relationship to fitness in this species (e.g. Atkinson and Ramsay 1995, Derocher and Stirling 1996, Derocher et al. 2010).

3.3 Materials and Methods

3.3.1 Sample collection, storage, and handling

Hair was obtained from 185 SH polar bears captured during Ontario Ministry of Natural Resources research programs in 2007 (*n*=59 bears), 2008 (*n*=93 bears), and 2009 (*n*=33 bears) [research goals summarized in Obbard et al. (2006, 2007)]. The study area extended along the Ontario coastline from Hook Point (~54° 50´N 82° 15´W) on northwestern James Bay to the Hudson Bay coast at the Ontario-Manitoba border and included: offshore spits, small islands, and inland areas up to 40 km from the coast. The geological and ecological characteristics of this location along with the major components of the diet of SH polar bears are reported in detail in Obbard et al. (2007). In all years, bears were captured between September 16 and October 13 via remote drug delivery from helicopter (independent animals) or using a pole syringe (cubs of the year) [capture and handling protocols summarized in Obbard et al. (2006, 2007)]. For polar bears, the timing and duration of active hair growth is poorly understood but is believed to begin

in spring (April) and last into autumn (September or October) (Amstrup 2003). As such, hair collected for this investigation should represent most of the hair growth period for the SH subpopulation. Hair was collected by shaving as evenly and close to the skin as possible with electric clippers (2007 and 2008) or by plucking with haemostats (2009). In 2007, hair was obtained from the rump. In 2008, hair was gathered from the rump in 73 bears and from the neck, shoulder, rump and abdomen in 20 bears. In 2009, was collected from the shoulder (Macbeth et al. 2010). All hair samples were stored in dry paper envelopes at room temperature in the dark (Macbeth et al. 2010). Cortisol in guard hair was the focus of this study and we only explored HCC in undercoat to examine the effect of hair type in 15 polar bears from which both types in all body regions were available. Hair from SH polar bears was uniformly clear-white to light yellow and no blood was apparent on any sample. However, the surface of most samples was contaminated by varying quantities of dirt and debris and approximately 20% were stained dark yellow (B. Macbeth, personal observation). The origins of yellow staining could not be determined but may represent the effects of weathering (bleaching) or the incorporation of oils from prey into the hair shaft (Amstrup 2003). Hair was also obtained opportunistically from the carcasses of an emaciated polar bear family group killed 400 km inland at Deline, Northwest Territories, Canada (Southern Beaufort Sea subpopulation; Thiemann et al. 2008, CBC 2008) in April, 2008. These samples were contaminated with blood and lipid (Macbeth et al. 2010, Contamination Category 2: 25% to 75% of the hair surface covered with biologicals), had been stored at -20°C for 2 months prior to HCC determination, and were not used in direct comparative analysis with SH bears.

3.3.2 Removal of surface contamination, hair preparation, and steroid extraction

Hair decontamination, preparation, and steroid extraction methods were modified after Macbeth et al. (2010). Following guidelines and analysis reported in detail in [Chapter 2, Macbeth et al. (2010)], we developed and validated a methanol-based protocol to remove surface contamination from polar bear hair in which 100 mg hair samples were washed three times with 4 ml methanol for three minutes per wash on a slow rotator (Haematology and Chemistry Mixer 346, Fisher Scientific, Ottawa, Ontario). This protocol was effective in decontaminating polar bear hair but did not remove intrinsic cortisol from the hair shaft (see section 3.4.1.2). Procedures for the subsequent grinding of washed and dried hair along with steroid extraction

and EIA based analysis were as described in [Chapter 2, Macbeth et al (2010)]. However, unlike grizzly bears, cortisol was assessed in triplicate 50 µl aliquots of reconstituted hair extract derived from 25 mg samples of powdered hair for all SH polar bears. Experimental approaches used to evaluate the effects of decontamination protocols (wash kinetics), sample quantity [comparison of HCC in 25 mg, 15 mg, 5 mg subsamples of powdered hair] and laboratory storage time (comparison of HCC at time 0 vs HCC 24 months later) on HCC in polar bears were also similar to those reported in [Chapter 2, Macbeth et al. (2010)] for grizzly bears.

3.3.3 Data analysis

Data was analyzed using SPSS 18.0.0 (PASW Statistics Version 18.0.0, July 30, 2009) with the level of significance set at $P \leq 0.05$ to assess the effects of decontamination protocols, sample quantity, laboratory storage time, body region, hair type, sex, family group status, and capture period. The level of significance was set at $P \leq 0.10$ to examine HCC measured within polar bear family groups along with factors influencing growth among all SH polar bears. To meet assumptions of normality and homogeneity of variance all data was log-transformed prior to analysis.

Statistical tests used to evaluate the effects of decontamination protocols (wash kinetics, paired samples *t*-tests), sample quantity [repeated measures analysis of variance (ANOVA)] and laboratory storage time (paired samples *t*-test) on HCC in polar bears were similar to those reported in Chapter 2 [Macbeth et al. (2010)] for grizzly bears. The effects of body region and hair type on HCC in SH polar bears were assessed with a two-way repeated measures analysis of variance (ANOVA) with body region and hair type as the two factors. We evaluated the effects of sex, and family group status by grouping all polar bears into one of five categories based on these parameters (sex-reproductive class; solitary male or female, female with dependent cubs, male or female dependent juvenile) and comparing HCC levels among groups with a one-way ANOVA. Simple linear regression was used to investigate the association of HCC in female polar bears and their dependent offspring as well as the association of HCC and age. HCC measured in females with cubs of the year and with yearlings, and HCC in cubs of the year and yearlings was then contrasted with independent samples *t*-tests. Independent samples *t*-tests were also used to compare HCC measured in polar bears with different sized litters. We evaluated the effect of capture period by using a one-way ANOVA to compare HCC levels in polar bears

sampled onshore over five, 8 day periods occurring after the date of 50 % sea-ice break-up (Period 1: date of capture 78-85 days after break-up, Period 2: date of capture 86-93 days after break-up, Period 3: date of capture 94-101 days after break-up, Period 4: date of capture 102-109 days after break-up, and Period 5: date of capture 110-117 days after break-up). When hair from more than one body region was available from an individual, the average HCC value of all regions was used (Macbeth et al. 2010). Hair samples determined to be below detection limits were assigned HCC values of 0.16 pg/mg. This value represents a point half-way between the 0 ng/ml cortisol standard provided with the EIA kit and the kit limit of detection (0.04 ng/ml; Macbeth et al. 2010) converted to pg/mg in 25 mg powdered hair.

A combination of hypothesis testing and information theory was used to develop and assess models explaining variation in polar bear length, mass, and body condition index (BCI; Cattet et al. 2002). First, potential predictor variables were divided into four model sets representing polar bear biology, time, early life sea-ice conditions, and HCC. Sex, age, and reproductive status are known to influence body size and body condition in polar bears (e.g. Atkinson and Ramsay 1996, Derocher and Stirling 1996, Thiemann et al. 2006) and measurement variables in the biology model set included sex-reproductive class and age. The potential relationship between age and dependent variables was evaluated as both a linear and quadratic association in order to assess: 1) the direct effect of age, and 2) how the effect of age on dependent variables changed as SH polar bears got older. The time model set included: year of capture (2007-2009) and year of birth (1979-2009). We assumed that all bears were born on January 1st in the year of birth. The sea-ice model set incorporated four measures of sea-ice conditions in the year a bear was born: 1) Julian day of ice break-up at birth (151-190), defined as 50% sea-ice concentration in spring of the birth year (Etkin 1991), 2) Julian day of ice freeze-up at birth (330-365), defined as 50% sea-ice concentration in fall of the birth year (Etkin 1991), 3) number of days of $\geq$ 50% ice cover at birth (ranging from 163-224 days between freeze-up in the previous year to break-up in the birth year), and 4) number of days of $\geq$ 50% ice cover after birth (ranging 163 -222 days between freeze-up in the birth year to break-up in year after birth). We considered the entire area of sea-ice enclosed by the SH subpopulation boundary in calculations of annual sea-ice extent and duration (methods and locations reported in Obbard et al. 2007). In order to evaluate the explained variation (calculated as adjusted R^2) attributable to

biology, time, sea-ice, or HCC each model set was added to developing best-fit models in a step-wise fashion. Within and between model sets, sample-size corrected Akaike's Information Criterion (AICc) scores were calculated and used to determine which variables to include and retain in the developing models for each dependent variable. Best-fit models were based on ΔAICc values ≤ 2 with the fewest predictor variables (parsimony) and associated R^2 values.

3.4 Results

3.4.1 Hair processing and storage

3.4.1.1 Assay performance

For polar bear hair extract, intra-assay coefficient of variation (C.V.) was 9.74% ($n = 6$) while inter-assay C.V. was 11.44% ($n = 6$). HCC results from serially-diluted hair extract were parallel ($r^2 = 0.991$, $P = 0.004$) with those from serially-diluted cortisol standards from the EIA kit. Extraction efficiency was 95.55 ± 3.18% [$n = 6$ determinations of recovery from a 4.0 ng/ml spike of a hair extract solution containing ground hair (Fluka Hydrocortisone Reference Standard 31719, Sigma-Aldrich, Munich). Furthermore, no additional cortisol could be measured when a previously extracted hair sample was re-extracted with fresh methanol for an additional 24 or 48 hours (i.e. 48 hrs or 72 hours total extraction time, $n = 20$). Likewise, no extra cortisol could be recovered when an additional 0.2 ml of reconstitution buffer was added to leftover residue in the glass test tube (i.e. after 12 hours reconstitution) and reconstituted for a further 12 hours ($n = 20$).

3.4.1.2 Decontamination protocol

Three, 3 minute methanol washes with 4 ml methanol per 100 mg hair were required to remove all visible contamination (i.e. dirt, debris, blood or lipid) and measurable cortisol from the surface of polar bear hair (Table 3.1). No evidence of penetration of the hair shaft (Davenport et al. 2006, Macbeth et al. 2010, Bechshøft et al. 2011) was observed until the sixth and seventh wash in 2 of $n = 18$ hair samples and in the seventh wash in 1 of $n = 18$ hair samples examined (Table 3.1). A comparison of hair shaft cortisol concentration in polar bear hair washed three times or seven times also showed no difference (Guard hair: Paired samples *t*-test, $t_{(11)} = 0.513$, $P = 0.618$, $n = 12$; Undercoat: Paired samples *t*-test, $t_{(11)} = 1.605$, $P = 0.137$, $n = 12$).

Table 3.1. Wash cortisol concentration determined in guard hair or undercoat collected from *n* = 18 polar bears (*Ursus maritimus*) and exposed to seven 3-minute washes with 0.04 ml methanol per mg hair. The limit of detection of the cortisol EIA kit is 0.04 ng/ml (Macbeth et al. 2010). Evidence of probable penetration of the hair shaft is highlighted in bold.

Polar Bear	Hair Type	Sample Quality (contamination type and hair colour)	Wash Cortisol Concentration (ng/ml)						
			W1	W2	W3*	W4	W5	W6	W7
1	guard	blood and lipid, dark yellow	0.773	0.070	0.054	-	-	-	-
2	guard	dirt, dark yellow	0.058	-	-	-	-	-	-
3	guard	dirt, dark yellow	0.055	-	-	-	-	-	-
4	guard	dirt, dark yellow	-	-	-	-	-	-	-
5	guard	dirt, dark yellow	-	-	-	-	-	-	-
6	guard	dirt, off white	0.056	-	-	-	-	-	**0.052**
7	guard	dirt, clear to white	-	-	-	-	-	-	-
8	guard	clean, light yellow	0.053	-	-	-	-	**0.064**	**0.076**
9	guard	clean, light yellow	0.050	-	-	-	-	-	-
10	guard	clean, light yellow	-	-	-	-	-	-	-
11	guard	clean, off white	-	-	-	-	-	-	-
12	guard	clean, off white	-	-	-	-	-	-	-
13	guard	clean, clear to white	-	-	-	-	-	**0.049**	**0.083**
14	guard	clean, clear to white	-	-	-	-	-	-	-
15	guard	clean, clear to white	-	-	-	-	-	-	-
16	undercoat	dirt, clear to white	-	-	-	-	-	-	-
17	undercoat	clean, clear to white	-	-	-	-	-	-	-
18	undercoat	clean, clear to white	-	-	-	-	-	-	-

* In most samples, 3 washes were required to remove visible contamination (i.e. blood, lipid or dirt) from the surface of polar bear hair.

3.4.1.3 Minimum sample quantity required and laboratory storage time

Hair cortisol concentration decreased in subsamples of ground hair weighing 25 mg, 15 mg and 5 mg (one-way repeated measures ANOVA, $F_{(2,8)} = 13.801$, $P < 0.001$, $n = 9$; Tukey-Kramer, $P < 0.05$ for all comparisons). However, there was no difference in HCC measured in paired subsamples of ground hair weighing 50 mg and 25 mg (Paired samples *t*-test, $t_{(11)} = 1.476$, $P = 0.182$, $n = 12$). Hair cortisol concentration measured in polar bear hair stored intact in dry paper envelopes for 24 months remained similar (Paired samples *t*-test, $t_{(11)} = 0.823$, $P = 0.428$, $n = 12$).

3.4.2 Features of hair and polar bears

3.4.2.1 Range of hair cortisol values determined in Southern Hudson Bay polar bears

The median HCC in 185 SH polar bears was 0.48 pg/mg [range 0.16 pg/mg-2.26 pg/mg]. HCC was below detection limits in 22/185 (11.9%) of bears.

3.4.2.2 Hair type and body regions

Variation in HCC in SH polar bears was not explained by body region or hair type [two-way repeated measures ANOVA $F_{(\text{body region})\,(3,\,12)} = 0.873$, $P = 0.482$, $n = 15$ and $F_{(\text{hair type})\,(1,\,14)} = 0.864$, $P = 0.368$, $n = 15$].

3.4.2.3 Sex-reproductive class, age, and family groups

HCC varied among different polar bear sex-reproductive classes (one-way ANOVA, $F_{(4,\,180)} = 6.751$, $P < 0.001$) (Fig 3.1). We recorded higher HCC levels in dependent male polar bears and females with dependent offspring than in solitary males (Tukey-Kramer, $P \leq 0.05$) (Fig 3.1). However, HCC levels in these groups were not different (Tukey-Kramer, $P > 0.05$) from those recorded in dependent female and solitary female SH polar bears (Fig 1). We also identified no difference (Tukey-Kramer, $P > 0.05$) in HCC levels among dependent females, solitary females, and solitary male polar bears (Fig 3.1). HCC measured in dependant cubs of the year was directly associated with that of their mother (Pearson product moment correlation $r = 0.560$, $P = 0.024$, $n = 16$) (Fig. 2) while HCC in dependant yearlings was not (Pearson product moment correlation $r = 0.141$, $P = 0.563$, $n = 19$) (Fig 3.2). HCC in cubs of the year was marginally greater than that in yearlings (Independent samples *t*-test, $t_{(35)} = 1.74$, $P = 0.091$, $n = 16$ cubs of year, $n = 19$ yearlings). HCC measured in female polar bears with cubs of the year was also greater than in those with yearlings (Independent samples *t*-test, $t_{(35)} = 4.12$, $P < 0.001$, $n = 16$ with cubs of year, $n = 19$ with yearlings). However, there was no difference in HCC measured in adult females with one or two dependent offspring of either age group (Independent samples *t*-test, $t_{(19)} = 0.066$, $P = 0.948$, $n = 8$ with a single cub of year, $n = 13$ with 2 cubs of the year; Independent samples *t*-test, $t_{(13)} = -0.410$, $P = 0.689$, $n = 10$ with a single yearling, $n = 5$ with 2 yearlings). HCC was also directly associated with polar bear age (Pearson product moment correlation, r = 0.156, $P = 0.034$, $n = 185$).

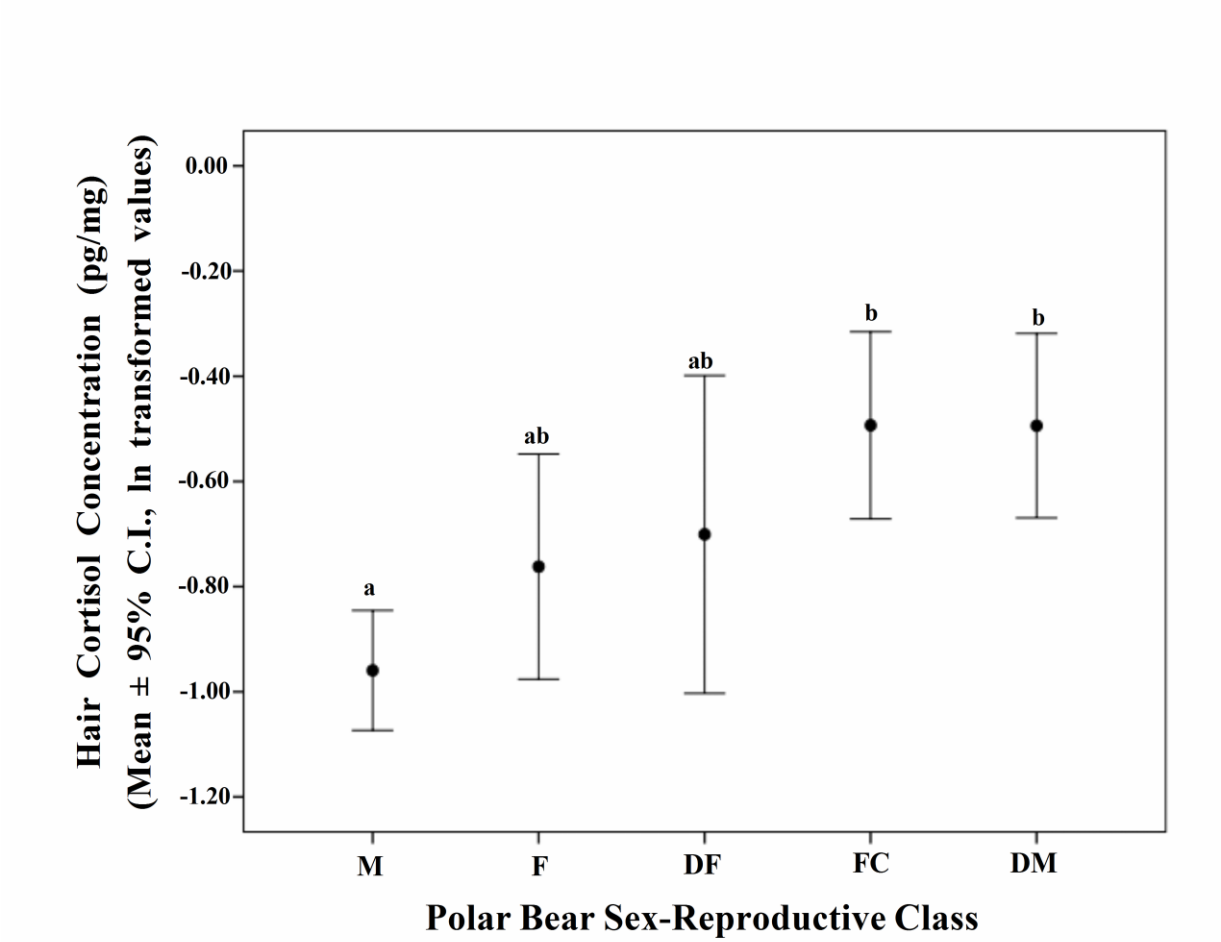

Figure 3.1 Comparison of hair cortisol concentration (HCC) measured in n = 185 polar bears (*Ursus maritimus*) captured in southern Hudson Bay and James Bay from 2007 to 2009. HCC varied among different polar bear sex-reproductive classes (solitary male: M, solitary female: F, dependent juvenile female: DF, female with dependent offspring: FC, dependent juvenile male: DM) (one-way ANOVA, $F_{(4, 180)} = 6.751$, $P < 0.001$). Significant differences (Tukey-Kramer, $P \leq 0.05$) in hair cortisol concentration among sex-reproductive classes are indicated by different letters.

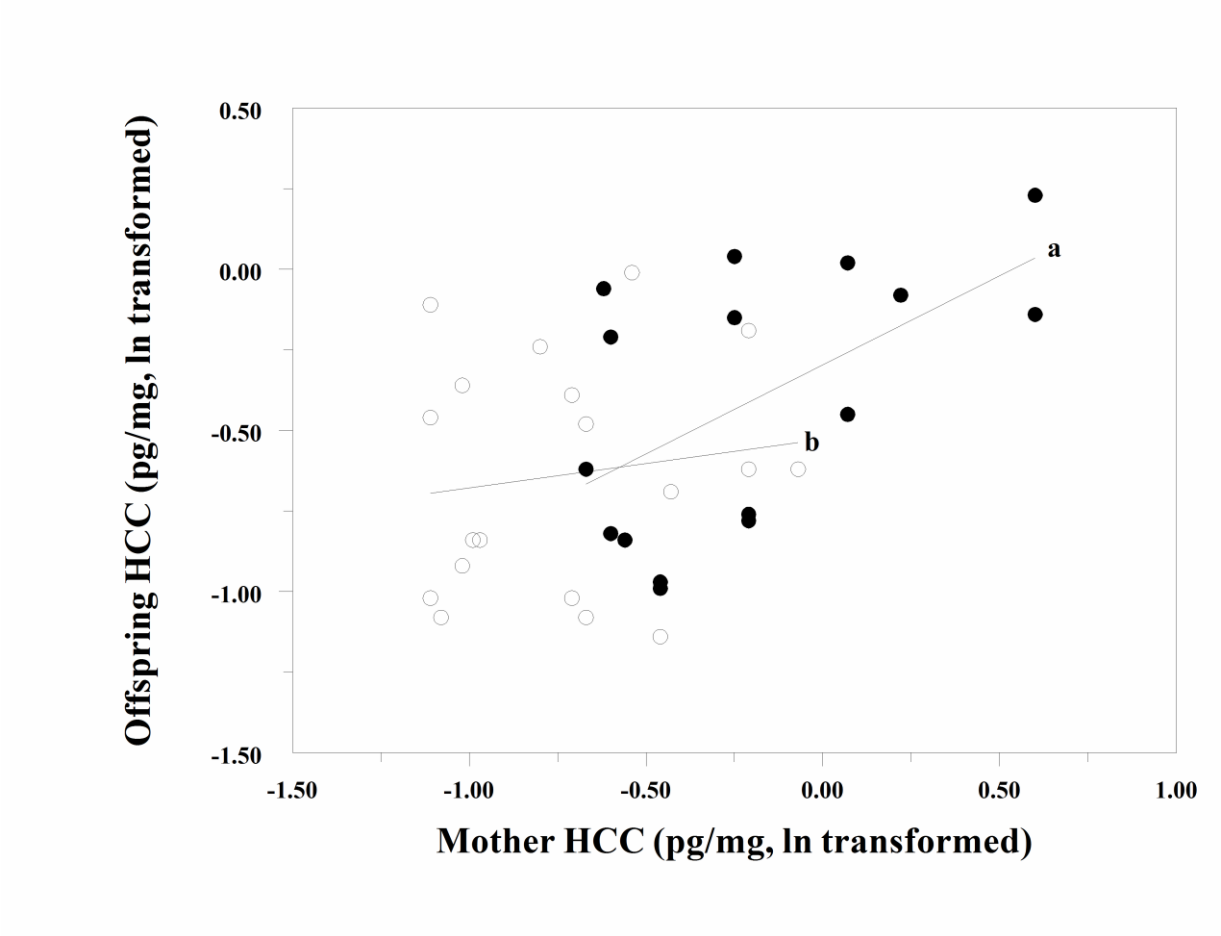

Figure 3.2 The association of hair cortisol concentration (HCC) in *n* = 35 polar bear (*Ursus maritimus*) family groups captured in southern Hudson Bay and James Bay from 2007 to 2009. HCC measured in female polar bears is directly associated with that of their dependent cubs of the year but not yearlings (Pearson product moment correlation $r = 0.560$, $P = 0.024$, $n = 16$ family groups with cubs of year, Pearson product moment correlation Pearson product moment correlation $r = 0.141$, $P = 0.563$, $n = 19$ family groups with yearlings, $\bullet^{a}$ = cubs-of-year and $\circ^{b}$ = yearlings).

3.4.2.4 Capture period

HCC was lower in bears sampled at the start of the capture period (date of capture 78-85 days after the date of 50% sea-ice break-up) compared to those sampled near the end (date of capture 102-117 days after date of 50% sea-ice break-up) [one-way ANOVA, $F_{(4, 180)} = 3.961$, $P = 0.004$ ($n = 25$ Period 1, $n = 11$ Period 2, $n = 32$ Period 3, $n = 51$ Period 4, $n = 66$ Period 5)] (Fig 3.3). HCC levels measured in bears sampled in the mid capture period (date of capture 86-

101 days after the date of 50% sea-ice break-up) were not different (Tukey-Kramer, $P > 0.05$) from those in bears captured earlier or later (Fig 3.3).

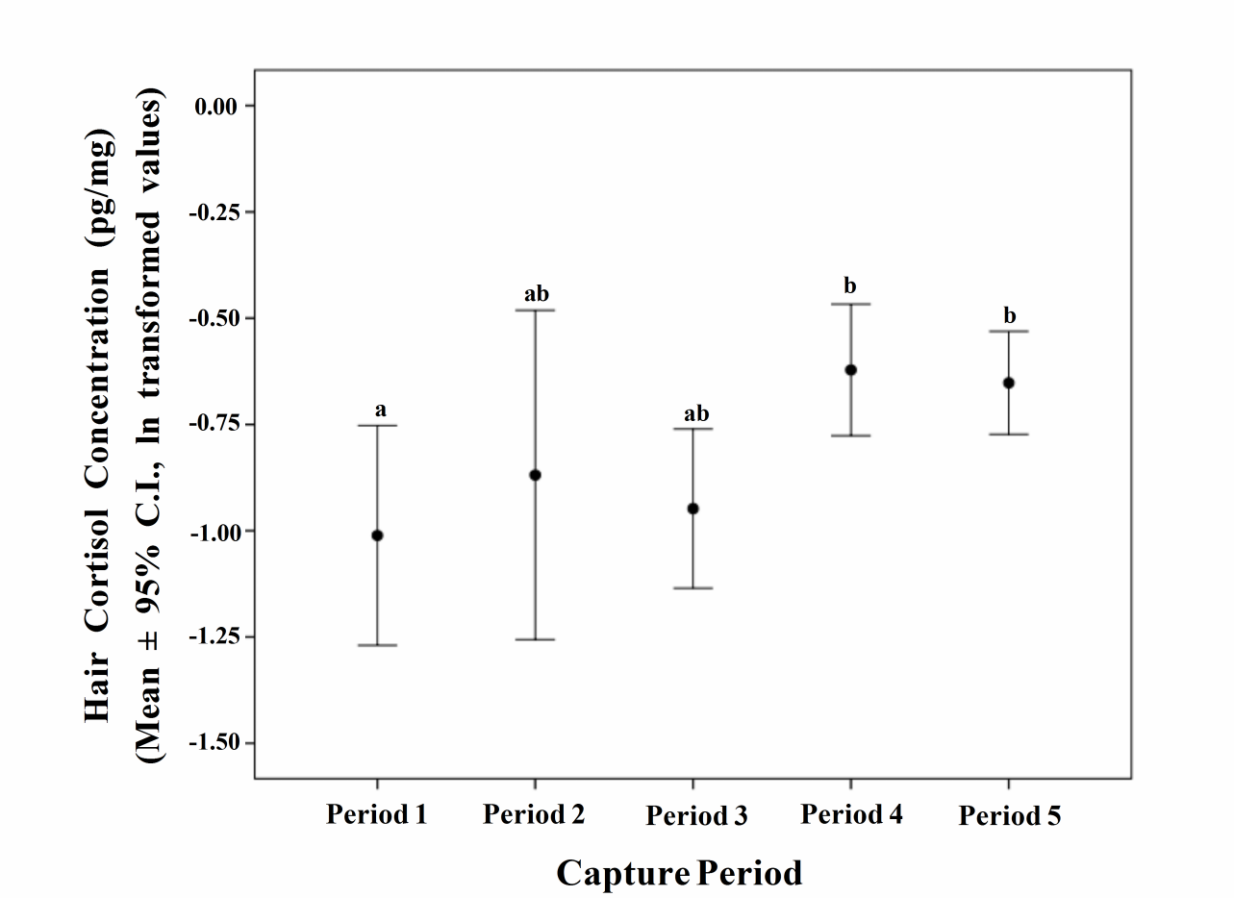

Figure 3.3 Comparison of hair cortisol concentration (HCC) measured in n = 185 polar bears (*Ursus maritimus*) captured in southern Hudson Bay and James Bay during fall 2007, 2008, and 2009: Period 1 (date of capture between 78-85 days after date of 50% sea-ice break-up), Period 2 (date of capture between 86-93 days after date of 50% sea-ice break-up), Period 3 (date of capture between 94-101 days after date of 50% sea-ice break-up), Period 4 (date of capture between 102-109 days after date of 50% sea-ice break-up), and Period 5 (date of capture between 110-117 days after date of 50% sea-ice break-up). HCC is lower in bears captured in Period 1 compared to those captured in Period 4 and 5 [one-way ANOVA, $F_{(4, 180)} = 3.961$, $P = 0.004$ (n = 25 Period 1, n = 11 Period 2, n = 32 Period 3, n = 51 Period 4, n=66 Period 5)]. Significant differences (Tukey-Kramer, $P \leq 0.05$) in hair cortisol concentration between capture periods are indicated by different letters.

3.4.3 Factors influencing growth in Southern Hudson Bay polar bears

3.4.3.1 Multivariate models of body length, body mass, and body condition in Southern Hudson Bay polar bears

Best-fit models explained 82 % and 86% of the variation in length and mass respectively and incorporated polar bear biology, time, sea-ice conditions, ± HCC (Table 3.2a, Table 3.2b). For BCI, the best fit model accounted for 41 % of the explained variation and included biology, time, and HCC (Table 3.2a, Table 3.2b). Body length and mass were greater in solitary male and female SH polar bears than in all other sex-reproductive classes (Table 3.2a). In addition, dependent juvenile females exhibited lower length and mass compared to their dependent male counterparts (Table 3.2a). Female polar bears with dependent offspring exhibited similar length but lower mass compared to all sex-reproductive classes except dependent juvenile females (Table 3.2a). We also found that BCI values were greatest in dependent juvenile males (Table 3.2a). We recorded the lowest BCI values in females with dependent cubs (Table 3.2a). Polar bear age showed both direct and positive curvilinear associations with all three growth indices (Table 3.2a). Polar bear biology explained 80 % of the variation in length, 83 % of mass, and 38% of BCI (Table 3.2b). When we considered measures of time, length and mass tended to be greater in bears born more recently, but these growth measures were not affected by year of capture (Table 3.2a). Conversely, BCI was not influenced by year of birth, but it did vary significantly between years of capture being lower in 2008 than in 2007 and 2009 (Table 3.2a). The addition of time to best-fit models added 1 %, 0 %, and 2 % to the explained variation in length, mass, and BCI respectively (Table 3.2b). However, based on AICc scores, models of polar bear mass containing birth year were superior (ΔAICc > 2) to those without (Table 3.2b). When we examined early life sea-ice conditions, length and mass were directly associated with the time of break-up and freeze-up in the year of birth along with the duration of ≥ 50% ice cover after birth (Table 3.2a). Length and mass were also inversely associated with the duration of ≥ 50% ice cover at birth (Table 3.2a). Although sea-ice conditions explained an additional 1% and 2 % of the variation in length and mass (Table 3.2b), there was no relationship between sea-ice conditions and BCI (Table 3.2a, Table 3.2b). HCC was inversely associated with all growth indices and added 0 %, 1 %, and 1 % to the explained variation in length, mass, and BCI respectively (Table 3.2a, Table 3.2b).

Table 3.2.1 Standardized coefficients (StD β) and significance (*P*) of model variables describing the best fit models for growth (body length, body mass, body condition index) in *n* = 185 polar bears (*Ursus maritimus*) captured in southern Hudson Bay and James Bay from 2007 to 2009. For potential predictor variables containing more than one category and also included in the best-fit model, non significant relationships (*P* > 0.10) are presented for comparative purposes.

Model set and measurement variables	*Length*		*Mass*		*Body condition index*	
	β	*P*	β	*P*	β	*P*
1) Biology						
sex-reproductive class						
male (solitary)	0.176	<0.001	0.375	<0.001	-0.744	<0.001
female (solitary)	0.081	<0.001	0.117	0.068	-0.574	<0.001
female (with dependent young)	0.026	0.327	-0.289	<0.001	-1.631	<0.001
female (dependent juvenile)	-0.042	0.059	-0.191	0.005	-0.289	0.072
male (dependent juvenile)	0	-	0	-	0	-
age (0-30 years)	0.053	<0.001	0.205	<0.001	0.237	<0.001
age^2	-0.001	<0.001	-0.005	<0.001	-0.008	<0.001
2) Time						
year of birth (1979-2009)	0.021	0.008	0.051	0.030		N.S.
year of capture (2007)		N.S.		N.S.	0.034	0.748
(2008)		N.S.		N.S.	-0.196	0.040
(2009)		N.S.		N.S.	0	-
3) Sea-ice conditions in early life						
Julian day of 50% ice break-up at birth (151-190)	0.003	0.004	0.009	0.001		N.S.
Julian day of 50% ice freeze-up at birth (330-365)	0.003	0.002	0.009	0.004		N.S.
Number of days ≥ 50% ice cover at	-0.002	0.002	-0.007	<0.001		N.S.

birth (163-224)						
Number of days ≥ 50% ice cover after birth (163-222)	0.001	0.001	0.003	0.002		N.S.
4) Hair cortisol concentration	-0.017	0.080	-0.087	0.003	-0.155	0.022

Table 3.2.2 Sample-size corrected Akaike's Information Criterion (AICc) values, change in Akaike's Information Criterion (ΔAICc) values, Akaike weights (w_i), associated R^2-values, and change in R^2-values (ΔR^2) for models describing A) body length, B) body mass, and C) body condition index in n = 185 polar bears (*Ursus maritimus*) captured in southern Hudson Bay and James Bay from 2007 to 2009. Best fit models [based on ΔAICc ≤ 2, fewest predictor variables (parsimony), and associated R^2 value] are highlighted in bold.

