Stress Physiology of Bears:
Cortisol Dynamics and Identification of Novel Serum Proteins

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

There is a need to understand how free-ranging animals respond and adapt to stress. However, little is currently known regarding the physiologic adaptations to stress in bears, and there are few tools available to wildlife managers to assess the health and stress status of free-ranging animals, including ursids. The hypothalamus-pituitary-adrenal (HPA) axis plays major roles in the physiological adaptation to stress, leading to the increased secretion of glucocorticoids (e.g. cortisol in most mammals) that mediate adaptive changes in physiology and behaviour. The vast majority of glucocorticoids are bound to its primary carrier protein, corticosteroid-binding globulin (CBG), in most animals, and only the unbound fraction is bioavailable. Thus, CBG plays a major role in modulating glucocorticoid dynamics, and this protein must be characterized to build a more complete understanding of the adaptive role that the HPA axis plays in mitigating stress in bears. The overall objective of this thesis was to characterize the HPA axis activity and CBG levels in bears, and develop tools targeted towards the monitoring of the health and stress status of American black bear (*Ursus americanus*), grizzly bear (*U. arctos*), and polar bear (*U. maritimus*).

The binding characteristics of cortisol to CBG in bears were studied via saturation binding experiments, and this information was used to estimate free cortisol concentrations based on CBG concentrations. To quantify CBG concentrations in bears, an enzyme-linked immunosorbent assay (ELISA) was developed. Grizzly bear CBG cDNA was cloned and sequenced, and an antibody was developed against a peptide sequence of the deduced amino acid sequence. The antibody showed good cross-reactivity against black, grizzly, and polar bear CBG, and the ELISA based on this antibody found differences in the mean CBG levels between species. Using this data, free cortisol levels were estimated, and mean levels were elevated in polar bears relative to black and grizzly bears.

Having developed these tools, the roles that corticosteroid-binding globulin (CBG) and bioavailable cortisol played in the physiological adaptation to major life history traits and environmental challenges faced by ursids were investigated. Importantly, CBG was not modulated by the acute stress of capture and handling, despite the large differences in the

magnitude of acute cortisol responses that are induced by these methods, suggesting that CBG levels may reflect the chronic health and stress status of bears. Altogether, there were few changes in CBG levels throughout much of the annual life cycle of bears, implying that CBG does not play a major adaptive role in the life history traits of bears and, instead, metabolic and environmental factors may be the key modulators of cortisol dynamics. However, CBG was not significantly associated with our measures of dietary patterns and nutrition, including body condition, seasonal dietary patterns, and fasting. The majority of the observed variation in the levels of this protein in bears remains unexplained. However, stress-induced free cortisol levels were negatively associated with urea to creatinine ratio (an indicator of dietary protein content and fasting status in grizzly and polar bears, respectively) and positively associated with lactation in hibernating black bears, suggesting that the variation in adrenal function may be playing an important role in the adaptation to adverse environmental conditions and/or metabolic stress in bears.

In addition to serum cortisol dynamics, other proteins were also hypothesized to play adaptive roles in maintaining the hibernating phenotype in bears. Changes in the serum proteome during hibernation in black bears were assessed as a means to discover novel proteins that may be indicative of metabolic stress in bears. The serum proteomes of active and hibernating black bears were compared and analyzed for significant changes by two-dimensional electrophoresis and tandem mass spectrometry. Proteins involved with immune-related function were significantly altered during hibernation, leading to the proposal that the serum protein changes are essential for maintaining immune competence, wound healing, and bone structure.

Altogether, this thesis developed a method to quantify CBG and estimated free cortisol concentrations in bears, and characterized their roles in the physiological adaptations associated with the major life history traits and environmental challenges faced by ursids. Also, novel serum proteins were identified as potential markers of immune function and health status in bears. These tools may be tremendously useful for wildlife managers and conservationists in determining how chronic stressors, including anthropogenic activities and climate change, may impact the stress and health performances of individual and populations of free-ranging bears.

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HPA	Hypothalamus-Pituitary-Adrenal
CBG	Corticosteroid-Binding Globulin
2DE	Two Dimensional Electrophoresis
DIGE	Difference Gel Electrophoresis
MS/MS	Tandem Mass Spectrometry
K_d	Equilibrium Dissociation Constant
K_a	Equilibrium Association Constant
ELISA	Enzyme-linked Immunosorbent Assay
B_{max}	Maximum Binding Capacity
HD	Heli-darting; Remote Administration of Anesthetic from a Helicopter
LGS	Leg-hold Snaring; Physical Immobilization by Leg-hold Snare
CT	Culvert Trapping; Physical Immobilization by Barrel Trap
SF	Solitary Adult Female
AF	Adult Female with Dependent Juveniles
AM	Adult Male
JF	Juvenile Female (Age < 5)
JM	Juvenile Male (Age < 5)
UC	Urea to Creatinine Ratio
BCI	Body Condition Index
PRE	Pre-hibernation
HIB	Hibernation
POST	Post-hibernation

CHAPTER 1:
GENERAL INTRODUCTION

1.1. Stress in Free-ranging Bears and Animals

Organisms must adapt to environmental disturbances to maintain fitness. Animals possess the ability to cope with these challenges (unpredictable conditions, situations, or stimuli; hereafter referred to as stressors) to prevent or mitigate the loss of health. These disturbances do not necessarily contribute directly to increased mortality, but may cause sublethal effects that weaken the health of the animal and detrimentally impact the persistence of the species [1, 2]. Two animals studied in this thesis – grizzly (*Ursus arctos*) and polar bears (*U. maritimus*) – are threatened by rapid environmental changes, due in part to human influences. These species are closely related [3] yet inhabit tremendously different environments. Grizzly bears are generalist omnivores that historically were distributed throughout the Nearctic ecozone, but their range is now greatly reduced [4]. Some grizzly bear populations in Alberta, Canada inhabit areas that have experienced widespread landscape changes due to expanding resource extraction activities and human populations. These landscape changes have resulted in the fragmentation of and increased human access to core grizzly bear habitats, which are linked to the unsustainable mortality rates of grizzly bears in this province [5]. The survival of reproductive females is critical for the maintenance of stable and increasing populations [6], and population trends may be sensitive to small changes in the survival rates of this reproductive class [8]. Moreover, grizzly bears slowly reproduce due to late sexual maturity, have small litter sizes and long reproductive intervals [6], and the reproductive rates of some populations within Alberta, Canada are lower than populations elsewhere [7, 8]. These factors are thought to play roles in the poor performance and, combined with small population sizes [5], the uncertain persistence of the grizzly bear populations in this province [5, 8, 9, 10, 11].

Similarly, polar bear populations inhabiting the southern areas of their range in the Hudson Bay region of Canada are threatened by environmental change. Polar bears are an Arctic maritime species and are almost entirely carnivorous [12]. They are dependent on sea ice as a platform upon which they hunt their primary food sources, and populations that inhabit areas where sea ice is seasonal (e.g. in the southerly portions of their range) are forced ashore during the open water season [12]. Moreover, inclement weather during the winter may also reduce or prevent hunting success even when sea ice is present [13]. During these periods of open water and unfavorable hunting, polar bears largely fast and rely on endogenous fat stores to provide substrates for energy metabolism [14]. Furthermore, pregnant female polar bears undergo gestation, parturition, and the first several months of lactation while fasting, so it is critical that they maximize their endogenous stores prior to reproduction [15]. Thus, polar bears are sensitive to changes in sea ice conditions, and declines in the extent and duration of seasonal ice cover during the peak hunting season (spring and early summer) that are associated with climatic warming may negatively impact the reproductive success and health of this species [16, 17, 18, 19]. Indeed, long-term declines in the body condition of polar bears in the Western [18] and Southern Hudson Bay regions [20] are linked to climatic warming and declining sea ice conditions [16]. Furthermore, most polar bear populations are forecast to decline over the long term due to projected declines in ice conditions [21]. Thus, there is a need to better understand the effects of rapidly changing environments on bear species, including on the health of individuals and populations.

Health relates to the state of well being and functioning of the organism. There are several overlapping definitions of the health of an organism, including the states of 1) normally functioning systems, 2) maintenance of homeostasis within their optimal ranges in the face of internal and external variation [22], 3) physical and psychological well-being, and 4) productivity [23]. Stress can be defined as the disruption of health, which may lead to a pathological state if not restored. However, in terms of physiology, stress is specifically the disruption of homeostasis [24], while the stimulus is termed a stressor. The series of physiological responses evoked by stressors is termed the stress response, and this is essential to reestablish and/or maintain homeostasis. Failure to adapt to stress may result in the disruption in the functioning of the affected systems (i.e. the development of pathology) [1], with downstream effects on the health of the animal. Chronic or long-term stress may affect

measures of population health, including decreased natality, birth rate and/or increased mortality, which may lead to population decline [1].

While population estimates and trends are important conservation tools to monitor populations, wildlife managers lack tools that can assess the biological factors that affect population health [2]. Glucocorticoids, including cortisol (the predominant glucocorticoid in most mammals) and corticosterone (in rodents and birds), are steroid hormones that play important roles in the physiological response and adaptation to stress, and their measurement is an established method to assess the stress and health status of animals [2]. Elevations in serum glucocorticoid levels in free-ranging animals are linked to increased mortality and decreased reproductive success and this has been reported in Galápogos marine iguanas (*Amblyrhynchus cristatus*) [25], snowshoe hares (*Lepus americanus*) [26] and elk (*Cervus canadensis*) [27]. Thus, the measurement of glucocorticoids and other markers of stress may provide a tool with which wildlife managers can investigate the physiological mechanisms underlying stress-related health effects, and will assist in developing tools to assess and predict the impact of environmental changes on wildlife populations [2].

The focus of this thesis is to examine and better understand ursid stress physiology with the aim to develop tools that can be used to assess the stress and health status of these animals. The sections below provide a background on stress physiology in general and glucocorticoid dynamics in particular, as well as the use of proteomics tools to discover novel serum protein markers of stress in wildlife.

1.2. The Stress Response

The stress response involves changes at the molecular, cellular, tissue, and whole organism levels that allow the animal to cope with the stressor(s) and maintain or reestablish homeostasis [28, 29]. The stress response is energetically expensive, and coping with stress occurs at the expense of other physiological processes, including growth, reproduction, and immunity [30,

31]. The prolonged induction of the stress response, particularly chronically elevated glucocorticoid levels may result in slower growth, poorer body condition, decreased reproductive success, and/or increased susceptibility to diseases. At the population level, chronically elevated glucocorticoids can negatively impact measures of population health, including natality and mortality [1]. Thus, chronic stress has detrimental consequences on animal health and may be one mechanism by which long-term environmental changes affect individual and population health.

The central nervous system is instrumental in the initiation, maintenance, and termination of the stress response. Two parallel systems are activated in synchrony with the initiation of the stress response: the fight-or-flight response and the hypothalamic-pituitary-adrenal (HPA) axis. Catecholamine hormones (CA) – norepinephrine (NE) and epinephrine (EPI) – mediate the fight-or-flight response. These hormones are released from post-ganglionic sympathetic nervous system (SNS) neurons and the adrenal medulla, respectively, although the adrenal medulla may also release significant amounts of NE [32]. Upon perception of stress, the paraventricular nucleus of the hypothalamus stimulates the sympathetic nervous system to release CA into general circulation [24], which act on the α- and β-adrenergic receptors (AR). These receptors are found in almost all tissues, and the two types may mediate contradictory effects (e.g. α-AR mediate vasoconstriction whereas β_2-AR mediate vasodilation). AR are G-coupled proteins that respond rapidly to ligand binding to exert their physiological effects [32], including the enhancement of cardiovascular tone, alterations in blood circulation, and the stimulation of glycogenolysis and lipolysis [24, 31]. These effects prime the organism for immediate reaction against stressors and promote short-term survival.

While CA undoubtedly play important roles in the short-term adaptation to stress in animals, the measurement of CA in free-ranging animals is generally not feasible due to their rapid appearance and disappearance from circulation [24]. Instead, most studies on stress in wildlife examine the physiological effects of glucocorticoids, which are secreted in response to activation of the HPA axis following SNS stimulation.