A.	*Length*				
Model set and measurement variables	AICc	ΔAICc	w_i	R^2	ΔR^2
Intercept-only	-155.77	308.90	0.0000	-	-
1) Biology	-449.68	14.99	0.0006	0.80	-
(sex-reproductive class, age, age²)					
2) Biology-Time	-451.86	12.81	0.0017	0.81	0.01
(sex-reproductive class, age, age², birth year)					
3) Biology-Time-Sea-ice conditions	**-463.98**	**0.69**	**0.7082**	**0.82**	**0.01**
(sex-reproductive class, age, age², birth year, Julian day 50% break-up at birth, Julian day 50% freeze-up at birth, days ≥ 50% ice cover at birth, days ≥ 50% ice cover after birth)					
4) Biology-Time-Sea-ice conditions -Hair cortisol concentration	**-464.67**	**0.0**	**1.0000**	**0.82**	**0.0**
(sex-reproductive class, age, age², birth year, Julian day 50% break-up at birth, Julian day 50% freeze-up at birth, days ≥ 50% ice cover at birth, days ≥ 50% ice cover after birth, HCC)					
B.		*Mass*			
Model set and measurement variables	AICc	ΔAICc	w_i	R^2	ΔR^2

Intercept-only	286.18	343.25	0.0000	-	-
1) Biology	-34.69	22.38	0.0000	0.83	-
(sex-reproductive class, age, age²)					
2) Biology-Time	-35.72	21.35	0.0000	0.83	0.0
(sex-reproductive class, age, age², birth year)					
3) Biology-Sea-ice conditions	-45.60	11.47	0.0032	0.84	0.01
(sex-reproductive class, age, age², Julian day 50% break-up at birth, Julian day 50% freeze-up at birth, days ≥ 50% ice cover at birth, days ≥ 50% ice cover after birth)					
4) Biology- Sea-ice conditions -Hair cortisol concentration	-54.74	2.33	0.3119	0.85	0.01
(sex-reproductive class, age, age², Julian day 50% break-up at birth, Julian day 50% freeze-up at birth, days ≥ 50% ice cover at birth, days ≥ 50% ice cover after birth, HCC)					
5) Biology-Time-Sea-ice conditions	-50.72	6.35	0.0418	0.85	0.02
(sex-reproductive class, age, age², birth year, Julian day 50% break-up at birth, Julian day 50% freeze-up at birth, days ≥ 50% ice cover at birth, days ≥ 50% ice cover after birth)					
6) Biology-Time-Sea-ice conditions -Hair cortisol concentration	**-57.07**	**0.0**	**1.0000**	**0.86**	**0.01**
(sex-reproductive class, age, age², birth year, Julian day 50% break-up at birth, Julian day 50% freeze-up at birth, days ≥ 50% ice cover at birth, days ≥ 50% ice cover after birth, HCC)					

C.	*Body condition index*				
Model set and measurement variables	AICc	ΔAICc	w_i	R^2	ΔR^2
Intercept-only	347.52	88.28	0.0000	-	-
1) Biology	266.84	7.60	0.0224	0.38	-
(sex-reproductive class, age, age²)					
2) Biology-Time	262.18	2.94	0.2300	0.40	0.02

(sex-reproductive class, age, age^2, capture year)					
3) Sea-ice conditions	-	-	-	-	-
4) Biology-Time-Hair cortisol concentration (sex-reproductive class, age, age^2, capture year, HCC)	**259.24**	**0.0**	**1.0000**	**0.41**	**0.01**

3.5 Discussion

3.5.1 Assay performance, decontamination, sample quantity, and laboratory storage

Highly significant parallelism with cortisol standards agrees with our previously published finding in grizzly bears and indicates that this technique is highly specific for cortisol in both species (Macbeth et al. 2010). Assay performance in polar bears [intra-assay C.V. 9.74%, inter-assay C.V. 11.44%] was somewhat less than that reported by Macbeth et al. (2010) using the same technique in grizzly bears [intra-assay C.V. 3.58%, inter-assay C.V. 5.75%]. Decontamination findings also agree with those reported for other species [grizzly bears (Macbeth et al. 2010), caribou-reindeer (Ashley et al. 2011), and mule deer (*Odocoileus hemionus*) (Dean Jeffery, personal communication)], and indicated that a series of short methanol washes are effective in cleaning polar bear hair without removing intrinsic cortisol from the hair shaft (Davenport et al. 2006, Macbeth et al. 2010, Bechshøft et al. 2011). Indeed, it is probable that the standardized protocol employed here (3, 3-minute washes with 0.04 ml methanol per mg hair) could have even been extended to seven washes in most hair samples if required (Macbeth et al. 2010).

Macbeth et al. (2010) found that HCC could be measured in as little as 5 mg of powdered grizzly bear hair (approximately 5-10 guard hairs) and suggested that remote barb-wire snagging may be a viable method of obtaining hair for cortisol analysis in this species. In contrast, we could not reliably measure HCC in SH polar bears in less than 25 mg of powdered hair. 25 mg powdered hair represents approximately 25-35 unprocessed polar bear guard hairs which can be obtained through established barb-wire techniques in other bear species (B. Macbeth, personal observation). We also found no differences in HCC measured among body regions or hair types in SH polar bears. Together, these finding suggest that the use of non-invasive techniques to collect hair for cortisol analysis in SH polar bears may warrant further investigation.

We also determined that HCC levels determined in polar bear hair did not change for approximately 2 years when samples were stored as intact hair in dry paper envelopes in the dark. This finding also agrees with work on other species and suggests that HCC in an intact polar bear hair shaft may remain stable for months to years (Macbeth et al. 2010, Webb et al. 2010, Gonzalez de la Vara et al. 2011). Indeed, Bechshøft et al. (2012) recently measured HCC in historical hair samples collected from East Greenland polar bears. Nonetheless, further work is recommended to better define the effects of long-term storage on HCC in polar bears and other species.

3.5.2 Factors influencing hair cortisol concentration in Southern Hudson Bay polar bears

In any species, HCC should serve as an integrated record of: 1) HPA activity related to life cycle stage, 2) energetic demands related to life cycle events, and 3) persistent, stress-induced elevations in cortisol which occur during the period of hair growth (Landys et al. 2006, Busch and Hayward 2009, Macbeth et al. 2010). In this investigation, cortisol was measured in hair collected in September or October which should represent the majority of the hair growth period for the SH polar bear subpopulation and may reflect HPA activity associated with: 1) the spring-early summer foraging period (Obbard et al. 2007), 2) the breeding season (Ramsay and Stirling 1986), 3) the onshore fast (Stirling and Parkinson 2006, Obbard et al. 2007), and 4) lactation-offspring care (Arnould and Ramsay 1994). Differences in basal or seasonal HPA activity (Øskam et al. 2004, Hamilton 2007, Chow et al. 2011) along with divergent energetic demands, stressors, or response to stressors are well documented at different stages of the polar bear life cycle (Derocher et al. 2004). Together, these factors may explain the patterns of HCC we recorded in SH polar bears (Landys et al. 2006, Busch and Hayward 2009, Wingfield et al. 2011).

We found that, unlike solitary female polar bears, female polar bears with cubs had higher HCC levels than solitary males. These findings suggest that the presence or absence of dependent offspring may have a relatively strong influence on HCC levels in independent SH polar bears. Energy deficits are known to be associated with increased HPA activity (Busch and Hayward 2009), and the demands of lactation may be a key factor in this observation (Owen et al. 2005, Chow et al. 2011,Wingfield et al. 2011). Lactation is known to be especially costly in polar bears which fast during the first 2-3 months of lactation, produce milk with a high fat

content, and rely solely on maternal fat reserves to support mother and offspring until they return to the sea-ice to hunt (Arnould and Ramsay 1994). Polar bear cubs may also consume milk for 1.5 to 2.5 years and over multiple fasting seasons (Derocher et al. 1993b). Recently, elevated levels of corticosteroid binding globulin (CBG) have been recorded in adult female SH polar bears with cubs compared to those without (Chow et al. 2011). This may be evidence of an adaptive mechanism for buffering the catabolic effects of increased cortisol levels during the lactation period (Chow et al. 2011, Wingfield et al. 2011). Our hypothesis is also supported by elevated glucocorticoid levels recorded in other bears (giant panda, *Ailuropoda melanoleuca*) during the lactation-offspring attendance period. (Owen et al. 2005). Furthermore, female polar bears with cubs of the year produce milk of the highest fat and energy content (Derocher et al. 1993b, Arnould and Ramsay 1994), and we found that these bears also had higher HCC levels than their counterparts with yearlings. This finding agrees with reports of elevated glucocorticoid levels in early lactation compared to weaning in other mammals (e.g. Goymann et al. 2001, Fukasawa et al. 2008, Dantzer et al. 2010), and may provide additional evidence that lactation is an important determinant of HCC levels in SH polar bears.

In addition to functioning as effectors of the stress response, glucocorticoids play an important role in energy metabolism and catabolic processes related to growth and development (Sapolsky et al. 2000, Landys et al. 2006, Busch and Hayward 2009). As such, the energetic demands of rapid growth in early life (Atkinson et al. 1996, Derocher and Stirling 1998a, Derocher et al. 2005) may account for the elevated HCC levels we recorded in dependent male polar bears compared to solitary males. Polar bears are also a sexually dimorphic species in which the growth rate, size, and mass of males overtakes that of females around 1 year of age (Atkinson et al. 1996, Derocher and Stirling 1998a, Derocher et al. 2005). Although we found no statistically significant difference in HCC levels among dependent polar bears of either sex, we did observe a trend toward higher HCC levels in dependent males than females. We also found that dependent male SH polar bears were longer and weighed more than their female counterparts. These observations may offer added support for the importance of the juvenile growth period as a contributor to HCC levels measured in male polar bear cubs.

Hair cortisol levels in dependent polar bears may also be influenced by the energetic benefits of milk consumption (Arnould and Ramsay 1994, Derocher and Stirling 1996, Busch

and Hayward 2009). Milk is readily available, requires little effort to obtain, and may buffer cubs from nutritional deficits; at least to the point at which the quantity or quality of their mother's milk decreases (Arnould and Ramsay 1994, Derocher and Stirling 1996). Relatively high HCC levels in dependent males, despite the availability of milk, may further highlight the importance of rapid growth in this group. In contrast, the lack of difference in HCC among dependent female polar bears and other sex-reproductive classes may reflect an interaction between milk consumption and slower growth compared to dependent males (Atkinson et al. 1996, Busch and Hayward 2009). Other aspects of milk consumption may also contribute to HCC levels measured in polar bear cubs. For example, a combination of relatively rapid growth and the direct transfer of maternal cortisol in milk may explain our observation of marginally higher HCC levels in polar bear cubs of the year than in dependent yearlings (Arnould and Ramsay 1994, Sullivan et al. 2010). Competition for limited maternal resources among littermates may also be relevant (Derocher and Stirling 1996, Derocher and Stirling 1998b, Hudson et al. 2011).

When compared to solitary polar bears, HCC levels measured in females and their cubs may also reflect the added demands of increased exposure to or decreased tolerance of exogenous stressors related to life history or anthropogenic disturbances (Landys et al. 2006, Derocher et al. 2004, Maestripieri et al. 2008). For instance, nutritional stress during the onshore fasting period is considered the principal life history stressor faced by the SH subpopulation (Obbard et al. 2006, 2007). Here, female polar bears with cubs of the year may have fasted for up to 8 months before they return to the sea-ice in the year of their cub's birth (Obbard et al. 2007). Similarly, females with older cubs arrive onshore before other bears and both groups also generally return to the sea-ice later (M. Cattet, personal communication). Prolonged fasting along with the concurrent demands of lactation and offspring care may indicate that female polar bears with cubs are subject to greater energetic deficits in the onshore period than other sex-reproductive classes (Ramsay and Stirling 1988, Thiemann et al. 2006, Busch and Hayward 2009). Indeed, we observed that this group was in relatively poor body condition compared to all other bears. The demands of lactation while fasting may also be linked with increased mobilization of adipose tissue (Thiemann et al. 2006) and an increase in exposure to bio-available contaminants in mother and offspring (Sonne 2010, Bytingsvik et al. 2012). Elevated levels of some organochlorine pesticides may be associated with increased HPA activity in polar

bears (Øskam et al. 2004), and these contaminants have been recorded in tissues from SH polar bears (McKinney et al. 2011).

Female polar bears with dependent offspring also avoid or interact aggressively with adult male bears which are known to kill and consume cubs (Ramsay and Stirling 1986, Derocher and Stirling 1990). As such, social stress related to repeated or prolonged harassment by males may contribute to elevated HCC in family groups (Goymann et al. 2001, Bartolomucci et al. 2005). Among anthropogenic disturbances, human activity has also been shown to alter the movement and behaviour of female polar bears with cubs more than other bears (Andersen and Aars 2008). Locomotion in this species is energetically inefficient (Hurst et al. 1982) and the demands of long-distance travel to find food and avoid males or humans may further influence HCC levels in female polar bears and their dependent cubs (Landys et al. 2006, Derocher and Stirling 1990).

Compared to family groups, solitary female polar bears may exhibit reduced energetic costs related to: 1) growth or lactation-offspring care, and 2) stressors possibly more prominent in family groups (e.g. toxins) (Derocher et al. 1993b, Arnould and Ramsay 1994, Sonne 2010). Despite this, we found that HCC values in solitary female polar bears, females with dependent offspring, and dependent juveniles were similar. Unlike females with cubs [which enter a period of lactational anestrous (Amstrup 2003)] or dependent juveniles [which are not sexually mature (Obbard et al. 2007)], solitary female polar bears may undergo prolonged and repeated periods of estrous during the breeding season (Amstrup 2003). Increasing levels of estrogens which occur during estrous may be associated with up-regulation of CBG production (Chow et al. 2010). This may indicate that elevated levels of cortisol also occur in solitary female polar bears in this period. As part of an integrated measure of HPA axis activity, it is possible that this seasonal increase may supersede the effects of other factors which may reduce HCC levels in this group.

Reproductive condition may also influence HCC in solitary male polar bears which incur relatively high energetic costs in the breeding season during long distance travels to locate females and in male-male contest competition (Hurst et al. 1982, Ramsay and Stirling 1986, Derocher et al. 2010). Furthermore, breeding behaviour may reduce the time available for foraging and induce fasting (Derocher et al. 1990, Cherry et al. 2009) which, in turn, may elevate both cortisol and CBG levels in this group (Hamilton 2007, Chow et al. 2011). In the same

period, elevated testosterone may also increase free cortisol levels by down regulating CBG production (Howell-Skalla et al. 2002, Chow et al. 2010). Nevertheless, unlike other SH polar bears, solitary males are known to delay their return to shore which may provide increased foraging opportunities and energy reserves just prior to the critical fasting period (M. Cattet, personal communication). In addition, this group may be able to minimize energetic deficits throughout the year by stealing food from other bears (Stirling 1974) or consuming a wider variety of energy rich prey not available to smaller conspecifics [(e.g. walrus (*Odobenus rosmarus*) and bearded seals (*Erignathus barbatus*) Calvert and Stirling 1990, Thiemann et al. 2011)]. Large size and social dominance (Derocher and Stirling 1990, Derocher et al. 2010) may also allow solitary males to control prime foraging areas on sea-ice as well as opportunistic food sources (e.g. whale carcasses) and near-shore habitat during the onshore fasting period (Derocher and Stirling 1990, Miller et al. 2006). Since the majority of the hair growth period for the SH subpopulation is believed to occur during the onshore fasting period, advantages related to enhanced foraging success or social dominance prior to or in this period may best explain the trend toward lower HCC levels we observed in solitary males.

The direct association between HCC and age may indicate that older SH polar bears are less able to cope with the energetic demands of natural life history processes or stressors (McEwen 2002), may be more reactive to these stimuli (Rothuizen et al. 1991, Smith et al. 2005), or may recover more slowly from energetic challenges than their younger counterparts (Reeder and Kramer 2005). This observation also agrees with age related HPA axis deregulation that has been previously recorded in other species (Rothuizen et al. 1991, Smith et al. 2005, Reeder and Kramer 2005).

Interestingly, HCC values we determined in live-captured polar bears from SH were less than HCC levels reported in two recent studies of hunter harvested polar bears from East Greenland [mean 9.5 pg/mg range 5.5- 19.9 pg/mg, n = 17 (Bechshøft et al. 2011), mean 12.8 pg/mg range 3.98- 24.42 pg/mg, n = 88 (Bechshøft et al. 2012)]. Unlike our study, Bechshøft et al. (2011) and (2012) also identified no difference in HCC levels among polar bears of different ages, sexes, or age classes (subadult, adult). We believe procedural differences were unlikely to have greatly altered HCC measured among studies, and these discrepancies may be evidence that HCC reflects the effects of different conditions experienced by polar bear populations.

Presently, climatic warming and related decreases in the duration and extent of annual sea ice are considered the principal factors which threaten SH polar bears (Obbard et al. 2006, 2007). In this investigation all bears were captured during the onshore fast, and we found that HCC levels were lowest in animals captured earlier in this period. This finding agrees with previous studies which identified increasing serum cortisol levels in fasting SH polar bears (Hamilton 2007), and suggests that long-term stress levels may increase as the onshore period progresses. Longitudinal studies of hair growth (including ACTH and dexamethasone challenges) in conjunction with HCC analysis in fasting polar bears are required to confirm this hypothesis. However, this may signify that as the length of the ice-free season increases (ACIA 2005) SH polar bears could experience greater or more prolonged elevations in cortisol during the onshore period. Importantly, the patterns of HCC we recorded indicate that increasing stress levels may have the greatest impact on female polar bears and cubs with potential consequences for the long-term sustainability of this subpopulation (Obbard et al. 2006, 2007).

For example, long-term stress may inhibit the secretion or release of essential sex hormones (Habib et al. 2001) which may diminish reproductive rates in females by reducing reproductive behaviours (Sapolsky et al. 2000), conception rates, or pregnancy success (Ebbesen et al. 2009, Latendresse 2009, Sheriff et al. 2009). Maternal stress during gestation may also increase HPA activity and cortisol levels in offspring (HPA axis programming; Seckel 2004). HPA axis programming may be an adaptive response which prepares offspring to cope in stressful environments (Love et al. 2013, Dantzer et al. 2013). However, HPA axis programming may also lead to abnormal fetal development along with reduced growth and diminished viability in neonates (Meaney et al. 2007, Sheriff et al. 2009). These effects may be multigenerational with persistent increases in HPA activity and reduced survival or fecundity also affecting the litters of offspring born to stressed females (Sheriff et al. 2010, Dunn et al. 2010). In some mammals, declining condition and increasing cortisol may also play a role in reducing milk production and in the abandonment of offspring (Guinet et al. 2004). If similar responses occur in polar bears, the survival rates of cubs could diminish with possible implications for population recruitment (Derocher and Stirling 1996). Long-term stress may also reduce growth potential in surviving juveniles (Habib et al. 2001) which may adversely impact their fitness (Atkinson and Ramsay 1995, Derocher and Stirling 1996, Derocher et al. 2010).

Importantly, we observed a direct association between HCC in female polar bears and their dependent cubs of the year along with a direct (but non-significant) association between HCC in females and dependent yearlings. These findings agree with HCC relationships recently described in captive non-human primates (Fairbanks et al. 2011) and may provide evidence that HPA axis programming occurs in SH polar bears. While not mutually exclusive, these observations may also reflect: 1) similar exposure and response to stressors in family groups (Stenius et al. 2007), (2) the genetic component of HPA activity in polar bears (Fairbanks et al. 2011), or 3) the effects of cub age on cortisol transfer in milk (Arnould and Ramsay 1994, Sullivan et al. 2010), and may underscore the potential importance of long-term stressors encountered by SH polar bears in early life.

3.5.3 Factors influencing growth in Southern Hudson Bay Polar Bears

Biological attributes are acknowledged to be closely tied to body size and condition in polar bears (e.g. Derocher and Stirling 1994, 1998a, Derocher et al. 2005) and not surprisingly, we found that age and sex-reproductive class were the most important predictors of length (R^2 80 %), mass (R^2 83 %), and BCI (R^2 38 %) in the SH subpopulation. Direct and positive curvilinear associations between age and all growth indices also suggest that the effect of age on size becomes less as SH polar bears get older and agree with known patterns of growth in this species (Atkinson et al. 1996, Derocher and Wiig 2002, Derocher et al. 2005).

We also determined that time (birth year) and early life sea-ice conditions together added an additional 2 % to the explained variation in length and mass. Conversely, time (capture year) only predicted BCI but alone explained 2% of the variation in its measurement. These findings may indicate that, in SH polar bears, more recent environmental conditions have a stronger impact on BCI compared to length and mass and also agree with known patterns of growth in this species. In polar bears, body length reflects skeletal size and can only be affected by exogenous factors during the early life period when growth is occurring (Atkinson et al. 1996). Conversely, body mass reflects both skeletal size and soft tissue weight and is therefore subject to conditions encountered in early life as well as those which may vary on an seasonal basis (Atkinson and Ramsay 1995, Atkinson et al. 1996, Ramsay and Stirling 1988). BCI is defined as the combination of mass of fat and skeletal muscle relative to body size which reveals the impacts of recent conditions and can vary in animals of any age or body size (Cattet et al. 2002).

Significantly, we also found that HCC was inversely associated with all growth indices so that bears with higher HCC were smaller, lighter, and in poor body condition compared to other bears. However, HCC was a poor predictor of length (ΔR^2 0 %) and a relatively weak predictor of mass (ΔR^2 1 %) and BCI (ΔR^2 1 %). Given the seasonal nature hair growth (and thus HCC; Macbeth et al. 2010) along with the relative importance of skeletal size, the limited utility of HCC as a predictor of polar bear length and mass was not entirely unexpected. The low proportion of variation in BCI explained by HCC was more surprising. Presently, the SH polar bear subpopulation is considered to be stable (IUCN PBSG 2010), and it is possible that recent conditions in this region are within the reaction norm of this species (Landys et al. 2006). Accordingly, one explanation may be that few bears in this study were in truly poor body condition making the probability of detecting a strong association between these variables low.

BCI scores range from +3.00 (excellent) to -3.00 (poor) (Cattet et al. 2002) and all but one bear in this study had BCI scores of greater than -1.90 (mean BCI score -0.21, range -2.19 to 1.26, *n*=185). Of bears with the 5 lowest BCI scores, 4/5 had HCC values in the highest 10% of all HCC values we determined in SH polar bears (HCC $\geq$ 0.93 pg/mg). It is likely that the importance of HCC as a predictor of BCI would increase if a larger group of polar bears representing greater extremes in BCI scores were examined. Adding support to this hypothesis were the HCC levels we also measured in three polar bears killed 400 km inland at Deline, Northwest Territories, Canada (Southern Beaufort Sea subpopulation; Thiemann et al. 2008). HCC levels in this family group were among the highest we have recorded in polar bears, and BCI scores in these individuals were also exceptionally poor [(adult female: HCC 5.92 pg/mg, BCI -3.24), (dependent female: HCC 1.01 pg/mg, BCI -2.25), and (dependent male: HCC 19.98 pg/mg, BCI -2.07)]. All considered, these findings provide preliminary evidence that long-term stress may be associated with diminished growth in SH polar bears, and that HCC may also show potential as a biomarker of long-term stress in this subpopulation (Busch and Hayward 2009).

As in the Southern Hudson Bay subpopulation (Obbard et al. 2006, Obbard et al. 2007), diminished size or body condition has been recorded in polar bears from Western Hudson Bay (Derocher and Stirling 1994, Atkinson et al. 1996a, Derocher and Stirling 1998a), the Southern Beaufort Sea (Rode et al. 2010a) and at Svalbard (Derocher and Wiig 2002, Derocher 2005). The precise cause of these declines have not yet been fully elucidated and a variety of potential

sources of long-term stress (or combination of sources) including climatic warming (Atkinson et al. 1996, Rhode et al. 2010a), marine ecosystem productivity, population density, prey availability (Derocher and Wiig 2002, Derocher 2005), or chemical contaminants (Derocher and Wiig 2002, Derocher et al. 2003) could be important.

Wildlife managers seeking to understand linkages between ecological conditions, long-term stress, health, and population performance in polar bears may benefit from the development of techniques to remotely assess these parameters. Our findings suggest that the determination of HCC may have the potential to be a practical and cost effective tool with which to achieve these objectives (Busch and Hayward 2009, Macbeth et al. 2010). We found that HCC in SH polar bears was influenced by age, sex, family group status, and hair sampling period but not by body region or hair type. HCC also did not change during extended periods of laboratory storage and could be measured in quantities of hair which may be collected opportunistically or through the use of non-invasive barb-wire snagging methods in bears. These novel findings identify key considerations which may guide future HCC studies in polar bears. We also found preliminary evidence that HCC measured in polar bears from the SH subpopulation may be inversely associated with growth indices directly related to fitness in this species. Nonetheless, our work may have been limited by the small range of ancillary data available for statistical analyses, and possibly by the fact that the overall health status of the SH polar bear subpopulation may be within normal limits at the present time. More research is therefore necessary to fully validate HCC as a biomarker of long term stress in polar bears. Our study provides baseline stress data for the SH subpopulation and regional studies tracking annual changes in HCC levels along with possible relationships between HCC and sea-ice conditions, weather patterns, marine productivity, prey or population density, and toxins may be useful in this regard (Derocher and Wiig 2002, Derocher et al. 2003, Derocher 2005, Rosing-Asvid 2006). The consideration of health status in individual polar bears including factors related to disease (Burek et al. 2008, Kirk et al 2010a, Kirk et al. 2010b), injury (Ramsay and Stirling 1986), or repeated capture for research (Cattet et al. 2008a) may also be warranted. Comparative HCC analysis among polar bear subpopulations that are stable, decreasing, or increasing (IUCN PBSG 2010) will also be required to fully evaluate the utility of this technique as a tool for polar bear conservation. These objectives are feasible as hair from polar bears is often collected and archived as part of ongoing

research or hunting, and may also be gathered remotely (via barb-wire snagging) (Herreman and Peacock 2011).

CHAPTER 4[a]

HAIR CORTISOL CONCENTRATION AFTER ADRENOCORTICOTROPIC HORMONE (ACTH) CHALLENGE IN CAPTIVE ALASKAN CARIBOU (*Rangifer tarandus granti*) AND REINDEER (*Rangifer tarandus tarandus*)

This investigation was performed as the third of four studies included in this thesis and was undertaken to evaluate the utility of hair cortisol analysis in caribou-reindeer (*Rangifer tarandus* sp.). As for grizzly bears (*Ursus arctos*) and polar bears (*Ursus maritimus*), long-term stress related to landscape or climatic conditions may be an important consideration in the health and conservation status of free-ranging *Rangifer* populations across their circumpolar range. In this study, hair was collected from captive Alaskan caribou (*R. t. granti*) and captive domestic reindeer *(R. t. tarandus)*. Hair cortisol techniques developed for use in grizzly bears (Chapter 2) and polar bears (Chapter 3) were then modified for use in this species. Laboratory validation was performed to determine if hair cortisol could be accurately and reliably measured in *Rangifer* hair, the effects of procedural considerations and selected biological factors which may influence hair cortisol concentration were explored, and considerations for the prudent use of hair cortisol analysis in caribou-reindeer were developed. Critically, this study expanded on previous work by evaluating the effect of acute stress (simulated by the administration of Adrenocorticotropic hormone, ACTH) on hair cortisol concentration. The results of this study also guided the application of hair cortisol analysis in free-ranging caribou from West Greenland (Chapter 5).

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*In this study B.J. Macbeth was responsible for study design, analysis, and interpretation related to hair cortisol.

4.1 Introduction

Human-caused climatic warming and landscape change have been implicated in the synchronous declines now being recorded in many caribou and reindeer herds (Vors and Boyce 2009). While evidence that anthropogenic factors negatively impact *Rangifer* is unambiguous (Vistnes and Nellemann 2008, Vors and Boyce 2009, Gunn 2011), the overall importance of and biological mechanisms behind these relationships remain poorly understood. This knowledge gap is further complicated by interactions between human-caused factors, natural population oscillations (Gunn 2003) and density dependent processes (Couturier et al. 2009a, Couturier et al. 2010, Mahoney et al. 2011) along with the highly variable life history strategies (Festa-Bianchet et al. 2011) and environmental conditions encountered by *Rangifer* across their vast distributional range (Vors and Boyce 2009, Tyler 2010, Joly et al. 2011). With the sustainability of many populations now at risk (Vors and Boyce 2009, Festa-Bianchet et al. 2011, CARMA 2012a), there is a pressing need to enhance our general understanding of *Rangife*r population dynamics and our ability to distinguish the relative importance of different drivers of population performance in this ecologically, economically, and culturally important species (Boudreau and Payette 2004, Mason et al. 2007, Brook et al. 2009).

Long-term physiological stress (occurring over weeks to months) is increasingly being recognized as an important factor which may link ecological change with impaired health and population performance in wildlife (Wikelski and Cook 2006, Sheriff et al. 2010, Ellis et al. 2012). For caribou, human-caused ecological change may act as a persistent source of stress above and beyond the more predictable demands of normal life history processes (e.g. migration, reproduction, feeding) (Whitfield and Russell 2005, Landys et al. 2006, Gunn et al. 2009), and the study of long-term stress may provide a useful approach with which to achieve these goals (Freeman 2008, Ashley et al. 2011, Wasser et al. 2011).

The mammalian stress response is mediated by the Hypothalamic-Pituitary Adrenal (HPA) axis and its glucocorticoid effector hormones (cortisol in *Rangifer*; Säkkinen et al. 2004, Omsjoe et al. 2009). The measurement of cortisol in hair has recently been recognized as a promising and potentially advantageous biomarker of long-term stress in free-ranging wildlife (Koren et al 2002, Macbeth et al. 2010, Bechshøft et al. 2011). However, this technique has not yet been evaluated in *Rangifer*. Hair samples from this species are likely to be readily available

from animals collected or captured as part of ongoing research programs (B. Elkin, personal communication, Cuyler and Ørtisland 2002, Duffy et al. 2005, Drucker et al. 2010). Caribou and reindeer are also an important food source in many northern communities and gathering hair from harvested animals may offer a strategy with which to engage local communities in conservation initiatives (Anderson 2000, Mahoney 2007, Brook et al. 2009). Importantly, the relative accessibility of captive *Rangifer* herds may provide unique opportunities to explore HCC analysis under controlled conditions not readily available for other large wild species.

Hair cortisol analysis is a rapidly emerging field (Gow et al. 2010). However, a controlled test of the potential effects of short-term stress on HCC has not yet been evaluated in any species. The incorporation of cortisol from the blood stream into hair is thought to occur over a period of approximately 3 days (Pragst and Balikova 2006, Kidwell and Smith 2007) and is believed to be restricted to the phase of active hair growth (anagen) in individual hair follicles (Davenport et al. 2006, Pragst and Balikova 2006, Gow et al. 2010). Accordingly, HCC should not be as sensitive to short-term stress as are other measures of HPA axis activity commonly employed in wildlife studies [e.g. serum cortisol (Säkkinen et al. 2004, Omsjoe et al. 2009), salivary cortisol (Millspaugh et al. 2002) or glucocorticoids measured in urine (Constable et al. 2006) and feces (Wasser et al. 2011)]. Nonetheless, human hair follicles (*in vitro*) have been shown to display a functional equivalent to the HPA axis and synthesize cortisol (Ito et al. 2005). Furthermore, while this HPA axis analogue may operate independently, it may also be influenced by systemic HPA axis activity (Ito et al. 2005, Arck et al. 2006). These findings may indicate that the effects of both short-term stress [e.g. capture and handling (Omsjoe et al. 2009)] and localized irritants [e.g. ectoparasites (Dove and Cushing 1933, Welch et al. 1990), skin disease (Rehbinder and Mattson 1994, Ayroud et al. 1995), or radio collars (Krausman et al. 2004)] on HCC may be greater than are currently recognized (Macbeth et al. 2010).

Adrenocorticotropic hormone (ACTH) has been used successfully in *Rangifer* to artificially elevate plasma cortisol concentration and effectively mimics an episode of acute stress in this species (Säkkinen et al. 2005, Freeman 2008). Measuring HCC in captive *Rangifer* subjected to an ACTH challenge may provide insight into the importance of short-term stress on HCC with broad implications for the utility of this technique in caribou and other mammals. The identification of factors which may influence cortisol in *Rangifer* hair is also a critical first step

in the evaluation of HCC as a potential biomarker of long-term stress and conservation tool for use in free-ranging *Rangifer* populations (Busch and Hayward 2009, Macbeth et al. 2010).

4.2. Objectives

The purpose of this investigation was: 1) to modify HCC techniques developed in grizzly bears and polar bears (Macbeth et al. 2010, Chapter 3) for use in *Rangifer*, 2) to examine the effects of body region, sex, and subspecies on HCC in captive Alaskan caribou (*Rangifer tarandus granti*) and reindeer (*Rangifer tarandus tarandus*), and 3) to determine if short-term activation of the HPA axis (caused by ACTH administration) alters HCC measured in *Rangifer*.

4.3 Materials and Methods

4.3.1 Animals

Twelve captive Alaskan caribou (n = 6 males, n = 6 females) and 12 captive reindeer (n = 6 males, n = 6 females) maintained at the University of Alaska, Fairbanks, Robert G. White Large Animal Research Facility, were used in this investigation. A detailed description of the origin of study animals, their housing, diet, and care is reported in Ashley et al. (2011). All reindeer were 0.8 years old at the start of this investigation, and females were maintained separately from males and were not pregnant. Conversely, female caribou ranged in age from 4 to 14 years (mean 9.0 years) at the start of the study, while male caribou were between 1.8 and 7.8 years old (mean 4.0 years). All female caribou had also been held with males in the preceding breeding season and at least five of six were pregnant throughout the study period (Ashley et al. 2011).

4.3.2 ACTH challenges

ACTH challenges were performed by P. Barboza and colleagues (Institute of Arctic Biology, University of Alaska Fairbanks, Fairbanks, Alaska, USA) and the following description of protocols used is modified after that reported in detail in Ashley et al. (2011). In February, 2008 all animals were separated into outdoor pens based on sex and subspecies. Animals were then trained to move from holding pens to a chute complex-handling area. ACTH challenges were initiated (Trial 1) on March 4, 2008 for reindeer and on March 5, 2008 for caribou. 15 minutes prior to ACTH challenge, all animals were moved to the chute complex-handling area. Five adult male caribou-reindeer and 5 adult female caribou-reindeer were then injected with 2 IU/kg ACTH [Meds for Vets Inc. Sandy, Utah, USA; intramuscular (IM) injection (18 gauge

needle: total volume divided equally between right and left shoulder)]. In both subspecies, 1 male and 1 female were injected with physiological saline (Butler Animal Health, Dublin, Ohio, USA) and served as controls. All injections were administered sequentially. Following sampling, all animals were returned to holding pens. A second ACTH challenge (Trial 2) with an increased dose (8 IU/kg) was performed in the same reindeer on June 17, 2008. Due to logistical and budgetary constraints, caribou were not re-examined in Trial 2. Reindeer were halter trained and would stand to be tethered and restrained for ACTH or saline administration and later for blood and hair sampling. In contrast, caribou were not tractable and had to be held in a squeeze chute or manually restrained for both procedures (P. Barboza, personal communication).

4.3.3 Hair sample collection, storage, and handling

Hair samples used in body region, sex, and subspecies analyses were collected prior to ACTH or saline administration in March, 2008 (Trial 1). For the first ACTH trial in both subspecies, hair was sampled one week before and one week after ACTH or saline injection from three body regions: 1) the neck 2) the shoulder, and 3) the rump (Ashley et al. 2011). For the second ACTH trial in reindeer, hair was sampled from the shoulder only (but from different sites than in the first trial) one week before and two and 16 weeks after ACTH or saline injection (Ashley et al. 2011). In both Trial 1 and Trial 2, hair was collected by shaving as close to the skin as possible with electric clippers. Clipped hair was then stored in clear plastic bags (tops left open to prevent condensation), at room temperature, and in the dark. After ACTH or saline administration, 100 hairs were also plucked from each body region in each animal to obtain intact hair shafts for growth stage analysis using trichograms and special staining techniques (Diaz et al. 2004a, Baden et al. 1979). Hair was sampled from the left side of the body before ACTH administration and from the right side of the body afterwards (Ashley et al. 2011).

In both subspecies, some hair samples originating from the neck were predominantly white while those collected from the shoulder and rump were a more uniform mixture of white-brown, white-grey, or grey brown (B. Macbeth, personal observation). However, in one caribou, shoulder and rump hair samples appeared predominantly red to brown (B. Macbeth, personal observation). In most cases, hair samples obtained pre and post ACTH challenges (i.e. from each body region in each individual) were grossly similar in colour (B. Macbeth, personal observation). All hair samples appeared somewhat weathered (B. Macbeth, personal

observation). The surface of all hair samples (from both subspecies) was also covered in a small quantity of dirt and dust but was free of visible contamination with blood or feces (B. Macbeth, personal observation). Unlike reindeer, most hair samples from caribou were fragmented in many places across the length of the hair shaft (a probable artifact of shaving intractable animals) making direct comparison of hair morphology between subspecies difficult (B. Macbeth, personal observation). We also found that it was not practical to separate guard hair and undercoat in either subspecies [due to large hollow guard hairs with very small, fine, and adherent undercoat (B. Macbeth, personal observation)]. Similar difficulties have been reported in other *Rangifer* hair studies (Cuyler and Ørtisland 2002). As a result, HCC analyses for all *Rangifer* investigations in this research program (Chapter 4 and Chapter 5) were performed on samples containing both hair types.

4.3.4 Removal of surface contamination

The decontamination protocol used in this study was modified after those reported in detail in Macbeth et al. (2010) and Chapter 3 for grizzly bears (*Ursus arctos*) and polar bears (*Ursus maritimus*), and a standardized wash protocol using 3-three minute washes with 0.10 ml methanol per mg hair was developed for use in *Rangifer*. Procedural efficacy and wash kinetics were examined in clean (no visible blood contamination), moderately contaminated (approximately 25% hair surface covered with blood) and severely contaminated caribou hair (> 80% of hair surface covered with blood) obtained from three barren ground caribou (*R. t. groenlandicus*) killed during research hunts on Southampton Island, Nunavut, Canada (n = 2) and in West Greenland (n = 1). To explore wash kinetics, 100 mg hair samples were exposed to an extended wash protocol using 6 washes only (3-minutes per wash/0.10 ml methanol per mg hair). To confirm the standardized wash protocol did not extract intrinsic cortisol from the hair shaft HCC was then compared in clean hair from 11 captive caribou washed three or 6 times (Macbeth et al. 2010).

4.3.5 Hair preparation and steroid extraction

Hair preparation and steroid extraction protocols were also modified after techniques reported in Macbeth et al. (2010) and Chapter 3 for grizzly bears and polar bears. For *Rangifer*, 100 mg hair samples were washed three times with 10 ml methanol for three minutes per wash on a slow rotator (Haematology and Chemistry Mixer 346, Fisher Scientific, Ottawa, Ontario,

Canada). After washing and drying (48 hours/room temperature), hair was ground for three minutes in a Retsch MM 301 Mixer Mill (Retsch Inc, Newtown, Pennsylvania, USA; 30 Hz; 10 ml stainless steel grinding jars; single 12 mm stainless steel grinding ball). Steroids were then extracted from the hair shaft by immersing 50 mg powdered hair in 1.0 ml of methanol (EMD Chemicals, Gibbstown, New Jersey, USA) for 24 hours. Following extraction, each sample was centrifuged, the supernatant collected, and transferred it to a glass test tube. Next, each sample was rinsed two separate times (1.0 ml of fresh methanol/rinse) and the additional supernatant was collected, pooled and dried at 38 ˚C under nitrogen gas. Dried extract was then concentrated by rinsing the sides of the test tube twice with 0.4 ml of methanol and once each with 0.3 ml and 0.2 ml of methanol. Concentrated and dried extract was then reconstituted with 0.4 ml of phosphate buffer and cortisol was assessed in duplicate 50 µl aliquots of reconstituted extract with an enzyme-linked immunoassay kit (Oxford EA-65 Cortisol EIA kit, Oxford Biomedical, Lansing, Michigan, USA). Cross reactivity of the antibody used in this EIA kit is reported in Macbeth et al. (2010).

4.3.6 Data Analysis

Data were analyzed using SPSS 17.0 (SPSS Statistics Version 17.0.0, August 23, 2008) with the level of significance set at $P \leq 0.05$. To meet assumptions of normality and homogeneity of variance all data were log transformed prior to analysis. Simple linear regression was used to assess parallelism between cortisol standards provided in the EIA kit and serially diluted hair extracts run in the same assay. In both subspecies, HCC among different body regions was assessed using one-way repeated measures analysis of variance (ANOVA) tests. The effects of sex and subspecies designation were evaluated with Independent samples *t*-tests using HCC values measured in hair collected prior to ACTH or saline administration in Trial 1 (March, 2008). Paired samples *t*-tests were used to compare HCC levels measured pre or post ACTH administration. Where hair samples from many body regions are available an average HCC value may be the best estimate of long-term HPA activity in an individual animal (Macbeth et al. 2010). As such, the effects of sex and ACTH were explored using whole body HCC and HCC from each body region where:

.....Whole body HCC =Σ (neck HCC+ shoulder HCC+ rump HCC)/3.....(4.1)

The effect of subspecies was explored using whole body HCC only. Importantly, control animals were used only as reference points and not statistically compared to ACTH treated animals owing to low samples size (n = 1 of each sex in each subspecies).

4.3.7 Hair growth stage analysis

Hair growth follows a cyclical pattern of alternating periods of active growth (anagen), transition (catagen) and quiescence (telogen) and the morphology of plucked hair may be useful in determining the stage of hair growth (Stenn and Paus 2001, Diaz et al. 2004a, Müntener et al. 2011). Trichograms use hair bulb morphology to identify stages of the hair cycle (Diaz et al. 2004a) and were performed on 100 plucked hairs from each body region in each animal to aid in the interpretation of HCC levels determined pre and post ACTH or saline administration. In an attempt to confirm results generated from this analysis, a randomly selected subsample of 25 hairs from each body region in each animal was then stained with 4-dimethylaminocinnamaldehyde (DOCA) (Baden et al. 1979).

4.4 Results

4.4.1 Hair processing

All *Rangifer* hair samples contained predominantly large, wide, and hollow guard hairs and were not easily immersed in wash solvent. Consequently, in order to achieve uniform submersion of hair during decontamination, the volume of methanol had to be increased (0.1 ml methanol /mg hair) relative to grizzly and polar bears (0.04ml methanol/mg hair). Severe static also caused difficulties in recovering ground hair from both caribou and reindeer (not encountered in grizzly bears or polar bears). As a result, the minimum quantity of ground hair which could be generated with any consistency was (50 mg) (B. Macbeth, personal observation), and the minimum quantity of unprocessed hair required to achieve this amount was 100 mg (approximately a 2 cm by 2cm plucked or shaved patch of hair) (B. Macbeth, personal observation). Owing to the larger quantity of ground hair, the volume of methanol used in extraction and amount of buffer used to reconstitute dried extract (1.0 ml and 0.4 ml) also had to be increased relative to grizzly bears and polar bears (0.5 ml and 0.2 ml). Furthermore, after extraction and centrifuging a distinct layer of thick, opaque, white to yellow material ranging in depth from < 1mm to approximately 3 mm was apparent on the surface of many samples of *Rangifer* hair extract. This unidentified material was noted in many samples from all body

regions (and in both sexes) of both subspecies (B. Macbeth personal observation). However, it appeared to occur with the greatest frequency in hair samples from the neck and in those collected after ACTH or saline injection (B. Macbeth, personal observation). It was also most prominent in hair samples obtained from caribou and within this subspecies it was observed in the greatest number of samples and in the largest quantities in hair from the neck and shoulder compared to the rump (B. Macbeth, personal observation). Similar body region based patterns were observed in some reindeer (B. Macbeth, personal observation) but were not as consistent. After drying affected samples, a significant quantity of a flakey, white to yellow, waxy substance remained at the bottom of the test tube (B. Macbeth, personal observation). In most samples, this material was easily returned to solution and (after vortexing) was evenly distributed within the reconstituted extract used to measure HCC. However, in some samples this material was difficult to return to solution and white flocculent material remained in the reconstituted extract (B. Macbeth, personal observation). Overall, the occurrence of this material was unique to the first extraction of hair and it was not generally observed (in extract or after drying) if the same hair sample was re-extracted for an additional 24 hours or 48 hours with fresh methanol (B. Macbeth, personal observation).

In the initial stages of this investigation we explored the possibility of removing this material using a variety of micro-centrifuge filtration tubes. However, the material blocked the filtration grid and this approach was inconsistent or unsuccessful even with prolonged centrifuging (≥ 30 minutes) at relatively high speed (≥ 5000 rpm) (B. Macbeth, personal observation). We also attempted manual removal (by pipette) but full recovery was not practical or possible (B. Macbeth, personal observation). Given these findings, the unknown composition, and since *Rangifer* hair extract had not previously been described, we ultimately elected to retain the material in samples used for HCC analysis in this study. A general quality control strategy was then adopted in which hair samples containing large quantities of flocculent material after reconstitution were not used. Instead, new hair samples from the same animal (and body region) were reprocessed in their entirety. Despite this, we could not eliminate all flocculent material from all hair samples in this study.

4.4.1.1 Assay performance

For *Rangifer* sp. hair extract, intra-assay coefficient of variation (C.V.) was 6.04% (n = 6) while inter-assay C.V. was 18.39% (n = 6). 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. Extraction efficiency was 94.53 ± 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). Furthermore, no additional cortisol could be measured when a previously extracted hair sample was re-extracted with fresh methanol for an additional 24 or 48 hours (i.e. 48 hrs or 72 hours total extraction time, n = 15 hair samples). Likewise, no extra cortisol could be recovered when an additional 0.4 ml of reconstitution buffer was added to leftover residue in the glass test tube (i.e. after 12 hours reconstitution) and reconstituted for a further 12 hours (n = 15 hair samples).

4.4.1.2 Decontamination protocol

Three, 3-minute washes with 0.1 ml methanol per mg hair were effective in removing all visible contamination (i.e. all visible dirt, debris or blood) from *Rangifer* sp. hair. Three washes also removed all measurable cortisol from the surface of clean (i.e. no visible blood contamination) and moderately contaminated (approximately 25% hair surface covered with blood) hair but did not remove all measurable cortisol from the surface of severely contaminated hair (> 80% of surface covered with blood) (Fig 4.1). Regardless of the degree of contamination, a single wash was effective at removing most (> 85%) of the measurable cortisol associated with the hair surface (Fig 4.1). A second wash was required to remove hair surface cortisol from moderately blood contaminated hair (Fig 4.1). Following one or two washes, cortisol was not detected in up to 5 additional washes of clean or moderately blood contaminated hair while hair surface cortisol was measurable in all six washes in severely blood contaminated hair (Fig 4.1). A comparison of hair shaft cortisol concentration in clean *Rangifer* hair washed three times or six times showed no difference (Paired samples t-test, $t_{(10)}$= 2.018, P = 0.0713, n = 11).

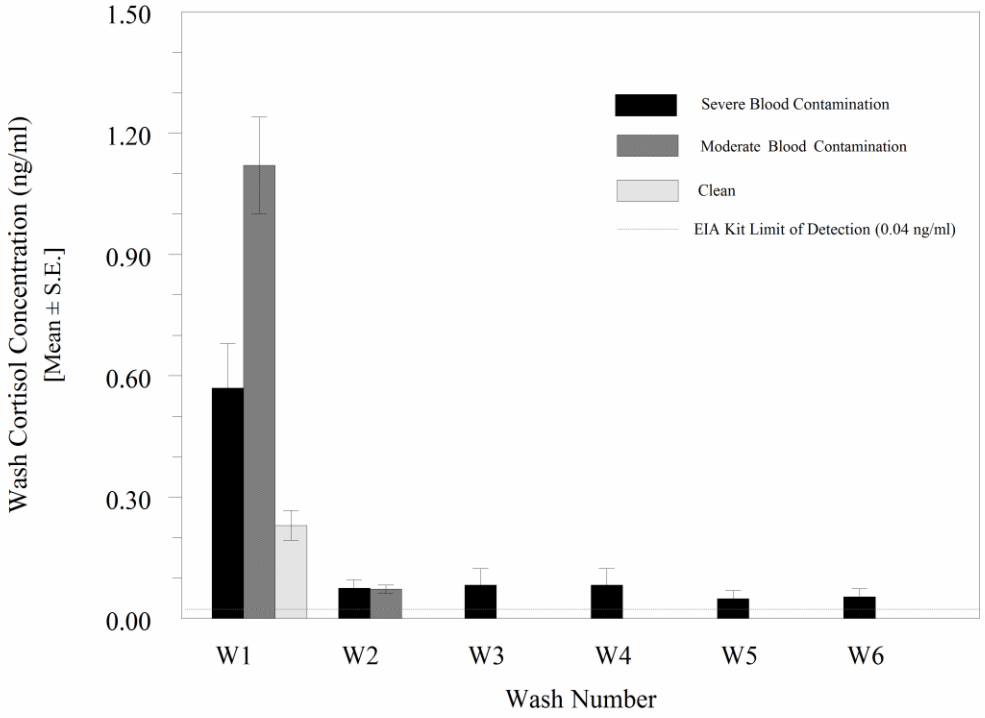

Figure 4.1 Dynamics of a wash procedure in caribou (*Rangifer tarandus groenlandicus*) hair exposed to six, 3-minute washes with 0.1 ml methanol per mg hair. Each wash specific cortisol concentration is the mean ± S.E. of 3 independent determinations in each category.

4.4.2 Features of hair and caribou-reindeer

4.4.2.1 Body regions

Hair cortisol concentration varied among samples collected from different body regions in caribou (one-way repeated measures ANOVA, $F_{(2.22)} = 118.89$, $P \leq 0.001$, $n = 12$) with highest values in the neck (Tukey-Kramer, $P < 0.001$) and lower values in the shoulder and rump (Fig 4.2). For caribou, within body regions, variation in HCC was greatest among samples taken from the neck (mean C.V. 75.54%, S.D. 21.90 %, $n = 12$), least in those collected from the shoulder (mean C.V. 11.52%, S.D. 9.13%, $n = 12$), and intermediate in samples from the rump (mean C.V. 17.04%, S.D. 13.78%, $n = 12$). Among body regions, HCC exhibited the opposite pattern in reindeer (one-way repeated measures ANOVA, $F_{(2, 20)} = 11.658$, $P \leq 0.001$, $n = 11$)

with the lowest levels in the neck (Tukey-Kramer, $P \leq 0.05$) and higher values in the shoulder and rump (Fig 4.3). However, within body regions, variation in HCC was also greatest for reindeer neck hair (mean C.V. 51.14%, S.D. 15.98 %, $n = 12$) and lower in hair from the shoulder (mean C.V. 16.04%, S.D. 6.89%, $n = 12$) and rump (mean C.V. 17.80%, S.D. 9.73%, $n = 12$).

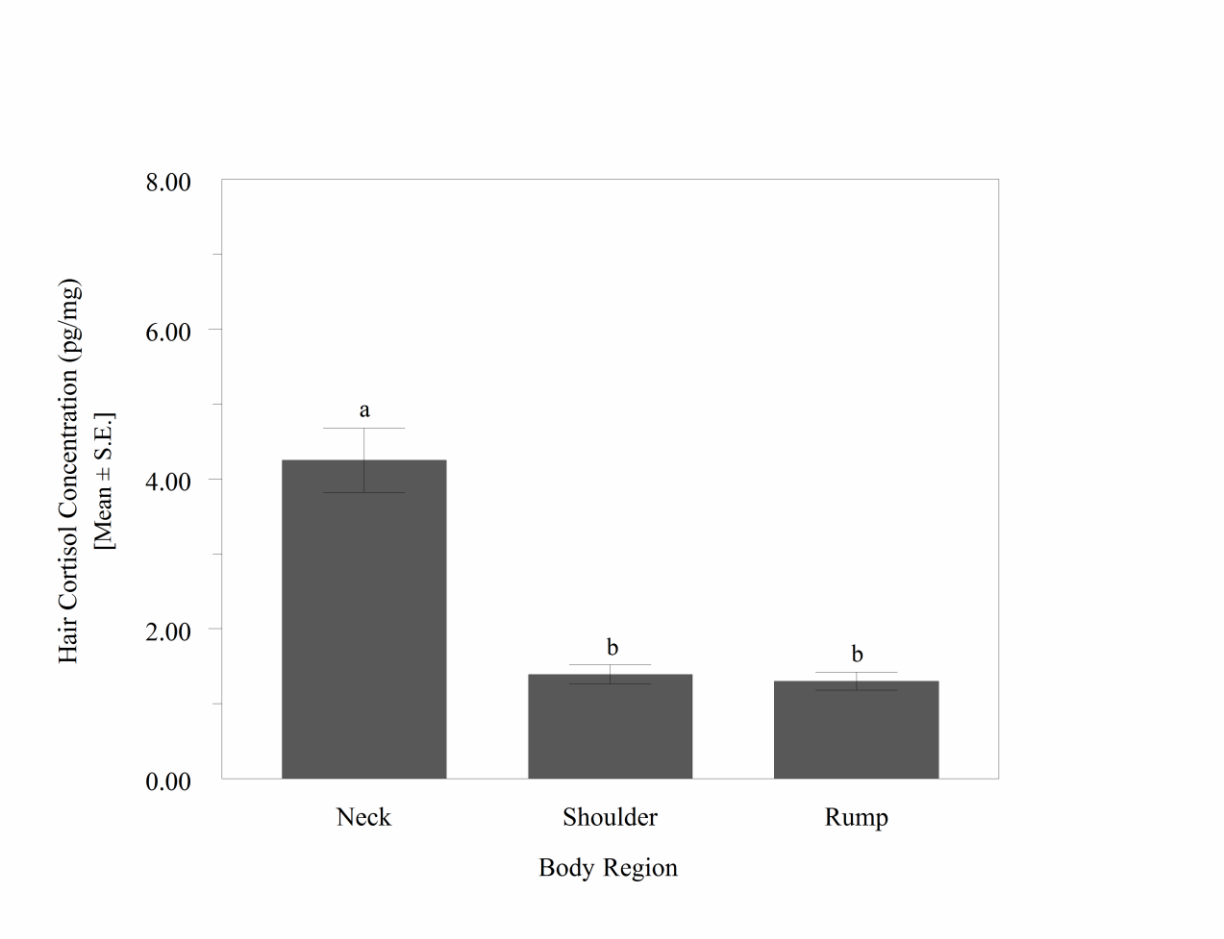

Figure 4.2 Comparison of hair cortisol concentration between neck, shoulder, and rump in the hair of $n = 12$ caribou (*Rangifer tarandus granti*). Mean values are represented by bars and S.E. represented by error bars. Significant differences (Tukey-Kramer, $P \leq 0.05$) in hair cortisol concentration between body regions are indicated by different letters.

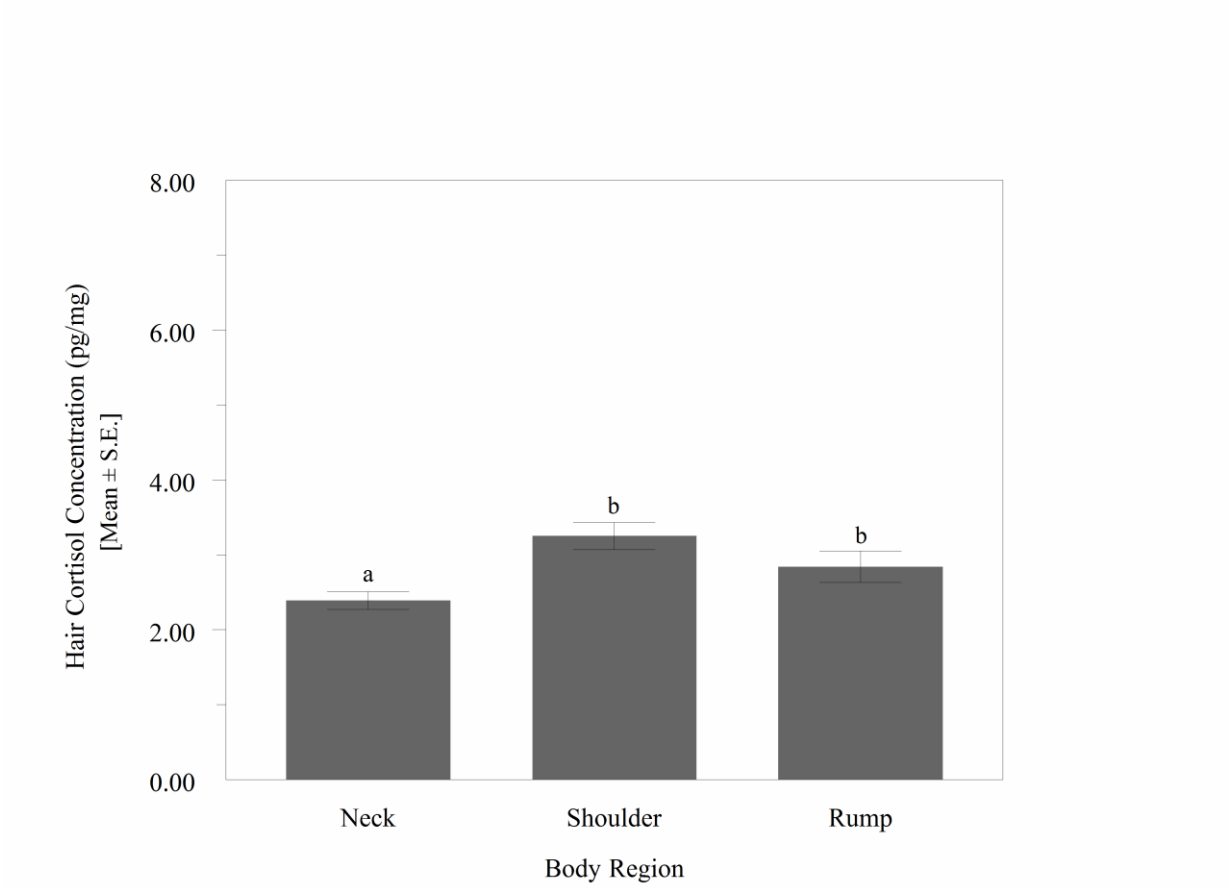

Figure 4.3 Comparison of hair cortisol concentration between neck, shoulder, and rump in the hair of $n = 11$ reindeer (*Rangifer tarandus tarandus*). Mean values are represented by bars and S.E. represented by error bars. Significant differences (Tukey-Kramer, $P \leq 0.05$) in hair cortisol concentration between body regions are indicated by different letters.

4.4.2.2 Range of hair cortisol values determined in captive caribou and reindeer

The mean HCC determined in caribou was 2.31 pg/mg (range 1.57-3.86 pg/mg), $n = 12$. The mean HCC determined in reindeer was 2.88 pg/mg (range 2.21-3.40 pg/mg), $n = 12$.

4.4.2.3 Sex

Whole body HCC levels were not different in male and female caribou (Independent samples *t*-test $t_{(10)} = 9.375^{-19}$, $P > 0.999$, $n = 12$). There was also no difference in HCC levels measured in either sex in any body region (all Independent samples *t*-tests, $P \geq 0.774$). Likewise, whole body HCC levels were not different in male and female reindeer (Independent samples *t*-

test $t_{(9)} = 1.626$, $P = 0.138$, $n = 11$) nor were differences observed in any body region (all Independent samples *t*-tests, $P \geq 0.125$).

4.4.2.4 Subspecies

Whole body HCC in reindeer was greater than whole body HCC in caribou (Independent samples *t*-test $t_{(21)} = 3.663$, $P = 0.002$, $n = 11$ reindeer, $n = 12$ caribou).

4.4.3 Hair cortisol concentration before and after ACTH or saline administration

4.4.3.1 Trial 1: caribou measured 7 days after injection

When whole body HCC was considered, HCC increased 7 days after ACTH administration (Paired samples *t*-test, $t_{(9)} = 6.237$, $P < 0.001$, $n = 10$). However, a similar pattern was observed in $n = 2$ saline injected controls (male control HCC: pre ACTH 0.82 pg/mg, post ACTH 1.64 pg/mg; female control HCC: pre ACTH, 0.83 pg/mg, post ACTH 1.33 pg/mg). When body regions were considered separately, no difference in HCC was apparent pre vs post ACTH injection in the neck (Paired samples *t*-test $t_{(9)} = 1.348$, $P = 0.211$, $n = 10$) or rump (Paired Samples *t*-test $t_{(9)} = 2.039$, $P = 0.072$, $n = 10$) (Fig 4.4). Nonetheless, in caribou shoulder hair HCC remained elevated post ACTH administration (Paired samples *t*-test $t_{(9)} = 9.284$, $P < 0.001$, $n = 10$) (Fig 4.4). The marginal result for rump hair also trended towards increased HCC post ACTH administration. However, similar patterns were observed in $n = 2$ saline injected controls (Fig 4.4).

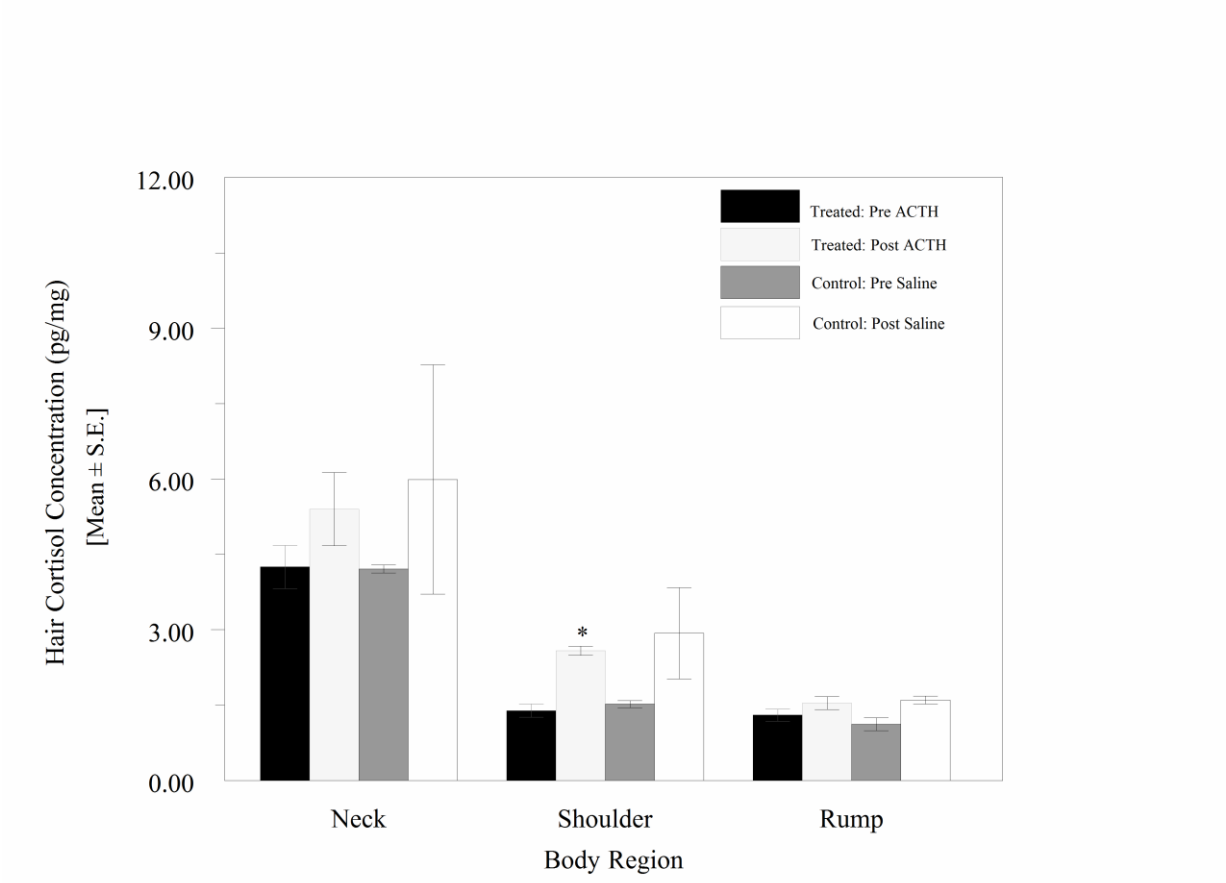

Figure 4.4 Hair cortisol concentration (HCC) pre and post Adrenocorticotropic hormone (ACTH) or saline administration in neck, shoulder, and rump hair of captive Alaskan caribou (*Rangifer tarandus granti*). Data are presented as mean HCC ± S.E. of hair cortisol determinations in $n = 10$ caribou. There is no significant difference (Paired samples *t*-tests, $P > 0.050$) in the cortisol concentration of caribou neck or rump hair pre and post ACTH administration. *There is a significant increase in HCC in shoulder hair (Paired samples *t*-test, $P < 0.001$) post ACTH administration. In all body regions the pattern of HCC pre and post injection in $n = 2$ control animals (injected with saline) is similar to that of ACTH treated animals.

4.4.3.2 Trial 1: Reindeer measured 7 days after injection

In reindeer, there was no difference in HCC measured before or 7 days after ACTH administration when whole body HCC [(Paired samples *t*-test $t_{(9)} = 0.932$, $P = 0.378$, $n = 10$) (male control HCC: pre ACTH 1.22 pg/mg, post ACTH 0.99 pg/mg; female control HCC: pre ACTH, 1.18 pg/mg, post ACTH 1.16 pg/mg)] or HCC among individual body regions were considered (neck: Paired samples *t*-test $t_{(9)} = 1.465$, $P = 0.177$, $n = 10$, shoulder: Paired samples

t-test $t_{(8)} = 1.090$, $P = 0.308$, $n = 9$, rump: Paired samples *t*-test $t_{(9)} = 2.238$, $P = 0.052$, $n = 10$) (Fig 4.5). Furthermore, in reindeer rump hair, a marginally non significant result trended to less HCC measured post ACTH administration. As in caribou, similar patterns were observed in $n = 2$ saline injected controls (Fig 4.5).

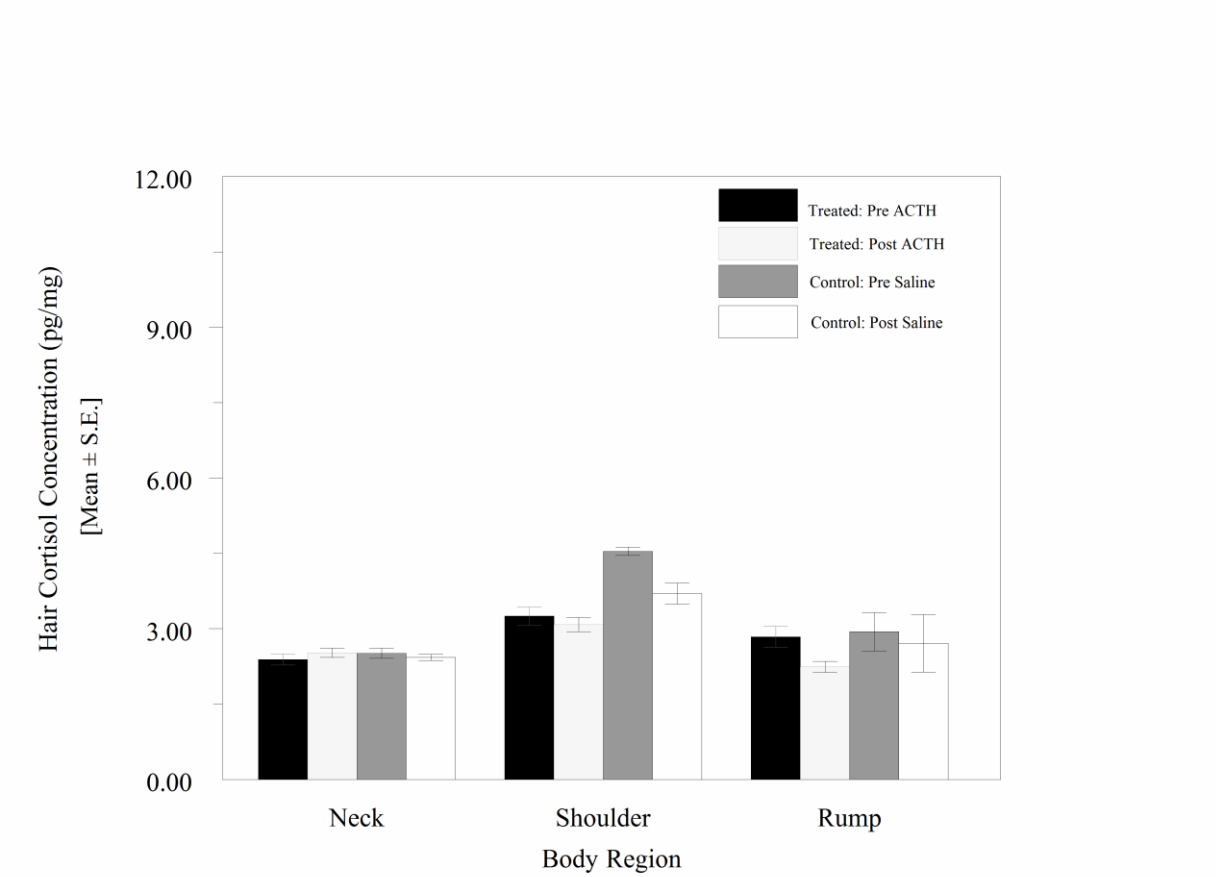

Figure 4.5 Hair cortisol concentration (HCC) pre and post Adrenocorticotropic hormone (ACTH) or saline administration in neck, shoulder, and rump hair of captive reindeer (*Rangifer tarandus tarandus*). Data are presented as mean HCC ± S.E. of hair cortisol determinations in $n = 10$ reindeer. There is no significant difference in the cortisol concentration of reindeer hair (Paired samples *t*-tests, $P > 0.050$) pre and post ACTH administration in any body region. In all body regions the pattern of HCC pre and post injection in $n = 2$ control animals (injected with saline) is similar to that of ACTH treated animals.

4.4.3.3 Trial 2: reindeer measured 16 days and 134 days after injection

Hair cortisol concentration decreased 16 and 134 days after ACTH injection in reindeer (one-way repeated measures ANOVA, $F_{(2, 18)} = 95.926$, $P < 0.001$, $n = 10$), and significant differences (Tukey-Kramer $P < 0.001$) in HCC levels were observed at all time periods (Fig 4.6). Similar patterns were apparent in $n = 2$ saline injected controls (Fig 4.6).