1.3. Glucocorticoid Physiology

1.3.1. Regulation of Glucocorticoid Secretion

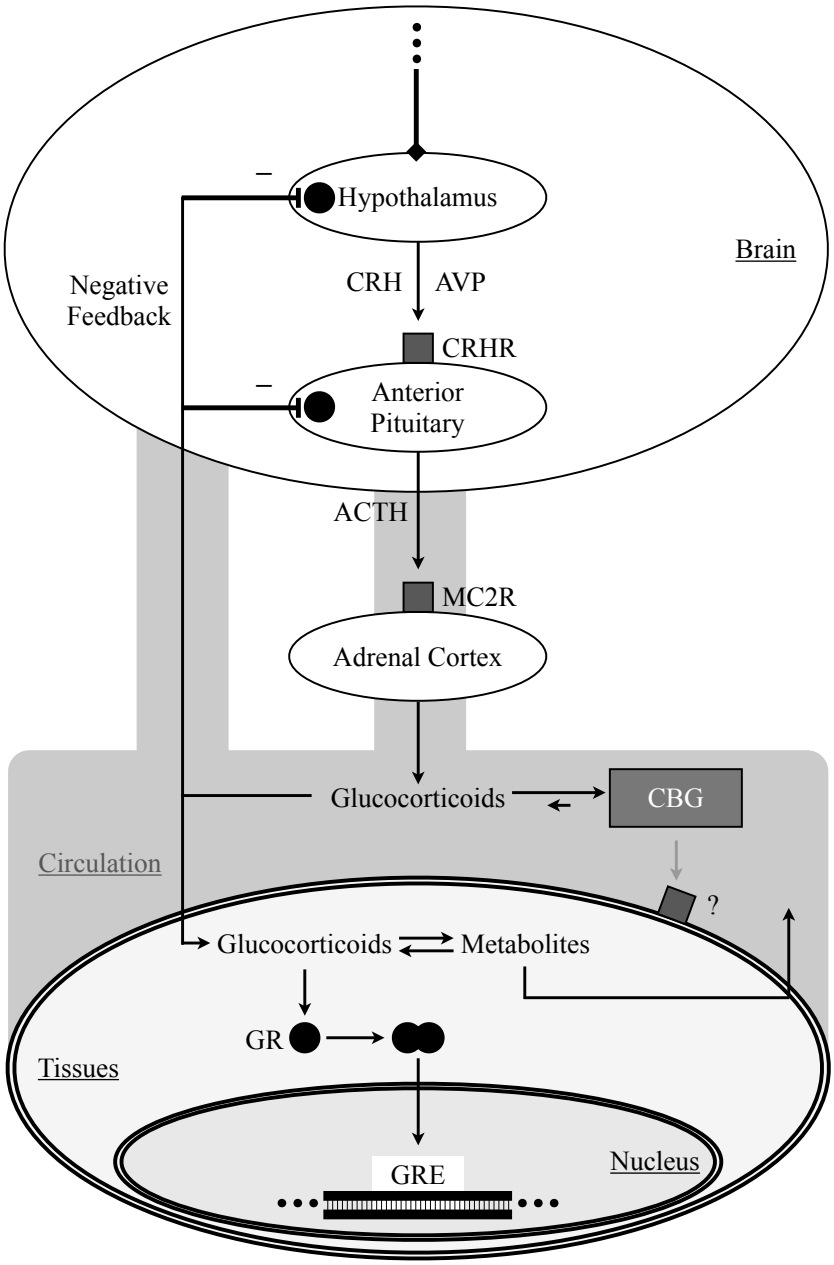
The regulation of HPA axis activity is illustrated in **Figure 1.1**. Upon stimulation of the HPA axis by stressors, corticotropin-releasing hormone (CRH) is released from parvocellular neuroendocrine neurons in the paraventricular nucleus in synchrony with the activation of the SNS response. CRH diffuses through the hypophyseal portal system to act on CRH receptors on corticotropes in the anterior pituitary. The activation of CRH receptors leads to the release of adrenocorticotropin hormone (ACTH), a product formed by post-translational modification from its precursor protein proopiomelanocortin (POMC) into general circulation. ACTH may also be released in response to CA and arginine vasopressin (AVP) stimulation; however, CRH is the major hormone that stimulates the release of ACTH in response to acute stress. ACTH binds to melanocortin 2 receptors (MC2R) on the steroidogenic cells in the zona fasciculata of the adrenal cortex. MC2R stimulation triggers the upregulation of enzymes that catalyze the rate-limiting steps in the corticosteroid biosynthesis pathway, including steroidogenic acute regulatory protein (StAR) and cytochrome P450 cholesterol side chain cleavage (P450scc) enzyme. The stimulation of steroid biosynthesis results in the elevation of serum corticosteroid concentrations (i.e. "stress-induced" levels) within minutes of stressor perception [31, 33, 34, 35].

1.3.2. Regulation of Glucocorticoid Action

1.3.2.1. Mineralocorticoid and Glucocorticoid Receptors

The effects of glucocorticoids on cells are mediated via mineralocorticoid (MR) and glucocorticoid receptors (GR), generally through their regulatory effects on gene transcription. Both receptors are transcription factors, and upon binding of glucocorticoids translocate to the nucleus and interact with response elements in the promoters of glucocorticoid-responsive genes to influence their transcriptional activity [36]. GR are present in virtually all tissues and are responsible for most of the effects of glucocorticoids on cells, while MR are restricted to

Figure 1.1: A Schematic Overview of the Hypothalamic-Pituitary-Adrenal (HPA) Axis and Glucocorticoid Dynamics in Higher Vertebrates.
The HPA response to stress is driven by the secretion of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from the parvocellular neuroendocrine cells of the hypothalamus into the hypophyseal portal system. CRH binds to CRH receptors (CRHR) on corticotrope cells of the anterior pituitary, which stimulates the release of adrenocorticotropic hormone (ACTH) into general circulation. ACTH binds to melanocortin-2 receptors (MC2R) on the cell surfaces of the zona fasciculata in the adrenal cortex, which together with the action of AVP stimulates steroid biosynthesis and glucocorticoid secretion. In general circulation, glucocorticoids are tightly bound to corticosteroid binding globulin (CBG). Bound glucocorticoids are generally biologically unavailable and only a small fraction of glucocorticoids are unbound (free) in serum under baseline conditions. There may exist a cell-surface receptor for CBG-glucocorticoid complexes that stimulates the internalization of this complex or the activation of a second messenger system. Otherwise, the major route of glucocorticoid action is the diffusion of free steroid into effector cells to complex with intracellular glucocorticoid receptors (GR). GR-glucocorticoid complexes homodimerize, translocate into the nucleus, and bind to glucocorticoid response elements (GRE) to modulate transcription of responsive genes. The HPA axis is negatively regulated by glucocorticoids, where free glucocorticoids act on GR in the hypothalamus and pituitary to downregulate CRH and ACTH secretion, respectively.


Hypothalamus
Brain
Negative
Feedback
CRH
AVP
CRHR
Anterior
Pituitary
ACTH
MC2R
Adrenal Cortex
Glucocorticoids
CBG
Circulation
?
Glucocorticoids
Metabolites
GR
Tissues
Nucleus
GRE

tissues involved in Na^+/K^+ balance and in some brain regions [36]. MR appear to act as a high-affinity glucocorticoid receptor in some tissues [38], while other MR-containing tissues are insensitive to glucocorticoids and respond only to aldosterone, the mineralocorticoid hormone in mammals. In the hypothalamus and pituitary gland, MR play an important role in the diurnal variation and negative feedback of glucocorticoid secretion [36]. 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) is expressed in the aldosterone sensitive tissues and catalyzes the conversion of cortisol to the inactive metabolite cortisone to protect against the activation of MR by glucocorticoids [39].

Corticosteroids are always present in circulation and show a diurnal pattern in their baseline levels; peak levels occur around the time of arousal from sleep and the lowest levels during sleep. At the lowest point of baseline levels, glucocorticoids mediate their cellular effects through the occupation of its high-affinity MR in tissues that lack 11B-HSD2. At peak baseline and stress-induced elevated concentrations, the steroid exerts its effect by binding to the lower-affinity, high-capacity GR that is expressed in almost all tissues [33, 40]. However, only free glucocorticoids are thought to cross plasma membranes to act on its intracellular receptors, and corticosteroid binding globulin (CBG) is the major circulating protein that regulates the bioavailability of this steroid hormone for target tissue usage.

1.3.2.2. Corticosteroid Binding Globulin

CBG is a ~50 kDa serum glycoprotein that belongs to the serpin proteinase inhibitor superfamily [41] and binds glucocorticoids and progesterone with high affinity in circulation [42]. In birds, which lack a sex hormone binding globulin, CBG also exhibits high affinity for testosterone [43]. Albumin is another major contributor to glucocorticoid binding, albeit with much lower affinity, and the two proteins in concert bind up to 95% of the total glucocorticoid in blood.

CBG buffers tissues against the deleterious consequences of excessively elevated glucocorticoid levels because only free, unbound glucocorticoids are thought to diffuse across plasma membranes to act on its intracellular receptors [43, 44]. The serum levels of CBG may be modulated by stress. For instance, some acute stressors result in a decrease in serum CBG concentrations, leading to elevated free cortisol levels that are sustained for several hours to

days post-stress [43, 45, 46]. Also, CBG may be rapidly released from the liver in response to acute stress to mediate a short-term (~ 20 min) delayed rise in free glucocorticoid levels in the face of rapid elevations in total glucocorticoid levels [47]. CBG bound glucocorticoids can be rapidly released under specific conditions. For example, the affinity of CBG for its ligands is sensitive to changes in the conformation of this protein, and elastases secreted by activated neutrophils are able to cleave the reactive loop of CBG, leading to conformational changes that result in a decreased affinity for glucocorticoids and its release [48, 49]. Furthermore, CBG is a temperature sensitive protein, and localized elevations in body temperature (e.g. at sites of inflammation) may also reduce binding affinity for cortisol [50]. These examples of the specific release of glucocorticoids are thought to increase local concentrations of this hormone to modulate immune function at sites of inflammation [48].

High-affinity membrane receptors for glucocorticoid-CBG complexes may also exist [51, 52, 53, 54], although these receptors have yet to be identified. It is proposed that CBG is internalized after binding to these receptors, after which the complexes are cleaved to release bound glucocorticoids to increase intracellular glucocorticoid levels in tissues that express this receptor [43]. Glucocorticoid-bound CBG may also activate a cyclic AMP mediated second messenger response when bound to cell membranes [54, 55]. Despite these findings and continued research into this area, no clear biological evidence is forthcoming yet on the interactions between glucocorticoid-bound CBG and membranes [43]. Thus, the contributions of glucocorticoid-bound CBG on cellular actions are unknown, and the diffusion of the free fraction of hormone into cells is generally held to be the primary mode of action for glucocorticoids.

1.3.2.3. Chronic Glucocorticoid Elevation

Some stressors or series of stressors may disturb homeostasis for a prolonged period of time. The persistence of the stressor may result in a sustained stress response, including chronically elevated serum glucocorticoid levels. The chronic elevation of glucocorticoids is generally held to be detrimental to animals [28]. Chronic stress is associated with alterations in the regulation of the HPA axis [40], including the attenuation of the negative feedback inhibition of glucocorticoids by suppression of GR expression in the brain [56]. This disrupted negative feedback may result in a greater total adrenal release of glucocorticoids, leading to sustained

elevated baseline levels of the steroid. Chronic glucocorticoid elevation is also associated with thymus involution and adrenal hypertrophy in most species, and the latter may be associated with increased glucocorticoid secretion in response to ACTH at both baseline and stress-induced conditions [24]. Serum CBG concentrations may also decline, exacerbating the effects of elevated cortisol levels because a decrease in buffering capacity may lead to the increased bioavailability of this steroid hormone to tissues [28].

Chronically elevated glucocorticoid levels are linked with a range of pathologies, including stunted growth and development [57, 58], elevated arterial blood pressure [59], hyperinsulinemia and insulin resistance [60], hyperglycemia [61], skeletal muscle [62] and bone atrophy [63], inhibition of immune system function [64], and reproductive failure [26]. Moreover, chronic stress may increase susceptibility to other diseases due to the detrimental effects of chronically elevated glucocorticoid levels on immunity and metabolism [65]. In free-ranging animals, the detrimental effects of chronic stress may include impaired reproduction [26], reduced growth rate and/or food conversion efficiency (i.e. increased food consumption per unit growth compared to unstressed animals) [66], and suppressed immunity [64].

Clearly, chronic stress and sustained elevation of glucocorticoid levels may be a major challenge faced by wildlife. However, while these physiological responses to chronic stressors may be pathological, the consequences of the changes that are mediated by chronically elevated glucocorticoids, including altered behaviour, metabolism, and physiology are not necessarily maladaptive (i.e. it does not always reduce fitness) unless the severity and/or type of stressor is beyond the capability of the animal to cope. These effects may ultimately enhance fitness by redirecting resources and maximizing effort towards survival at the expense of physiological processes that are unnecessary for short-term survival, but these effects are highly dependent on the unique combination of life history traits and evolutionary history of each species [67]. Thus, study of the interactions of the stress response with the life history and evolution of animals is required to better understand the roles of stress response in maintaining health and fitness.

1.4. The Biological Effects of Glucocorticoids in Wildlife

Glucocorticoids have widespread effects on physiology and behaviour. It should be noted that this steroid hormone has quite different effects at baseline and stress-induced levels. Permissive actions are mediated by baseline levels of glucocorticoids and prepare an animal for potential stressors [31]. These permissive actions may include synergistic and antagonistic interactions with glucoregulatory hormones to regulate energy availability for normal functions, including the regulation of feeding and adipose tissue metabolism [68, 69]. Glucocorticoids may also permissively enhance immunity by upregulating hormone, growth factor, and cytokine receptors [31], which may enhance the immune response to an immune stimulus.

Stimulatory, suppressive, and preparatory effects generally manifest at stress-induced levels of glucocorticoids [31], and serve to repartition energy resources to aid in fueling the increase in metabolic demand that is associated with the stress. The stimulatory and suppressive actions of glucocorticoids mediate some of the prototypical effects of the stress response, which include the stimulation of gluconeogenesis and inhibition of peripheral glucose uptake [30], stimulation of lipolysis in adipose cells, and proteolysis of muscle to provide amino acid substrates for gluconeogenesis [68]. Stress-induced levels of glucocorticoids are a powerful inhibitor of immune function [31], the growth hormone (GH)/insulin-like growth factor 1 (IGF1) axis (a key mediator of growth and metabolism in animals) [70], and the hypothalamus-pituitary-gonadal axis that regulates sexual function [31]. Together, these many actions of glucocorticoids, whether at baseline or stress-induced levels, mediate the biological changes that enable an organism to cope with stress.