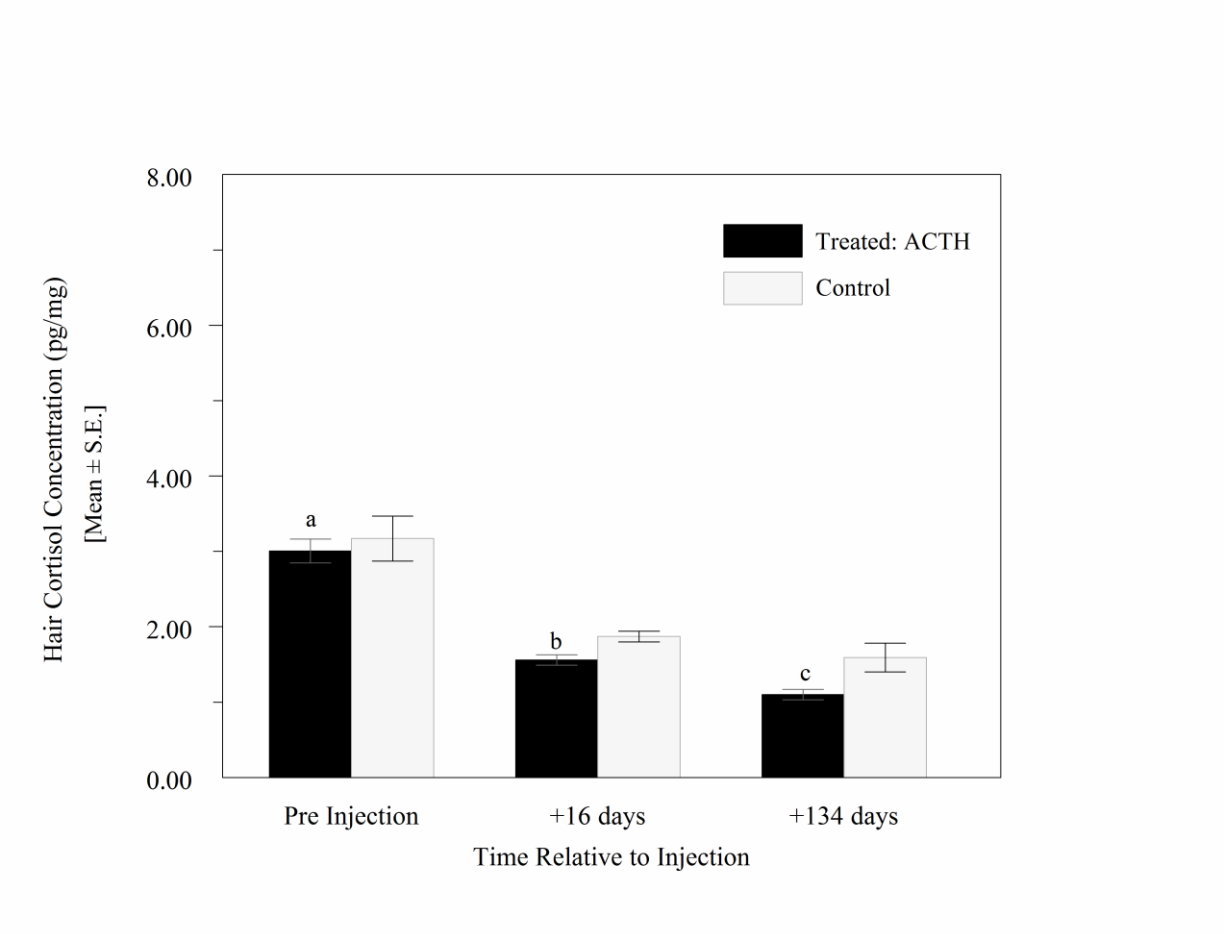

Figure 4.6 Hair cortisol concentration (HCC) pre and post Adrenocorticotropic hormone (ACTH) or saline administration in reindeer (*Rangifer tarandus tarandus*) rump hair. Data are presented as mean HCC ± S.E. of hair cortisol determinations in $n = 10$ reindeer. For ACTH treated animals, different letters designate significant differences (Tukey-Kramer, $P < 0.001$) in HCC measured at each time period. The pattern of HCC pre and post injection in $n = 2$ control animals (injected with saline) is similar to that of ACTH treated animals at all time periods.

4.4.4 Hair growth stage analysis

4.4.4.1 Trichograms

Most hairs from all body regions in all animals were club shaped (Table 4.1, Table 4.2). A variable percentage of hairs were broken (relatively more in reindeer than caribou) and bulb morphology could not be assessed (Table 4.1, Table 4.2). Likewise, plucked hair from the neck of one caribou and 2 reindeer and from the shoulder of 3 reindeer was not available in sufficient quantity or quality (i.e. crushed or broken) for growth stage analysis.

Table 4.1 Hair bulb morphology in hair samples from n = 12 captive Alaskan caribou (*Rangifer tarandus granti*) plucked after injection with Adrenocorticotropic hormone (ACTH) or saline. Where possible, bulb morphology was assessed in a subsample of n = 100 randomly selected hair shafts from each body region in each animal.

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

Table 4.2 Hair bulb morphology in hair samples from n = 12 captive reindeer (*Rangifer tarandus tarandus*) plucked after injection with Adrenocorticotropic hormone (ACTH) or saline. Where possible, bulb morphology was assessed in a subsample of n = 100 randomly selected hair shafts from each body region in each animal.

Body Region	Mean % Club Shaped Hair Bulbs	Mean % Broken Hairs	Number Hair Shafts Examined
Neck	81	19	1000
Shoulder	91	9	900
Rump	99	1	1200

4.4.4.2 Staining with 4-dimethylaminocinnamaldehyde (DOCA)

All hairs from all animals (both subspecies) stained light pink to orange (Fig 4.7).

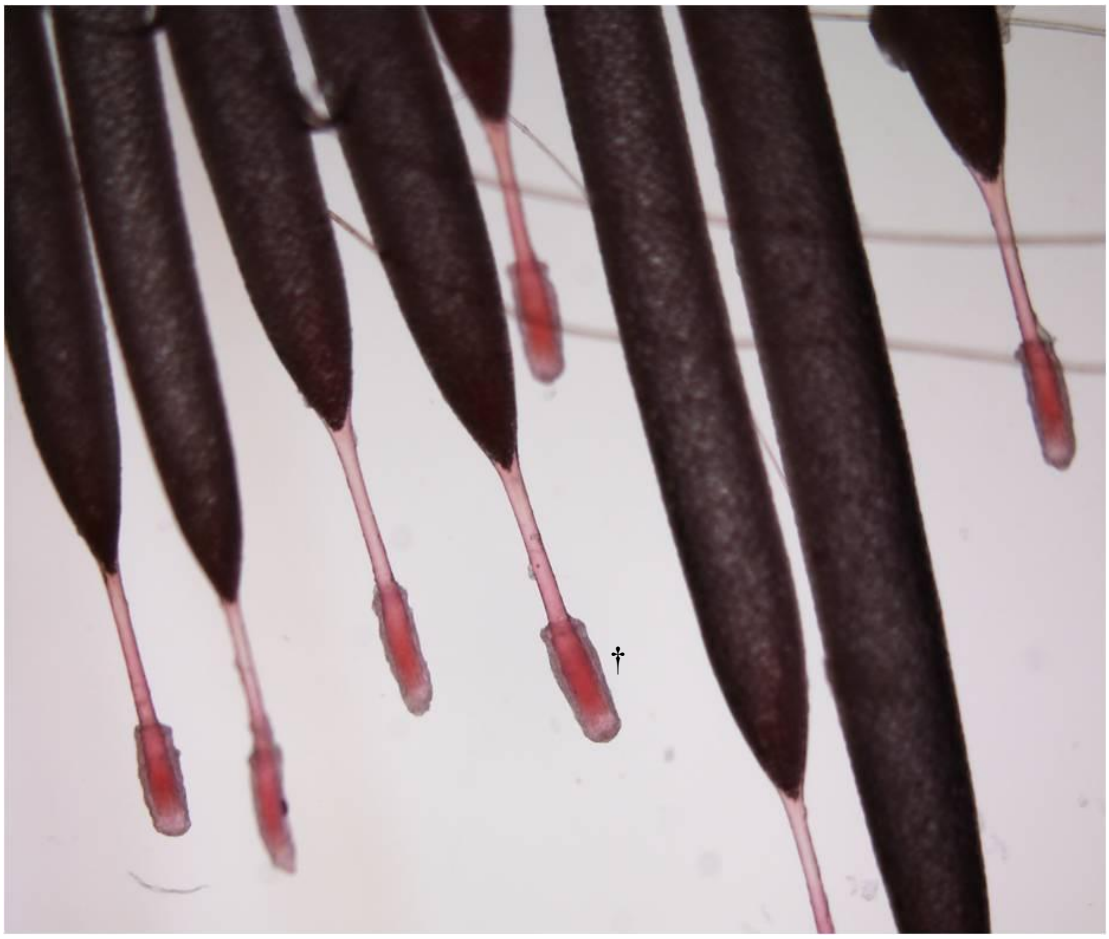

Figure 4.7 Example of plucked *Rangifer* hair stained with 4-dimethylaminocinnamaldehyde (DOCA) at 100X magnification showing club shaped hair bulbs and light pink to orange colouration (e.g. †).

4.5 Discussion

4.5.1 Assay performance and decontamination protocol

In general, assay performance in *Rangifer* was within acceptable limits and agrees with our work in grizzly bears (Macbeth et al. 2010) and polar bears (Chapter 3). The EIA kit used in this investigation was also sensitive across the range of *Rangifer* HCC values determined in this study. These findings indicate that, overall, HCC can be accurately and reliably determined in caribou and reindeer. Nonetheless, the marginal inter assay C.V. (18.39%) suggests that the

precision of HCC values determined in individual *Rangifer* may decrease if assessed on different EIA plates.

Decontamination findings also agree with those reported for other species [grizzly bears (Macbeth et al. 2010), polar bears (Chapter 3), and mule deer (*Odocoileus hemionus*) (Dean Jeffery, personal communication)], and indicate that a series of short methanol washes are effective in cleansing clean or moderately contaminated (blood) *Rangifer* hair without removing intrinsic cortisol from the hair shaft (Davenport et al. 2006, Macbeth et al. 2010, Bechshøft et al. 2011). However, the standardized wash protocol employed in this study was not effective in removing surface cortisol from *Rangifer* hair that had been heavily contaminated with blood. While not directly applicable to this study (no hair samples were contaminated with blood), hair samples collected from *Rangifer* killed by hunting are commonly covered in varying quantities of this material (B. Macbeth, personal observation). We currently recommend that HCC analysis in this species should be restricted to clean or moderately contaminated (blood) hair. Furthermore, the marginal but non-significant decrease in HCC measured in *Rangifer* hair washed 3 or 6 times suggests that caution may also be warranted in extending wash protocols past 6 methanol washes in this species (Macbeth et al. 2010).

4.5.2 Features of hair and caribou-reindeer

4.5.2.1. Body regions

Elevated HCC values observed in caribou neck hair compared to the shoulder and rump agree with HCC patterns previously recorded in grizzly bears and mule deer (Macbeth et al. 2010, D. Jefffery personal communication). Although the configuration was dissimilar, differences in HCC levels measured in the neck, shoulder, and rump hair of reindeer were also not unexpected. As in other species, the divergence of HCC levels among body regions may be related to differences in energetic demands or exposure to stressors overlapping with periods of active hair growth in each region (Macbeth et al. 2010). Observations of free-ranging reindeer in Alaska suggest that, in many animals, moulting of neck hair may be completed prior to the shoulder or rump (B. Macbeth, personal observation). Since visible moult is influenced by the presence of actively growing hair in the hair follicle (Harkey 1993, Pragst and Balikova 2006), this may support an earlier initiation of growth in this body region. In turn, this may indicate that

(within each subspecies) factors contributing to HCC levels in different body regions were most divergent relatively early in the hair growth period.

In addition to their role as effectors of the stress response, glucocorticoids play an important role in energy metabolism and are often elevated in juvenile animals during their active growth phase (Landys et al. 2006). Hypo-responsiveness of the HPA axis has also been associated with the early neonatal period in a variety of species, where it is believed to minimize the negative impact of glucocorticoids on growth and development (Sapolsky and Meaney 1986, Panagiotaropoulos et al. 2004). Hair samples used in this analysis were collected prior to ACTH-saline administration in March, 2008 and should represent the entire period of hair growth from the year preceding collection (2007) (Miller 2003, Macbeth et al. 2010). All reindeer were born in spring 2007 and were 0.8 year old juveniles at the time hair samples were collected (Ashley et al. 2011). Accordingly, diminished HCC in the neck of reindeer compared to other body regions could reflect both the effects of early neck hair growth and HPA axis hypo-responsiveness in the early neonatal period. In turn, elevated HCC levels in the shoulder and rump may be related to an interaction between the gradual maturation of the HPA axis and the energetic demands of rapid growth in the later juvenile period (Panagiotaropoulos et al. 2004, Landys et al. 2006, Busch and Hayward 2009).

Conversely, the majority of caribou used in this study were not actively growing and elevated neck HCC in this group may instead reflect relatively high energetic demands early in the hair growth period for this subspecies. Five of 12 caribou used in this study were pregnant and the costs of gestation or lactation in female caribou may have been important in this regard (Parker et al. 1990, Ropstad 2000, Gjostein et al. 2004). In both sexes, elevated neck hair may also reflect the effects of energy use or allocation during late winter or early spring when hair growth is likely to begin (but is not yet visible as active moult) (Stenn and Paus 2001, Barboza and Parker 2006, Barboza and Parker 2008). For example, neck HCC in male caribou may have been influenced by the effects of reproductive effort in the preceding fall rut and subsequent loss of condition not regained over the course of the winter (Barboza et al. 2004, Busch and Hayward 2009).

While not mutually exclusive, some structural feature of neck hair (Bubenik and Bubenik 1985, Cuyler and Ørtisland 2002, Davis et al. 2010), or functional characteristics of the neck's

integument (e.g. glandular secretions containing cortisol) may also explain these findings (Pragst and Balikova 2006). In this regard, we found that hair extract originating from *Rangifer* neck hair contained greater quantities of the unidentified waxy material compared to other body regions and that this material was most prevalent in samples from caribou (see section 4.4.1). In both subspecies, HCC levels were also most variable in this body region.

Hair colour has also been proposed as a potential confounder of HCC analysis in animals (Bennett and Hayssen 2010, Macbeth et al. 2010). However, the general importance of this factor remains unclear in animals. Among ungulates, higher HCC has been recently been identified in the white vs black hair from domestic cattle (*Bos taurus*) (Gonzalez de la Vera et al. 2011). However, dark grey hair has been found to contain more cortisol than light grey or grey brown hair in free-ranging mule deer (D. Jefferey, personal communication). Interestingly, we found that hair obtained opportunistically from the neck of a free-ranging adult male, barren ground caribou (*R. t. groenlandicus*) killed on Southampton Island, Nunavut was pure white and also contained less cortisol than hair from the shoulder, back, side, rump, or abdomen which were uniformly grey-brown (Appendix 4).

As such, it is possible that differences in hair colour may have contributed to some of the variation in patterns of HCC observed among (and within) body regions in each animal. This may have been especially important for neck hair where colour within each sample was most variable (i.e. hair shafts ranging from pure white to a mixture of white-brown to grey-brown) compared to other body regions where hair colour was relatively uniform (B. Macbeth, personal observation). Further research is recommended to improve our understanding of the effects of hair colour on HCC in *Rangifer*. Based on these knowledge gaps, we currently recommend that hair from the neck is not used for routine HCC analysis in *Rangifer* and that future work in this species sample from the shoulder or rump in areas of relatively uniform hair colour. Of these sites, the shoulder may be preferable due to the possibility of fecal contamination (containing glucocorticoid metabolites) on rump hair (Freeman 2008, Macbeth et al. 2010, Wasser et al. 2011).

4.5.2.2 Subspecies

Rangifer growth rate is highest in the juvenile period (Miller 2003), and elevated serum cortisol levels have also been observed in free-ranging juvenile *Rangifer* compared to older

animals (Miller 2003, Omsjoe et al. 2009). Accordingly, age-related differences in the two study groups may best explain the higher whole body HCC levels we observed in reindeer compared to caribou (Landys et al. 2006, Busch and Hayward 2009). Among captive *Rangifer* maintained at the University of Alaska Fairbanks, both moult and the timing of key energetic demands (e.g. parturition and lactation) are also known to occur earlier in the year in reindeer compared to caribou (Barboza and Parker 2008, Ashley et al. 2011), and temporal differences in hair growth may also have influenced HCC levels in both groups.

In general terms, HCC levels in caribou and reindeer may have been affected by differences in social interactions in the year proceeding sampling (Miller 2003, Bartolomucci et al 2005, Fauchald et al. 2007). This may have been especially important for caribou which were held in mixed-sex herds during the 2007 rutting season (Hirotani 1989, Hirotani 1990, Barboza et al. 2004, Holand et al. 2004). HPA axis activity in caribou may also have been directly influenced by the effects of reproductive hormones, behaviour, or lactation in this group (Barboza et al. 2004, Boonstra 2005, Kajante and Phillips 2006, Chow et al. 2011) while maternal effects such as HPA axis programming (Seckel 2004) or cortisol transfer in milk (Sullivan et al. 2010) may have contributed to HCC levels in reindeer. Hair colour in caribou and reindeer was similar (B. Macbeth, personal observation), and any influence on whole body HCC determined in each subspecies was likely minimal. However, hair from caribou used in this study is known to be shorter and finer than that of the reindeer (Ashley et al. 2011) and structural differences in hair may have influenced cortisol deposition or retention in the hair of both subspecies (Kidwell and Smith 2007). Genetic differences between subspecies may also be relevant (Cronin et al. 2006, Solberg et al. 2006).

4.5.2.3 Sex

We observed no difference in HCC levels measured in male and female caribou or reindeer. These findings agree with other studies in captive or free-ranging *Rangifer* (Nilssen et al. 1985, Bubinek et al. 1998), and may indicate that over the course of the active hair growth period male and female caribou and reindeer are exposed to a similar array of stressors. However, in *Rangifer*, seasonal differences in peak cortisol levels have been recorded with the highest values recorded in June and October for males and females respectively (Bubinek et al. 1998). Unlike males, female reindeer in good body condition may also not experience elevated

cortisol in winter when activity is low whereas male reindeer may demonstrate a significant increase in plasma cortisol levels during the rut when food intake is low and activity is high (Barboza et al. 2004, Barboza and Parker 2006, Ashley et al. 2011). These differences in energetic demands occur primarily outside of the hair growth period (Miller 2003) and limited overlap between hair growth and major sex-related energetic demands or stressors may explain the lack of difference in HCC levels we observed in male and female caribou and reindeer. Presently, the timing of the hair cycle and rates of hair growth, in *Rangifer* are poorly understood. Studies examining these factors in captive caribou or reindeer herds would enhance our understanding of HCC in this species.

4.5.3 ACTH Challenges

In Trial 1, we found that a single administration of ACTH (2 IU/kg) did not influence cortisol levels measured 7 days after injection in caribou neck and rump hair, or in reindeer, neck, shoulder, and rump hair. Conversely, HCC measured in caribou shoulder hair increased 7 days after ACTH administration. However, a similar increase was also observed in saline injected controls. Cortisol from the systemic circulation is believed to enter the hair shaft primarily by passive diffusion and only during periods of active hair growth (Davenport et al. 2006, Gow et al. 2010). We found that the majority of hair bulbs collected from *Rangifer* shortly after ACTH administration were club shaped which is generally considered to be indicative of non-growing (telogen) hairs (Stenn and Paus 2001, Müntener et al. 2011). This observation agrees with current understanding of temporal patterns of moult in *Rangifer* which suggest that hair collected (both pre and post ACTH-saline administration) for this trial (March, 2008) should not have been actively growing (Miller 2003). We did attempt to confirm these findings by staining sub samples of hairs from each body region in each animal with 4-dimethylaminocinnamaldehyde (DOCA). DOCA distinguishes growing hair (which contains the root sheath protein citrulline) from non-growing hair which does not, and growing hair stains bright red while non-growing hair stains pale orange or not at all (Baden et al. 1979). Unfortunately, the results of this experiment were inconclusive with all hairs from all animals staining light pink to orange (Fig 4.7). DOCA turns pink on contact with air (B. Macbeth, personal observation; B. Sarauer, personal communication), and 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 (B. Macbeth, personal observation). It is most likely that the pale pink observed in all samples was a result of this phenomenon and was not due to the presence of growing hairs. The roots of all hair samples were also somewhat dry (owing to transport and storage prior to analysis) which may have influenced the efficacy of the staining procedure itself (Baden et al. 1979). Nonetheless, morphological findings along with similar patterns of HCC observed in control animals suggest that the difference in shoulder HCC observed pre and post challenge in caribou was not due to ACTH administration. Rather, these findings were more likely an artefact of the protocols employed in this study.

Hair collected after ACTH administration was gathered from the right side of the body while that obtained before ACTH challenge was collected from the left. While general patterns of moult may be consistent among body regions, within each region patterns of follicular activity in individual hair shafts (or groups of hair shafts) may be more variable (Stenn and Paus 2001, Müntener et al. 2011). In *Rangifer*, subsamples of hair taken from the same body region may also contain hair shafts of varying colour (B. Macbeth, personal observation). These factors may have influenced pre-existing cortisol levels in hair from sites sampled before and after challenge and may also explain why increased HCC was observed in saline injected controls. Likewise, in some species, cortisol levels may vary along the length of the hair shaft (Kirschbaum et al. 2009, Xie et al. 2012). Hair collected in the ACTH Trials was shaved as close to the skin as possible. However, unlike reindeer, caribou were not tractable and the uniformity of hair samples was somewhat difficult to control (P. Barboza, personal communication). As such, sample heterogeneity may have influenced HCC levels determined before and after ACTH injection. Unfortunately, an accurate assessment of HCC along the length of the hair shaft in caribou could not be performed using the hair samples available for this experiment. Research published after the completion of this study may provide additional insight into the cause of the elevated cortisol levels we observed in caribou shoulder hair after ACTH administration.

Gonzalez de la Vara et al. (2011) examined the effect of ACTH injection on HCC in domestic cattle and found that after 3 sequential intravenous (IV) injections of ACTH (0.15 IU/kg; spaced seven days apart), elevated HCC could be identified 14 days and 28 days after the first administration. The process of incorporation of substances from the blood stream into the shaft of actively growing hair is believed to take up to three days (Pragst and Balikova 2006)

with an additional week or more required for that hair to emerge at the skin surface (Kidwell and Smith 2007). Gonzalez de la Vara et al. (2011) measured HCC after ACTH challenge in hair that had been shaved and was actively growing. As such, the time-lag between injection and the observation of elevated HCC agrees with the incorporation of blood borne cortisol into growing hair (Gow et al. 2010). Even so, repeated doses of ACTH were required to identify elevated HCC, which also supports the hypothesis that HCC may not be as sensitive to short-term elevations in circulating cortisol as are other measures of HPA axis activity.

Unlike Gonzalez de la Vara et al. (2011), we measured HCC in *Rangifer* hair samples that were not growing (Trial 1, March 2008) and found that (with the exception of caribou shoulder hair) a single injection (2 IU/Kg, IM) of ACTH did not increase HCC. In Trial 2 (July-October 2008), reindeer were actively shedding their winter coat and were also simultaneously growing new hair. As in Trail 1, hair samples collected prior to ACTH administration and 2 weeks after (July and August 2008) represented hair growth from the 2007 season, and a single ACTH challenge at an increased dose (8 IU/kg) failed to increase HCC measured in this period. In contrast, hair collected 16 weeks after ACTH administration (October 2008) was new growth from the year of sampling (2008) that had been actively growing during the time of ACTH administration. However, ACTH challenge still did not lead to a detectable increase in HCC levels after administration. In fact, HCC actually decreased over the time course of the experiment in Trial 2; a finding possibly related to the effects of weathering in pre administration vs 2 week hair (Miller 2003), or to variation in seasonal HPA axis activity between 2007 and 2008 (pre administration and 2 weeks vs 16 weeks). These discrepancies may indicate that repeated dosing with ACTH is required to elevate HCC in *Rangifer*. Likewise, deposition of steroid may have occurred in our study but this increase may have been too small relative to the total cortisol in the entire hair shaft to be identified. Indeed, Gonzalez de la Vara et al. (2011) failed to detect elevated HCC after 44 days which could indicate that a dilution effect may occur as hair growth progress. Refining techniques in *Rangifer* to evaluate potential changes in HCC occurring along the length of the hair shaft may be useful to clarify these knowledge gaps (Kirschbaum et al. 2009, Dettenborn et al. 2010, Xie et al. 2012).

We believe these differences also highlight the importance of hair growth stage in HCC analysis, and propose that non-growing hair may be more resistant to the effects of short-term increases in levels of circulating cortisol than growing hair. We had intended to explore this hypothesis in *Rangifer* using serial ACTH administration (at 4 and 8 weeks intervals) and collecting hair for HCC analysis throughout the growth cycle. However, a lack of funds and logistical problems limited this study to a single ACTH injection and collection in each trial and to the use of reindeer only in the second trial (Ashley et al. 2011). We did attempt to measure HCC in the new hair growth that had occurred at previously sampled sites in the period between the end of the first trial (March, 2008) and the initiation of the second (July, 2008) in reindeer. Unfortunately, hair had not re-grown in sufficient quantities (all hair samples < 30 mg) to accurately determine cortisol concentration. Nevertheless, we believe these findings support the assertion that mechanisms not related to the incorporation of blood borne cortisol may account for elevated HCC measured in caribou shoulder hair after ACTH administration.

At the time of our investigation (2008) the human hair follicle had also been shown (*in vitro*) to display a functional equivalent to the HPA axis and synthesize cortisol (Ito et al. 2005). Later, limited evidence arose to suggest that an acute stressor (painful stimuli) may be associated with a transitory and localized increase in HCC measured in humans (Sharpley et al. 2009, Sharpley et al. 2010a). Sharpley et al. (2010b) also reported a lag in salivary cortisol attributed to the same stimuli, and interpreted this finding as evidence that the response in hair was localized and independent of central HPA axis activity The single ACTH injection used in our study was administered via intramuscular injection using a relatively large (18 gauge) needle and divided equally between the left and right shoulder. In animals, lasting tissue damage and localized irritation may be associated with IM injections (Ramussen et al. 1980, Nouws 1990, Fajt et al. 2011), especially when given to intractable animals [B. Macbeth, personal observation]. Importantly, hair sampled post ACTH administration was collected from the right shoulder and it is possible that ACTH injection itself could have led to localized irritation and production of cortisol in the shoulder skin of caribou (Ito et al. 2005, Sharpley et al. 2009, Sharpley et al. 2010a). Precisely how such a localized stress response in skin may have influenced HCC in caribou shoulder hair is less clear.

Since elevated HCC was not identified in the majority of non-growing *Rangifer* hair samples, direct incorporation from the blood stream appears unlikely. Substances may also enter the hair shaft via diffusion from tissues surrounding actively growing hair (Harkey 1993, Pragst and Balikova 2006) or as the result of glandular apocrine, sebaceous and sweat secretions in and around the follicle (Henderson 1993, Cone 1996, Kidwell and Smith 2007). Evidence from the forensic drug field suggests that of these pathways, glandular secretions may represent the most important alternate mechanism (Raul et al. 2004, Pragst and Balikove 2006, Kidwell and Smith 2007). We observed that the occurrence and amount of the unidentified lipid like material in *Rangifer* hair extract was greatest in caribou and in hair samples obtained after ACTH administration (B. Macbeth, personal observation). Secretory activity in some types of skin glands (e.g. sebaceous and sweat glands) may be enhanced by mediators of the stress response (e.g. corticotrophin releasing hormone, CRH) or may increase in response to short-term stress (Zouboulis et al. 2002, Krause et al. 2007, Scharf et al. 2008). As such, it is plausible that this material could represent a glandular secretion containing increased cortisol levels related to the localized effects (irritation) of injection (or due to ACTH administration itself) in the shoulder. In this study, *Rangifer* hair also appeared somewhat weathered (B. Macbeth, personal observation) which may have facilitated penetration of the hair shaft by this material, reduced the efficacy of the wash procedure, and artificially elevated hair cortisol concentration in the affected region (Macbeth et al. 2010). Presently, the distribution and types of skin glands in the *Rangifer* shoulder along with their response to short-term stress, and the cortisol content of their secretions are poorly understood (Quay 1955, Kallquist and Mossing 1977, Müller-Schwarze et al. 1977). Additonal research is necessary to more clearly define the effect of localized HPA axis activity on HCC measured in *Rangifer*, and the potential importance of hair growth stage and glandular secretions deserve special attention. Given this knowledge gap, we currently recommend that hair for cortisol anaylsis in *Rangifer* (or other species) is not collected from locations in which potential irritants such as ectoparasites (Dove and Cushing 1933, Welch et al. 1990), skin disease (Rehbinder and Mattson 1994, Ayroud et al. 1995), injury, or radio collars (Krausman et al. 2004) are present. Further research is also necessary to establish HCC as a valid biomarker of long-term stress in *Rangifer*. In this regard, relationships between HCC and life history traits, parasites, diseases, and environmental conditions known to be associated with population

performance in free-ranging *Rangifer* should be thoroughly investigated (Vors and Boyce 2009, Busch and Hayward 2009, Gunn 2011).

CHAPTER 5

AN EVALUATION OF HAIR CORTISOL CONCENTRATION AS A BIOMARKER OF LONG-TERM STRESS IN FREE-RANGING CARIBOU (*Rangifer tarandus groenlandicus*) FROM WEST GREENLAND

This investigation was performed as the last of four studies included in this thesis and applied techniques developed in Chapters 2, 3, and 4 to evaluate the utility of hair cortisol analysis in free-ranging *Rangifer*. In this study, hair was collected from the Akia-Maniitsoq and Kangerlussuaq-Sisimiut caribou (*Rangifer t. groenlandicus*) populations of West Greenland. In these regions, divergent ecological conditions and population trajectories are recognized. However, a lack of potential confounders related to migration behaviour, risk of predation, and inter specific competition (typical of other *Rangifer* populations in North American and Eurasia) offered a unique opportunity to evaluate hair cortisol as a potential biomarker of long–term stress and conservation tool in this species. A combination of hypothesis testing and information theory was used to develop and assess models to explore potential relationships between hair cortisol concentration, life history traits, environmental conditions (parasitism), and indices of body size (length and girth) and body condition (mass, cold carcass weight, and body fat) closely related to survival and fecundity in this species. Hair cortisol levels determined in Akia-Maniitsoq and Kangerlussuaq-Sisimiut caribou may also provide baseline long-term stress data in two *Rangifer* populations which face increasing conflict with human interests and anthropogenic ecological change in the near future.

5.1 Introduction

A scientific consensus suggests that populations of caribou and reindeer (*Rangifer tarnadus* sp.) are now declining throughout much of the circumpolar north (Vors and Boyce 2009, Callaghan et al. 2010, Festa-Bianchet et al. 2011, Gunn 2011). Population dynamics in this species are driven by a complex suite of natural and anthropogenic influences (Gunn 2003, Vors and Boyce 2009, Joly and Klein 2011), and the occurrence of long-term physiological stress related to these factors has been proposed as a mechanism linking prevailing ecological conditions with diminished health and poor population performance in declining *Rangifer* herds (*Rangifer tarandus* sp) (Reimers 1983, Whitfield and Russell 2005, Gunn et al. 2009, Ashley et al. 2011). The origin and relative importance of these influences may be highly variable among different *Rangifer* populations and across the species' vast distributional range (Vors and Boyce 2009, Joly et al. 2011, Festa-Bianchet et al. 2011, CARMA 2012a). Thus, the development of practical techniques to assess long-term stress in free-ranging *Rangifer* may advance our general understanding of biological mechanisms linking environmental conditions with population dynamics in this species as well as our ability to distinguish the relative importance of natural and human-caused factors as drivers of health and performance in specific caribou and reindeer populations (Freeman 2008, Ashley et al. 2011, Wasser et al. 2011).

The determination of hair cortisol concentration (HCC) has recently shown promise as a biomarker of long-term stress in captive caribou and reindeer (Ashley et al. 2011). However, prior to its widespread application in free-ranging populations, HCC must first be validated as a biologically relevant biomarker of long-term stress by: 1) identifying factors influencing HCC in *Rangifer* (Macbeth et al. 2010, Busch and Hayward 2009), and 2) establishing linkages between HCC, life history traits, and environmental conditions associated with rates of survival or fecundity in this species (Busch and Hayward 2009). To date, these factors have not been thoroughly investigated.

In West Greenland, caribou (*Rangifer tarandus groenlandicus*) and reindeer (*Rangifer tarandus tarandus*) are found in ten relatively isolated populations that are separated by natural landscape features and inhabit ecosystems with distinct climates, precipitation patterns, and food resources (Cuyler and Østergaard 2005, Witting and Cuyler 2011). However, unlike many North American populations (Couturier et al. 2010, Festa-Bianchet et al. 2011), *Rangifer* in West

Greenland occur in relatively simple systems where no mass seasonal migrations occur, no natural predators exist, and there is limited inter specific competition (Cuyler 2007, Witting and Cuyler 2011). Accordingly, density dependent effects such as intra specific competition and range capacity are considered to be the primary drivers of caribou population performance in this region (Cuyler 2007).

The Akia-Maniitsoq (AK) and Kangerlussuaq-Sisimiut (KQ) caribou populations are two of the largest in West Greenland (Cuyler 2007). Both are known to exhibit natural cycles of abundance characterized by decades of scarcity followed by an exponential increase in caribou numbers, a population peak, and then a rapid decline (Cuyler 2007, CARMA 2012b, CARMA 2012c). Although the AK and KQ caribou populations have been increasing since the 1960's, recent population surveys (2005) have identified poor juvenile recruitment in both groups (Cuyler and Østergaard 2005, Cuyler 2007, CARMA 2012b, CARMA 2012c). In the AK herd, a simultaneous decrease in caribou abundance was also recorded between 2001 and 2005 and again between 2005 and 2010 (Cuyler 2007, CARMA 2012b, CARMA 2012c), suggesting this population may have reached carrying capacity and the peak of its population cycle (CARMA 2012b). In contrast, the number of KQ caribou increased between 2005 and 2010 (Cuyler 2007, CARMA 2012c).

Although relatively limited at the present time, the importance of human-caused ecological change may also be growing in both regions (CARMA 2012b, CARMA 2012c). Road development and an increase in human land use (e.g. hunters and tourists) already disrupt key calving habitat for the KQ population (CARMA 2012c), and further disturbances are anticipated if a proposed all season highway crossing core KQ caribou habitat is completed (CARMA 2012c). Similarly, the planned construction of an Aluminum smelter on the West Greenland coast along with associated hydro power plants, transmission lines, and other infrastructure may threaten both populations in the immediate future while the proposed construction of an iron ore mine near the Greenland Ice Cap, an associated harbour, and roads and pipelines running between may threaten what is suspected to be sensitive calving habitat for the AK population (CARMA 2012b). A proposed bridge linked to this project and to be built near an important seasonal river crossing may also affect this population (CARMA 2012b). Moreover, in the last decade, unpredictable winters characterized by extended periods with little snow covering or

ground icing events have been observed on AK and KQ range (CARMA 2012b, CARMA 2012c), and the earlier arrival of spring has been recorded. Together, these observations suggest that human-caused ecological change may play an increasingly important role as a driver of West Greenland caribou population cycles in the near future (CARMA 2012b, CARMA 2012c).

The existence of divergent ecological conditions and population trajectories along with the lack of potential confounders related to migration behaviour, predation, or inter specific competition suggest that an evaluation of hair cortisol in West Greenland caribou may offer a unique opportunity to assess the utility of HCC as a biomarker of long–term stress and conservation tool in free-ranging *Rangifer*. Moreover, the determination of HCC levels in AK and KQ caribou may also facilitate the establishment of baseline HCC data in two *Rangifer* populations which face increasing conflict with human interests and anthropogenic ecological change in the near future.

5.2 Objectives

The purpose of this investigation was to: 1) examine the effects of age, reproductive status, body condition, parasite burden, and herd designation on HCC in free-ranging AK and KQ caribou from West Greenland, and 2) to explore the association of HCC and selected measures of body size and body condition in these caribou populations. Body size and body condition are important determinants of survival and reproductive success in *Rangifer* (Helle et al. 1987, Cameron et al. 1993, Weladji et al. 2003, Barboza et al. 2004). Furthermore, growth measures related to these parameters are known to be affected by prevailing environmental conditions in this species (Weladji et al. 2003, Weladji et al. 2005, Couturier et al. 2009a, Couturier et al. 2009b, Mahoney et al. 2011), and diminished growth may also be linked with declining caribou health (Weladji et al. 2003, Weladji and Holand 2003, Olofsson et al. 2011) and population performance (Weladji and Holand 2006, Vors and Boyce 2009, Mahoney et al. 2011).