1.4.1. HPA Axis and Wildlife Reproduction

Reproduction is a metabolically expensive process in most animals, and glucocorticoids generally suppress reproductive activity at multiple levels. Many components of the stress response, including sympathetic nervous system activity, CRH, pre-proopiomelanocortin

cleavage products, and stress-induced levels of glucocorticoids inhibit reproductive function and behaviour by suppressing the synthesis and/or release of reproduction hormones [31]. In birds, stress-induced levels of glucocorticoids during reproductive seasons are associated with altered behaviour that disrupts reproduction, including reduced or abandoned territoriality [71], courtship [72], and parental care [73]. Pied flycatchers experimentally implanted with corticosterone-containing capsules abandoned their nests, and this response may allow these animals to escape severe stressors and resume reproduction when conditions allow for more successful reproduction [71].

Despite these detrimental effects of stress on reproduction, baseline and stress-induced glucocorticoid levels tend to peak during breeding for many animals [74]. One hypothesis posits that seasonal changes in glucocorticoid levels, including increases during reproduction, reflect temporal changes in the probability and/or severity of stressors. Also, since reproduction is energetically demanding, enhanced glucocorticoid secretion may play a role in mobilizing energy to meet this demand, but species must balance the energy mobilizing effects of glucocorticoids with its suppressive effects on reproduction. However, elevated levels of glucocorticoids do not inhibit the gonadal axis during reproduction in some species, including in small semelparous marsupials (e.g. red-tailed phoscogale [*Phascogale calura*] [75]) and partially semelparous species (e.g. Arctic ground squirrels [*Urocitellus parryii*] [76]). There is no evidence that these animals interrupt reproduction with behaviours that mediate immediate survival (i.e. the emergency life history stage, which may include altered behaviour such as increased foraging and decreased parental care [77]). Semelparous males have only one or very few opportunities to mate, and compete vigorously and die shortly after the breeding season [28]. This is thought to be due to pathologies generally associated with chronic stress, including immunosuppression and organ failure. Moreover, the altered HPA axis function of these species during reproduction is indicative of chronic stress, including elevated free glucocorticoid levels, decreased CBG concentrations, adrenal hypertrophy, and attenuated negative HPA feedback. Thus, these species may have evolved mechanisms to avoid the suppression of reproduction by enhanced glucocorticoid stimulation and, instead, expend maximum effort on reproduction at the price of long-term survival. In contrast, males of iteroparous species that have multiple opportunities to breed may trade off short-term reproductive success for the long-term maintenance of homeostasis if conditions are

unsuitable, and exhibit glucocorticoid-mediated suppression of reproduction during breeding in response to acute stress [28]. Moreover, these animals generally do not exhibit symptoms of chronic stress and maintain normal CBG levels to prevent excessive glucocorticoid stimulation.

There may also be differences in HPA axis functioning between sexes and this may be mediated by the actions of sex steroids. Androgens suppress and estrogens stimulate HPA function [78], resulting in sex-specific differences in both baseline levels of glucocorticoids and the magnitude of increase due to induction by acute stressors. CBG expression is also under the regulation of the gonadal axis, and androgens and estrogens down and upregulate CBG biosynthesis, respectively [79]. The actions of these sex steroids may result in elevated free glucocorticoid and lowered CBG levels in males during courtship, which may be associated with intense intrasex competition between males for mates. In contrast, CBG and glucocorticoid levels are generally elevated during gestation for females due to elevated estrogen levels (e.g. [80, 81, 82, 83, 84, 85]). However, while baseline levels are elevated, levels of stress-induced glucocorticoids may be suppressed in pregnant and lactating animals [24], which together with the elevated CBG levels may protect the developing fetus from the detrimental effects of elevated glucocorticoids.

Furthermore, while estrogen levels decrease post-parturition, elevated baseline glucocorticoid levels are associated with lactation in female mammals (e.g. [86, 87]) and with nesting in birds (e.g. [73, 88, 89]), which are both energetically expensive processes leading to negative energy balance in the parents. While lactation is associated with the suppression of stress responses, nesting birds maintain robust glucocorticoid responses to acute stressors. This is associated with adaptive responses to poor environmental conditions, including the nest abandonment that accompanies the activation of the Emergency Life History Stage [77]. CBG may play a key role in modulating glucocorticoid action in nesting birds. Love et al [89] found that while total baseline glucocorticoid levels were not different between nesting stages in female European starlings, free glucocorticoid levels were highest in the final, chick rearing stage because CBG levels decreased during nesting while baseline glucocorticoid levels did not change. The increased free glucocorticoids were deemed to be necessary to meet the energetic demands of

provisioning for chicks without the stimulation of adrenal activity that is associated with nest abandonment.

Glucocorticoids also play a role in the fetal and neonate development of the HPA axis. Prenatal exposure to elevations in maternal glucocorticoids (e.g. due to experimentally induced stress or administration of exogenous glucocorticoids) or postnatal stress at critical points during development may have far-reaching effects on subsequent HPA axis functioning of the progeny in later life [90, 91]. Generally, such effects include increased baseline and stress-induced glucocorticoid levels, and attenuated HPA axis negative feedback. Many species also exhibit a stress hyporesponsiveness period during the neonatal period that may function to protect certain glucocorticoid-sensitive tissues during a critical period during their development, and during which only severe stressors elicit an HPA response [92].

Altogether, the HPA axis interacts with reproduction and life history traits to maximize reproductive success. In particular, the suppression of the stress response and/or some of its effects at key points during reproduction avoids the deleterious effects of glucocorticoids on reproductive physiology and behaviour, and the baseline secretion of this hormone or its bioavailability (by decreases in CBG expression) may be enhanced at other times to meet the elevated metabolic demands of reproduction.

1.4.2. HPA Axis and Wildlife Metabolism

Seasonal fasting is a challenge faced by many species with temporally limited foraging opportunities, including during breeding and molting in king penguins (*Aptenodytes patagonicus*) [93, 94] and migration in some sea bird species [95]. The HPA axis plays a key role in modulating the supply and type of energy substrates utilized during extended fasting [93, 96]. At the initiation of fasting (phase I fasting), the primary metabolic fuel is stored glucose (e.g. glycogen in muscle and liver). These stores are likely to be depleted rapidly, upon which animals switch to phase II fasting. This phase is characterized by the utilization of lipids from adipose stores as the major metabolic fuel, and also by the downregulation of peripheral proteolysis (i.e. protein sparing). The characteristic decrease in glucocorticoid levels during

phase II fasting likely plays a major role in this protein sparing. This is because the transition to phase III fasting, which is marked by the utilization of amino acids from protein stores to meet energy demands, is associated with significant increases in the circulating levels of this steroid hormone. These findings are supported by observations in other animals of the association between the state of endogenous adipose stores (e.g. body condition) and glucocorticoid levels. Animals in good condition generally exhibit low baseline and normal stress-induced increases in glucocorticoid levels, while those in poor condition exhibit elevated baseline and stress-induced levels of this hormone (e.g. in mammals: [87, 96, 97, 98, 99]) and birds: [73, 100, 101]). Thus, glucocorticoids may play a major role in the selection of metabolic fuels during fasting to maintain fitness by sparing protein stores until all other sources of metabolic fuels are exhausted. Little is currently known regarding the role of CBG in modulating glucocorticoid bioavailability during prolonged fasting in wildlife.

HPA axis function may also be modulated by the quality of nutrition, as measured by the macronutrient profile of an animal's diet, independent of energy intake. Dietary protein deficiency in the face of adequate energy intake is associated with elevated HPA axis activity in mammals (e.g. [102, 103, 104, 105]) and birds (e.g. [106, 107]). Glucocorticoids mediate the mobilization of amino acids from protein stores (e.g. skeletal muscle) to fulfill obligate requirements elsewhere in the body. Elevated HPA axis activity in response to low fat diets is observed in species with limited lipid reserves, including in black- [108] and red-legged kittiwake (*Rissa brevirostris*) chicks [109]. In the case of the kittiwake chicks, elevated glucocorticoid levels are associated with aggressive behavior, including increased begging behaviour towards parents and aggression towards littermates [108]. In contrast to the response of kittiwake chicks to food shortages, in bird species where the parents either cannot or are not willing to respond to chick begging for food, chicks respond by suppressing baseline and stress-induced glucocorticoid levels to avoid the detrimental catabolic effects of elevated levels of this hormone [110]. Thus, while energy balance and nutrition are key modulators of the HPA axis, the demands of some life history stages and environmental conditions may result in the suppression of the typical glucocorticoid response to achieve situational goals.

The HPA axis also exhibits altered activity during metabolic depression. There exists a range of the extent and duration of this depression, from the mild diurnal depression in daily torpor in

many animals to the months-long frozen hibernation state in the wood frog (*Rana sylvatica*) [111]. In daily torpor, body temperature is reduced proportionally to ambient temperature to reduce energy expenditure during periods of low activity and possibly low predation risk [112]. Hibernation, in contrast, is a specialized fasting state that is associated with wide spread physiological and cellular changes leading to a profoundly depressed metabolic state to decrease energetic demands during periods of extreme environmental conditions or prolonged food shortages [111, 113, 114]. In the Rufous hummingbird (*Selasphorus rufus*), which undergoes daily torpor, glucocorticoid concentrations are associated with food consumption, and the experimental dilution of nectar resulted in increased torpor length and elevated glucocorticoid levels [115]. However, the relationship between glucocorticoids and daily torpor in birds is not completely understood. Many small hibernators do not hibernate continuously, but periodically and briefly (≤ 24 h) return to a euthermic state between bouts of deep metabolic depression [114]. The adrenal glands atrophy and glucocorticoid secretion is suppressed during episodes of hibernation in red-cheeked ground squirrels (*Spermophilus erythrogenys*), but bursts of adrenal activity are associated with the periodic arousals that may play a role in stimulating gluconeogenesis to replenish carbohydrate stores that are depleted during hibernation [116], and in maintaining immune competence [117]. In other animals that do not show periodic arousal, including the little brown bat (*Myotis lucifugus*) [118] and American black bear [119, 120], hibernation is associated with elevated glucocorticoid levels and this is thought to mediate the mobilization of adipose stores [119, 120].

Prior to hibernation, many animals enter a hyperphagic state to accumulate adipose stores upon which to survive the hibernation period [24]. Elevated glucocorticoid levels play a role in mediating hyperphagic behaviour and increased adiposity (in conjunction with the action of elevated insulin levels [68]) in several species, including the little brown bat [121], yellow-bellied marmot (*Marmota flaviventris*) [122], and European (*Spermophilus citellus*) [123] and golden-mantled ground squirrels (*Callospermophilus lateralis*) [124]. Hibernators that do not survive on their endogenous stores, but instead rely on cached food, do not exhibit pre-hibernation elevations in glucocorticoid levels [82, 125]. Thus, seasonal changes in glucocorticoid secretion appear to play a vital role in the preparation for and maintenance of winter hibernation and torpor, but the mechanisms involved are not well understood.

1.5. The Annual Life Cycle of Bears

Despite the diversity in environments that the American black bear (*U. americanus*), grizzly bear, and polar bear inhabit, there are remarkable similarities in the annual life cycle of these species. Mating occurs in the spring for all three species; polar bears mate during March to May, followed by grizzlies from April to July and black bears from May to August. However, conspecific interactions and mating may occur outside of these seasons for grizzly bears [126, 127, 128]. There also exists a period of embryonic diapause for these species, in which the implantation of fertilized blastocyst(s) is delayed for six to eight months [129]. It is thought that this delayed implantation allows the pregnant female some flexibility in choosing whether to commit resources towards the development of the fetus depending on her foraging success and state of accumulated energy stores [130]. This is important because much of the gestation period, parturition, and the first few months of lactation occur during denning, a period of fasting [131, 132, 133, 134]. For black and grizzly bears, females fast for the entirety of the hibernating season, which persists from mid/late autumn to early spring [135]. Additionally, female polar bears may fast for up to 8 months in the southern reaches of the range for this species because the sea ice breaks up a few months prior to den entry [136]. Consequently, seasonality and nutritional status play important roles in the reproductive success and population dynamics of these species. Females must accumulate enough energy stores to survive the denning season, especially when the interval between reproductive cycles may be quite long in unfavourable environments, and may contribute to poor population performance. For grizzly bears in some populations in Alberta, Canada, this interval may be 4 – 4.5 years long [5, 8], while this interval may be as low as 2.4 years in other populations of grizzlies [4, 129] and 2 years for black bears [129].

Another remarkable commonality between these bear species is their metabolic responses to predictable, seasonal food deprivation. Black and grizzly bears enter dens in the winter and assume a hypometabolic, hibernating state [137]. Similarly, fasting polar bears during the open-water season also exhibit a hypometabolic state but, unlike the terrestrial species, may remain active outside of dens ("walking hibernation" [138]) except for pregnant females [136,

137]. Bear hibernation is best studied in the American black bear, and is characterized by a profound metabolic depression to approximately 25% of the basal active rate, yet core body temperatures are only mildly depressed [139]. In contrast, small hibernators exhibit cooling to a few degrees above ambient temperatures [113], but despite these differences in body temperature, the mass-adjusted metabolic rates of black bears and small hibernators are comparable [139]. Hibernating bears may lose up to 40% of their initial body mass, with higher losses observed in reproductive females [140, 141, 142, 143, 144]. Bears rely almost exclusively on fat metabolism to supply energy to survive through the denning season, and during this period exhibit elevated levels of blood serum lipids [137] and upregulation of genes related to lipid catabolism and carbohydrate synthesis [145, 146]. Accordingly, pre-hibernation hyperphagia is observed in ursids, wherein large quantities of body fat are accumulated over a span of 2 – 4 months and subsequently utilized during hibernation [4, 138]. Furthermore, fat may continue to be lost after arousal from hibernation during the spring hypophagic period in some reproductive classes, including adult males (i.e. males competing for mating opportunities) and lactating females [147]. The forage and prey availability during this time may be inadequate to support routine metabolic rates, and may be fueled in part by endogenous stores.

Unlike other hibernators that undergo periodic arousal during the winter season, including hibernating sciurids [113, 148, 149], bears stay in a hypometabolic state for the entire duration [137]. They neither eat nor drink and seldom excrete nitrogenous wastes during the denning season [137, 150]. It is thought that while amino acids are catabolized during hibernation, nitrogen is not excreted but recycled in the gut by urease-expressing bacteria [151, 152, 153]. The lean body mass of hibernating bears, including skeletal muscle and bone are largely spared and do not exhibit atrophy due to prolonged unloading and disuse during the denning season [154, 155, 156, 157, 158, 159, 160, 161]. In contrast, the atrophy response of lean tissues to prolonged disuse in most other animals result in osteoporosis and muscle wasting [113, 162]. Recent studies suggest that the up and downregulation of genes encoding proteins involved in the anabolic and catabolic processes, respectively, in liver, heart, skeletal muscle and bone may be contributing to the sparing of lean body mass during hibernation in bears [145, 146, 163]. However, lean tissue sparing is not absolute but dependent on the adiposity of the animal, with leaner animals catabolizing proportionally more lean tissues to fuel metabolism during denning

[164, 165, 166]. Thus, hibernating bears exhibit remarkably efficient metabolism that largely preserves the functional bone and skeletal muscle of the animal and relies on fat to supply energy for metabolism.

Little is currently known about cortisol dynamics and how it may be modulated with respect to the annual life cycle of bears. Total cortisol levels during hibernation are generally elevated relative to the summer season nadir in black bears [119, 120, 154, 167]. Conceptually, elevated glucocorticoid levels may be contributing to lean body tissue catabolism during denning, so some yet to be characterized aspects of glucocorticoid dynamics, including changes in CBG concentration, may be modulating cortisol bioavailability during this period to prevent these potentially detrimental effects on these tissues. Furthermore, the role of glucocorticoids in fat deposition during the hyperphagic period in bears is also unclear. Additionally, since there are broad changes in gene expression profiles in hibernating black bears [145, 146, 163], there may also be corresponding changes in the proteins that are secreted into blood serum, but this has not been characterized in black bears.

In summary, the function of the HPA axis does not remain constant over the life history stages of an animal, but is modulated in the face of changing seasonal and environmental factors, including nutrient availability. These facts constitute a challenge for the measurement of glucocorticoid concentrations as a tool to assess and monitor the health and stress status of free-ranging animals given that the baseline and stress-induced levels of this steroid change seasonally. Thus, the factors underlying these seasonal changes and its implications on animal physiology must be characterized in order to assess the feasibility of using HPA axis activation as a tool for wildlife management.

1.6. Serum Markers of Stress

The sampling of blood is commonly employed to assess the health and stress status of animals. Blood sampling is minimally invasive compared to taking tissue biopsies, and the protein and

metabolite components of blood are often reflective of the health status of the animal (e.g. [168, 169]). Many pathological states may result in altered protein or metabolite patterns in blood or may result in the presence of molecules that normally are not present in this medium. Moreover, given that glucocorticoids play an important role in mediating the adaptation to stress and the maintenance of health in free-ranging animals, the measurement of serum levels of this steroid may be used by wildlife managers to aid in the monitoring and conservation of threatened species.

However, there are a number of challenges involved in the collection of blood from wild animals, including bears [24]. The response to acute stressors, including capture and handling, results in the elevation of blood glucocorticoid levels above baseline levels rapidly in many species [170], necessitating the collection of blood within a relatively short time frame after capture (<10 min). While possible for small animals, this is not a feasible strategy for large, potentially dangerous animals such as bears, which require immobilization prior to sampling and handling. Moreover, immobilization by anesthesia may itself induce a stress response and lead to elevated glucocorticoid levels independently of capture stress [171]. The use of physical traps may also preclude rapid sampling if these are placed in remote areas or are not constantly monitored, and different trapping methods may induce different stress profiles [172]. In free-ranging grizzly bears, the use of leg-hold snares have been associated with muscle injury, dehydration, and elevated glucocorticoid levels relative to other capture methods [172]. Helicopter darting is an alternative capture method in which anesthetic is remotely injected via darting from a helicopter, and is considered less stressful in terms of the glucocorticoid response and its effects on the white blood cell profile [172]. However, this capture method results in the animal attempting to flee from the helicopter prior to the induction of the effects of the anesthetic, leading to a stress response [172]. Thus, with currently employed capture and sampling methods, blood sampling in bears will inevitably result in the induction of a stress response and elevated glucocorticoid levels. The magnitude of this glucocorticoid response may provide information regarding the stress and health status of free-ranging animals, but these analyses are complicated by the lack of a true control group to which to compare and which can be used as a benchmark.

Some techniques have been employed to circumvent these issues with capture stress and/or to test the functioning of the HPA axis [24, 28]. Terminal sampling is a technique where the sampled animal is shot and sampled before it can mount a stress response (e.g. [76]), but this is unsuitable for a threatened species such as bears. A challenge protocol involves taking a baseline blood sample prior to subjecting the animal to a standardized stressor (e.g. placing in a holding trap [82] or the administration of anesthetic [173]), followed by serial sampling. This technique provides information on the changes in glucocorticoid levels over a period of time, which may reveal abnormalities in HPA axis function. In bears, this may be complicated by the variable timing of the initiation of a capture, administration of anesthetic, and sampling.

The dexamethasone/ACTH challenge test is another technique that is employed in the field to test HPA axis function [28]. After capture and anesthetization, dexamethasone, an artificial glucocorticoid analog, is administered. This drug acts on the GR receptors in the hypothalamus and pituitary to powerfully suppress the release of ACTH and, subsequently, glucocorticoid secretion from the adrenals. A blood sample taken after dexamethasone administration may reveal the extent of this induced negative feedback and the possible effects of chronic stress if this negative feedback is attenuated compared to healthy animals. A standardized dose of ACTH can then be administered to determine the ability of the adrenal glands to respond to this peptide hormone, and the presence of an abnormal response may indicate, for example, alterations due to chronic stress.

There are concerns regarding invasive techniques utilized for monitoring wildlife programs, including the long-term health effects of capture and sampling [174]. A variety of non-invasive, remote methods have been proposed, in development, or are in use to assess the stress status of wild animals, including the measurement of glucocorticoids and/or its metabolites in feces, urine, and hair (e.g. [175, 176, 177]). The measurement of glucocorticoids in these samples may provide an integrated measure of the secretion of this hormone over a period of time, from hours to days for urine and feces (e.g. [178, 179]) to several weeks to months for hair [180], rather than a point measurement as with blood glucocorticoids, and collection can be completely non-invasive. There are a number of technical and analytical challenges to overcome in using these methods, including the presence of multiple glucocorticoid metabolites that exist in proportions that may differ between sexes or may be immunologically

similar to metabolites of other steroid hormones [181]. Fecal glucocorticoids may also be metabolized by gut bacteria, or otherwise degraded or concentrated by exposure to the elements [178]. However, the ability to non-invasively examine the stress status of an animal is an attractive proposition, and the measurement of glucocorticoids and metabolites in these media may complement blood serum assessment leading to a greater understanding and better monitoring of the health of free-ranging species. While the measurement of glucocorticoids in blood serum is a well-established technique, the role of serum proteins as markers of stress and/or health status in wildlife has not received much attention. The characterization of serum proteome in wildlife may provide the basis for discovery of novel protein markers that may predict chronic stress and/or altered health status.

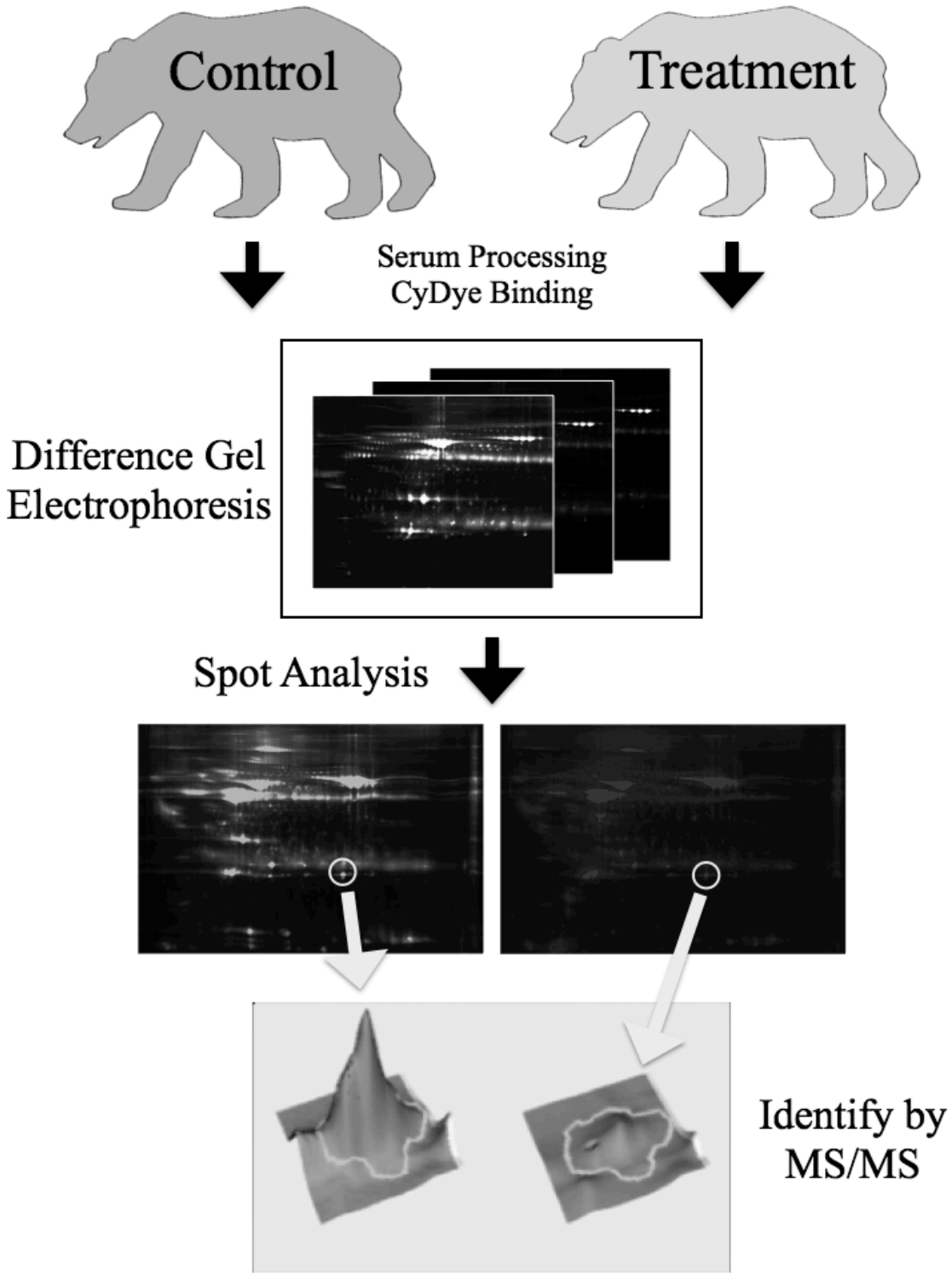
1.7. The Utility of Proteomics in Wildlife Biology

Proteomics is the quantitative study of the proteins expressed by a genome (i.e. the proteome) in a particular cell or tissue at a particular time [182]. The proteome of each tissue in an organism is likely to be unique because the complex pattern and timing of gene expression, protein translation, post-translational protein modification, and metabolism in that tissue gives rise to its form and function. Moreover, each proteome is complex because each step of the production of a protein from the genome introduces variation (e.g. alternative splicing of mRNA, variable post-translational modifications), resulting in isoforms of a protein that may appear biochemically different but share a common function, or vice versa. Despite these complexities, the study and comparison of the proteome of a tissue under different states (e.g. comparing an experimentally treated or diseased tissue to a control) may give insights into the proteins and the related metabolic pathways that are affected. Thus, proteomics is a powerful tool for research that can elucidate widespread changes that may occur in a tissue under different conditions and treatments, which may form the bases for hypotheses that are tested by subsequent experiments and for discovery of novel proteins.

Presently, the most commonly used methods to analyze proteomes utilize some form of biochemical protein separation followed by mass spectrometry-based identification of the protein. Two dimensional gel electrophoresis (2DE) is one commonly employed technique that separates proteins based on their isoelectric point (pI) in the first dimension using isoelectric focusing (IEF), and by molecular mass in the second dimension using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [183, 184]. The pattern of protein spots is visualized by staining after separation or by tagging the proteins with a dye prior to separation [185]. The latter method allows for the separation of multiple samples that are tagged with different dyes on the same gel, which forms the basis for difference gel electrophoresis (DIGE) [186]. Samples are labeled at the ε-amino group of lysine residues with different cyanine dyes (Cy Dyes) that fluoresce at different wavelengths. Moreover, an internal standard sample that is composed of a pool of an aliquot of each sample can also be labeled with a unique Cy Dye and separated on each gel to facilitate the matching of spot patterns between gels. The samples and standards are loaded onto and separated on the same gel. The protein spot patterns on the gel are then visualized at the fluorescence wavelength of each dye, and the intensity of a spot is proportional to the protein in that spot. This DIGE method streamlines the workflow by reducing the number of gels that are run and the amount of post-processing to match protein spots across gels. The internal standard also facilitates spot matching between gels because its composition and pattern of protein should be identical, notwithstanding the differences introduced by variation in the gel. 2DE and DIGE allows the analysis of all proteins within a range of pI and molecular mass, but generally, only a proportion of proteins exhibit significantly changed expression by an experimental treatment. The goal of the proteomic analysis is to analyze the 2D pattern of proteins for the significant changes due to experimental treatment, to identify these differentially expressed proteins, and to categorize these identified proteins into functional pathways. An overview of the 2DE workflow is shown in **Figure 1.2**.