5.3 Materials and Methods

5.3.1 Overview of the Akia-Maniitsoq and Kangerlussuaq-Sisimiut caribou populations

The following account provides a general description of the AK and KQ caribou populations which may be found in detail at CARMA (2012b, 2012c). Briefly, the AK caribou population is located in an approximately 15 300 km² area of West Greenland (between 64° and

65°N) bound in the North by the Kangerlussuaq fjord and the Sukkertoppen ice cap, in the South by the Godthåbsfjord, in the East by the Greenland Ice Cap and in the West by Davis Strait (CARMA 2012b). AK habitat contains coastal plain, open upland, mountains, and alpine tundra and is characterized by relatively high precipitation levels and an abundance of macro lichens (Cuyler and Østergaard 2005, CARMA 2012b). This population is composed of wild Greenland caribou (*R.t. groenlandicus*) and feral-semi domestic reindeer (*R. t. tarandus*) with some genetic mixing (CARMA 2012b). Conversely, The KQ population consists of wild Greenland caribou (*R.t. groenlandicus*) only and inhabits an approximately 26, 000 km² region near the Arctic Circle (67°N). KQ range is bound in the North by the Nodre Strømfjord, in the south by the Kangerlussuaq fjord and the Sukkertoppen ice cap, in the east by the Greenland Ice Cap, and in the West by Davis Straight (CARMA 2012c). However, habitat use is predominantly restricted to the inland portion of this area, which consists of open upland, mountains, and alpine tundra, and is characterized by a relatively dry climate and lack of lichens (Cuyler and Østergaard 2005, CARMA 2012c).

Unlike many North American *Rangifer* populations, AK and KQ caribou travel in small group of 3-4 animals during short distance annual migrations between wintering and calving grounds (CARMA 2012b, CARMA 2012c). Total distances travelled by AK caribou are generally ≤ 150 km, and some AK caribou do not migrate at all, but remain within relatively small areas (10 km radius) throughout their lives (CARMA 2012b). Distances travelled by KQ caribou generally do not exceed 60 km (CARMA 2012c). There are also no large predators in terrestrial West Greenland ecosystems, and inter specific competition is primarily limited to ptarmigan (*Lagopus mutus*), arctic hare (*Lepus arcticus*), and (in some areas) muskox (*Ovibos moschatus*) (Pedersen and Aastrup 2000, Witting and Cuyler 2011). Overpopulation is currently considered the greatest threat to AK and KQ caribou population performance. As such, there is no quota for commercial or sport hunting of AK or KQ caribou at the present time (CARMA 2012b, CARMA 2012c).

5.3.2 Study area, sample collection, storage, and handling

Hair samples were collected from 94 caribou killed during two research harvests undertaken as part of an ongoing study of factors influencing health and reproduction in female caribou from West Greenland (C. Cuyler, Greenland Institute of Natural Resources, personal

communication). AK collection occurred in a wide area west of the Godthåbsfjord approximately 80-100 km North-Northeast of the city of Nuuk (e.g. 64°39’32.58” N/51°34’05.65 W) while KQ collection occurred in a wide area north of the Kangerlussuaq International Airport (e.g. 67°02’59.76” N/50°50’16.63W) (C. Cuyler, personal communication). Sampling was non-random and focused on adult females with animals harvested in an attempt to reflect a range of ages, body conditions, and reproductive states in both herds. Hair samples were not collected from any subadult male or adult male caribou from either population. All animals were killed via a gunshot to the neck. AK caribou [n = 34 adult females (≥ 3 years of age), n = 6 dependent calves (0.8 years old, n = 6 females), and n = 7 subadult females (1.5-2.5 years of age)] were shot between March 29 and April 13, 2008, while KQ caribou (n = 36 adult females, n = 9 dependent calves (n = 3 males, n = 6 females), n = 4 subadult females) were shot between March 3 and March 17, 2009 (C. Cuyler, personal communication). In *Rangifer,* the period of hair growth is believed to begin in spring and last into autumn (Miller 2003). Thus, all hair gathered for this investigation should represent the entire growth period in the year preceding sampling.

In the AK population hair samples were collected by cutting as close to the skin as possible with scissors or a sharp knife during carcass processing (C. Cuyler, personal communication). Sampling techniques were subsequently improved for KQ caribou and hair was collected before other biological samples by shaving as close to the skin as possible with a razor-sharp knife within minutes of an animal being shot (Fig 5.1). However, all sampling occurred prior to the development of recommendations for HCC analysis in *Rangifer* (reported in Ashley et al. 2011) and hair from the AK herd was collected from the neck while hair from the KQ herd was obtained from the shoulder (C. Cuyler, personal communication).

Hair from AK caribou ranged in colour from uniformly white to a mixture of white and grey-brown. Owing to the timing of sampling, dried blood was also visible on the hair surface in all samples. While the precise quantity of blood could not be quantified, it was estimated to cover ≥ 50% of the hair surface in most samples (B. Macbeth, personal observation). Hair from KQ caribou was also a mixture of white and grey-brown (Fig 5.1), however, no significant blood contamination was apparent on the surface of any sample (B. Macbeth, personal observation). All hair samples were stored in dry paper envelopes, at room temperature, and in the dark. Techniques used in the collection of ancillary data related to age, reproductive status, body size,

body condition, and parasite burden followed standardized protocols developed by the CircumArctic *Rangifer* Monitoring and Assessment (CARMA) Network which are reported in detail in CARMA (2008a, 2008b, 2008c).

Figure 5.1 Recommended sampling methodology for hair collection from *Rangifer* carcasses. Hair is collected before carcass processing and within minutes of a caribou being harvested by shaving as close to the skin as possible with a razor-sharp knife. Note bloodless collection and uniformity of hair samples at the hair-skin interface. Method developed by and photo courtesy of C. Cuyler, Greenland Institute of Natural Resources.

Figure 5.2 Example of typical colour of hair collected from caribou (*Rangifer tarandus groenlandicus*) of the Kangerlussuaq-Sisimiut herd, West Greenland in March, 2009. Photo courtesy of C. Cuyler, Greenland Institute of Natural Resources.

5.3.3 Decontamination protocol, hair preparation, and steroid extraction

Hair decontamination, preparation, and steroid extraction protocols were as reported in [Chapter 4, Ashley et al. (2011)]. However, where blood contamination was extensive (AK caribou only), subsamples of the cleanest hair shafts available from each animal were selected for use in HCC analysis. It should also be noted that the unidentified flocculent material previously reported in *Rangifer* hair extract (see Chapter 4 section 4.4.1) was not observed in extract from as many animals or in as large quantities in West Greenland caribou (B. Macbeth, personal observation). Furthermore, in the relatively few cases where the substance was present in this study it was easily returned to solution (and was evenly distributed in solution) after the reconstitution process (B. Macbeth, personal observation).

5.3.4 Data analysis

Data were analyzed with SPSS 18.0.0 (PASW Statistics Version 18.0.0, July 30, 2009) with the level of significance set at $P \leq 0.10$. To meet assumptions of normality and homogeneity of variance all data were log transformed prior to analysis. We employed a combination of hypothesis testing and information theory to develop and evaluate models explaining variation in HCC and selected indices of caribou body size and body condition. First we divided potential predictor variables into six model sets representing caribou biology, body size, body condition, parasite burden, herd designation, and HCC. In *Rangifer*, age and reproductive stage may be important determinants of both HPA axis activity (Bubenik et al. 1998, Omsjoe et al. 2009, Ashley et al. 2011) and growth parameters (Thing et al. 1986, Nieminen and Peterson 1990, Couturier et al. 2009a, Couturier et al 2009b, Taillon et al. 2011). As such, measurement variables in the biology model set included the constructed variable age-reproductive class and age in years. Age-reproductive class positioned all caribou into one of six descriptive categories based on their age and reproductive status (dependent calf of either sex, subadult female, adult female not pregnant and not lactating, adult female not pregnant but lactating, adult female pregnant but not lactating, adult female pregnant and lactating). The potential relationship between age and dependent variables was evaluated as both a linear and quadratic association in order to assess: 1) the direct effect of age, and 2) how the effect of age on dependent variables changed as caribou got older. The body size model set included total body length and girth (methods for measurement reported in CARMA 2008b), while the body condition model set included total body weight, cold carcass weight [weight without head, skin, viscera, or lower legs (CARMA 2008b)], and an additional constructed variable designated as body fat reserves (BFR) which was calculated as:

....BFR= Σ (rump fat + omental-gut fat mass + mandibular fat mass + metatarsal fat mass)....(5.1)

The parasite burden model set considered caribou in one of four standardized categories based on the intensity of warble (*Hypoderma tarandi*) or nasal-bot (*Cephenemyia trompe*) infection [(0 per animal), (1-20 per animal), (21-100 per animal), (101-1000 per animal)](CARMA 2008a). Herd designation reflected population of origin.

The HCC model evaluated the importance of caribou biology, body condition, parasite burden, and herd designation as potential predictors of HCC. For models of body condition, the body size model set replaced the body condition model set and vice versa. HCC was also added as an additional predictor variable to models for all growth indices. In order to evaluate the explained variation (calculated as adjusted R^2) attributable to caribou biology, body size or condition, parasite burden, herd designation, or HCC we added each model set to developing best fit models in a step-wise fashion. Within and between model sets, sample-size corrected Akaike's Information Criterion (AICc) scores were calculated and used to determine which variables to include and retain in the developing models for each dependent variable. We based best fit models on ΔAICc values ≤ 2 with the fewest predictor variables (parsimony) and associated R^2 values. We also used simple linear regression to assess the relationship between female caribou and their dependent calves at heel.

5.4 Results

5.4.1 Range of hair cortisol values determined in West Greenland caribou

The mean HCC determined in caribou from the AK herd was 2.17 pg/mg [range 0.60 pg/mg-6.90 pg/mg, $n = 46$]. The mean HCC determined in caribou from the KQ herd was 2.26 pg/mg [range 1.21 pg/mg-3.87 pg/mg, $n = 48$].

5.4.2 Factors influencing hair cortisol concentration in West Greenland caribou

5.4.2.1 Multivariate models of hair cortisol concentration in West Greenland caribou

The best fit model explained 31% of the variation in HCC and incorporated caribou biology, body condition, parasite burden, and herd designation (Table 5.1.1, Table 5.1.2). Caribou age and reproductive status explained most (21%) of the variation in HCC that could be accounted for (Table 5.1.2). There was a direct association between caribou age (mean 5.4 years, range 0.8-11.9 years, $n = 94$) and HCC (Table 5.1.1). Relative to subadult females and dependent calves in which levels were similar, HCC was lower in adult female caribou regardless of their pregnancy or lactation status (Table 5.1.1). There was no association between HCC levels measured in adult female caribou and their dependent calves (Pearson product moment correlation $r = 0.047$, $P = 0.869$, $n = 15$ cow calf pairs). There was also no relationship between HCC and total body weight or cold carcass weight (Table 5.1.1). Nevertheless, HCC was inversely associated with BFR, and body condition accounted for an additional 3 % of the

explained variation in HCC (Table 5.1.1, Table 5.1.2). Based on AICc scores, the HCC model incorporating caribou biology, body condition, and parasite burden was similar ($\Delta AICc < 2$) to the model containing only caribou biology and body condition (Table 5.1.2). However, the HCC model containing caribou biology, body condition, parasite burden, and herd designation was superior ($\Delta AICc > 2$) to the model containing only caribou biology, body condition, and herd designation (Table 5.1.2). There was no relationship between HCC and the intensity of warble infection (Table 5.1.1). However, caribou with low nasal bot burdens (1-20 larvae per animal) also had lower HCC than their counterparts with relatively high burdens (101-1000 larvae per animal) (Table 5.1.1). Overall, parasite burden accounted for an additional 2% of the explained variation in HCC (Table 5.2.2). HCC levels in AK caribou were lower than those measured in KQ caribou, and herd designation accounted for 5% of the explained variation in HCC (Table 5.1.1, 5.1.2).

Table 5.1.1 Standardized coefficients (StD β) and significance (*P*) of model variables describing the best fit model for hair cortisol concentration (HCC) in free-ranging caribou (*Rangifer tarandus groenlandicus*) from the Akia-Maniitsoq herd (n = 46) and the Kangerlussuaq-Sisimiut herd (n = 48) killed in West Greenland in 2007 and 2008. For potential predictor variables containing more than one category and also included in the best-fit model, non significant relationships ($P > 0.10$) are presented for comparative purposes.

Model set and	*HCC*	
measurement variables	β	*P*
1) Biology		
age-reproductive class		
calf (dependent)	0.191	0.186
adult female (not pregnant and not lactating)	-0.388	0.041
adult female (not pregnant but lactating)	-0.397	0.012
adult female (pregnant but not lactating)	-0.266	0.077
adult female (pregnant and lactating)	-0.334	0.049
subadult female	0	-
age (0.8-11.9 years)	0.051	0.006

age^2			N.S.
2) Body Condition			
weight			N.S.
cold carcass weight			N.S.
body fat reserves		-0.129	0.025
3) Parasite Burden			
CARMA bot score			
(1-20 larvae)		-0.286	0.005
(21-100 larvae)		-0.057	0.557
(101-1000 larvae)		0	-
CARMA warble score			N.S.
4) Herd			
Akia-Maniitsoq		-0.222	0.006
Kangerlussuaq-Sisimiut		0	-

Table 5.1.2 Sample-size corrected Akaike's Information Criterion (AICc) values, change in Akaike's Information Criterion (ΔAICc) values, Akaike weights (w_i), associated R^2-values, and change in R^2-values (ΔR^2) for models describing hair cortisol concentration (HCC) in free-ranging caribou (*Rangifer tarandus groenlandicus*) from the Akia-Maniitsoq herd (n = 46) and the Kangerlussuaq-Sisimiut herd (n = 48) killed in West Greenland in 2007 and 2008. The best fit model [based on ΔAICc ≤ 2, fewest predictor variables (parsimony) and associated R^2 value] is highlighted in bold.

			HCC		
Model set and measurement variables	AICc	ΔAICc	w_i	R^2	ΔR^2
Intercept-only	96.94	19.71	0.0000	-	-

1) Biology (age-reproductive class, age)	83.52	6.29	0.0430	0.21	-
2) Biology-Body Condition (age-reproductive class, age, body fat reserves)	80.52	3.29	0.1930	0.24	0.03
3) Biology-Body Condition-Parasite Burden (age-reproductive class, age, body fat reserves, CARMA bot score)	81.80	4.57	0.1018	0.26	0.02
4) Biology-Body Condition-Herd	81.24	4.01	0.1347	0.25	0.01
5) Biology-Body Condition-Parasite Burden-Herd **(age-reproductive class, age, body fat reserves, CARMA bot score, herd)**	**77.23**	**0.0**	**1.0000**	**0.31**	**0.05**

5.4.3 Factors influencing growth in West Greenland caribou

5.4.3.1 Multivariate models of body size in West Greenland caribou

The best fit model explained 86 % of the variation in total body length and incorporated caribou biology, body condition, and herd designation (Table 5.2.1, Table 5.2.2). Total body length of dependent caribou calves was lower than that of subadult females (Table 5.2.1) and most age-reproductive classes of adult female caribou in which total body lengths were similar (Table 5.2.1). Caribou age was also directly associated with total body length (Table 5.2.1) Overall, caribou biology explained 81% of the variation in total body length that could be accounted for (Table 5.2.2). While there was no relationship between total body length and total body weight or body fat reserves in the best fit model, cold carcass weight was directly associated with this dependent variable (Table 5.2.1). Body condition explained an additional 4 % of the variation in caribou total body length (Table 5.2.2). AK caribou were longer than KQ

caribou (Table 5.2.1), and herd designation accounted for 1% of the explained variation in caribou total body length. There was no relationship between parasite burden or HCC and total body length in AK and KQ caribou (Table 5.2.1, 5.2.2).

The best fit model explained 63% of the variation in caribou girth and included body condition, and herd designation only (Table 5.2.1, Table 5.2.2). Caribou biology (age reproductive class) accounted for 50% of the explained variation in girth during early model development, and we found that girth in dependent AK and KQ calves was < girth subadults < girth adults (Table 5.2.1, Table 5.2.2). When measures of body condition were added, cold carcass weight and BFR were not associated with girth (Table 5.2.1). However, girth tended to increase as total body weight increased (Table 5.2.1). Moreover, based on AICc scores, the girth model containing body condition (weight) only was superior to the model containing caribou biology and body condition together (ΔAICc >2) (Table 5.2.2). As a result, age reproductive class did not ultimately predict girth in the best fit model (Table 5.2.1, Table 5.2.2), and body condition alone explained 60% of the total variation in girth that could be accounted for (Table 5.2.2). There was no relationship between parasite burden and girth in AK or KQ caribou (Table 5.2.1). However, caribou from the AK herd had diminished girth relative to those from the KQ herd (Table 5.2.1), and the addition of herd designation to the best fit model added 3% to the explained variation in girth (Table 5.2.2). There was no relationship between HCC and girth in AK and KQ caribou (Table 5.2.1).

Table 5.2.1 Standardized coefficients (StD β) and significance (*P*) of model variables describing the best fit models for body size (total length, girth) in free-ranging caribou (*Rangifer tarandus groenlandicus*) from the Akia-Maniitsoq herd (*n* = 46) and the Kangerlussuaq-Sisimiut herd (*n* = 48) killed in West Greenland in 2007 and 2008. Measurement variables designated N.S.[#] were significant during model development but were not included in the best fit model. For potential predictor variables containing more than one category and also included in the best-fit model, non significant relationships (*P* > 0.10) are presented for comparative purposes.

Model set and	*Total length*		*Girth*	
measurement variables	β	*P*	β	*P*
1) Biology				
age-reproductive class				

calf (dependent)	-0.058	0.001		N.S.[#a]
adult female	0.003	0.872		N.S.[#a]
(not pregnant not lactating)				
adult female	0.031	0.032		N.S.[#a]
(not pregnant but lactating)				
adult female	0.009	0.508		N.S.[#a]
(pregnant but not lactating)				
adult female	0.016	0.291		N.S.[#a]
(pregnant and lactating)				
subadult	0	-		N.S.[#a]
age (0.8-11.9 years)	0.003	0.070		N.S.
age^2		N.S.		N.S.
2) Body Condition				
total body weight		N.S.	0.329	<0.001
cold carcass weight	0.184	<0.001		N.S.
body fat reserves		N.S.		N.S.
3) Parasite Burden				
CARMA bot score		N.S.		N.S.
CARMA warble score		N.S.		N.S.
4) Herd				
Akia-Maniitsoq	0.017	0.014	-0.039	0.008
Kangerlussuaq-Sisimiut	0	-	0	-
5) Hair Cortisol		N.S.		N.S.

[a] In the Biology model for girth:

Block category and	*Girth*	
measurement variables	β	*P*
age reproductive class		
calf (dependent)	-0.060	0.068
adult female (not pregnant not lactating)	0.146	0.001
adult female (not pregnant but lactating)	0.138	<0.001
adult female (pregnant not lactating)	0.144	<0.001
adult female (pregnant and lactating)	0.144	<0.001
subadult	0	-

Table 5.2.2 Sample-size corrected Akaike's Information Criterion (AICc) values, change in Akaike's Information Criterion (ΔAICc) values, Akaike weights (w_i), associated R^2-values, and change in R^2-values (ΔR^2) for models describing A) total length and B) girth in free-ranging caribou (*Rangifer tarandus groenlandicus*) from the Akia-Maniitsoq herd (n = 46) and the Kangerlussuaq-Sisimiut herd (n = 48) killed in West Greenland in 2007 and 2008. The best fit models [based on ΔAICc ≤ 2, fewest predictor variables (parsimony) and associated R^2 value] are highlighted in bold.

A.			*Total length*		
Model set and measurement variables	AICc	ΔAICc	w_i	R^2	ΔR^2
Intercept-only	-197.57	167.59	0.0000	-	-
1) Biology (age-reproductive class, age)	-341.11	24.05	0.0000	0.81	-
2) Biology-Body Condition (age-reproductive class, age, cold carcass weight)	-361.82	3.34	0.1882	0.85	0.04
3) Parasite Burden	-	-	-	-	-
4) Biology-Body Condition-Herd (age-reproductive class, age, cold carcass	**-365.16**	**0.0**	**1.0000**	**0.86**	**0.01**

weight, herd)					
5) Hair Cortisol Concentration	-	-	-	-	-
B.			*Girth*		
Model set and measurement variables	AICc	ΔAICc	w_i	R^2	ΔR^2
Intercept-only	-137.16	78.94	0.0000	-	-
1) Biology (age-reproductive class)	-186.66	29.44	0.0000	0.50	-
2) Biology-Body Condition (age-reproductive class, weight)	-204.05	12.05	0.0024	0.59	0.09
3) Body Condition (weight)	-211.61	4.49	0.1059	0.60	0.10
4) Parasite Burden	-	-	-	-	-
5) Body Condition- Herd (weight, herd)	**-216.10**	**0.0**	**1.0000**	**0.63**	**0.03**
6) Hair Cortisol Concentration	-	-	-	-	-

5.4.3.2 Multivariate models of body condition in West Greenland caribou

The best fit models explained 94% and 90% of the variation in total body weight and cold carcass weight and included caribou biology, body size, and herd designation (Table 5.3.1, Table 5.3.2). Adult female caribou weighed more than subadult female caribou and dependent calves in which total body weights were similar (Table 5.3.1). There was also a trend towards pregnant adult female caribou weighing more than their non pregnant counterparts regardless of lactation status (Table 5.3.1). The cold carcass weights of subadult female caribou were also greater than those of dependent caribou calves (Table 5.3.1), and were similar to those of adult female caribou that were not pregnant but lactating (Table 5.3.1). However, the cold carcass weights of subadult females were less than those of adult female caribou that were not pregnant and not lactating or pregnant regardless of lactation status (Table 5.2.1). There was no relationship between total body weight or cold carcass weight and caribou age (Table 5.2.1). Overall, biology explained 86% and 82% of variation in total body weight and cold carcass weight that could be accounted for (Table 5.3.2). Total body weight and cold carcass weight also increased as total length and girth increased (Table 5.3.1). However, adult female caribou from the AK herd weighed less and had lower cold carcass weights than those from the KQ herd (Table 5.3.1). Body size and herd designation explained an additional 5% and 3% and 6 % and 2% of the variation in total body weight and cold carcass weight respectively (Table 5.3.2). There was no relationship between parasite burden or HCC and total body weight or cold carcass weight in AK and KQ caribou (Table 5.3.1, Table 5.3.2).

Equivalent best fit models explained 69 % or 70 % of the variation in BFR and included caribou biology, body size, parasite burden, herd designation, ± HCC (Table 5.3.1, Table 5.3.2). Body fat reserves were similar in subadult female caribou, dependent calves, and adult females that were not pregnant and not lactating (Table 5.3.1). However, body fat reserves in these groups were lower than those of adult female caribou that were pregnant regardless of lactation status (Table 5.2.1). Although the relationship was not significant, there was also a trend towards adult female caribou that were not pregnant but lactating having fewer body fat reserves than all other age-reproductive classes (Table 5.3.1). Overall, biology explained 64% of the variation in BFR that could be accounted for (Table 5.3.2). In the best fit model, total body length was directly associated with BFR (Table 5.3.1) and body size accounted for an additional 3% of the

explained variation in BFR (Table 5.3.2). Those caribou with low intensity warble infections (21-100 larvae per animal) were in better body condition relative to those with higher intensity infections (101-1000 larvae per animal), and parasite burden also added 2% to the explained variation in BFR (Table 5.3.1, 5.3.2). There was no relationship between nasal-bot infection or herd designation and BFR (Table 5.3.1). HCC was inversely associated with BFR in AK and KQ caribou, and accounted for an additional 1% of the explained variation in its measurement (Table 5.3.1, 5.3.2).

Table 5.3.1 Standardized coefficients (StD β) and significance (*P*) of model variables describing the best fit models for body condition (total body weight, cold carcass weight, body fat reserves) in free-ranging caribou (*Rangifer tarandus groenlandicus*) from the Akia-Maniitsoq herd (*n* = 46) and the Kangerlussuaq-Sisimiut herd (*n* = 48) killed in West Greenland in 2007 and 2008. For potential predictor variables containing more than one category and also included in the best-fit model, non significant relationships (*P* > 0.10) are presented for comparative purposes.

Model set and	*Total body weight*		*Cold carcass weight*		*Body fat reserves*	
measurement variables	β	*P*	β	*P*	β	*P*
1) Biology						
age-reproductive class						
calf (dependent)	-0.165	<0.001	-0.200	<0.001	0.171	0.587
adult female	0.156	<0.001	0.122	0.016	0.430	0.148
(not pregnant not lactating)						
adult female	0.132	<0.001	0.037	0.313	-0.292	0.220
(not pregnant but lactating)						
adult female	0.242	<0.001	0.141	<0.001	0.935	<0.001
(pregnant but not lactating)						
adult female	0.240	<0.001	0.129	0.001	0.717	0.004
(pregnant and lactating)						
subadult	0	-	0	-	0	-
age (0.8-11.9 years)		N.S.		N.S.		N.S.

age^2		N.S.		N.S.		N.S.
2) Body Size						
total length	1.061	<0.001	1.279	<0.001	5.351	0.001
girth	0.218	0.019	0.270	0.023		N.S.
3) Parasite Burden						
CARMA bot score		N.S.		N.S.		N.S.
CARMA warble score						
(21-100 larvae)		N.S.		N.S.	0.303	0.027
(101-1000 larvae)		N.S.		N.S.	0	-
4) Herd						
Akia-Maniitsoq	-0.100	<0.001	-0.077	<0.001		N.S.
Kangerlussuaq-Sisimiut	0	-		N.S.		N.S.
5) Hair Cortisol		N.S.		N.S.	-0.324	0.033

Table 5.3.2 Sample-size corrected Akaike's Information Criterion (AICc) values, change in Akaike's Information Criterion (ΔAICc) values, Akaike weights (w_i), associated R^2-values, and change in R^2-values (ΔR^2) for models describing A) total body weight, B) cold carcass weight, and C) body fat reserves in free-ranging caribou (*Rangifer tarandus groenlandicus*) from the Akia-Maniitsoq herd (n = 46) and the Kangerlussuaq-Sisimiut herd (n = 48) killed in West Greenland in 2007 and 2008. The best fit models [based on ΔAICc ≤ 2, fewest predictor variables (parsimony) and associated R^2 value] are highlighted in bold.

A.	*Total body weight*				
Model set and measurement variables	AICc	ΔAICc	w_i	R^2	ΔR^2
Intercept-only	17.17	216.56	0.0000	-	-

1) Biology (age-reproductive class)	-160.99	72.71	0.0000	0.86	-
2) Biology-Body Size (age-reproductive class, total length, girth)	-200.62	33.08	0.0000	0.91	0.05
3) Parasite Burden	-	-	-	-	-
4) Biology-Body Size-Herd (age-reproductive class, total length, girth, herd)	**-233.70**	**0**	**1.0000**	**0.94**	**0.03**
5) Hair Cortisol Concentration	-	-	-	-	-

B.	*Cold carcass weight*				
Model set and measurement variables	AICc	ΔAICc	w_i	R^2	ΔR^2
Intercept-only	15.54	203.28	0.0000	-	-
1) Biology (age-reproductive class)	-139.13	48.61	0.0000	0.82	-
2) Biology-Body Size (age-reproductive class, total length, girth)	-175.66	12.08	0.0024	0.88	0.06
3) Parasite Burden	-	-	-	-	-
4) Herd (age-reproductive class, total length,	**-187.74**	**0**	**1.000**	**0.90**	**0.02**

girth, herd)					
5) Hair Cortisol Concentration	-	-	-	-	-
C.	*Body fat reserves*				
Model set and measurement variables	AICc	ΔAICc	*wi*	R^2	ΔR^2
Intercept-only	259.07	89.17	0.0000	-	-
1) Biology (age-reproductive class)	173.97	13.07	0.0015	0.64	-
2) Biology-Body Size (age-reproductive class, total length)	166.37	5.47	0.0649	0.67	0.03
3) Biology-Body Size-Parasite Burden (age-reproductive class, total length, CARMA warble score)	**162.80**	**1.90**	**0.3867**	**0.69**	**0.02**
4) Herd	-	-	-	-	-
5) Biology-Body Size-Parasite Burden-Hair Cortisol Concentration (age-reproductive class, total length, CARMA warble score, HCC)	**160.90**	**0.0**	**1.0000**	**0.70**	**0.01**

5.5 Discussion

5.5.1 Factors influencing hair cortisol concentration in West Greenland caribou

HCC levels were higher in dependent calves and subadults compared to adult female caribou. Growth is an energetically costly process in ungulates (Gaillard et al. 2000, Parker et al.

2009), and, in *Rangifer*, relatively rapid growth is known to occur in the first year of life, and may continue in female animals (at a reduced rate) until approximately 5 years of age (Nieminen and Peterson 1990, Miller 2003). In addition to their role as effectors of the stress response, glucocorticoids play an important part in energy metabolism and are often elevated in juvenile animals during their active growth phase (Landys et al. 2006). Thus, elevated HCC in dependent calves and subadults compared to adults may reflect the demands of growth in the juvenile period (Landys et al. 2006, Ashley et al. 2011). However, juvenile *Rangifer* may also be particularly susceptible to density dependent effects (Weladji et al. 2005, Couturier et al. 2009a, Couturier et al. 2010), environmental conditions (Weladji and Holand 2003, Hegel et al. 2010, Bårdsen and Tverra 2012), and parasitic infection (Folstad et al. 1989, Weladji et al. 2003); and this finding may also reflect the effects of increased exposure to or decreased tolerance of stressors in juvenile animals (Gaillard et al. 2000, Parker et al. 2009). Importantly, elevated serum cortisol has been previously recorded in free-ranging juvenile *Rangifer* compared to adult females (Omsjoe et al. 2009) while elevated HCC has also been observed in juvenile reindeer (*R.t. tarandus*) compared to adult caribou (*R.t. granti*) held in captivity (Ashley et al. 2011). Together, these findings suggest that the consequences of increasing long-term stress are likely to impact the health and survival of juvenile AK and KQ caribou before other groups. Thus, increasing long-term stress levels could have important consequences for the continued sustainability of the AK and KQ caribou populations.

In the best fit model, we also observed a direct association between caribou age and HCC. This findings agrees with relationships identified in polar bears from southern Hudson Bay (Chapter 3) but disagrees with HCC studies in other species where no relationship between these parameters has been identified [e.g. grizzly bears (*Ursus arctos)* (Macbeth et al. 2010); East Greenland polar bears (*Ursus maritimus)* (Bechshøft et al. 2011); humans (Manenschijn et al. 2011a)]. Nonetheless, deregulation of the HPA axis is known to occur with age in some species and may also be associated with increasing levels of circulating glucocorticoids (Rothuizen et al. 1991, Reader and Kramer 2005, Smith et al. 2005). As for Southern Hudson Bay polar bears, older *Rangifer* 1) may be less able to cope with the energetic demands of natural life history processes or stressors (McEwen 2002), 2) may be more reactive to these stimuli (Rothuizen et al. 1991, Smith et al. 2005), or 3) may recover more slowly from energetic challenges than their

younger counterparts (Reeder and Kramer 2005). However, age, body size, and body mass are known to be directly associated with social dominance in female *Rangifer* (Hirotani 1990, Miller 2003, Holand et al. 2004a) and the direct association of HCC and age in this species may reflect the stress of social dominance that has been recorded in other herd dwelling ungulates (Mooring et al. 2006, Berg et al. 2008, Forristal et al. 2012). Overall, patterns of HCC observed in West Greenland caribou appear to reflect changes in HPA axis activity related to caribou life history, and provide evidence in support of HCC as a biologically relevant index of long-term stress in this species (Busch and Hayward 2009).

Body condition (reported as total body weight, carcass weight, body fat reserves and sometimes body protein reserves in *Rangifer*; Olofsson et al. 2011, Taillon et al. 2011) is an index of growth that is considered a key determinant of health, survival, and fecundity in female *Rangifer* and their calves (Cameron et al. 1993, Rönnegård et al. 2002, Taillon et al. 2011, Bårdsen and Tverra 2012). Using available ancillary data, we evaluated the importance of total body weight, cold carcass weight, and body fat reserves (BFR) as potential predictors of HCC in AK and KQ caribou. We observed no relationship between HCC and total body weight or cold carcass weight in the best fit model. However, we did find that HCC was inversely associated with BFR such that higher HCC levels were recorded in those caribou with diminished body fat reserves.

In *Rangifer*, total body weight and cold carcass weight reflect both skeletal size and soft tissue mass (Olofsson et al. 2011,Taillon et al. 2011) and are therefore influenced by conditions encountered in early life as well as those which may vary on an annual basis (Couturier et al. 2009b, Couturier et al. 2010, Taillon et al. 2011, Bårdsen and Tverra 2012). Conversely, body fat reserves may be strongly influenced by more recent energetic challenges (Chan-McLeod et al. 1999, Olofsson et al. 2011, Taillon et al. 2011). HCC levels in *Rangifer* are considered to represent an integrated measure of HPA axis activity during the period of active hair growth (Ashley et al. 2011). As a result, it is also likely that HCC is foremost a seasonal measure of HPA axis activity in this species (Ashley et al. 2011) and this relationship was not unexpected. Overall, these results suggest that long-term stress may be associated with diminished health in caribou (Reimers 1983, Whitfield and Russell 2005, Ashley et al. 2011). These findings also agree with similar relationships previously identified in free-ranging grizzly bears (M. Cattet,

personal communication) and Southern Hudson Bay polar bears (Macbeth et al. 2012), and may provide additional support for the measurement of HCC as a valid biomarker of long-term stress in *Rangifer* (Busch and Hayward 2009). Nonetheless, further research is necessary to more precisely define relationships between body condition and HCC in caribou. Male *Rangifer* also exhibit different temporal patterns of energy use and allocation compared to females and juveniles (Barboza et al. 2004, Parker et al. 2009) making an exploration of changes in HCC with body condition in this group an essential component of any future research effort. Controlled studies tracking changes in body condition and HCC in captive *Rangifer* herds would be especially informative.

More complex relationships may also exist between levels of circulating cortisol and body condition in *Rangifer*. For example, maternal stress during gestation may permanently alter HPA axis activity and negatively impact growth and development in affected fetuses (HPA axis programming: Seckel 2004, Kapoor et al. 2006, Meaney et al. 2007). The negative effects of maternal stress during gestation and HPA axis programming in calves could represent one mechanism responsible for diminished body size recorded in the offspring of female caribou in poor body condition (Cameron et al. 1993, Couturier et al. 2009b, Bårdsen and Tverra 2012, Taillon et al. 2012). We did investigate HCC levels in the limited number of cow-calf pairs available for this study, but did not observe any relationship between HCC levels in mothers and dependent offspring. Nonetheless, maternal stress and diminished body size are considered important consequences of overpopulation in *Rangifer* (Reimers 1983, Couturier et al. 2009a, Couturier et al. 2009b, Mahoney et al. 2011) and we suggest that further study of these relationships in a larger group of animals is warranted.

To prove useful as a conservation tool, HCC must also be associated with environmental conditions related to determinants of survival and fitness in caribou (Busch and Hayward 2009). In this study, available data allowed us to explore the effects of parasitic infection with Oestrid fly larva [warble flies (*Hypoderma tarandi*); life cycle reviewed in (Nilssen and Haugerud 1994), and nasal-bot flies (*Cephenemyia trompe*); life cycle reviewed in (Nilssen and Haugerud 1994, Nilssen and Haugerud 1995)]. In caribou, insect harassment may reduce foraging efficiency and milk production while simultaneously increasing energy expenditure in avoidance behaviours (Toupin et al. 1996, Weladji et al. 2003, Witter et al. 2012). Infection with warbles and nasal-

bots may also be associated with significant localized pathology and sometimes overt clinical disease in heavily infected hosts (Dove and Cushing 1933, Cogley et al. 1987, Nilssen and Haugerd 1995). Furthermore, larval Oestrid flies inhabit their host for extended periods (weeks to months) (Nilssen and Haugerud 1994, Nilssen and Haugerd 1995) suggesting these parasites may be an important source of long-term stress in *Rangifer* (Reimers 1983, Weladji et al. 2003, Whitfield and Russell 2005). Diminished body condition (Thomas and Kiliaan 1990, Weladji et al. 2003, Cuyler et al. 2012) along with a reduced probability of pregnancy (Hughes et al. 2008, Cuyler et al. 2012) have also been observed in heavily infected animals, and long-term stress due to increased insect harassment and infection is believed to be an important factor contributing to the decline of some *Rangifer* populations (Weladji et al. 2003,Vors and Boyce 2009, Witter et al. 2012).

In the best fit HCC model, we found that animals infected with relatively few nasal bot larvae (1-20) had lower HCC levels than those with higher intensity (21-100 or 101-1000 larvae) infections. However, we observed no relationship between the intensity of warble infection and HCC. Interestingly, the observed pattern is opposite of what may be expected based on known differences in the extent of infection and overall pathology caused by the larvae of each fly species. For example, *C. trompe* infections are localized (nasopharyngeal region only) and generally believed to cause limited pathology in *Rangifer* (Nilssen and Haugerd 1995, Colwell 2001, Cuyler et al. 2012). In contrast, infection with *H. tarandi* larvae may occur in a wide area along the dorsal aspect of the animal (Dove and Cushing 1933, Nilssen and Haugerud 1994, Colwell 2001). At different stages of their development, warble fly larvae may also be associated with the skin-hair follicle and migrate extensively in subcutaneous tissue (Dove and Cushing 1933). Moreover, this species causes extensive pathology in the skin and subcutaneous tissue and related damage may persist for extend periods (>1 year) (Dove and Cushing 1933, Colwell 2001). In addition, Cuyler et al. (2012) found that infection with warbles may represent a subtle but important energy cost which may threaten the reproductive success or survival of AK and KQ caribou. Together, these characteristics suggest that long-term stress should also be related to heavy warble burdens in caribou; either due to energetic deficits (Busch and Hayward 2009), a generalized stress response, a more localized reaction to warble fly larvae and tissue damage, or a combination of these factors (Ito et al. 2005, Sharpley et al. 2009a, Ashley et al. 2011).

Importantly, the intensity of warble infection in free-ranging *Rangifer* may be affected by age, sex-class, social status (Folstad 1989, Thomas and Kiliaan 1990, Fauchald et al. 2007) and even hair colour (Rodven et al. 2009). These factors may also influence HCC in caribou (Omsjoe et al. 2009, Ashley et al. 2011, Chapter 4) and may be one reason why we did not observe elevated HCC levels in caribou with high warble burdens. Overall, our findings provide preliminary evidence that long-term stress may be associated with parasite burden (an environmental condition also related to diminished health or population performance in *Rangifer*; Weladji et al. 2003, Hughes et al. 2008, Cuyler et al. 2012) and offer further support for the utility of HCC analysis in this species (Busch and Hayward 2009). Moreover, these results suggest that infection with nasal bots may be more costly for *Rangifer* than is presently understood. However, experimental infections with both parasites are necessary to determine causal relations between parasite burden and elevated HCC in this species (Booth et al. 1993).

Other natural or anthropogenic factors may contribute to long-term stress levels and may also be highly variable among different caribou populations (Vor and Boyce 2009, Festa-Bianchet et al. 2011, Gun 2011). In West Greenland, the AK and KQ caribou populations are separated by natural landscape features, exhibit distinct population characteristics, and inhabit regions with divergent environmental conditions (Cuyler 2007, CARMA 2012b, CARMA 2012c, Witting and Cuyler 2011). We observed that AK caribou had lower HCC levels than KQ caribou. Ashley et al. (2011) found that HCC levels measured in neck hair of captive *R. t. granti* were higher than in other body regions (shoulder or rump). Hair from AK caribou was obtained prior to guidelines established in Ashley et al. (2011) and was collected from the neck. In contrast, hair from KQ caribou was collected from the shoulder (C. Cuyler, personal communication), and this discrepancy may account for our findings. An alternate explanation (which is not necessarily mutually exclusive) may be that hair samples from each herd were obtained in different years which may indicate that divergent energetic demands or stressors existed in the two populations during the hair growth season preceding sampling (Macbeth et al. 2010, Ashley et al. 2011). For example, population density may be an important determinant of stress levels in caribou (Skogland 1983, Couturier et al. 2009b, Mahoney et al. 2011), and caribou density in both the AK and KQ populations is considered to be above sustainable levels (1.2 caribou/km^2; CARMA 2012b). However, caribou density is known to be far greater in the

KQ population (7 caribou/km^2; CARMA 2012c) than in the AK population (2 caibou/km^2; CARMA 2012b). Moreover, the KQ caribou population is found in a less productive region and is also subject to a greater amount of anthropogenic landscape disturbance than the AK population at the present time (CARMA 2012b, CARMA 2012c).

Although we were able to establish relationships between HCC and caribou life history, health, and environmental conditions we were unable to explain 69% of the variation in its measurement. Had additional supporting data been available in this study it is likely we could have developed more robust models for HCC. The relationships between HCC in *Rangifer* and prevailing ecological conditions such as precipitation, snow depth, predation risk, and temporal patterns of forage availability deserve further investigation (Gunn et al. 2009, Gunn 2011). Free-ranging *Rangifer* herds may also be compromised by a variety of other parasitic infections [e.g. abomasal nematodes (Hughes et al. 2008), *Besnoitia* sp : (Wobeser 1976)] or chronic diseases [e.g. *Brucella suis* biovar 4; (Ferguson 1997), alpha herpes virus infection (Evans et al. 2012)]. Potential relationships between these factors and HCC also warrant further study. The direct impacts of human activity (Freeman 2008, Wasser et al. 2011, Nellemann 2012) and caribou population density (Couturier et al. 2009b, Mahoney et al. 2011) on HCC should also be evaluated.

5.5.2 Factors influencing growth in West Greenland caribou

Biological attributes are known to be closely related to body size and condition in free-ranging female caribou and their calves (Festa-Bianchet et al. 1998, Couturier et al. 2009a, Couturier et al. 2009b, Taillon et al. 2011) and not surprisingly, we observed that these traits were the most important predictors of total body length (R^2 81 %), total body weight (R^2 86 %), cold carcass weight (R^2 82 %), and body fat reserves (R^2 64 %) in AK and KQ caribou. Our observation of a direct association between age and length as well as between indices of body size and body condition also agree with known patterns of growth in this species (Nieminen and Peterson 1990, Festa-Bianchet et al. 1998, Rettie 2004, Olofsson et al. 2011,Taillon et al. 2011). For example, larger female caribou are known to accumulate greater fat reserves compared to smaller females (Fest-Bianchet et al. 1998) and we found that BFR in AK and KQ caribou were directly associated with an animal's total length. Similarly, we observed that pregnant female *Rangifer* weighed more, had greater cold carcass weights, and more body fat reserves than those

that were not pregnant regardless of lactation status. These findings also agree with previous studies which report that heavier and fatter female caribou are more likely to reproduce than their lighter counterparts since larger stores of body fat may ensure sufficient energy for survival and reproduction (Cameron et al. 1993, Rönnegård et al. 2002, Bårdsen and Tverra 2012). An inverse association between body fat reserves and intensity of warble infection has also been previously reported in this species (Hughes et al. 2008, Cuyler et al. 2012).

Although we identified a variety of relationships in support of HCC as a biologically relevant biomarker of long-term stress in our HCC model, the full validation of this technique also requires that HCC be revealed as a useful predictor of health status in *Rangifer* (Busch and Hayward 2009). To more vigorously evaluate this criterion, we examined HCC as a potential predictor of selected indices of body size (total length, girth) and body condition (total body weight, cold carcass weight, body fat reserves). These indices of growth are closely related to health, fitness and population performance in female caribou and their calves (Cameron et al. 1993, Weladji et al. 2003, Taillon et al. 2011).

We found that HCC did not account for any of the explained variation in total body length or girth among AK and KQ caribou. In *Rangifer,* these parameters reflect skeletal size which is primarily related to the effects of genetics and environmental conditions encountered during the period of active growth in early life (Barboza et al. 2009, Mahoney et al. 2011, Taillon et al. 2011). Our sample of caribou was dominated by adult females and, since HCC may best account for the effects of relatively recent stressors (Macbeth et al. 2012), the lack of association between HCC and these indices of skeletal size was not entirely unexpected. These findings also agree with the lack of HCC-length relationship reported previously in polar bears (Macbeth et al. 2012).

Similarly, we also determined that HCC did not predict total body weight or cold carcass weight in our study animals. As in polar bears (Macbeth et al. 2012), the lack of association between HCC and total body weight or cold carcass weight may be explained by the fact that skeletal size is an important component of these growth indices (Nieminen and Peterson 1990, Rettie 2004, Olofsson et al. 2011,Taillon et al. 2011). Short-term variation in rumen mass or alimentary fill may also have masked any relationship between total body weight and HCC levels (CARMA 2008a, Taillon et al. 2011). These relatively crude indices of condition may also

be insensitive to subtle changes related to HCC levels (Taillon et al. 2011). Findings for indices of body weight are also similar to those reported in polar bears (Macbeth et al. 2012).

Significantly, we did observe that HCC was inversely associated with BFR such that those AK and KQ caribou with higher HCC levels also had fewer body fat reserves than other caribou. Nonetheless, HCC was a relatively weak predictor of BFR (ΔR^2 1 %) in these caribou populations. Given the seasonal nature of hair growth (Macbeth et al. 2010) and the inverse association between BFR and HCC identified in the HCC model, the low proportion of variation in BFR explained by HCC was somewhat surprising. However, a similar relationship has been observed in polar bears (Macbeth et al. 2012).

In cervids, body fat stores are used sequentially beginning with subcutaneous fat, followed by mesenteric-kidney associated fat, and finally bone marrow fat (Mautz 1978, Taillon et al. 2011). Fat reserves are also generally depleted by late winter and spring (Taillon et al. 2011). In this study, all caribou were sampled in late winter/early spring (March-April) and most caribou were in poor body condition (CARMA Condition Score; Skinny, C. Cuyler, personal communication) with diminished fat reserves recorded in the majority of animals (C. Cuyler, personal communication). As in polar bears (Macbeth et al. 2012), one explanation for this finding may be that a narrow range of body fat levels in study animals reduced the probability of detecting a strong association between BFR and HCC in the growth model (Macbeth et al. 2012).

An alternate explanation may be that restricted overlap between periods of fat deposition or use and active hair growth in *Rangifer* may have prevented an accurate evaluation of this relationship in our study (Couturier et al. 2009a, Couturier et al. 2009b, Taillon et al. 2011). For example, adult female caribou may not deposit fat until after weaning their calves and body fat reserves may more accurately reflect body condition in adult female caribou from late fall to early spring (Couturier et al. 2009b , Taillon et al. 2011). Hair growth in caribou occurs primarily in the spring through early fall (Miller 2003, Ashley et al. 2011) and limited overlap between hair growth and the deposition or use of fat reserves may explain why HCC only accounted for 1% of the explained variation in BFR. The most accurate indices of body condition may also vary with age in *Rangifer* (Taillon et al. 2011) and our results may also have been influenced by the high numbers adult females considered in our analysis. For adult female caribou, body protein reserves may actually be the best indicator of body condition in adult female caribou

during the hair growth period (Taillon et al. 2011), and changes in protein reserves (e.g. peroneus muscle mass; Taillon et al. 2011) may be more relevant to HCC levels measured in this group (Landys et al. 2006, Macbeth et al. 2010, Taillon et al. 2011). Further research in captive *Rangifer* herds is recommended to address these knowledge gaps.

We also observed differences in the total body length, girth, weight, and cold carcass weight of AK and KQ caribou. Although they were marginally longer, AK caribou exhibited diminished girth, weighed less, and had lower carcass weights than KQ caribou. However, we did not identify any differences in body fat reserves between the two populations. In *Rangifer*, reduced body size and body condition may reflect density dependent processes (Skogland 1983, Couturier et al. 2009a, 2009b, Mahoney et al. 2011). Caribou numbers are currently above sustainable levels in both populations, however caribou abundance in the KQ population is increasing while that in the AK population has been declining since at least 2001 (Cuyler 2007, CARMA 2012b, CARMA 2012c). These differences may indicate that, unlike KQ caribou, the AK population may already have reached carrying capacity and the peak of its population cycle (CARMA 2012b). A time lag effect is recognized between increasing population density and diminished body size or body condition in *Rangifer* (Olofsson et al. 2011, Mahoney et al. 2011) and, while genetics (Cronin et al. 2006, CARMA 2012b, CARMA 2012c), other environmental characteristics (Couturier et al. 2010), or factors related to sampling (e.g. rumen or gut fill; CARMA 2008a, Taillon et al. 2011) may also be contributing factors, population trends may account for the diminished growth indices we recorded in AK caribou (Reimers 1983, Couturier et al. 2009a, 2009b, Mahoney et al. 2011). Interestingly, we also found that AK caribou exhibited lower HCC levels than KQ caribou. As previously described, low HCC levels in the AK population may be explained by sampling protocols, environmental conditions, or the relatively low caribou abundance compared to the KQ population. However, these differences may also be evidence of a declining density dependent effect related to the decreasing population abundance now being recorded in the AK population (CARMA 2012b). As such, it is possible that diminished body size and condition as well as lower HCC levels may be anticipated in the KQ population in the near future (Cuyler 2007, CARMA 2012c, Mahoney et al. 2011).

Overall, we found no evidence to suggest that HCC is not a biologically relevant biomarker of long-term stress in free-ranging *Rangifer* from the AK and KQ populations of West

Greenland. With further research, HCC may also have potential as a broadly applicable and practical conservation tool for caribou and reindeer elsewhere. By incorporating longitudinal hair sample collection into ongoing research programs and harvest reporting procedures already in existence (CARMA 2012a, CARMA 2012b), the continued study of HCC levels caribou inhabiting the relatively simple systems of West Greenland may further advance the development of this technique. I also recommend additional research in other free-ranging and captive *Rangifer* herds to address current knowledge gaps and to more vigorously evaluate the utility of HCC analysis in this species.

CHAPTER 6

GENERAL DISCUSSION AND RECOMMENDATIONS FOR FUTURE WORK

6.1 Hair Cortisol Assay

The first objective of this research program was to develop and apply a reliable and accurate method to measure cortisol levels in hair collected remotely or opportunistically from free-ranging grizzly bears, polar bears and caribou. In the first stage of this investigation an EIA-based technique for the determination of HCC in non-human primates (Davenport et al. 2006) was modified for use in grizzly bears, and was found to be precise (intra-assay C.V. 3.58%, inter-assay C.V. 5.75%), accurate (extraction efficiency: 94.16±5.12%), and specific for cortisol (parallelism of grizzly bear hair extract with cortisol standards $r^2 = 0.998$, $P < 0.001$) in this species (Macbeth et al. 2010). The estimated sensitivity of this commercially available EIA kit (approximate limit of detection 0.32 pg/mg) also agreed with (0.30 pg/mg; Renaud 2012) or exceeded values reported using other EIA's in other HCC studies (0.90 pg/mg; Davenport et al. 2006).

Methods developed in grizzly bears were then altered for use in polar bear hair (Chapter 3, Macbeth et al. 2012). Assay precision (intra-assay C.V. 9.74%, inter-assay C.V. 11.44%) in SH polar bears was somewhat less than that measured in grizzly bears in this study and was also less than that recorded in East Greenland polar bears (intra-assay C.V. 3.0 %, inter-assay C.V. 7.0 %) by Bechshøft et al (2011). The performance of enzyme immunoassays frequently decreases near the lower (and upper) limits of detection, and, unlike grizzly bears and East Greenland polar bears, HCC levels in SH polar bears were often near the detection limit of the EIA kit employed in this study. This discrepancy may account for the minor differences in assay precision observed between bear species and studies. Nonetheless, these parameters remained within generally accepted limits (intra-assay C.V. $\leq$ 10% and inter-assay C.V. $\leq$ 15%), and estimates of accuracy (extraction efficiency: 95.55 ± 3.18%) and specificity (parallelism of polar bear hair extract with cortisol standards $r^2 = 0.991$, $P = 0.004$) were similar to values determined in grizzly bears in this study.

In general, assay performance in *Rangifer* [intra-assay C.V. 6.04%, extraction efficiency (94.53 ± 3.52%), parallelism ($r^2 = 0.998$, $P < 0.001$)] also agreed with findings in both bear species and with those of a more recent HCC-based *Rangifer* study (Renaud 2012). Nonetheless, a marginal inter-assay C.V. (18.39%) suggests that the precision of HCC values determined in this species may decrease if assessed on different EIA plates. It is probable that the presence of

the unidentified flocculent material in some samples of *Rangifer* hair extract (Chapter 4, section 4.4.1) contributed to this finding.

Logistical and budgetary restraints did not permit chemical or structural identification of this substance. However, similar material was observed infrequently (and in much smaller quantities) in hair extract from a few grizzly bears and polar bears (B. Macbeth, personal observation). In these species, this material was most frequently associated with hair samples obtained from carcasses and contaminated with large quantities of lipid (and blood) (B. Macbeth, personal observation). Together with gross morphology, this may indicate that the unidentified material in *Rangifer* hair extract is a lipid possibly related to the structural makeup of caribou hair or to the presences of glandular secretions on the hair surface (Cuyler and Ørtisland 2005, Pragst and Balikova 2006). It should also be noted that this material was not observed in hair extract from as many animals or in as large quantities in caribou from West Greenland (Chapter 5). Furthermore, in all cases where the substance was present in the Greenland study it was easily returned to solution after the reconstitution process (B. Macbeth, personal observation). Possible explanations for these discrepancies may include: 1) unintended procedural differences between the two studies (e.g. inadvertent over-drying of hair extract prior to reconstitution in the ACTH study), or 2) some difference in the composition of the unidentified material (possibly related to study animals, sampling protocols, or investigative techniques). To improve assay precision, further research is recommended to identify this material and its overall importance to HCC analysis in *Rangifer*.

Despite minor differences, overall, HCC assay performance in all study species also agreed with or exceeded values reported for humans and domestic animals (e.g. Davenport et al. 2006, Sauvé et al. 2007, Accorsi et al. 2008, Gow et al. 2010). These findings suggest that the first objective of this research program was achieved, and demonstrate that, with minor modification, methods developed in this study may be broadly applicable in other mammals.

6.2 Hair Cortisol Concentration as a Biomarker of Long-term Stress and Potential Conservation Tool in Grizzly Bears, Polar Bears, and Caribou

The second objective of this research program was: 1) to evaluate the utility of HCC as a biomarker of long-term stress in individuals of each study species, and 2) to assess the potential of HCC to serve as a practical conservation tool in free-ranging grizzly bear, polar bear, and

caribou populations. In order to be revealed as a defensible biomarker of long-term stress, factors influencing HCC must first be identified in target species and unambiguous linkages must then be established between HCC, prevailing environmental conditions, and the biology or health of individual animals (Busch and Hayward 2009). In order to be considered a practical tool for wildlife conservation, hair collection for HCC analysis should be easily accomplished and also relatively cost effective. HCC analysis should also integrate well with current research techniques and management goals related to the species of interest. Most importantly, HCC must also be associated with parameters directly related to rates of survival or reproduction at the population level (Busch and Hayward 2009). The approach towards objective 2 was guided by the availability of ancillary data and varied among study species.

6.2.1 Grizzly bears and polar bears

In grizzly bears, this study was limited to an exploration of procedural, structural, and basic biological characteristics which may have bearing on the measurement or interpretation of HCC in individual animals. Building on these results, similar relationships were explored in polar bears. However in the latter, more supporting data was available which allowed potential relationships between HCC, biology, health, and prevailing environmental conditions to be more thoroughly evaluated. Interestingly, procedural considerations for HCC analysis, the range of HCC values, and the relationship between HCC and biological traits identified in each bear species were somewhat different.

Although hair surface contamination with cortisol-containing biologicals was revealed as a potential confounder of HCC analysis in both species, the probability was far greater in grizzly bears than in polar bears; a factor most likely reflecting differences in life history (i.e. terrestrial vs marine) and the capture methods employed in each study (Chapter 2, Chapter 3, Appendix 2). In grizzly bears, HCC could be reliably assessed in small quantities of hair (approximately 5-10 guard hairs) representative of samples which may be easily obtained through remote (e.g. barb wire snagging; Woods et al. 1999) or opportunistic (e.g. hunting and research archives) methods in this species (Chapter 2, Macbeth et al. 2010). Conversely, HCC could not be reliably measured in less than 25-35 polar bear guard hairs (Chapter 3). Furthermore, HCC was influenced by hair type and body region in grizzly bears (Chapter 2, Macbeth et al. 2010), while in polar bears these factors had no effect (Chapter 3). HCC levels in Alberta grizzly bears (mean

2.84 pg/mg, range 0.62-43.33 pg/mg) also greatly exceed those of SH polar bears (mean 0.48 pg/mg, range, 0.16-2.26 pg/mg) (Independent sample t-test, $t_{(334)} = 27.843$, $P < 0.001$, $n = 336$).

The ability to measure HCC in small quantities of bear hair may be a function of the EIA cortisol detection limit, the amount of cortisol in a hair sample, and the lowest volume of reconstituted extract required for HCC analysis. In this study, a minimum of 0.2 ml phosphate buffer was necessary to achieve submersion of concentrated-dried hair extract and also to reliably obtain the 0.15 ml of reconstituted extract needed for triplicate HCC analysis in polar bears (B. Macbeth, personal observation). Given this fact and the generally low values recorded in SH polar bears it is not surprising that HCC could not be consistently measured in small quantities of hair from this subpopulation. However, 25-35 guard hairs can be obtained through established barb-wire collection techniques in other bear species (B. Macbeth, personal observation). Moreover, HCC values determined in three polar bears from the Beaufort Sea subpopulation (adult female: 5.92 pg/mg, dependent female: 1.01 pg/mg, dependent male: 19.98 pg/mg) in this study and in polar bear from East Greenland (Bechshøft et al. 2011, Bechshøft et al. 2012) were similar to or exceed those determined in grizzly bears. Together, these findings suggest that, as in grizzly bears, barb-wire snagging may prove useful for the collection of hair for HCC analysis in SH polar bears and in other polar bear subpopulations.

The option of remote sample collection indicates that HCC analysis may have great potential as a practical research and conservation tool for use in free-ranging grizzly and polar bear populations (Macbeth et al. 2010, Macbeth et al. 2012). Compared to capture and immobilization, established barb wire snagging techniques (Woods et al. 1999) are generally considered to be cost effective and non-invasive (Waits and Paetkau 2005, Proctor et al. 2010). Capture-mark-recapture based estimates of bear abundance have also been undertaken using DNA collected from hair bulbs to identify individual bears, their gender, patterns of movement, habitat use (Mowat et al. 2005, AGBRP 2008, Proctor et al. 2012), and family relationships (Zeyl et al. 2009, Swenson et al. 2011). Hair cortisol data generated from bear hair may have the potential to be evaluated in the context of these parameters as well as with estimates of exposure to environmental contaminants (Christensen et al. 2007, Dietz et al. 2009, Jaspers et al. 2010), assimilated diet (Felicetti et al. 2003a, Mowat and Heard 2006, Horton et al. 2009), and other conservation-relevant hormones (e.g. sex steroids or thyroid hormones; D. Janz, personal

communication) generated from the same hair sample. These considerations, along with the fact that hair from bears can easily be transported over long distances without refrigeration (B. Macbeth, personal observation), and the observation that HCC in bear hair may be relatively stable in the environment and in the laboratory (Macbeth et al. 2010, Chapter 3) provide additional support for the potential utility of this technique. Furthermore, the set up and collection of bear hair from barb wire snags requires limited technical training and may represent a unique approach with which to engage local communities in science-based initiatives in bear conservation (Aryal 2012). Most bear species are widely distributed, and face markedly different life history constraints or anthropogenic challenges across their geographic ranges (Thiemann et al. 2008, Zedrosser et al. 2011, Proctor et al. 2012). Collaborative studies of HCC in different bear populations using remote or opportunistic sampling techniques and incorporating health and environmental data are recommended to further evaluate HCC as a defensible biomarker of long-term stress and conservation tool in ursids.

General differences in HCC levels and patterns of HCC recorded between bear species are also noteworthy and may reflect divergent structural features of hair, patterns of weathering, inherent differences in glucocorticoid physiology, or patterns of exposure to stressful stimuli. Although grizzly bears and polar bears have similar hair types (guard hair and undercoat) with comparable morphological characteristics (Grojean et al. 1980, Elkmørk and Riiser 1990), hair colouration is vastly different in these species. In grizzly bears pelage varies from light blond to black and often along the length of the hair shaft (Pearson 1975, Harting 1987, Schwartz et al. 2003) while polar bear hair is uniformly unpigmented (Grojean et al. 1980, Amstrup 2003). In the field of forensic hair analysis, the proportion of drugs or xenobiotics incorporated into hair is known to be influenced by hair colour (Potsch et al. 1997, Kronstand et al. 1999, Rollins et al. 2003) which is dependent on the quantity and type of melanin (i.e. eumelanin vs. pheomelanin) in the hair shaft (Kidwell and Blank 1996). Each type of melanin is chemically distinct and has a unique binding affinity for different blood borne substances (Rollins et al. 2003). In humans and laboratory species, the highest xenobiotic concentrations have generally been reported in black hair, intermediate levels in other hair colours (e.g. red/brown), and the lowest in non pigmented (white) hair (Rollins et al. 2003). This relationship has also been demonstrated along the length of the hair shaft in grizzled (grey) hair which is a mixture of pigmented and non pigmented hair)

(Rothe et al. 1997). Significantly, hair colour may also influence the persistence of substances within the hair shaft, with relatively rapid loss occurring in non pigmented hair where substances may be less firmly bound to the hair matrix (Gygi et al. 1997). These factors suggest that the lack of pigment in polar bear hair may account for the low HCC levels recorded in this species. However, the importance of hair colour in HCC analysis is poorly understood at the present time.

In grizzly bears, hair colour did not influence HCC levels when compared across different bears. This finding agrees with previous work in humans where no effect of hair colour on HCC has been recorded across several studies (e.g. Raul et al. 2004, Sauvé et al. 2007, Manenschijn et al. 2011a). In both species, these observations may be attributable to the fact that cortisol is generally considered to be a neutral molecule (Borges et al. 2001) and should therefore not be preferentially bound by melanin (Raul et al. 2004). However, in grizzly bears, there was some evidence that, within individuals (and body regions), dark hair may contain more cortisol than light hair in this species (Macbeth et al. 2010). Elevated cortisol levels have also been recorded in dark grey, compared to light grey, and grey-brown hair samples in free-ranging mule deer (*Odocoileus hemionus*) (D. Jeffery, personal communication). Conversely, hair from black domestic dogs has been found to contain less cortisol than non black dogs (Bennett and Hayssen 2010), and, within individuals, black (eumelanin) hair (collected from the same body region) may contain less cortisol than yellow (pheomelanin) hair while agouti hairs may have intermediate cortisol levels (Bennett and Hayssen 2010). White hair has also been shown to contain more cortisol than dark hair in domestic cattle (Gonzalez de la Vera et al. 2011). Further research is clearly necessary to increase our understanding of the effects of hair colour on HCC in bears and in other animals.

Polar bears also spend a significant proportion of their time travelling in a marine environment and exposed to the effects of UV radiation while on sea-ice (Amstrup 2003, Derocher et al. 2004, Pagano et al. 2012). Water and UV exposure have recently been described as important weathering agents that may facilitate the loss of cortisol from the hair shaft (Hamel et al. 2011, Lie et al. 2012). On average, polar bear hair may therefore be subject to more intense weathering effects than hair from grizzly bears. This factor may explain the low HCC levels in SH polar bears as well as the lack of difference in HCC recorded among hair types and body regions in this species (i.e. due to a wash out effect). Weathering may also account for the

apparent decline in HCC levels recorded in dark and light grizzly bear gathered from the same individual and body region. Nevertheless, it is doubtful that low HCC levels in SH polar bears were the result of either hair colour or weathering alone. This is further supported by the recent determination of HCC levels comparable to those of Alberta grizzly bears in polar bears from East Greenland (Bechshøft et al. 2011, Bechshøft et al. 2012), by our measurement of relatively high levels of other steroid hormones (corticosterone) in the same samples of SH polar bear hair in which low cortisol values were determined (B. Macbeth, unpublished data), and by the fact that relatively high HCC values were identified in hair from the three Southern Beaufort Sea polar bears evaluated in this investigation.

Although further evaluation of the effects of weathering on HCC in free-ranging bears (and other wildlife) is necessary, a more plausible explanation for these findings may be the occurrence of divergent patterns of glucocorticoid physiology during the period of active hair growth in each species. Seasonal moult in grizzly bears and polar bears occurs once annually beginning spring and lasting into late summer or early fall (Harting 1987, Amstrup 2003) and cortisol incorporation from the blood-stream into the hair shaft is likely to occur primarily during this period (Macbeth et al. 2010, Macbeth et al. 2012). However, in Alberta grizzly bears, this period overlaps with the species' active foraging season, while in SH polar bears hair growth is believed to occur primarily during the onshore fasting period when no (or little) food is consumed and metabolic activity is reduced (Obbard et al. 2006, Macbeth et al. 2010, Macbeth et al. 2012). Recent research indicates that blood levels of corticosteroid binding globulin (CBG) may be elevated in fasting SH polar bears where they are believed to be an adaptation to reduce free cortisol levels and conserve body protein while onshore (Chow et al. 2011). Importantly, CBG levels in SH polar bears have also been found to be greater than those of Alberta grizzly bears (B. Chow, personal communication). CBG binds between 80-90% of circulating corticosteroids (Henley and Lightman 2011) and relatively high levels of CBG overlapping with the period of active hair growth in SH polar bears may explain the divergence of HCC levels in the two bear species (Breuner and Orchinik 2002). Although other factors are likely to be important (e.g. environmental contaminants; Øskam et al. 2004, Sonne 2010, McKinney et al. 2011), differences in the timing or duration of fasting may also explain the low HCC levels recorded in SH polar bears (Macbeth et al. 2012) compared to East Greenland polar bears

(Bechshøft et al. 2011, Bechshøft et al. 2012). Interestingly, B. Chow (personal, communication) also identified lower CBG levels in black bears (*Ursus americanus*) compared to either polar or grizzly bears. Further research into the relationships between CBG, free cortisol levels, and HCC in bears is encouraged.

An interaction between the timing of hair growth and seasonal changes in CBG or cortisol levels may also account for the dissimilar HCC patterns observed among different hair types and body regions in grizzly and polar bears. For example, the relatively linear demands of the fasting period may explain the uniformity of HCC levels among hair types and body regions in polar bears. In contrast, HCC levels in grizzly bears may be influenced by more dynamic changes in exposure (or responsiveness) to sources of long-term stress over the course of the hair growth period. Changing patterns of food availability throughout the active season may be one such factor. For instance, elevated serum cortisol has been recorded in ursids both in the spring and in years of poor food availability (Harlow et al. 1990, Hellgren et al. 1993), while relatively low levels have been observed during the summer active period (Palumbo et al. 1983). The growth of grizzly bear guard hair is believed to begin in the spring while undercoat may not begin its active growth phase until later in the summer (Jones et al. 2006, Macbeth et al. 2010). As such, higher HCC levels in grizzly bear guard hair may reflect blood cortisol levels elevated due to limited food availability in period of hypophagia (or as a residual effect of hibernation; Harlow et al. 1990), while the lower HCC levels recorded in undercoat may indicate a shift towards a more favourable energetic balance during hyperphagia when food is more readily available (Nielsen et al. 2004a, Busch and Hayward 2009, Macbeth et al. 2010). Similar relationships may also explain the pattern of HCC observed in different body regions in grizzly bears (Macbeth et al. 2010).

In some animals, elevated levels of circulating glucocorticoids may be linked with the consumption of carbohydrate rich foods which may provide a rapidly available source of energy (Landys et al. 2006). Unlike polar bears which preferentially consume fat (Stirling and McEwen 1975), plants (often high in carbohydrates, Felicetti et al. 2003b, McLellan 2011) and relatively lean sources of animal protein form the majority of the diet for continental grizzly bear populations (Hobson et al. 2000, Mowat and Heard 2006, McLellan 2011). As such, differences

in HCC levels or patterns of HCC recorded here may also reflect food preferences or seasonal dietary constraints in grizzly and polar bears (Landys et al. 2006).

Among natural considerations, changes in food resources may in fact be the most important environmental factor contributing to long-term stress in individual bears. Food resources are also important determinants of morphology, health, density, and overall performance in free-ranging bear populations (e.g. Hilderbrand et al. 1999, Derocher et al. 1992, Mowat and Heard 2006, McLellan 2011, Robbins et al. 2012), and potential relationships between HCC and the preferred or seasonal diets of grizzly and polar bears clearly warrant further attention. In bears, ratios of naturally occurring stable isotopes of carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$), and sulfur ($\delta^{34}S$) are commonly evaluated in hair to generate estimates of assimilated diet (Felicetti et al. 2003c, Robbins et al. 2004, Mowat and Heard 2006). This approach has also been used successfully to evaluate trophic relationships (Jacoby et al. 1999, Hobson et al. 2000) and geographic or seasonal patterns of resource use in bear populations (Hilderbrand et al. 1999, Mowat and Heard 2006, Jones et al. 2006, Belant et al. 2006) as well as to identify the relative importance of specific natural (Hobson et al. 2000, Felicetti et al. 2003a, Edwards et al. 2011) or anthropogenic (Narita et al. 2011, Hopkins et al. 2012) food sources to individual bears. The concurrent evaluation of cortisol levels and stable isotope ratios in hair may therefore provide valuable insight into relationships between long-term stress, diet, and even patterns of habitat use (Nielson et al. 2008) in individual bears as well as within and among free-ranging bear populations. Such studies may also have the potential to be applied in long archived hair samples to evaluate changes in these parameters in bear populations recently affected by human-caused or other ecological changes (Hilderbrand et al. 1996, Webb et al. 2010). Controlled studies in captive bears exploring direct linkages between HCC levels and dietary fat, protein or carbohydrate content would also be useful. In this regard, refining HCC analysis in bears to more accurately measure the distribution of cortisol along the length of individual hair shafts (Macbeth et al. 2010) would be informative, and could facilitate the identification of seasonal associations of long-term stress, diet or other factors in these species (Kirschbaum et al. 2009, Dettenborn et al. 2010, Xie et al. 2012).

Differences between bear species were also noted when relationships between HCC and life history traits such as age, reproductive class, and family group status were evaluated. In

grizzly bears, HCC levels were not related to age, sex, or reproductive status (Macbeth et al. 2010). Conversely, HCC levels were directly associated with age and highest in dependent male polar bears and in female polar bears with cubs (Macbeth et al. 2012). These findings may indicate that Alberta grizzly bears face similar demands, encounter similar stressors, or are uniformly reactive to stressors throughout their life cycle. However, life history stage and HPA axis activity (and reactivity) are known to be closely associated in a wide variety of species (Boonstra 2005, Reeder and Kramer 2005, Landys et al. 2006), and this discrepancy may simply reflect the low number of dependent juvenile bears and family groups examined in the grizzly bear study. Further evaluation of the potential relationship between HCC and life history stage is recommended for Alberta grizzly bears.