Proteins are commonly identified by mass spectrometry. Briefly, the protein of interest is excised from the gel, and is fragmented into peptides by trypsin digestion. This digestion

Figure 1.2: A Schematic Overview of the Two-dimensional Gel Electrophoresis (2DE) Proteomics Workflow.
Serum samples are processed and proteins labeled with Cy Dyes. A set of differentially labeled control, treatment, and pooled internal standard samples are separated in a single run by isoelectric focusing in the first dimension and sodium dodecyl sulfate polyacrylamide gel electrophoresis in the second dimension. The gels are scanned at the respective absorption/emission wavelength of each Cy Dye, and the resulting images are analyzed in DeCyder. During spot analysis, the internal standard facilitates the matching of spots between gels because gel-to-gel variability may lead to protein spots migrating at different rates. Additionally, the internal standard provides a means of normalizing spot volumes between gels. The spot volumes of each matching spot are compared between control and treatment samples, and protein spots that are differentially expressed are subsequently identified by tandem mass spectrometry (MS/MS).


Control
Treatment
Serum Processing
CyDye Binding
Difference Gel
Electrophoresis
Spot Analysis
Identify by
MS/MS

results in the specific hydrolysis of a polypeptide chain at the carboxyl side of lysine or arginine, except where a proline residue is attached to this cleavage site. After the digest is cleaned and suspended in an appropriate solution, the mass spectra of the peptides are analyzed. The mass spectra of a digest (i.e. the peptide mass fingerprint) are sometimes sufficient to identify its parent protein. Otherwise, tandem mass spectrometry is employed, where each peptide in the digest is subjected to fragmentation within the mass spectrometer, and the mass spectra of the collision fragments is analyzed to determine its amino acid sequence or matched against a database of possible fragmentation spectra to deduce the identity of the parent protein.

This proteomic workflow has been successfully employed to characterize changes in the proteomes of tissues under experimental treatments in model animals, including biomarker discovery for detecting cancer (e.g. [187, 188, 189]), chronic alcoholism [190], schizophrenia [191], and neurological diseases [192]. However, the application of these techniques in non-model organisms is more complicated because of the paucity of gene and protein sequence information, limiting the successful identification of proteins [193]. However, modern mass spectra database search software may still be capable of assigning significant matches based on conserved sequences, and provide reasonable identities for unknown proteins. Proteomics analyses have been used to characterize the changes in some tissues associated with hibernation in non-model animals, including the liver [194, 195, 196, 197], muscle [198], and kidney [199] proteomes of sciurids, and the skeletal muscle proteome of greater tube-nosed bats (*Murina leucogaster*) [200]. These studies found widespread remodeling of tissue proteomes in the hibernating state that are consistent with previous studies on the gene expression, protein, and metabolic changes in these animals, including the switch to lipid-fueled and protein-sparing metabolism. The recent work of Fedorov et al [145, 146, 163] characterized the transcriptomic profile in liver, skeletal and cardiac muscle, and bone of hibernating bears. These studies found that, in general, genes involved in lipid catabolism and protein biosynthesis were upregulated, while genes related to most catabolic pathways were downregulated in hibernating relative to active black bears. However, there is a lack of information on the changes in serum protein expression during hibernation relative to the active state in bears. Some studies have profiled such changes in a limited number of proteins, including some peptide hormones and proteins in bear serum [167, 201, 202]. The

characterization of the changes in the entire serum proteome associated with hibernation may elucidate some of the mechanisms that promote and maintain this depressed metabolic state, which may have important implications not only for the monitoring of health and stress in bears but also for human health and medicine.

1.8. Thesis Objectives

CBG plays an important role in the dynamics of glucocorticoid action by regulating the bioavailability of this steroid hormone to tissues. The quantification of this protein and the subsequent estimation of free, bioavailable cortisol concentrations may provide information regarding the health and stress status of free-ranging animals. The primary objectives of this thesis were to characterize the dynamics of glucocorticoids, including total and free cortisol and CBG concentrations, in the serum of three ursid species, the grizzly (*U. arctos*), polar (*U. maritimus*), and American black bear (*U. americanus*). The black bears used in these studies were captive animals, while samples from the other two species were taken from free-ranging animals. Furthermore, the changes in the serum proteome due to hibernation of captive American black bears were assessed to elucidate the potential mechanisms behind the unique metabolic aspects of ursid hibernation and their contribution to health during periods of fasting and hypometabolism. This will also allow for the discovery of novel proteins as markers of health status in bears. Specific objectives included:

1. Characterize the binding properties of cortisol to CBG, and the development of methods to estimate free cortisol in ursids (Chapter 2);

2. The cloning and sequencing of grizzly bear CBG, and the development of a homologous CBG ELISA for grizzly bears (Chapter 3);

3. Characterize the effect of life-history traits and season on cortisol dynamics in free-ranging grizzly and polar bears (Chapter 4);

4. Determine the effect of hibernation on cortisol dynamics in captive American black bears (Chapter 5);

5. Characterize the serum proteome changes that are associated with hibernation in captive black bears (Chapter 6).

CHAPTER 2:
CHARACTERIZATION OF CORTISOL DYNAMICS IN URSID SERUM

2.1. Introduction

Corticosteroid-binding globulin (CBG) is a ~50 kDa serum glycoprotein in the serpin proteinase inhibitor superfamily [1] that binds glucocorticoids (e.g. cortisol) and progesterone with high affinity in circulation [2]. CBG plays a major role in the dynamics of glucocorticoid action because the CBG-bound fraction of this hormone cannot cross plasma membranes to activate the intracellular glucocorticoid (GR) and mineralocorticoid receptors (MR) that mediate the cellular effects of this steroid hormone [3]. Thus, the free, unbound fraction of serum glucocorticoids is the biologically relevant fraction of this steroid hormone. The measurement of serum free glucocorticoid levels may allow for a better understanding of the link between glucocorticoid effects on the health status of animals [4].

Free cortisol concentrations can be estimated by the free hormone equation of Barsano and Baumann [5] if CBG and total cortisol concentrations and the affinity of CBG for cortisol are known. This affinity is measured as the dissociation binding constant (K_d), which is usually translated as the concentration of cortisol at which half of the CBG binding sites in a sample are occupied. Alternatively, the equilibrium association constant (K_a, the reciprocal of K_d) is also commonly reported. The lower the K_d or higher the K_a, the higher the affinity of cortisol for CBG. There is a wide range of reported K_d values for animals [4], and the affinity of cortisol for CBG may be dependent on the amino acid sequence and post-translational modifications of CBG.

While total cortisol concentrations have been measured in black [6, 7, 8, 9], grizzly [10], and polar bears [11, 12], CBG and serum free cortisol levels have not. There is a need to assess and monitor the stress status of free-ranging animals to gain insights into the effects of rapid

ecological changes on the health of free-ranging animals. For example, grizzly bear populations in Alberta, Canada, are threatened by habitat fragmentation and increased human access of habitats, leading to increased mortality risk [13]. In combination with the low reproductive potential and population size of this species in this province [14, 15], these ecosystem changes may lead to population declines. Similarly, climatic warming is leading to decreases in the extent and duration of seasonal ice cover in the Hudson Bay region of Canada [16]. Polar bears are dependent on sea ice to hunt their primary prey, and declining ice conditions are linked to negative impacts on the body condition of animals in the southern portions of their range [17, 18]. A reduction in body condition index has been hypothesized to decrease reproductive performance, leading to population declines in this species [17, 18].

Environmental stressors may lead to sustained elevation in serum glucocorticoid levels and chronic effects on the health of these bears. For instance, chronically elevated free cortisol concentrations, due in part to elevated total cortisol and/or decreased CBG levels, may have detrimental impacts on growth, immunity, and reproduction [19, 20, 21]. Consequently, chronic stress may lead to negative impacts on population health and persistence, including increased mortality and decreased reproductive performance [22]. The development of tools for health and stress status monitoring, including the measurement of CBG and free cortisol levels, may provide wildlife managers with information to assess and predict population health [23]. For instance, elevations in glucocorticoid levels due to environmental stress may be linked to increased mortality or decreased reproductive success in animals [19, 24]. However, little is known regarding stress and cortisol dynamics in bears. Thus, the primary objective of this chapter was to characterize the binding affinity of CBG to cortisol by equilibrium saturation binding experiments, and CBG levels in black, grizzly, and polar bears. Using these data, free cortisol levels were estimated and compared to evaluate any potential differences in cortisol dynamics between these species.

2.2. Methods and Materials

2.2.1. Animals

2.2.1.1. Grizzly Bear Serum

Sera were collected from 28 female grizzly bears for the Foothills Research Institute Grizzly Bear Project (research goals are summarized by [25]) within a 150,000-km^2 area of western Alberta, Canada (49°00' – 55°50'N, 113°50' – 120°00'W). These animals were captured using Aldrich leg-hold snares (Aldrich Snare Co., Clallam Bay, Washington) and anesthetized by remote drug delivery using a combination of xylazine and zolazepam–tiletamine (XZT) administered intramuscularly as xylazine (Cervizine 300; Wildlife Pharmaceuticals, Inc., Fort Collins, Colorado) at 2 mg/kg and Telazol (Fort Dodge Laboratories, Inc., Fort Dodge, Iowa) at 3 mg/kg estimated body weight prior to handling [26]. Blood was collected by venipuncture from the jugular vein into sterile tubes, and samples were centrifuged within 8 h of collection to extract serum. Extracted serum samples were stored frozen at –20°C until analysis. At the conclusion of handling, atipamezole (Antisedan; Novartis Animal Health Canada Inc., Mississauga, Ontario, Canada) was administered at 0.15 – 0.20 mg/kg, half-volume intramuscularly and half-volume intravenously, to reverse the effects of xylazine. The capture and sampling protocol was reviewed and approved by the University of Saskatchewan's Committee on Animal Care and Supply, and was in accordance with guidelines provided by the American Society of Mammalogists' Animal Care and Use Committee [27] and the Canadian Council on Animal Care [28].

2.2.1.2. Polar Bear Serum

Sera were collected from 39 female polar bears that were captured and sampled in conjunction with a long-term research project in the Canadian Arctic conducted by the Ontario Ministry of Natural Resources [29]. These samples were taken from animals spotted by a helicopter and immobilized by remote injection with projectile syringes using a combination of xylazine and zolazepam-tiletamine (XZT) administered intramuscularly as xylazine (Cervizine 300H, Wildlife Pharmaceuticals, Inc., Fort Collins, Colorado, USA) at 2 mg/kg and Telazol (Fort

Dodge Laboratories, Inc., Fort Dodge, Iowa) at 3 mg/kg estimated body weight. Blood was collected by venipuncture from the jugular vein into sterile tubes, and the sample was centrifuged within 8 h of collection to extract serum. Sera were frozen within 8 h of collection and maintained frozen (–20°C) in long-term storage at the University of Saskatchewan. The capture and sampling protocol was reviewed and approved by the University of Saskatchewan's Committee on Animal Care and Supply, and was in accordance with guidelines provided by the American Society of Mammalogists' Animal Care and Use Committee [27] and the Canadian Council on Animal Care [28].

2.2.1.3. Black Bear Serum

Sera were collected from six captive female black bears held at the Virginia Polytechnic Institute Center for Bear Research. Animals were anesthetized with a 2:1 mixture of ketamine (100 mg/mL):xylazine (100 mg/mL) at a dosage of 1 cc of the mixture per 45.5 kg of body mass. Blood samples were drawn from the femoral vein while the animal was anesthetized, and the samples were transported to the laboratory in an ice-packed cooler. Immediately on return to the laboratory, the blood was spun to isolate the serum and was frozen at –20°C. The Virginia Polytechnic Institute and State University Animal Care Committee approved all bear handling protocols (#98-069-F&WS).

2.2.2. Cortisol Saturation Binding Assay

The binding characteristics of CBG for cortisol in black, grizzly, and polar bear sera and fish plasma were determined by saturation binding experiments as described previously [30]. Sera for bears were pooled from 4 females of each respective species into one sample for each species. Plasma from rainbow trout (*Oncorhynchus mykiss*) was used as a negative control because teleosts lack CBG [31]. Serum was stripped of steroids by incubation with washed activated charcoal (Sigma) for 4 h at 37°C. 100 µL of stripped serum was diluted 1:100 in phosgel (100 mM phosphate, 0.1% gelatin, pH 7.4), and incubated with 1.56 – 100 nM of serially diluted 1,2,6,7-^{3}H(N) hydrocortisone (^{3}H-F, Perkin-Elmer, Waltham, Massachusetts, USA) for 4 h at 37°C in glass tubes in duplicate. A parallel set of tubes with excess unlabeled

cortisol (2000 nM) along with the ^{3}H-F and diluted bear serum was incubated to determine non-specific binding (NSB). Additionally, another set of tubes with no added serum was incubated to determine total counts (TC). After the incubation, all tubes were cooled in an ethanol-ice-water bath, and unbound ^{3}H-F was separated by the addition of 700 µL of dextran T-70 (Sigma)-coated activated charcoal in phosgel. Tubes were incubated for 20 min, centrifuged at 2000 x G for 12 min at 4°C. 350 µL of the supernatant was transferred and mixed with 2 mL scintillation fluid. Samples were counted in a Beckmann LS-9600 liquid scintillation counter. Specific binding (SB) was calculated as the difference between the TB and NSB counts. K_d and B_{max} were first estimated by Scatchard analysis [32] and Rosenthal correction [33]. These estimates were then refined by non-linear least squares (NLS) regression analysis in R 2.14.0 [34], and was fit to the equation:

$$B_s = \frac{B_{max} * L}{K_d + L}$$

Where B_s is specific binding (cpm), B_{max} is the maximum binding capacity of cortisol to serum (cpm), and L is the free ^{3}H-F (nM). The K_d and B_{max} from the Scatchard analyses were used as seed values for NLS regression. B_{max} values were converted to nmol cortisol bound/L serum using the standard curves derived from the total counts (TC; linear regression of cpm versus ^{3}H-F).