Conversely, HCC findings in polar bears suggest that life history stage may be an important determinant of HPA axis activity in this species. The direct association of HCC and age indicates that older SH polar bears may be less able to cope with the energetic demands of natural life history processes or stressors (McEwen 2002), may be more reactive to these stimuli (Rothuizen et al. 1991, Smith et al. 2005), or may recover more slowly from energetic challenges than their younger counterparts (Reeder and Kramer 2005). This observation agrees with age related HPA axis deregulation that has been previously recorded in other species (Rothuizen et al. 1991, Smith et al. 2005, Reeder and Kramer 2005, Chapter 5). The patterns of HCC recorded in polar bears also agrees with the basic biology of this species where juvenile males are known to display relatively rapid growth rates and a prolonged growth period compared to other bears (Atkinson et al. 1996, Derocher and Stirling 1998a, Derocher et al. 2005), and where females with cubs may be subject to relatively high energetic demands related to lactation or parental care (Derocher et al. 1993, Arnould and Ramsay 1994). Together, these findings offer support for HCC as a biologically relevant index of long-term stress in polar bears (Busch and Hayward 2009). Interestingly, no relationships between HCC and age or reproductive status were recorded in East Greenland polar bears (Bechshøft et al. 2001, Bechshøft et al. 2012). As in grizzly bears, a limited number of dependent juveniles and family groups examined in East Greenland studies may have prevented an accurate evaluation of these parameters.

In polar bears, a direct association was also identified between HCC levels in family groups. Genetics and maternal effects (e.g. HPA axis programming or transfer of cortisol in

milk; Seckel 2004, Sullivan et al. 2007) are known to play an important role in HPA axis activity and reactivity (Solberg et al. 2006, Steinus et al. 2008, Fairbanks et al. 2011), as well as in the health or fitness related outcomes of exposure to long-term stress (Sheriff et al. 2009, Sheriff et al. 2010). While encouraging in the sense that this relationship provides further support for HCC as a biomarker of long-term stress in polar bears, these repercussions also indicate that a more detailed evaluation of the relationship between genetics and HPA axis activity is necessary in both bear species. The relatively long generation time of bears is likely to preclude studies in captive populations. However, genetic data from remotely collected or archived hair samples could be combined with HCC analysis and may provide an excellent opportunity to evaluate these relationships among individual bears (Zeyl et al. 2009, Swenson et al. 2011) and their association with prevailing ecological conditions, anthropogenic disturbance, and population performance in free-ranging bear populations.

In this research program, the development of analytical techniques, prudent use guidelines, and the identification of plausible relationships with the biology of individual animals established HCC as a potentially useful biomarker of long-term stress in both grizzly and polar bears. However, these studies could not fully evaluate the utility of HCC as none could demonstrate that HCC was related to and changed with prevailing environmental conditions or the health status of individual animals (Busch and Hayward 2009). In the next phase of this study we sought to explore these criteria using available ancillary data collected from both bear species.

HCC in bears is most likely to represent a seasonal measure of HPA axis activity and may therefore be most strongly affected by factors occurring in the year of hair growth (Macbeth et al. 2010, Macbeth et al. 2012). In this study the availability of such data was limited in both grizzly and polar bears. As a result, we did not attempt to develop statistical models to evaluate the importance of seasonal factors as predictors of HCC in either bear species. However, HCC levels did vary in individual grizzly bears captured in different seasons or in different years (Macbeth et al. 2010). HCC levels in polar bears also increased as the onshore fasting season progressed (Macbeth et al. 2012). These findings may be evidence that seasonal or annual changes in environmental conditions affect HCC levels in bears and may also provide support for HCC as a biomarker of long-term stress in both species (Busch and Hayward 2009).

To explore the association of HCC and health in SH polar bears, a combination of hypothesis testing and information theory was used to develop models to evaluate the importance of HCC as a predictor of selected growth indices [length, mass, and body condition index (BCI); Cattet et al. 2002] known to be closely associated with health and fitness in this species (Atkinson and Ramsay 1995, Derocher and Stirling 1996, Derocher et al. 2010). Significantly, HCC was inversely associated with all growth indices such that those SH polar bears with higher HCC levels were shorter, lighter, and in relatively poor body condition compared to other bears (Macbeth et al. 2012). These findings: 1) provided further support for HCC as a biomarker of long-term stress in polar bears, and 2) preliminary evidence that this technique may also have potential as a conservation tool for use in the management of polar bear populations (Busch and Hayward 2009). However, HCC accounted for relatively little of the explained variation in these parameters (0% length, 1% mass, and 1% BCI) (Macbeth et al. 2012). Of these findings, the limited explanatory power of HCC the BCI model was particularly surprising given that (like HCC) this parameter is believed to reflect the effects of seasonal environmental conditions in bears (Cattet et al. 2002, Macbeth et al. 2010, Macbeth et al. 2012).

Throughout this study anecdotal but compelling evidence suggested that elevated HCC levels should be closely associated with diminished health in individual bears. For example, of the 5 SH polar bears found to be in the worst body condition, 4/5 had HCC values in the highest 10% of all values determined in this subpopulation (HCC $\geq$ 0.93 pg/mg). Likewise, HCC levels in the emaciated family group of polar bears killed inland at Deline, Northwest Territories, Canada were among the highest recorded in polar bears in this study, and body condition index (BCI) scores (range -3.00-+3.00; Cattet et al. 2002) in these individuals were also extremely poor [(adult female: HCC 5.92 pg/mg, BCI -3.24), (dependent female: HCC 1.01 pg/mg, BCI -2.25), and (dependent male: HCC 19.98 pg/mg, BCI -2.07)]. Similarly HCC determined in an emaciated adult male grizzly bear killed near Yellowknife, Northwest Territories (10.16 pg/mg) was in the top ten percent of all grizzly bear HCC values determined in this investigation (Appendix 5), while HCC measured in a particularly unhealthy male black bear (*Ursus americanus*) (12.20 pg/mg) was only exceeded by HCC in 9 of the 151 Alberta grizzly bears (*Ursus arctos*) and 1 of the 188 SH polar bears (*Ursus maritimus*) examined. Given these relationships the most probable explanations for the relatively weak association of HCC and BCI

in SH polar bears may be that 1) recent conditions in the southern Hudson Bay region are within the reaction norm of this subpopulation (Landys et al. 2006), and 2) that few bears in this study were in truly poor body condition making the probability of detecting a strong association between these variables low (Macbeth et al. 2012). However, alternate explanations should also be considered.

In bears, BCI reflects the combined mass of fat and skeletal muscle in a bear relative to its body size (Cattet et al. 2002). It is possible that, while useful for generating a biologically relevant measure of a bear's health, BCI may be too crude an index to accurately reflect subtle changes in condition related to circulating cortisol levels. A similar relationship, further confounded by the influence of skeletal size, may also explain why HCC was a relatively weak predictor of body mass in SH polar bears (Cattet et al. 2002, Macbeth et al. 2012). Body fat stores provide the majority of energy used in a bear's response to life history challenges, however body protein may also be exploited, and both parameters may fluctuate widely throughout a bear's annual life cycle (Harlow et al. 1990, Barboza et al. 1997, Thiemann et al. 2006, McLellan 2011).Therefore, an alternate strategy to more vigorously evaluate HCC as a potential conservation tool in polar bears may be to examine relationships between HCC and body fat or body protein reserves separately. The association of HCC and body fat could be evaluated in free-ranging bears, by comparing HCC levels with the structural composition (lipid content) of fat biopsies collected from a standardized body region (Thiemann et al. 2006). Although it requires extensive training, bioelectrical impedance analysis (BIA) could also be useful in this regard. BIA is a rapidly accomplished, field applicable, and non-invasive method which measures an organism's resistance to a low level electrical current (Farley and Robbins 1994, Gau and Case 2002, Robbins et al. 2004). Body resistance is directly proportional to body fat content and is considered to provide a relatively accurate measure of body composition in bears (Farley and Robbins 1994, Hilderbrand et al. 1998, Gau and Case 2002, Robbins et al. 2004, Nakamura et al. 2011). A mathematical model (based on dynamic energy budget theory) which quantifies the amount of tissue (storage mass) from which energy can be drawn has also recently been developed and applied in polar bears (Molnár et al. 2009). This technique employs only measurements of body length and mass in calculations, and could provide an alternate strategy with which to evaluate HCC data in bears. Importantly, this model also estimates stored

energy density (in MJ/kg) and allows the relative proportions of storage mass related to fat, protein and water to be determined (Molnár et al. 2009). In addition, relationships between HCC and body composition may even be assessed in historical hair samples as long as estimates of length and mass are available (Molnár et al. 2009). Although not readily accessible, the use of dual energy x ray absorptimetry (DEXA) (Felicetti et al. 2003c, Robbins et al. 2004) has also been used to evaluate body composition in bears, and studies in captive animals employing this technique could be especially valuable in refining our understanding of relationships between HCC and body fat or protein levels.

Hair cortisol analysis is a rapidly emerging field, and a recent radiometabolism study published after the completion of this research program and tracking the incorporation of radiolabelled cortisol (^{3}H-cortisol; 1,2,6,7-[3H]-hydrocortisone) in the growing hair of guinea pigs (Keckeis et al. 2012) may provide further insight into the relatively weak relationship between HCC and BCI in SH polar bears. Keckeis et al (2012) employed a combination of high performance liquid chromatography (HPLC) and EIA based assays to evaluate hair extract both for a suite of corticosteroids (cortisol, cortisone and corticosterone) and the presence of radioactivity related to the systemic [intraperitoneal (IP)] administration of ^{3}H-cortisol. The authors found that little systemically injected ^{3}H-cortisol could be recovered from the growing hair of guinea pigs 7 days after administration (Keckeis et al. 2012). A prominent radioactive peak in the HPLC assay coinciding with a cortisone standard along with strong immunoreactivity of hair extract evaluated in a cortisone EIA were also observed (Keckeis et al. 2012). Keckeis et al (2012) attributed this finding to the presence of cortisone in hair, and suggested that the radiolabelled cortisol administered intraperitoneally may have been metabolized prior to incorporation into the hair shaft (thus explaining the low levels of ^{3}H-cortisol recovered) (Keckeis et al. 2012). This finding may suggest that relatively little systemic cortisol is actually incorporated from the blood stream into the hair shaft (i.e. without first being metabolized to cortisone). If true for bears, this phenomenon could account for the outcome of the BCI model in SH polar bears, and a combination of cortisol and cortisone may represent a superior index of long-term stress in this species than cortisol alone. Although this relationship clearly deserves further attention in controlled laboratory studies, the overall importance of these findings is not

yet clear and these outcomes may also be explained by procedures employed in the radiometabolism study.

In humans, the enzyme 11ß-hydroxysteroid-dehydrogenase (HSD) type 2 (which metabolizes cortisol to inactive cortisone) has been identified in eccrine sweat glands (those not directly associated with the hair follicle; see Fig 1.3 for review of hair follicle anatomy) and Raul et al (2004) suggested that the conversion of cortisol to cortisone in these glands and subsequent transfer into growing hair via sweat may explain relatively high levels of cortisone found in human hair. This mechanism of incorporation seems unlikely in polar bears which possess few sweat glands and which do not rely on sweating as a source of thermoregulation (Ørtisland 1970, Berg 1982, Hurst et al. 1982). Moreover, HSD type 2 has not been described in the hair follicle nor in the epidermis, apocrine sweat glands, sebaceous glands, or any structure more directly associated with the growing hair shaft in any species (Smith et al. 1996, Hirasawa et al. 1997, Tiganescu et al. 2011, see also Fig 1.3). Conversely, HSD type 1 (which converts cortisone to cortisol) has recently been recorded in the outer root sheath of the mouse hair follicle (Tiganescu et al. 2011)

Importantly, all animals used by Keckeis et al (2012) were shaved prior to the administration of ^{3}H-cortisol. In the field of dermatology, shaving is well recognized as a source of skin irritation and also stimulates active hair growth (Stenn and Paus 2001). The hair follicle is also known to contain a functional equivalent to the HPA axis and may synthesize cortisol locally (Ito et al. 2005). Recent evidence also suggests that the hair follicle and this HPA axis analogue may be easily stimulated which may result in a highly localized stress response and may culminate in artificially elevated HCC levels (Sharpley et al. 2009, Sharpley et al. 2010a). As such, it is possible that the combined effects of active growth and irritation related to the shaving of hair may have influenced general hair follicle activity and may explain the relatively high amount of unlabelled cortisol observed by Keckeis et al. (2012).

Even in growing hair, the incorporation of substances into the hair root is believed to take approximately 3 days with an additional week or more for that section of the hair root to emerge at the hair surface (Pragst and Balikova 2006, Kidwell and Smith 2007). These factors suggest that 7 days may not have been a long enough period to ensure an accurate detection of the systemically administered ^{3}H-cortisol in the hair shaft in the radiometabolism study. This may be

especially important considering that guinea pigs are known to exhibit a mosaic pattern of hair growth and replacement where each hair follicle may have a distinct growth pattern and rate of growth from even closely adjacent neighbours (Katz 1993, Stenn and Paus 2001). This effect may also have been enhanced by the unique multilayer basal lamina of the capillaries in the guinea pig dermal root sheath (Parakkel 1966), which Keckeis et al (2012) acknowledge may have promoted generally low ^{3}H-cortisol concentrations in the hair shaft.

To clarify these important knowledge gaps similar investigations are recommended using animals allowed to progress naturally through their hair cycle (i.e. not shaved to stimulate hair growth) and sampled after a longer waiting period (minimum 14 days; Gonzalez de la Vera et al. 2011). Some domestic sheep breeds (e.g. merino) have been artificially selected to have hair follicles in a nearly continuous state of anagen (Rogers 2006) and may prove particularly useful for this type of general HCC study.

The findings of Keckeis et al. (2012) also highlight the importance of increasing our basic understanding of hair follicle anatomy and hair biology in bears (and other wildlife species). In particular, the potential occurrence and activity of HSD type 2 in the hair follicle of bears should be investigated. The distribution, development, and functionality of eccrine sweat glands, apocrine sweat glands, and sebaceous glands [possible sources of cortisol (Pragst and Balikova 2006) or potential locations of HSD type 2 activity (Smith et al. 1996, Hirasawa et al. 1997)] should also be evaluated in detail. Skin samples from polar bears were not available for this study. However, a general histological evaluation of the grizzly bear integument and hair follicle was attempted in the early stages of this investigation, and it was clear from gross examination that this species possesses a compound hair follicle (Müntener et al. 2011) with a single primary guard hair and several small secondary hair emanating from the same follicular opening (B. Macbeth, personal observation). Unfortunately, all available skin samples from grizzly bear carcasses were too autolyzed to characterize hair follicle anatomy or perform a detailed analysis of the hair growth cycle (Müntener et al. 2011) in this species. Many dermatological techniques [e.g. trichograms (Dietz et al. 2004a)] and special staining protocols (Baden et al. 1979) are relatively non-invasive and may be easily applied in field based studies of bears (Macbeth et al. 2010). Future research efforts in these species (and in other wildlife) should also consider these topics.

An evaluation of relationships between HCC and grizzly bear health was not performed as part of this research program. Instead, based on encouraging findings reported in Macbeth et al. (2010), a large scale collaborative study was initiated between the Canadian Cooperative Wildlife Health Centre (CCWHC), the Foothills Research Institute Grizzly Bear Program (FRIGBP) and the Scandinavian Brown Bear Research Project (SBBRP) in which HCC levels were determined in and compared among a total of 785 grizzly bears [referred to as brown bears (*U. arctos*)] from four geographic areas (Alberta, Sweden, Nunavut, and Mongolia) across the species' distributional range (Cattet et al. 2011). Recently completed, this study identified a four-fold difference between the highest and lowest median HCC values determined among the four *U. arctos* populations. Relationships were also observed between HCC and sex, age, season of collection as well with physical attributes related to fitness in grizzly bears (body mass and body length) (M. Cattet, personal communication). Interestingly, the importance of these attributes varied among study populations which inhabit markedly different environments and whose conservation status ranges from relatively secure to critically endangered. In Alberta grizzly bears, HCC levels were also found to be elevated in individuals having worn radio-collars and/or ear-tag transmitters, and HCC increased as BCI declined in this population (M. Cattet, personal communication). Importantly, this study also found no evidence that short-term stress (due to capture methods or gunshot) influenced HCC in this species (M. Cattet, personal communication). Potential relationships between HCC and the location of a bear's home range were also evaluated in a companion study using data generated from the same animals. Interestingly, distinct clusters of high and low HCC levels were found in bears from both Alberta and Sweden (Cattet et al. 2011). Ongoing work in Alberta grizzly bears is now evaluating potential relationships between HCC and anthropogenic landscape features occurring within a bear's home range. When considered together with the findings of this research program, these studies provide compelling evidence that HCC represents a valid biomarker of long-term stress in *U. arctos* that may also have great potential as a practical conservation tool for this species. An evaluation of HCC across polar bear subpopulations and considering a wider array of factors potentially related to health in this species may be similarly informative and is highly recommended (Macbeth et al. 2012).

6.2.2 Caribou

As in grizzly and polar bears, an evaluation of HCC in *Rangifer* identified an array of prudent use guidelines and practical considerations for this species. Like grizzly bears, hair surface contamination (blood) was identified as a potential source of bias in *Rangifer* HCC studies. Severe interference from static (not observed in either bear species) also caused difficulties in recovering ground caribou and reindeer hair. As a result, a minimum sample quantity of 100 mg unprocessed hair (approximately a 2 cm by 2cm plucked or shaved patch of hair) was required to obtain the lowest quantity of ground hair (50 mg) that could be consistently generated with the hair grinding protocol used in this study (B. Macbeth, personal observation). Unlike bears, it is probable that hair samples for HCC analysis in *Rangifer* will be obtained primarily from hunting or research captures. As such, this sample size constraint is unlikely to reduce the potential utility of this technique in free-ranging *Rangifer* populations. Moreover, an effective and rapidly applied hair collection technique developed by C. Cuyler (Greenland Institute of Natural Resources) may be used to eliminate most blood contamination on hair samples obtained from *Rangifer* carcasses (see Chapter 5, Fig 5.1). As in bears, hair collection may be an ideal method to engage local communities in science-based conservation initiatives (Anderson 2000, Morton et al. 2007, Brook et al. 2009).

Differences in HCC levels among *Rangifer* body regions were similar to patterns observed in grizzly bears (i.e. significant difference in neck hair only; Chapter 4, Appendix 4) and may suggest that this species also experiences dynamic changes in HPA axis activity throughout its active hair growth period. As in bears, these patterns may reflect the effects of prevailing environmental conditions and offer support for HCC as a biomarker of long-term stress in this species (Busch and Hayward 2009). Like bears, the availability and nutritional quality of seasonal foods may be of particular importance (Heggberget et al. 2002, Joly et al. 2009, Waterhouse et al. 2011) and these relationships should be evaluated by considering HCC and stable isotope ratios in *Rangifer* hair (Drucker et al. 2010) or diet trials in captive animals. Hair colour in *Rangifer* also exhibits marked seasonal changes in colour (Miller 2003), and the effects of hair colour and weathering deserve further study in this species (Ashley et al. 2011).

The accessibility of captive caribou (*R. t. granti*) and reindeer (*R. t. tarandus*) provided a unique opportunity to explore the effects of ACTH administration on HCC (Ashley et al. 2011). There was no evidence that a single ACTH injection was related to an increase in HCC levels in

the hair of reindeer. With a single exception (most likely related to a procedural artefact; Chapter 4), similar findings were recorded in caribou. These observations indicate that HCC may be relatively resistant to the effects of acute increases in systemic cortisol levels, and offers distinct support for the utility of HCC as a biomarker of long-term stress in *Rangifer*. Nonetheless, contrasting the outcomes of this study with more recent investigations, indicates that factors such as the period of active hair growth (Gonzalez de la Vera et al. 2011) and the potential effects of localized skin irritation (Ito et al. 2005, Sharpley et al. 2010) should always be considered in HCC analysis and deserve further research attention in this and other species. Further studies of hair growth, as well as HCC response to ACTH (and also dexamethasone) challenges, are encouraged in *Rangifer* and in other species.

Among biological attributes no differences were identified in HCC levels among captive male or female reindeer or caribou. However, there was some evidence that younger captive *Rangifer* may have higher HCC levels than their older counter parts. Elevated HCC levels were also observed in subadult and dependent caribou calves vs adult females in two *R .t. groenlandicus* herds from West Greenland, and a direct association between age and HCC was identified in this population. The patterns of HCC identified in West Greenland caribou agree with higher levels of circulating cortisol previously recorded in juvenile compared to adult female caribou (Omsjoe et al. 2009), and with the direct association of HCC and age observed in SH polar bears. Findings in captive animals also agree with recent observations of similar HCC levels in male and female woodland caribou (*R. t. caribou*) (Renaud 2012), and both offer support for HCC as a biologically relevant indicator of long-term stress in this species *(*Busch and Hayward 2009).

HCC levels in $n = 6$ captive adult female *R. t. granti* were also similar to those recorded in $n = 69$ free ranging adult female *R. t. groenlandicus* from West Greenland (Independent sample t-test, $t_{(73)} = 1.088$, $P = 0.280$, $n = 75$). *Rangifer* held at the University of Alaska Fairbanks had been raised in captivity and were maintained under ideal nutritional and housing conditions (described in Ashley et al. 2011). Accordingly, this finding may indicate that free-ranging West Greenland caribou are not highly stressed at the present time (Coburn et al. 2010). In contrast, HCC values determined in $n = 124$ female and $n = 18$ male free-ranging woodland caribou (*R. t. caribou*) from Quebec, Canada were higher [mean 8.1 pg/mg (range 1.4 - 31.6

pg/mg)] than levels in either captive *Rangifer* subspecies or West Greenland caribou (Renaud 2012). Renaud (2012) also recorded higher HCC levels in animals living in regions impacted by human industrial or recreational activity than in those inhabiting relatively undisturbed areas. Although subspecies or sex-related differences may be contributing factors, dissimilarity of HCC levels recorded by Renaud (2012) and in this study may be due to the fact that anthropogenic influences (and predation risk) do not exist or are minimal for both captive *Rangifer* and West Greenland caribou (Cuyler et al. 2007, Witting and Cuyler 2011, Ashley et al. 2011). In aggregate, these findings offer additional support for the utility of HCC as a field applicable biomarker of long-term stress in free-ranging *Rangifer*, and for the potential importance of factors related to anthropogenic disturbance as sources of long-term stress in this species.

As in polar bears, a combination of hypothesis testing and information theory was used to develop models to evaluate the potential relationship between HCC, biological attributes and health in *Rangifer*. Only West Greenland caribou were considered in this analysis. Unlike polar bears, more relevant data from two distinct caribou populations was available and separate models were developed to evaluate: 1) factors influencing HCC, and 2) HCC as a possible predictor of an expanded and refined range of growth indices (total body length, girth, total body weight, cold carcass weight, and body fat reserves) known to be directly associated with health and fitness in this species (Cameron et al. 1993, Rönnegård et al. 2002, Taillon et al. 2011, Bårdsen and Tverra 2012). Moreover, both approaches were able to incorporate as potential predictor variables factors (parasite burden and herd designation) likely related to prevailing ecological conditions encountered by each caribou population.

The best fit model explained 31% of the variation in HCC and contained predictor variables related to caribou biology (age, age-reproductive status), body condition (body fat reserves), parasite burden (intensity of nasal bot infection) and herd designation (Chapter 5). Significantly, HCC was greatest in those caribou with relatively high nasal bot burdens and in those caribou that had the fewest body fat reserves. There was also a difference in HCC levels measured between the two caribou populations considered in this analysis. Inverse relationships between HCC, body fat reserves, and parasite burden indicate that HCC may increase as the health status of individual West Greenland caribou declines, and provides clear support for HCC as a biomarker of long-term stress in *Rangifer* (Busch and Hayward 2009). The inclusion of herd

designation in the best fit HCC model may also support this deduction as each population included in this analysis is subject to a dissimilar array of prevailing environmental conditions related to population density, abiotic factors, food availability, and anthropogenic influences (Busch and Hayward 2009, CARMA 2012b, CARMA 2012c).

When growth models were considered, HCC did not predict total body length, girth, total body weight or cold carcass weight in West Greenland caribou. However, in the best fit model for body fat reserves, HCC was inversely associated with this dependent variable. This finding provides further evidence that HCC is a valid biomarker of long-term stress in individual caribou (Busch and Hayward 2009). Nevertheless, as in the BCI model for SH polar bears, HCC accounted for little (1%) of the explained variation in caribou body fat reserves. *Rangifer* are adapted to wide ranging seasonal fluctuations in body fat (Barboza and Parker 2006, Vors and Boyce 2009, Couturier et al. 2009b). Therefore, this finding may indicate that conditions in West Greenland are within the reaction norm for this species at the present time (Landys et al. 2006), and may be further supported by the similarity of HCC levels in captive adult female *R. t granti* and free-ranging adult female *R. t groenlandicus* used in this analysis. As in SH polar bears, the index of seasonal body condition (body fat reserves) used in this evaluation may not have been refined enough to reveal a strong association between these parameters. As discussed in Chapter 5, body protein reserves (e.g. peroneus muscle mass) may actually be a more appropriate index of seasonal condition for adult female *Rangifer* during the active hair growth period (Taillon et al. 2011, Chapter 5). Other factors as described for polar bears may also be relevant, and a histological evaluation of hair follicle anatomy and hair biology is recommended for caribou and reindeer.

In the near future, West Greenland caribou will be affected by a variety of new industrial developments (CARMA 2012b, CARMA 2012c) and a longitudinal study of HCC levels is recommended to evaluate and track the impact of these likely sources of long-term stress (Renaud 2012). Building on the findings of this research program and Renaud (2102) further study of HCC in other free-ranging *Rangifer* populations is also encouraged to identify factors influencing long-term stress levels in this species. As a general consideration, relationships between HCC, health, and fitness have not have not yet been explored in intervention-based studies in any species (Russell et al. 2012), but would be particularly valuable to unequivocally

demonstrate that HCC can be used as an effective tool with which to monitor long-term stress in free-ranging wildlife populations. The accessibility of captive *Rangifer* may provide a unique opportunity to directly test these relationships in a large wild mammal.

This research program represents the first evaluation of HCC in free-ranging large mammals. Overall, evidence revealed in this study suggests that HCC may be a valid biomarker of long-term stress in individual grizzly bears, polar bears, and caribou. With further intervention-based studies in captive animals and additional research in free-ranging populations, HCC may also prove to be a useful conservation tool in these threatened species.

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APPENDIX 1

APPARATUS AND CONDITIONS OF GRIZZLY BEAR (*Ursus arctos*) HAIR ENVIRONMENTAL EXPOSURE TRIAL

Figure A1.1 Screen apparatus used to hold samples in the grizzly bear (*Ursus arctos*) hair environmental exposure trial (Macbeth et al. 2010). Sub samples of hair collected from the shoulder region of a single grizzly bear weighing approximately 500 mg were flattened then placed within 10 cm by 10 cm screen pouches and exposed to the elements in two mock barb-wire snag sets following the methods of Woods et al. (1999). Photo: B. Macbeth.

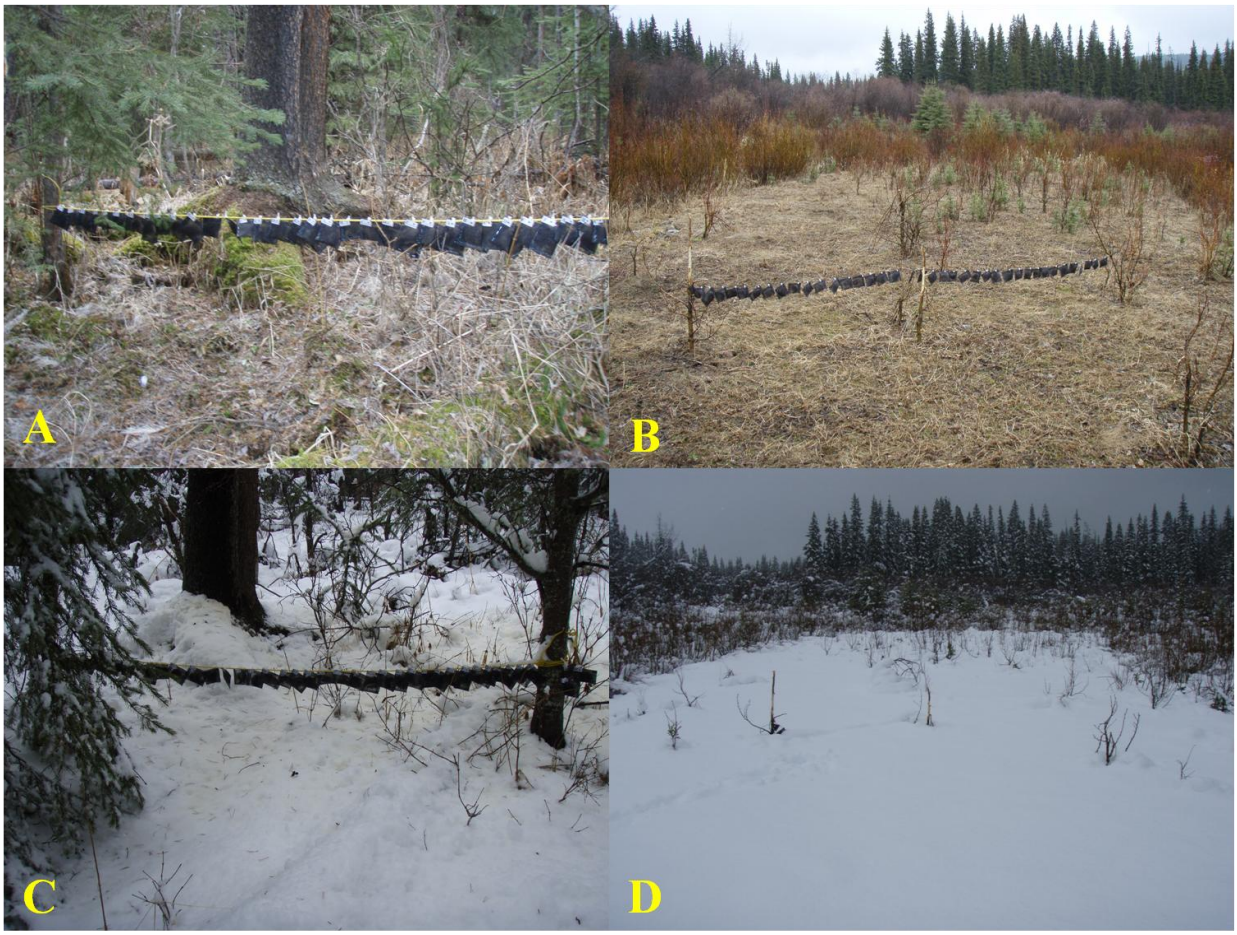

Figure A1.2 Example of weather encountered during exposure of grizzly bear (*Ursus arctos*) hair to ambient environmental conditions in Alberta grizzly bear habitat (latitude, longitude: 52°22′22” N, 119°45′31”W) for up to 18 days from May 7 to May 25, 2008. One mock snag was set in a forested site (A) and a second was set in an open meadow (B). Weather during the trial ranged from -1ºC with rain and snow to +15°C with sun. A severe spring blizzard deposited > 50 cm of wet snow on May 7, 2008 and while hair samples at the forested site were relatively protected from the elements (C), all open meadow site hair samples were snow covered for one week or more (D). Photos: B. Macbeth

APPENDIX 2

A PRELIMINARY TEST OF THE EFFECTS OF EXTERNAL CONTAMINATION (WITH CORTISOL SPIKED URINE AND FECES) ON CORTISOL CONCENTRATION IN GRIZZLY BEAR (*Ursus arctos*) HAIR

A2.1 Introduction

Hair collected from free-ranging wildlife may be exposed to a variety of biological materials known to contain cortisol such as blood (Cattet et al. 2003), lipid (Stimson et al. 2009), urine (Constable et al. 2006), feces (Hunt and Wasser 2003) or saliva (Millspaugh et al. 2002). Surface contamination with these substances may confound the measurement and interpretation of cortisol levels in hair (Macbeth et al. 2010), with the magnitude of effect influenced by: 1) hair collection and processing methods (Macbeth et al. 2010, Bechshøft et al. 2011), 2) the species and body region sampled (Gosling and McKay 1990, Whittle et al. 2000, Dawson et al. 2000, Drea et al. 2002), 3) the season of collection (Mossing and Damber 1981, Bowyer and Kitchen 1987, Olson et al. 2008), and 4) ambient weather conditions (Kidwell and Blank 1996, Kidwell and Smith 2007). Evidence from the forensic drug field also suggests that if the hair shaft is breached, some contaminants may become tightly bound in the hair matrix and impossible to remove with decontamination protocols (Kidwell and Blank 1996, Kidwell and Smith 2007).

For live captured grizzly bears, contamination of hair with feces and urine may be of particular concern and may be one factor explaining elevated hair cortisol levels recorded in some bears captured by culvert trap (Macbeth et al. 2010). Specifically, elevations in fecal and or urinary cortisol (due to the stress of capture) could influence hair cortisol concentration (HCC) when grizzly bears are held in culvert traps for extended periods (≥ 12 hours) (Hunt and Wasser 2003, Owen et al. 2005, Macbeth et al. 2010). This may be of particular importance for those grizzly bears with wet or damaged hair or those captured under conditions where the collection of clean and dry samples is not possible (Kidwell and Blank 1996, Kidwell and Smith 2007, Macbeth et al. 2010). To increase our understanding of factors influencing HCC in grizzly bears, the effects of surface contamination with cortisol containing biological materials must be explored in a controlled experiment. As well as enhancing standardized hair sampling protocols and the accurate interpretation of HCC data derived from grizzly bears, such work may also have broad implications for the advancement of HCC analysis in other species.

A2.2 Objectives

The objectives of this preliminary investigation were to: 1) determine if feces and urine (spiked with laboratory grade cortisol) when applied to the hair surface could alter HCC

measured in grizzly bear hair, and 2) to assess the specific effects of duration of exposure, hydration status (i.e. dry vs. wet hair), and contaminant concentration on this possible phenomenon.

A2.3 Materials and Methods

A2.3.1 Source and preparation of feces-urine-cortisol slurry and hair exposure

No feces or urine from grizzly bears captured in culvert traps were available for this study. As such, fecal-urinary contamination in culvert trapped grizzly bears were replicated by exposing 100 mg subsamples guard hair from $n = 6$ grizzly bears to a homogenous semi-solid mixture of 1g canine feces and 3 ml urine spiked with solution of $\geq$120 ng laboratory grade cortisol (Fluka Hydrocorisone Analytical Standard 3179; Sigma-Aldrich, Munich Germany) in 1 ml of phosphate buffer. Feces and urine were collected (free-catch) from a visibly healthy, castrated, 8 year old, male dog over a period of two weeks and held at -20°C prior to the manufacture of the test slurry. Due to logistical and budgetary restraints we did not measure glucocorticoid levels in the feces/urine samples used to make the test slurry. For both time-based and concentration-based experiments, the severity of fecal-urinary contamination was designed to mimic the gross appearance of heavy soiling with these materials previously observed in some grizzly bears capture in culvert traps (B. Macbeth, personal observation (Fig A2.1, Fig A2.2). To achieve this, 100 mg subsamples of dry hair (or wet hair: time-based experiment only; presoaked 24 hours in room temperature tap water prior to exposure) were placed in capped, 5 ml plastic test tubes containing the test slurry and gently shaken on a laboratory rocker (VWR Rocking Platform 200, VWR International, Edmonton, Alberta, Canada) at room temperature. For time-based experiments the duration of exposure was designed to mimic both short-term and longer holding periods in culvert traps and subsamples of grizzly bear hair were mixed with the test slurry for 2, 4, 8, or 16 hours. The addition of cortisol was intended to account for the effects of acute stress due to capture and confinement in culvert traps; and the amount of cortisol (120 ng) added to the slurry was chosen based on peak glucocorticoid levels measured in grizzly bear feces after ACTH Stimulation (~120 ng glucocorticoids /g feces, Hunt and Wasser 2003). For concentration-based experiments the amount of cortisol added was 120 ng, 1200 ng, and 12000 ng and all samples were exposed to the slurry for 8 hours. It is likely that the 1200 ng and 12000

ng challenges far exceed any real-life contamination scenario in live-captured grizzly bears (Hunt and Wasser 2003).