Hill plots were also constructed [35] to explore possible interactions between cortisol binding sites. The proportion of binding sites occupied (θ) was calculated from the estimated K_d and L:

$$\theta = \frac{L}{K_d + L}$$

$\log(\theta / (1 - \theta))$ was plotted against $\log(L)$, and the line of best fit through the points was calculated by linear least squares regression analysis.

2.2.3. Maximum Corticosteroid Binding Capacity Assay

The maximum corticosteroid binding capacity (MCBC) assay [36] was used to estimate the CBG binding capacity in six black, 28 grizzly, and 36 polar bear serum samples. The estimation of CBG levels by the MCBC assay is equivalent to such estimations by B_{max} in the saturation binding assays, but the former is a point assay that requires fewer materials. For the determination of total binding (TB), 10 µL of 1:100 diluted serum in phosgel was incubated with 0.105 ng of ^{3}H-F and 10 ng of unlabeled cortisol in 500 µL of phosgel for 4 h at 37°C in duplicate glass tubes. A parallel set of duplicate tubes with excess unlabeled cortisol (2000 nM) along with the same mass of ^{3}H-F and volume of diluted serum as the TB tubes was incubated to determine non-specific binding (NSB). After the incubation, all tubes were cooled in an ethanol-ice-water bath, and unbound ^{3}H-F was separated by the addition of 200 µL of dextran T-70-coated activated charcoal in phosgel. Tubes were incubated for 12 min and then centrifuged at 2000 x G for 12 min at 4°C. 500 µL of the supernatant was transferred and mixed with 2 mL scintillation fluid. Samples were counted in a Beckmann LS-9600 liquid scintillation counter. Specific binding (SB) was calculated as the difference between the TB and NSB counts, and the MCBC was calculated as the proportion of SB / TC multiplied by the amount of cortisol present (the sum of endogenous and added cortisol) per unit volume serum.

2.2.4. Serum Total Cortisol Assay

Serum total cortisol was measured by radioimmunoassay (RIA) using a commercial ^{125}I kit (MP Biomedicals, Orangeburg, NY) as described by Hamilton [37]. Grizzly and polar bear serum cortisol levels used in this study were previously reported by Hamilton [37].

2.2.5. Free Cortisol Calculations

Free cortisol concentrations were calculated by the equation of Barsano and Baumann [5]:

$$fCORT = \frac{-\left(CBG - tCORT + \frac{1}{K_a}\right) \pm \sqrt{\left(CBG - tCORT + \frac{1}{K_a}\right)^2 - 4\left(\frac{-tCORT}{K_a}\right)}}{2}$$

Where *fCORT* is free cortisol concentration (mol/L), CBG is CBG concentration (mol/L), tCORT is total cortisol concentration (mol/L), and $K_a = 1/K_d$ = equilibrium association constant (L/mol).

2.2.6. Statistics

For the visualization of saturation binding assay data, curves of best fit were fit to total binding (TB), non-specific binding (NSB), and specific binding (SB) data using four parameter logistic nonlinear regression models:

$$\text{Binding} = \frac{\text{A} - \text{D}}{1 + \left(\frac{L}{C}\right)^B} + D$$

Where Binding is TB, NSB, or SB; L is the concentration of free added ^{3}H-F; A is the minimum asymptote; B is the slope factor; C is the inflection point; and D is the maximum asymptote. The values for Binding and L were taken from the saturation binding assays, while A, B, C, and D were estimated by regression analysis.

Significant differences ($p < 0.05$) in MCBC, and total and free cortisol between the three bear species were determined by 1-way ANOVA, and data were log-transformed as necessary to meet the parametric assumption of normality. *Post hoc* significance testing was performed with Tukey's Honest Significant Differences test.

2.3. Results

2.3.1. Saturation Binding Experiments

Saturation binding assays were conducted on pooled grizzly, polar, and black bear sera to determine the K_d and B_{max} for ursid CBG. Plots of total counts of beta decays versus the concentration of added ^{3}H-F revealed a linear relationship between these variables (**Figure 2.1**). Specific binding was calculated as the difference between total binding and non-specific binding of 1:50 diluted serum with 0 – 100 nM ^{3}H-F, and free ^{3}H-F (L) was calculated as the product of ^{3}H-F concentration and the proportion of total binding to total counts (**Figure 2.2**). Specific binding was higher than non-specific binding in all the bear sera, but not rainbow trout plasma. Scatchard plots (specific binding divided by L versus specific binding) were constructed for each experiment (**Figure 2.3**), and K_d (negative inverse of the absolute value of the slope of the linear regression line) and B_{max} (x-intercept of the regression line) were estimated. These K_d and B_{max} values were used as starting estimates for the subsequent non-linear regression analyses of specific binding versus L to determine refined K_d and B_{max} (**Table 2.1**). The K_d and B_{max} for fish plasma could not be estimated because there was no detectable specific binding.

Hill plots were constructed from the saturation binding data. The fraction of binding sites occupied (θ) was calculated from L and the NLS-estimated K_d for each experiment and the linear least squares regression line of $\log(\theta / (1 - \theta))$ versus $\log(L)$ was calculated. The slopes of the regression lines were 1 for all experiments (an example is shown in **Figure 2.4**). The Scatchard plots were also linear by inspection, which suggests a single binding site for cortisol in bear serum.

2.3.2. Maximum Corticosteroid Binding Capacity and Free Cortisol Estimates

Maximum corticosteroid binding capacity (MCBC) was determined in an expanded sample set of black, grizzly, and polar bear sera (**Figure 2.5A**). The mean MCBC in polar bears was significantly elevated relative to black and grizzly bears ($F_{2,67} = 9.45$, $p < 0.001$). Serum total cortisol concentrations were quantified in each of the serum samples from black, grizzly, and polar bears (**Figure 2.5B**), and were elevated in grizzly and polar bears relative to black bears ($F_{2,67} = 18.0$, $p < 0.001$). To estimate free cortisol calculations in the samples used for the MCBC assay, the equation of Barsano and Baumann [5] was employed, using MCBC as the CBG concentration. Free cortisol concentrations (**Figure 2.5C**) were elevated in grizzly and polar bears relative to black bears ($F_{2,67} = 6.47$, $p < 0.01$).

2.4. Discussion

The primary aim of this study was to characterize the binding affinity (K_d) of cortisol to CBG in black, grizzly, and polar bear serum with saturation binding experiments, and to estimate CBG concentrations by the maximum corticosteroid binding capacity (MCBC) assay. The derived equilibrium dissociation constants (K_d) were used in conjunction with total cortisol and MCBC measurements to estimate free cortisol concentrations. Altogether, there were differences in MCBC and free cortisol levels between the bear species suggesting differences in cortisol dynamics.

The Scatchard and non-linear regression analyses of the saturation binding data were consistent with a high-affinity binding site for cortisol in the sera of black, grizzly, and polar bears. Generally, Scatchard plots are linear for single ligand binding sites, while the presence of multiple binding sites lead to non-linear plots. While, in most species, serum albumin is also a significant contributor to steroid binding in circulation, the affinity of cortisol to this protein is several orders of magnitude less compared to CBG. For example, in dogs (*Canis lupis*

Figure 2.1: β Decay Counts versus Tritium Labeled Hydrocortisone Concentration. Representative total counts (counts per minute) versus 0 – 100 nM ^{3}H-hydrocortisone for a saturation binding assay. The line of best fit was calculated by linear regression analysis and was used to convert counts per minute to nM cortisol.

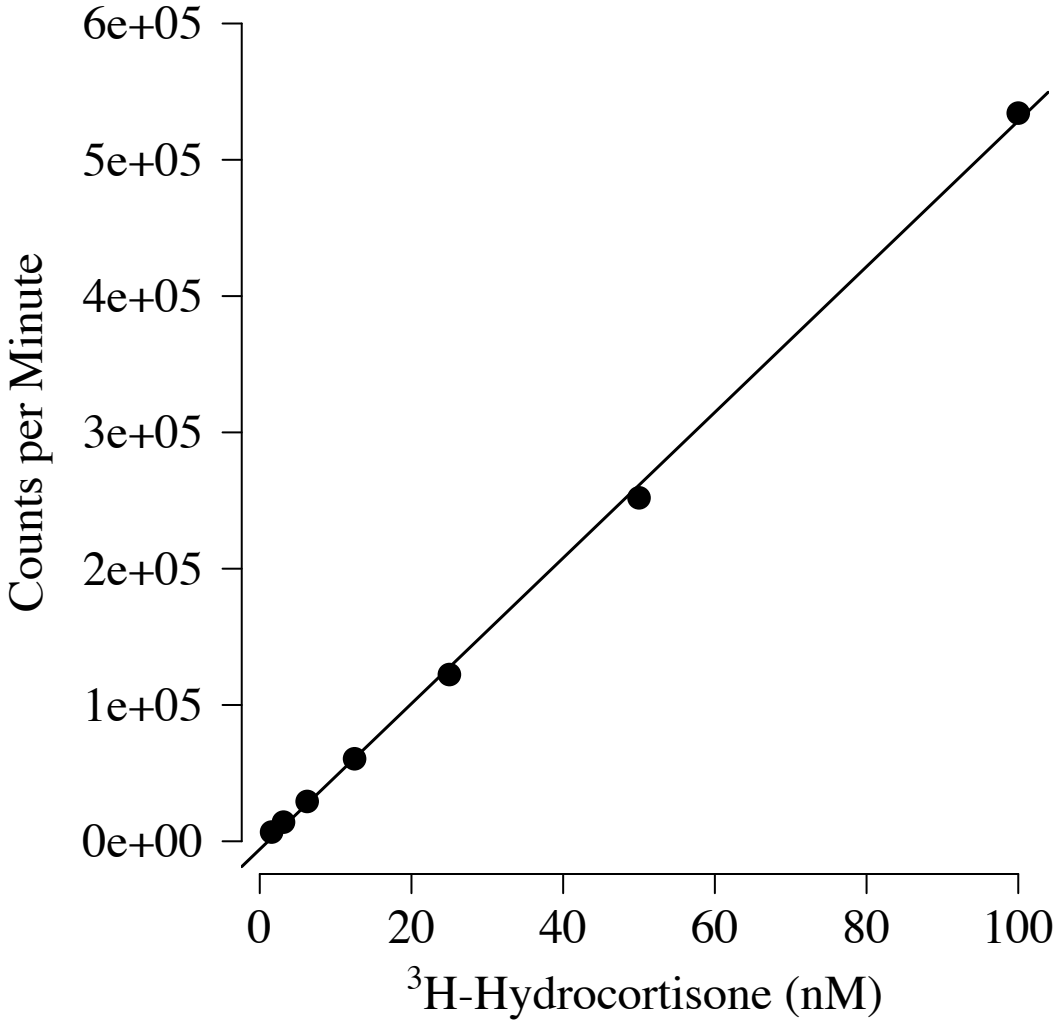

6e+05
5e+05
4e+05
3e+05
2e+05
1e+05
0e+00
Counts per Minute
0
20
40
60
80
100
3H-Hydrocortisone (nM)

Figure 2.2: Saturation Binding Curves of Cortisol to Bear Sera.
Total, non-specific, and specific binding curves for A) grizzly, B) polar, and C) black bear sera. Circles represent total binding, squares represent non-specific binding, and diamonds represent specific binding in counts per minute (cpm). Curves are fit through the data points by 4-parameter non-linear regression analysis.

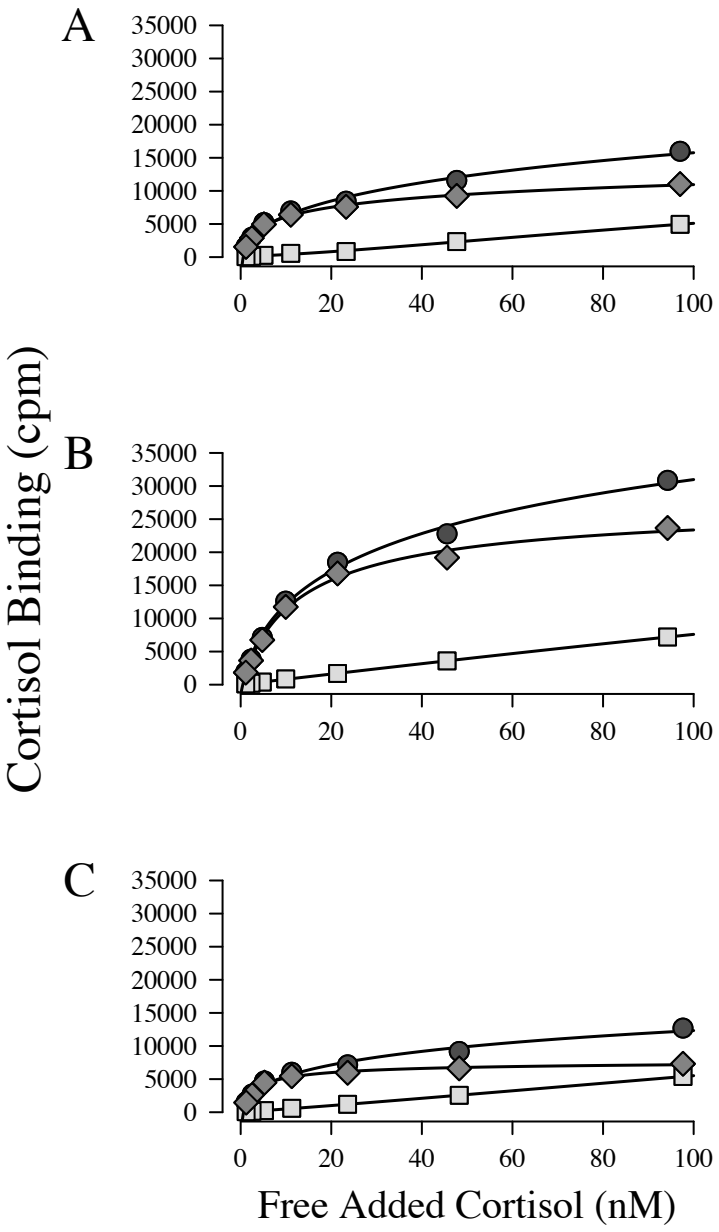

A
B
C
35000
30000
25000
20000
15000
10000
5000
0
0
20
40
60
80
100
Cortisol Binding (cpm)
Free Added Cortisol (nM)

Figure 2.3: Representative Scatchard Plot.
Representative Scatchard plot of cortisol binding to pooled grizzly bear serum. Data from the saturation binding experiments were transformed, and specific binding (cpm) divided by free ligand (^{3}H-labeled hydrocortisone, nM) was plotted against specific binding. The line of best fit was calculated by linear regression analysis.

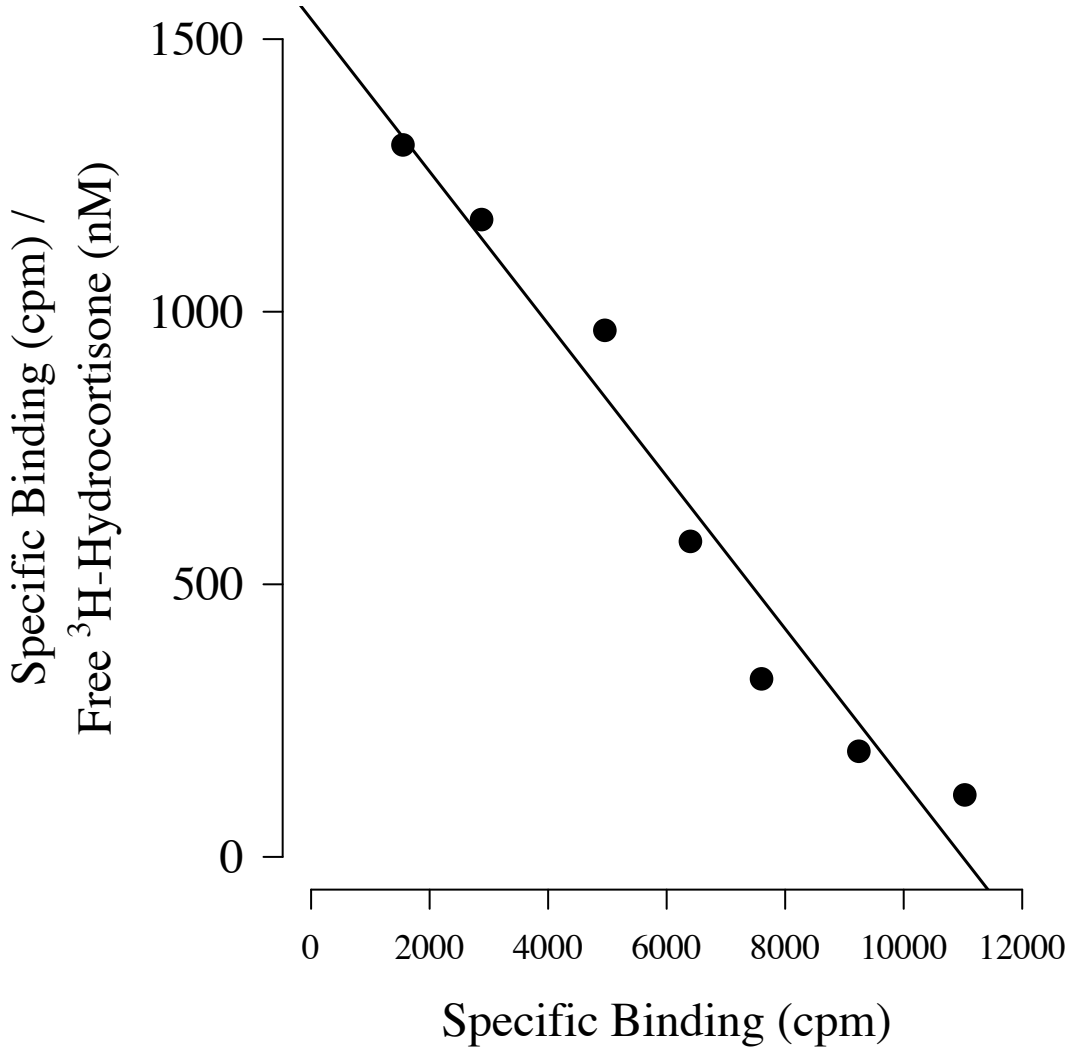

1500
1000
500
0
Specific Binding (cpm) /
Free ^{3}H-Hydrocortisone (nM)
0
2000
4000
6000
8000
10000
12000
Specific Binding (cpm)

Figure 2.4: Representative Hill Plot.
Representative Hill plot of cortisol binding to pooled grizzly bear serum.
Data from the saturation binding experiment were transformed, and θ represents the fraction of cortisol binding sites that are occupied at a given concentration of ^{3}H-F. The slope of the line of best fit, as determined by linear least squares regression analysis, is one.

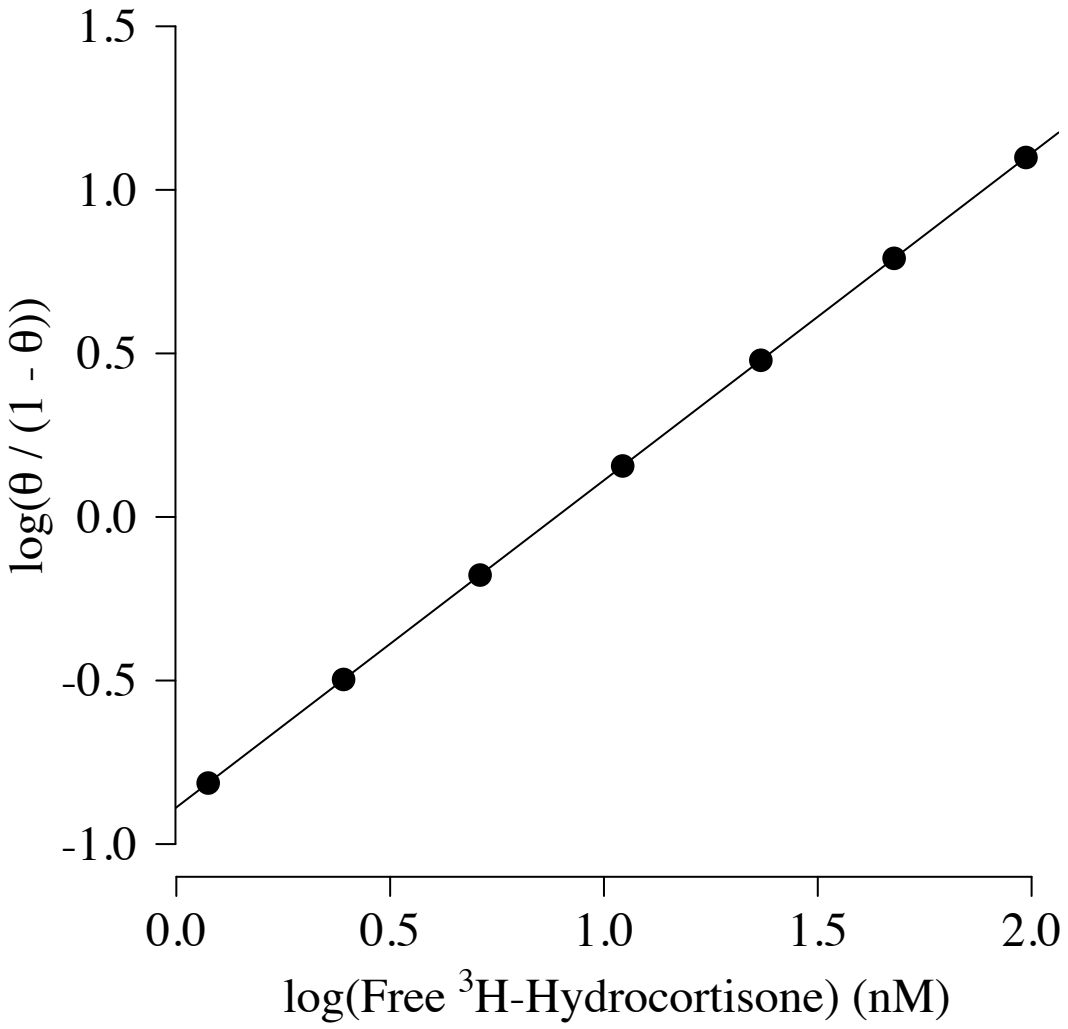
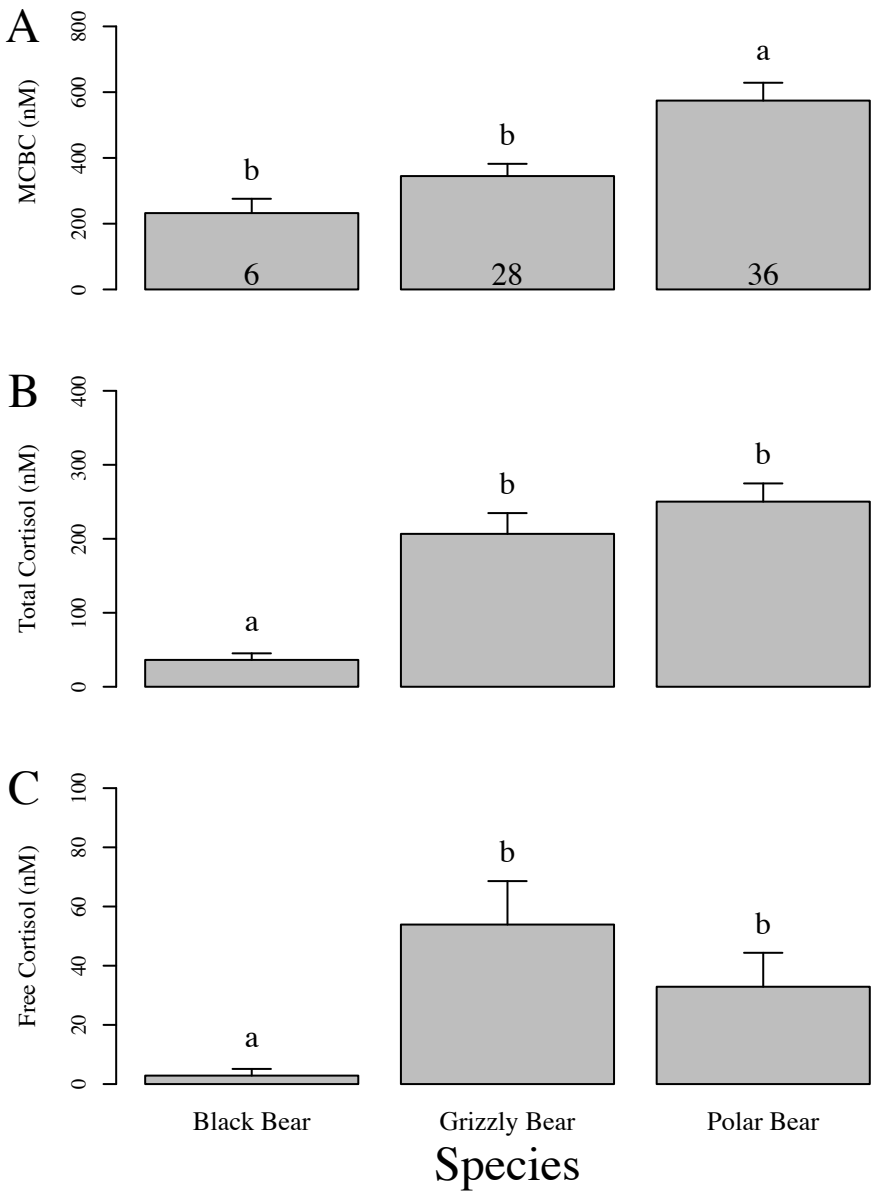

1.5
1.0
0.5
0.0
-0.5
-1.0
log(θ / (1 - θ))
0.0
0.5
1.0
1.5
2.0
log(Free 3H-Hydrocortisone) (nM)

Table 2.1: Binding Characteristics of Cortisol to CBG.
Estimates for B_{max} (nM) and K_d (nM) were determined by single-site non-linear regression analyses of saturation binding assay data.

Species	B_{max} (nM)	K_d (nM)
Grizzly Bear	316	7.72
Polar Bear	595	13.2
Black Bear	225	4.23
Rainbow Trout	ND	ND

Figure 2.5: Serum Cortisol Dynamics in Black, Grizzly, and Polar Bears.
Mean + SEM of A) maximum corticosteroid binding capacity (MCBC), B) total cortisol, and C) estimated free cortisol in black, grizzly, and polar bears. Sample sizes are shown as inset in panel A, and significantly different means (one-way ANOVA, $p < 0.05$) are indicated by different letters. MCBC was significantly higher in polar bears relative to black and grizzly bears, and total and free cortisol levels were higher in grizzly and polar relative to black bears.