Figure A2.1 View of culvert trap used in capture of grizzly bear 15 in the spring of 2009 (see Macbeth et al. 2010) demonstrating contamination with a semi solid mixture of feces, urine and trap bait. Photo: B. Macbeth.

Figure A2.2 Grizzly bear 15 (Macbeth et al. 2010) on removal from culvert trap in Fig A2.1. Note the nearly uniform wetting of hair and coverage of the hair coat with feces, urine, and trap bait. This level of contamination is not frequently encountered on grizzly bears captured in culvert traps as part of the Foothills Research Institute Grizzly Bear Program. Note also the relatively clean and dry area at the top of the shoulder (hump). Photo: B. Macbeth.

A2.3.2 Hair preparation, steroid extraction, and data analysis

To mimic field handling procedures that have been employed for heavily contaminated or wet grizzly bear hair (B. Macbeth, personal observation) all hair samples were gently blotted clean with paper towel and then allowed to air dry at room temperature for 8 hours in open paper envelopes. Hair samples were then washed, ground, extracted, and analyzed following methods reported in Macbeth et al. (2010). One-way repeated measures analysis of variance (ANOVA) tests (with the level of significance set at $P \leq 0.05$) were then used to compare, log transformed HCC values determined in unexposed and slurry exposed grizzly bear hair.

A2.4 Results

The contamination protocol employed in this study resulted in approximately 50% to 75% of the hair surface being covered in a thin coating of the test slurry in most samples. Five-3-minute washes with 4 ml methanol per 100 mg hair removed all visible contamination from the surface of all hair samples. Hair cortisol concentration increased in both dry-contaminated and wet-contaminated grizzly bear hair after 2 hours exposure to the canine urine-feces-cortisol slurry (One-way repeated measures ANOVA, $F_{(8, 40)} = 23.631$, $P < 0.001$,Tukey-Kramer, $P < 0.05$). There was also a trend towards cortisol increasing in hair over time, but this trend was not statistically significant (Fig A2.3). However, there was no difference (Tukey-Kramer, $P > 0.05$) in HCC measured in dry or wet hair exposed to the test slurry (Fig A2.3).

Hair cortisol concentration increased as the concentration of cortisol added to the test slurry increased (One-way repeated measures ANOVA, $F_{(2, 10)} = 318.07$, $P < 0.001$, Tukey-Kramer, $P < 0.05$ for all treatment groups) (Fig A2.4). In both experiments, only a small portion (< 1.0 %) of cortisol possibly attributable the cortisol spike was measured in the hair shaft.

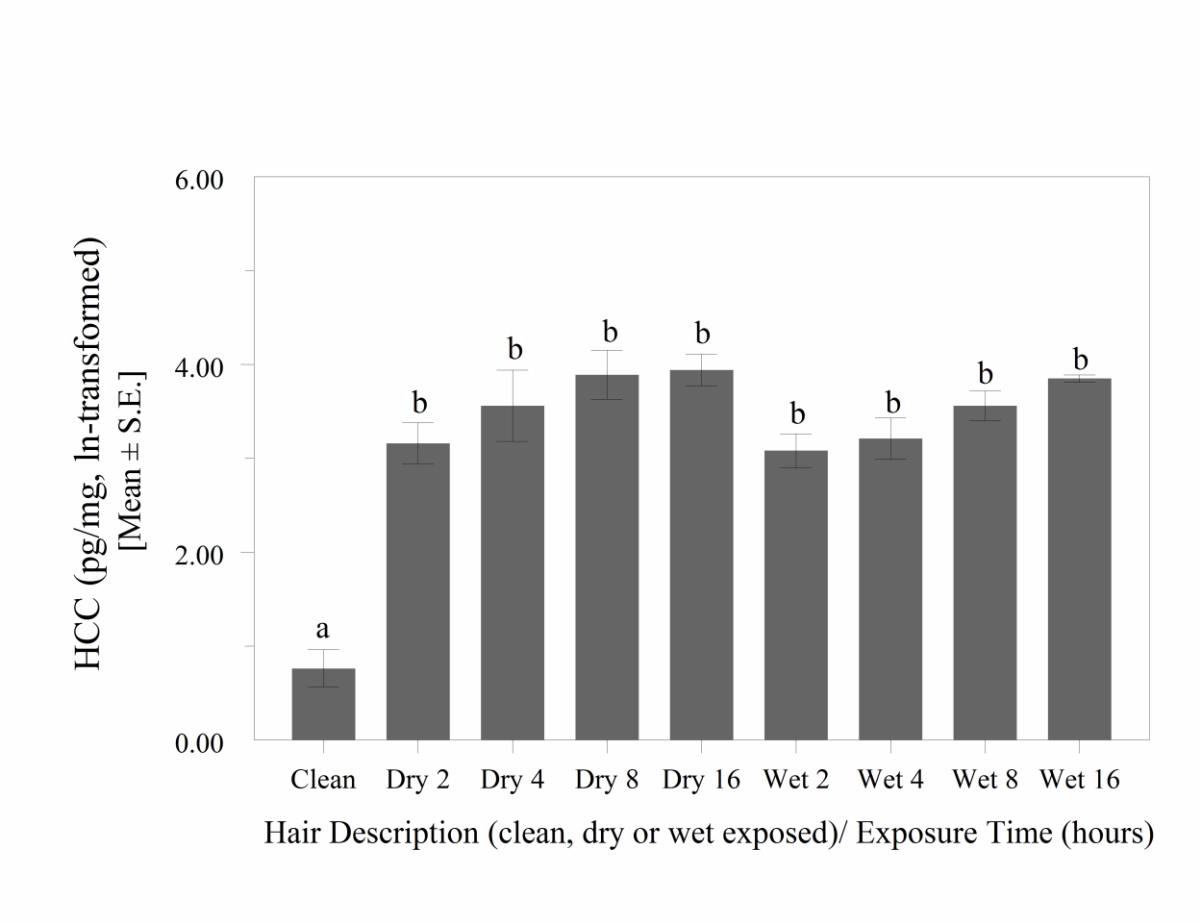

Figure A2.3 A comparison of hair cortisol concentration (HCC) in dry and clean guard hair from $n = 6$ grizzly bears (*Ursus arctos*) and dry or wet guard hair (from the same animals) exposed to a semi-solid slurry of canine urine and feces spiked with 120 ng of laboratory cortisol standard for 2, 4, 8 or 16 hours. Mean values are represented by bars and S.E. represented by error bars. Significant differences (Tukey-Kramer $P < 0.001$) in hair cortisol concentration are designated by different letters.

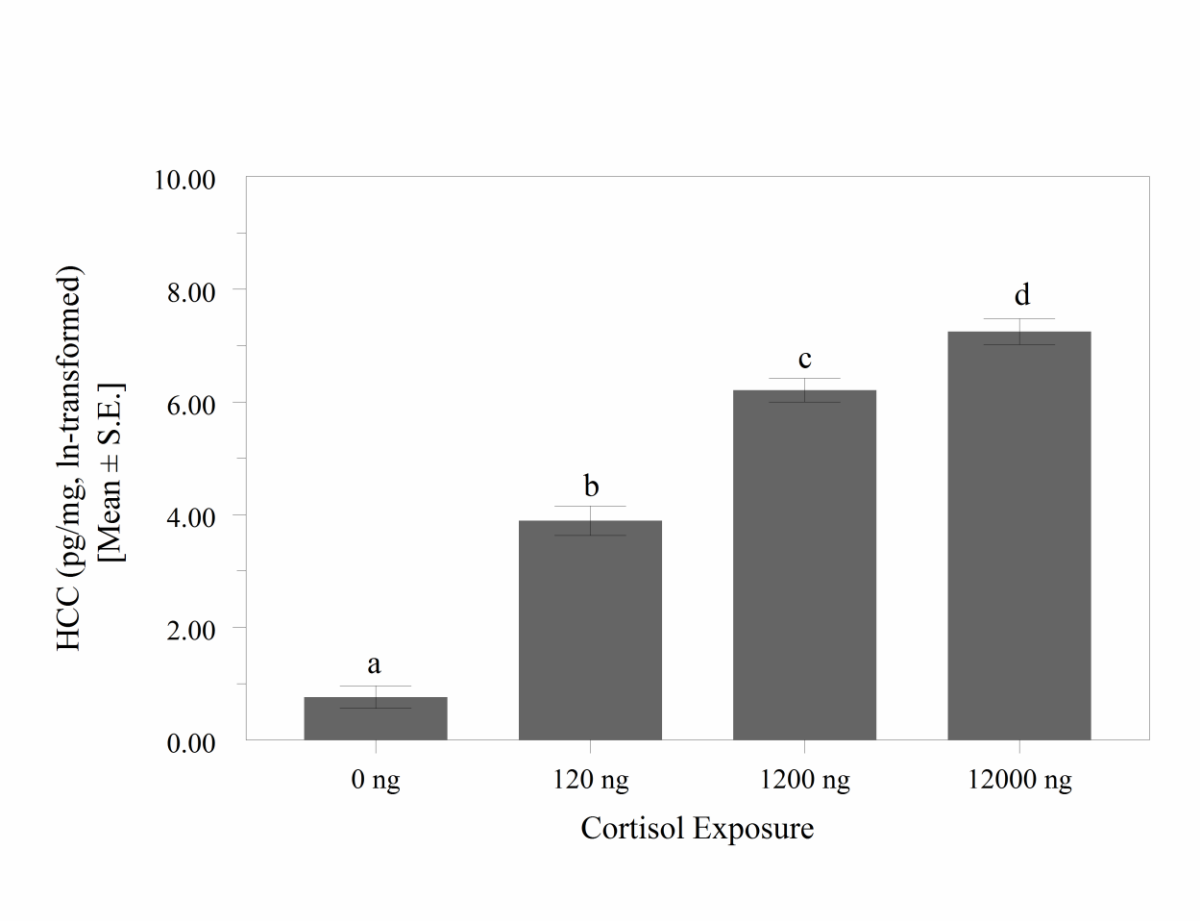

Figure 3.2 A comparison of hair cortisol concentration (HCC) in dry and clean guard hair from *n* = 6 grizzly bears (*Ursus arctos*) and dry guard hair (from the same animals) exposed to a semi-solid slurry of canine urine and feces spiked with 120 ng, 1200 ng or 12000 ng of laboratory cortisol standard for 8 hours. Mean values are represented by bars and S.E. represented by error bars. Significant differences (Tukey-Kramer, $P < 0.05$) in hair cortisol concentration among cortisol exposure groups are designated by different letters.

A2.5 Discussion

The results of this preliminary investigation demonstrate that some cortisol associated with large quantities of feces and urine may enter grizzly bear guard hair in as little as 2 hours. Importantly, this contamination could not be removed with established wash protocols (Macbeth et al. 2010). The amount of cortisol incorporated into the hair shaft may also be related to its concentration on the hair surface and possibly to the duration of exposure. These findings agree with reports from the forensic drug field that suggest surface contamination may influence xenobiotic concentrations measured in hair, and that once the hair shaft has been breached contaminants may become tightly bound in the hair matrix and impossible to remove (Kidwell

and Blank 1996). Given the significant contamination employed in this experiment, these findings were not unexpected. However, that no difference in HCC was measured in dry or wet hair exposed to the feces-urine-cortisol slurry was surprising. Unlike dry hair, where surface contamination is more easily removed, water is known to expand the hair cuticle as well as facilitate penetration of and provide a medium for diffusion into the hair shaft (Kidwell and Blank 1996, Kidwell and Smith 2007). This process is also known to be enhanced by damage to the hair shaft (Kidwell and Smith 2007). This discrepancy may be explained by hair shaft damage possibly related to the effects of acidic canine urine (Gevaert et al. 1991) (or other aspects of the test slurry) in both dry and wet samples. The general wetting effect of the semi-solid slurry itself or an overwhelming contaminant challenge may also be important. Prolonged exposure and increasing contaminant concentrations on the hair surface may also increase penetration and incorporation of substances into hair (Kidwell and Smith 2007). Our observation that HCC appeared to increase with duration of exposure and contaminant concentration on the hair surface agree with these reports.

The results of this investigation clearly indicate that caution should be used in interpreting HCC values in hair collected from live captured grizzly bears (or other species) where pelage is contaminated by feces or urine. However, it should emphasized that: 1) techniques used in this experiment only attempted to replicate high levels of fecal-urinary contamination seen in a few culvert trapped bears, and 2) most bears come out of culvert traps visibly clean or with minor hair surface contamination that can be easily avoided at the time of sampling. Moreover, many hair samples in this study were contaminated near the point of maximum efficacy of the wash protocol employed (i.e. near 75% of the hair surface covered in biological material, Macbeth et al. 2010). Due to budgetary restraints we did not measure cortisol levels in the washes themselves, and it is possible that reduced wash efficacy in these highly contaminated samples could have influenced results. However, studies in the forensic drug field suggest that the continued recovery of cortisol in many washes of heavily contaminated grizzly bear hair reported in Macbeth et al. (2010) may more accurately reflect the incorporation of surface contaminants into the hair shaft rather than unremovable surface contamination (Kidwell and Blank 1996). Further research is recommended to more precisely define the relationship between more typical fecal-urinary contamination levels and HCC in live captured grizzly bears.

In addition, Bechshøft et al. (2011) recently reported that HCC measured in hair obtained from polar bear (*Ursus maritimus*) carcasses and contaminated with blood and lipid had higher mean HCC values in than visibly uncontaminated hair samples obtained from the same population. In grizzly bears, blood and lipid may also occur on hair samples collected opportunistically from carcasses (B. Macbeth, personal observation). Likewise, these materials may compromise samples from animals having recently fed or scavenged or hair covered in attractants associated with barb wire snagging (blood lures) or live capture (animal carcass baits) (B. Macbeth, personal observation). Future research efforts should also consider these materials.

APPENDIX 3

A PRELIMINARY TEST OF THE EFFECTS OF TAXIDERMY ON HAIR CORTISOL CONCENTRATION IN GRIZZLY BEARS (*Ursus arctos*)

A3.1 Introduction

Hair is generally considered to be a robust medium whose structure may be relatively resistant to the effects of natural weathering (Kintz 2004, Webb et al. 2010, Macbeth et al. 2010) or chemical processes (Quadros and Monterio-Fiho 1998). Recently, the measurement of cortisol in hair has shown promise as a reliable index of long-term stress in grizzly bears (*Ursus arctos*) (Macbeth et al. 2010). Current evidence also suggests that cortisol in hair may be detectable after many years or centuries (Webb et al. 2010, Bechshøft et al. 2012), and that cortisol levels in hair may also remain unchanged for no less than many months to years (Macbeth et al. 2010, Gonzalez de la Vara et al. 2011, Macbeth et al. 2012). These features may signify that hair cortisol concentration (HCC) measured in hair from hides maintained in private collections or museums could provide an invaluable record of historical stress levels in grizzly bear populations. This may be especially relevant for conservation efforts in Alberta, Canada (Stenhouse and Graham 2011) where the species has recently been designated as "Threatened" (Festa-Bianchet 2010), and inhabits an environment in which significant human-caused landscape change has occurred in the last 20 years (Stelfox et al. 2005, AGBRP 2008).

Since 1971, the government of Alberta has kept systematic records of all known grizzly bears killed in the province and data from more than 1000 grizzly bear deaths have been collected since this program began (Stenhouse et al. 2003, ASRD 2010, ASRD 2011, ASRD 2012). Importantly, many of these deaths have been associated with a tightly regulated spring hunting season that existed until 2006, when all harvest of grizzly bears in Alberta was suspended (Festa-Bianchet 2010). Most often, grizzly bears were killed as highly valued "trophies" and it is probable that most hides from bears taken as part of legal hunting seasons have been preserved (tanned) and maintained by the original hunter or their family. Likewise, the hides of many Alberta grizzly bears killed accidently or as the result of human animal conflict have been mounted for public educational display or are archived in museum collections.

Only 5-10 guard hairs are required for HCC analysis in grizzly bears (Macbeth et al. 2010) and the removal of this quantity of hair would not alter a hide's appearance or decrease its value as a trophy or educational tool. The mandatory process of registering all legally harvested grizzly bears may also provide a means of locating and accessing hair from hides which reside in private collections. These factors make an investigation of historical stress levels in Alberta

grizzly bears a potential goal for future research programs. However, a wide variety of techniques exist for preserving and preparing animal hides, and many commonly used protocols involve harsh chemicals (e.g. Merkel 2007, Boren et al. 2009) which may alter the levels of substances measured in hair (Newman et al. 2004). The first step in evaluating the utility of preserved hides as viable source of hair for cortisol analysis is an assessment of the effects of taxidermy protocols on cortisol levels in grizzly bear hair.

A3.2 Objective

The purpose of this preliminary investigation was to determine the effects of a commonly employed, chemical-based taxidermy protocol on hair cortisol concentration (HCC) in grizzly bear hair.

A3.3 Materials and Methods

A3.3.1 Source and condition of grizzly bear hide

An intact, fresh-frozen hide from an adult female grizzly bear killed as the result of human-animal conflict in Alberta was used in this investigation. Prior to processing, the hide had been stored at -20°C for a period of ≥ 2 months and had likely been partially unthawed and refrozen at least twice during this period (B. Macbeth, personal observation). Overall, the condition of the hide was good to excellent with all hair still closely adhered to the underlying skin. Mild to moderate blood and or lipid contamination (Macbeth et al. 2010, Contamination Category 1 or 2) was probable in most body regions owing to a large amount of residual soft tissue (adipose tissue, muscle, liquid blood) and to the fact that the hide had been frozen in a rolled and folded position at the time of collection. The hair coat was also somewhat moist. In spite of this, significant blood-lipid contamination (Macbeth et al. 2010, Contamination Category 3) or moisture was restricted to a few discrete patches of hair on the head, the limbs, and the upper aspect of the rump (B. Macbeth, personal observation). These areas were not sampled for subsequent HCC analysis.

A3.3.2 General handling and taxidermy protocol

To prepare the hide for processing, it was laid flat and then bisected with a knife to create two approximately equal halves. The right half was then refrozen while the left half was subjected to a chemical-based taxidermy protocol commonly employed in the preservation and preparation of bear hides. The taxidermy process followed a series of standardized steps and was

performed by Link Taxidermy, Saskatoon, SK. First, excess tissue was removed from the hide with a fleshing knife. To prevent hair loss, the hide was then cured with high grade, non-iodized, granulated salt (NaCl) (AAA Supply House, Calgary, Alberta, Canada) for 12 hours. Next, the hide was soaked for 48 hours in an acid solution containing approximately 8 cups of salt, 38 litres of water, and 180 ml of 85% formic acid (Univar Canada Ltd., Richmond, British Columbia, Canada). This stage of the tanning process is commonly referred to as "pickling" and temporarily preserves and chemically prepares the skin for tanning (Merkel 2007). After 48 hours, 250 g of sodium bicarbonate ($NaHCO_3$) (AAA Supply House, Calgary, Alberta, Canada) was added to the pickling solution for one hour in order to neutralize the pickling solution and to raise the pH of the hide (Merkel 2007). The pickled hide was then rinsed in water, drained, and soaked for 24 hours in a tanning solution containing: 250 g of Lutan F-tanning powder™ (BASF Corporation, Florham Park, New Jersey, USA), 38 litres of water, and 8 cups of salt. After 24 hours, the tanned hide was washed with Tide™ laundry detergent (Proctor and Gamble, Cincinnati, Ohio). A liquid tanning oil [Liqua-soft™ (Knobloch Chemicals, Louisville, Colorado, USA)] was then applied to the underside of the skin and the hide was stretched out on a floor to air dry for 4 days at room temperature. Following air drying, the tanning process was completed by tumble drying in a commercial laundry dryer for approximately 6 hours. The finished hide was stored at the taxidermy studio for approximately one month where it was held at room temperature in the dark. On transfer to the University of Saskatchewan, it was stored on a laboratory bench top at room temperature for approximately three weeks prior to HCC analysis.

A3.3.3 Hair sample collection, processing and data analysis

200 mg hair samples were collected from 15 paired locations on each of the tanned and unprocessed portions of the grizzly bear hide including: the head, neck, shoulder, back, rump, side (cranial, middle and caudal aspect), front leg (proximal and distal aspect), hind leg (proximal and distal aspect), and abdomen (cranial, middle and caudal aspect). Hair samples were then washed, ground, extracted, and analyzed following methods reported in Macbeth et al. (2010). An independent samples *t*-test (with the level of significance set at $P \leq 0.05$) was used to compare, log transformed HCC values determined in the tanned and un-tanned portions of the hide.

A3.4 Results and Discussion

Hair cortisol concentration measured in hair from the tanned portion of a bisected grizzly bear hide was greater than HCC measured in hair in from the un-processed portion the same hide (Independent Samples *t*-test $t_{(28)} = 3.769$, $P < 0.001$, $n = 15$). This finding suggests that this commonly employed chemical-based taxidermy protocol may alter HCC in grizzly bear hair. Uniquely, hair extract from the tanned portion of the hide contained significant quantities of a white crystalline powder when dried (Fig A3.1) and this material was readily returned to solution when buffer was applied during the reconstitution process.

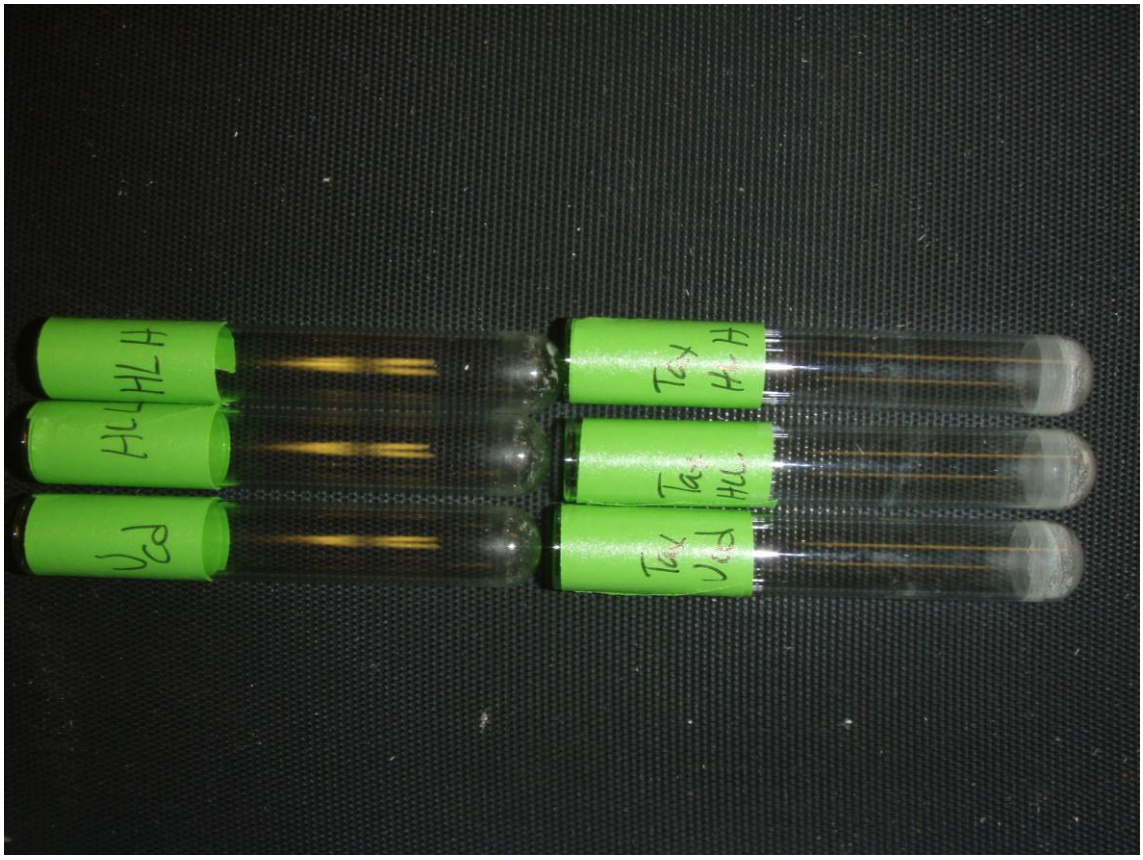

Figure A3.1 Example of white, crystalline powder (A) observed in dried hair extract from tanned grizzly bear (*Ursus arctos*) hair, but not in dried hair extract from unprocessed grizzly bear hair (B) taken from the same animal and body region. Photo: B. Macbeth

Lutan F-tanning powder™ was the only white-crystalline material used in this experiment (Link Taxidermy, personal communication), and it is probable that this material was incorporated into the hair shaft during the tanning process (Kidwell and Blank 1996, Kidwell and

Smith 2007). It is also likely that incorporation was facilitated by hair shaft damage related to the repeated exposure of processed hair to strong solvents (e.g. 85% formic acid) in combination with the extended periods (≤ 48 hours) of wetting in pickling, tanning, or cleaning solutions (Kidwell and Blank 1996, Kidwell and Smith 2007).

Lutan F-tanning powder™ is a mineral tanning agent composed of aluminum chloride ($AlCl_3$), sodium dihydrogen 4-sulphonatophthalate ($C_8H_5NaO_7S$), and other mineral salts (BASF 2012). One possible explanation for the elevated HCC levels recorded after tanning may be that the presence of cross reacting substances in this material artificially elevated HCC values in processed hair. Alternately, repeated exposure to acidic or basic solutions throughout the tanning process may have damaged the structural integrity of the hair shaft and enhanced total cortisol extraction. Overall, these findings suggest that the use of chemical-based tanning protocols may prevent the accurate determination of HCC levels in preserved grizzly bear hides. However, it is important to consider that only one hide and one tanning protocol were directly tested in this preliminary investigation. Studies using hair from more animals or different protocols could yield different results. Further research into the effects of other tanning methods which do not use harsh chemicals (e.g. brain or smoke tanning) may also be warranted.

Interestingly, this work disagrees with a previous anecdotal observation of a decrease in HCC after tanning (unknown protocol) (Paired Samples *t*-test $t_{(4)} = 3.457$, $P = 0.027$) in hair subsamples ($n = 5$ pre tanning, $n = 5$ post tanning) collected from an adult male grizzly bear (body regions unknown) killed near Yellowknife, Northwest Territories, Canada. However, white crystalline material was not observed in post tanning hair extract obtained from this grizzly bear (B. Macbeth, personal observation).

APPENDIX 4

HAIR CORTISOL CONCENTRATION IN AN ADULT MALE CARIBOU *(Rangifer tarandus groenlandicus)* FROM SOUTHHAMPTON ISLAND, NUNAVUT, CANADA

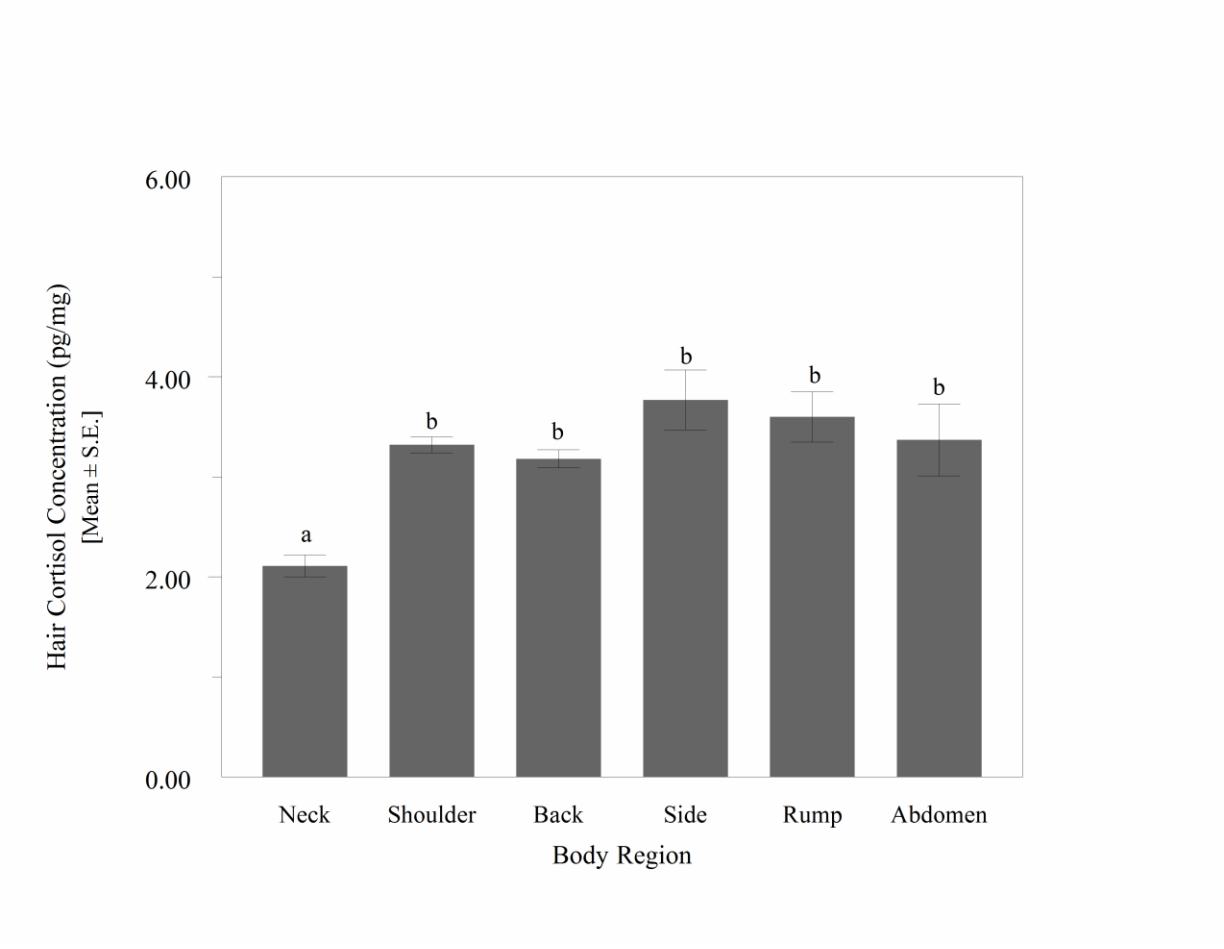

Figure A4.1 Comparison of hair cortisol concentration (HCC) determined in the neck, shoulder, back, side, rump, and abdominal hair of an adult male caribou (*Rangifer tarandus groenlandicus*) harvested on Southampton Island, Nunavut, Canada. Variation in HCC was explained by body region (one-way repeated measures ANOVA, $F_{(4, 20)} = 7.312$, $P < 0.001$). Bars and error bars represent the mean HCC values and S.E. of $n = 5$ hair subsamples from each body region. Significant differences (Tukey-Kramer, $P \leq 0.05$) in HCC between body regions are indicated by different letters. Hair from the neck was uniformly white while that collected from all other body regions was a similar mixture of grey to brown. No blood contamination was apparent on any hair sample. Hair provided by Brett Elkin, Wildlife Division Environment and Natural Resources, Government of the Northwest Territories.

APPENDIX 5

HAIR CORTISOL CONCENTRATION IN AN EMACIATED ADULT MALE BLACK BEAR (*Ursus americanus*) AND AN EMACIATED ADULT MALE GRIZZLY BEAR (*Ursus arctos*) FROM YELLOWKNIFE, NORTHWEST TERRITORIES, CANADA

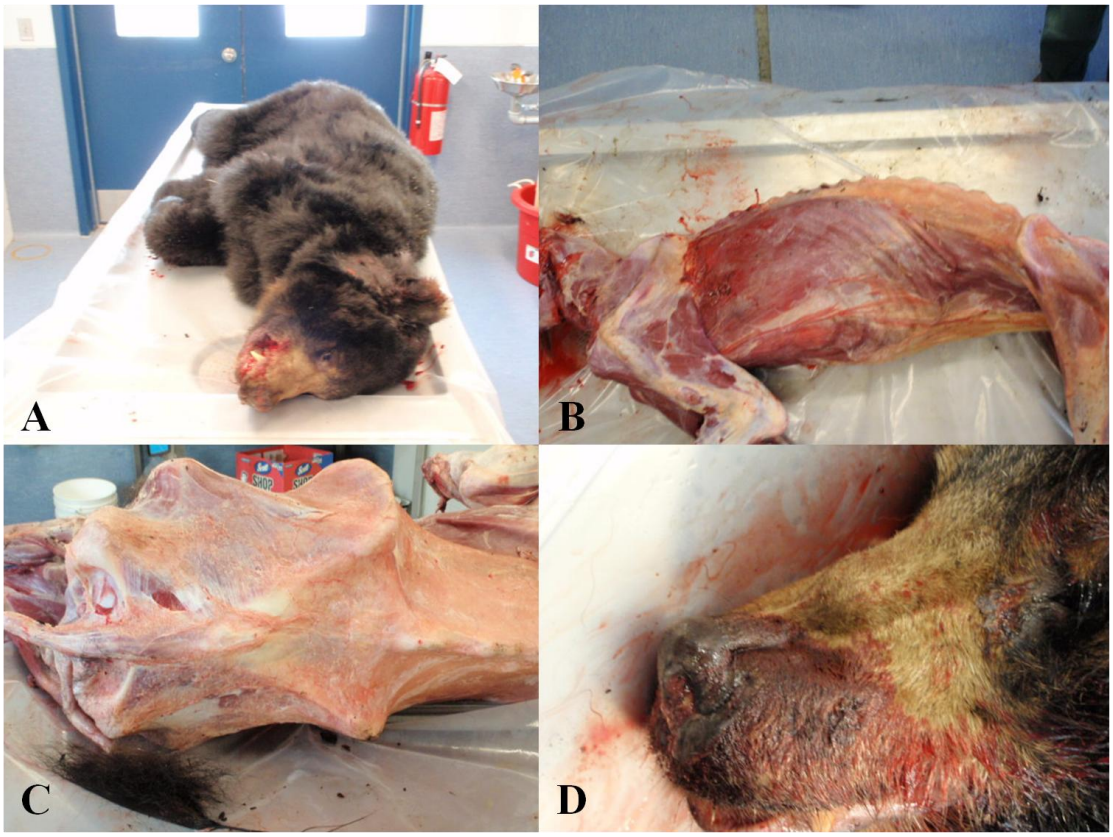

Figure A5.1 Hair cortisol concentration determined in an adult male black bear (*Ursus americanus*) in poor health collected near Yellowknife, Northwest Territories, Canada on November 3, 2009 (A). The bear weighed 58.4 kg, was severely dehydrated, and was emaciated. Extensive and severe muscle atrophy was also readily apparent (B), (C). Porcupine quills were found protruding through the intestinal walls and stomach. Quills were also found in the middle of the tongue, and in the head, abdominal area and, carpal joint. The lymph nodes were enlarged and the nasal bones of the skull appeared collapsed (D). There was also evidence of haemorrhaging under the right arm and ribs and associated with a tear on the liver. Using protocols from Macbeth et al. (2010) we determined that hair cortisol concentration (HCC) in this black bear was 12.20 pg/mg. This value was only exceeded by HCC levels in 9 of the 151 grizzly bears (*Ursus arctos*) and 1 of the 188 polar bears (*Ursus maritimus*) we examined in this study.

Using the same protocol, we also opportunistically evaluated HCC in an emaciated adult male grizzly bear (*Ursus arctos*) killed near Yellowknife in the fall of 2007. HCC (10.16 pg/mg) determined in this grizzly bear was in the top ten percent of all grizzly bear HCC values in this study. These finding may represent further anecdotal evidence of a potential link between elevated HCC levels and diminished health in bears. Hair samples, photos and black bear necropsy report courtesy of Brett Elkin, Wildlife Division Environment and Natural Resources, Government of the Northwest Territories.

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