A
MCBC (nM)
0
200
400
600
800
b
b
a
6
28
36
B
Total Cortisol (nM)
0
100
200
300
400
a
b
b
C
Free Cortisol (nM)
0
20
40
60
80
100
a
b
b
Black Bear
Grizzly Bear
Polar Bear
Species

familiaris) the K_d of cortisol to CBG is 7.9 nM, while the affinity of cortisol for albumin is 510.3 μM [38]. The concentration of ^{3}H-F used in these experiments was a maximum of 100 nM, so albumin was likely not a significant contributor to cortisol binding under these conditions. In addition, the Hill plots suggested a single cortisol binding site in bear sera because the lines of best fit based on the saturation binding of cortisol to ursid serum protein had slopes of 1, supporting the presence of a single high-affinity binding site in black, grizzly, and polar bears. This serum protein with high affinity binding site for glucocorticoids is likely CBG and this is well-established in animals [4], but never before been reported for ursids.

The K_d values for cortisol in bears were comparable to values reported for other mammalian species (see references [4, 39]). For instance, the reported K_d of dogs, range from 3.8 [40] to 7.9 nM [38]. Various phocid seals, which are also carnivorans like bears, possess CBG with K_d values from 11.0 to 27.0 nM [41]. New World monkeys, including squirrel (*Saimiri sciureus*) and Titi monkeys (*Callicebus moloch*) express CBG with exceptionally low affinity for corticosteroids (K_d = 500 and 250 nM, respectively [42]), and may contribute to the exceedingly high free cortisol concentrations in these species [42]. Similarly, the mean B_{max} and MCBC for bears were also within the range of reported values for mammals [4]. Dogs, for example, have reported cortisol binding capacity values ranging from 41 [40] to 82 nM [38], and humans have values ranging from 400 to 600 nM [38, 42]. Altogether, the binding characteristics of cortisol to high-affinity sites in bear sera protein were similar to observations in other mammals. The utility of characterizing this binding affinity is the capability to estimate free cortisol concentrations if CBG and total cortisol concentrations are also known, which may help to elucidate the dynamics of cortisol in sera and its potential action on tissues.

We found differences in MCBC and total cortisol levels between black, grizzly, and polar bears, and this may play a role in differing free cortisol concentrations among these species. However, cross-species comparisons of total and free cortisol levels are subject to caveats (discussed below) that may make clear interpretations of the data difficult. Thus, these comparisons of cortisol levels between species were provided as a proof-of-concept for the estimation of free cortisol in ursids.

The total cortisol levels reported in this investigation were likely above baseline, unstressed levels due to the acute stress of capture and time delay in sampling [43]. These factors may be reasons for the elevated serum total cortisol levels in grizzly and polar bears compared to black bears. In the captive black bears used in this study, blood sampling were likely more rapid relative to the free-ranging animals, resulting in levels more representative of the basal levels. However, the administration of anesthetic may itself induce a stress response that could result in the elevation of cortisol levels above baseline [44]. Thus, the cortisol values reported in this study likely reflect stress-induced levels despite the lower cortisol levels in black bears compared to the other two species. There were also likely differences in cortisol levels that are not due to species differences. For instance, total serum cortisol levels in animals captured by leg-hold snaring are higher than in animals captured by helicopter darting [10, 37]. Since the grizzly bears studied in this investigation were all captured by the former method and the polar bears by the latter method, the capture-induced cortisol levels in grizzly bears were likely inflated due to the capture method. In contrast, while CBG expression may be modulated by acute stress [45], changes in the serum levels of this protein generally manifest several hours after the initial stress response. For example, MCBC levels significantly decrease 24 h after the initiation of fasting in white-crowned sparrows (*Zonotrichia leucophrys gambelii*) [46], after 1 h post-capture in Richardson's ground squirrels (*Urocitellus richardsonii*) [47], and 24 h post-capture in Japanese quail (*Coturnix japonica*) [48]. Consequently, the B_{max} and MCBC values that are presented in this investigation may reflect pre-sampling levels of CBG, but further research is required to confirm this hypothesis.

Altogether, the saturation binding experiments revealed that cortisol binding to CBG in ursids is similar to that observed in other mammals, including a single high-affinity binding site per CBG molecule. The results of this investigation provided a proof-of-concept for the estimation of free cortisol concentrations in ursids, and the technique can be applied in subsequent studies to further characterize glucocorticoid physiology and its modulation by life history factors and environmental stressors in these species. However, a major weakness of MCBC and saturation binding assays are their reliance on radioisotope labeled-glucocorticoids. Additionally, MCBC and B_{max} are indirect measures of CBG in that these assays measure the amount of cortisol bound by this protein rather than the amount of CBG itself. The development of an assay that quantifies CBG directly and rapidly without the use of these hazardous and expensive

materials may be advantageous for the routine monitoring of CBG and free cortisol levels in bears.

2.5. Acknowledgements

Bear samples were provided from the Foothills Research Institute, the Ontario Ministry of Natural Resources, and Michigan Technical University. Funding was provided from NSERC and Alberta Innovation and Science grants to M.M. Vijayan.

CHAPTER 3:
THE DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR MEASURING URSID-SPECIFIC CORTICOSTEROID-BINDING GLOBULIN

3.1. Introduction

Free glucocorticoids are, in general, the biologically available fraction of this steroid hormone in animals [1], and their levels in circulation are regulated in part by corticosteroid binding globulin (CBG), a ~50 kDa glycoprotein that belongs to the serpin proteinase inhibitor superfamily [2]. CBG binds glucocorticoids with high affinity, and the majority of serum glucocorticoids are bound to this protein [3, 4]. Albumin is another contributor to glucocorticoid binding, albeit with low affinity, and in conjunction with CBG bind up to 95% of the total glucocorticoids in blood [4]. This bound glucocorticoid fraction may act as a reserve pool that can be locally and rapidly released. For example, elastases secreted by activated immune cells, including granulocytes, can cleave CBG, leading to conformational changes in the protein that result in a decreased affinity for glucocorticoids [5, 6]. This elastase-mediated release of glucocorticoids is thought to increase the concentrations of free steroid in a localized area to modulate immune function. Additionally, there may exist a cell surface CBG receptor that may bind and mediate the uptake of CBG-bound glucocorticoids [7]. Aside from these contributions, CBG-bound glucocorticoids are in general considered biologically unavailable because these bound steroids cannot freely cross plasma membranes. Thus, free, unbound glucocorticoids are the primary mediators of the action of this hormone, and changes in serum CBG concentration may have major effects on glucocorticoid bioavailability.

While serum total cortisol levels have been previously measured in bears [8, 9, 10], neither serum CBG nor free cortisol levels have been measured in black, grizzly, and polar bears.

Since CBG plays an important role in modulating the bioavailable free fraction of cortisol, the measurement of this protein may help to elucidate the roles that cortisol play in the adaptation to stress in ursids. The objective of this study was to develop and validate a homologous enzyme-linked immunosorbent assay (ELISA) for grizzly bear CBG to accurately quantify this protein in bear serum, and to examine the feasibility of using this ELISA to measure CBG in other ursids.

3.2. Methods and Materials

3.2.1. Animals

3.2.1.1. Tissues

For molecular work, RNA was extracted from a testicle collected from a grizzly bear killed by Alberta Government Fish and Wildlife Officers in defense of human life or property in September 2005.

3.2.1.2. Grizzly Bear Serum

For the validation of the anti-grizzly bear CBG (gbCBG) antibody and CBG ELISA, serum was collected from a representative mature male five year-old grizzly bear (bear ID G078). This animal was captured using an Aldrich leg-hold snare (Aldrich Snare Co., Clallam Bay, Washington) on May 16, 2004 for the Foothills Research Institute Grizzly Bear Project (research goals are summarized by [11]) within a 150,000-km^2 area of western Alberta, Canada (49°00’ – 55°50’N, 113°50’ – 120°00’W). He was immobilized using a combination of xylazine and zolazepam–tiletamine (XZT) administered intramuscularly as xylazine (Cervizine 300; Wildlife Pharmaceuticals, Inc., Fort Collins, Colorado) at 2 mg/kg and Telazol (Fort Dodge Laboratories, Inc., Fort Dodge, Iowa) at 3 mg/kg estimated body weight [12]. Blood was collected by venipuncture from the jugular vein into sterile tubes, and the sample was centrifuged within 8 h of collection to extract serum. This sample was stored at –20°C until

analysis. At the conclusion of handling, atipamezole (Antisedan; Novartis Animal Health Canada Inc., Mississauga, Ontario, Canada) was administered at 0.15 – 0.20 mg/kg, half-volume intramuscularly and half-volume intravenously, to reverse the effects of xylazine. The capture and sampling protocol was reviewed and approved by the University of Saskatchewan's Committee on Animal Care and Use, and was in accordance with guidelines provided by the American Society of Mammalogists' Animal Care and Use Committee [13] and the Canadian Council on Animal Care [14].

For the determination of the mean CBG, total cortisol, and glucose levels in this species, sera were used from an additional 47 grizzly bears. These animals were spotted from a helicopter, and were immobilized by the remote injection of anesthetic with a syringe dart ("heli-darting"). The anesthetics used were a combination of xylazine and zolazepam–tiletamine (XZT) administered intramuscularly as xylazine (Cervizine 300; Wildlife Pharmaceuticals, Inc., Fort Collins, Colorado) at 2 mg/kg and Telazol (Fort Dodge Laboratories, Inc., Fort Dodge, Iowa) at 3 mg/kg estimated body weight [12]. Blood sampling occurred as detailed above. Thirty-one of these animals were female, and 16 were male. No two samples were taken from the same animal (i.e. no repeated measures), and if an animal was sampled more than once, serum collected from the most recent capture of the animal was used in this study. Additionally, to validate the results of the CBG ELISA, CBG concentrations were measured in the 28 grizzly bears for which maximum corticosteroid binding capacities (MCBC) were determined in Chapter 2.

3.2.1.3. Polar Bear Serum

For the validation of the use of polar bear sera with the gbCBG antibody and ELISA, we obtained an archived frozen serum sample from a mature 13-year old female polar bear (bear ID X19562). This animal was captured on October 4, 2004 in conjunction with a long-term research project in the Hudson Bay and central Canadian Arctic regions conducted by the Ontario Ministry of Natural Resources. The sample was collected and processed similarly to grizzly bears captured by heli-darting (above), with serum frozen within 8 h of collection and maintained frozen (–20°C) in long-term storage at the University of Saskatchewan. The research protocols for this project were approved by the animal care committees at the

University of Saskatchewan and Ontario Ministry of Natural Resources, and were in accordance with guidelines provided by the American Society of Mammalogists' Animal Care and Use Committee [13] and the Canadian Council on Animal Care [14]. For the determination of the mean CBG, total cortisol, and glucose levels in polar bears, serum samples from an additional 348 polar bears were collected as detailed above. Altogether, 169 samples were collected from females and 179 from males. As with the grizzly bears, only samples from the most recent capture were used.

3.2.1.4. Black Bear Serum

For the validation of the use of black bear sera with the CBG ELISA, a serum sample from a captive, mature 5-year old female black bear was obtained. She was held at the Virginia Polytechnic Institute Center for Bear Research and was sampled on November 30, 2007. Prior to sampling, this animal was anesthetized with a 2:1 mixture of ketamine (100 mg/mL):xylazine (100 mg/mL) at a dosage of 1 cc of the mixture per 45.5 kg of body mass. Blood samples were drawn from the femoral vein while the animal was anesthetized, and the samples were transported to the laboratory in an ice-packed cooler. Immediately on return to the laboratory, the blood was spun to isolate the serum and was frozen at –20°C. The Virginia Polytechnic Institute and State University Animal Care Committee approved all bear handling protocols (#98-069- F&WS). For the determination of the species mean CBG, total cortisol, and glucose concentrations, serum samples from an additional eight mature female black bears were collected as detailed above. All animals were sampled prior to hibernation in early October through late November.

3.2.2. Cloning and Sequencing Grizzly Bear CBG cDNA

3.2.2.1. RNA Extraction and cDNA Synthesis

Approximately 50 mg of tissue sample (kept frozen at –80°C) was used for total RNA extraction using RNeasy Mini Kit (Qiagen; Mississauga, ON, Canada). The sample was first treated with DNase (Qiagen) to remove genomic DNA. RNA was quantified at 260/280 nm using a Nanodrop spectrophotometer (Wilmington, DE, USA), and 5 μL was loaded on an

RNA denaturing gel to visually assess RNA integrity. First strand cDNA was synthesized using a commercial kit (MBI Fermentas; Burlington, ON, Canada), where 1 μg of total RNA was reverse transcribed using M-MuLV reverse transcriptase in a total volume of 20 μL according to manufacturer's instruction.

3.2.2.2. PCR Amplification and Sequencing of CBG

Primers were designed to amplify two overlapping sections of grizzly bear CBG (**Table 3.1**) that were found to be conserved in dog (*Canis familiaris lupus*: GenBank accession XM_547960) and human (*Homo sapiens*; GenBank accession NM_001756) CBG (hCBG) sequences using Primer3 v0.4.0 software. CBG RT-PCR amplification consisted of an initial denaturing period of 95 °C for 3 min, followed by 40 cycles of: 1) denaturing at 95 °C for 30 s; 2) annealing at 60 °C for 30 s; and 3) extension at 72 °C for 30 s. This was followed by a 10 min extension period at 72°C. The PCR reaction products were fractionated in 1.5% agarose gels along with DNA molecular weight standards (Fermentas Life Sciences, Glen Burnie, Maryland), stained with ethidium bromide, and images were captured under UV light. Amplified products were excised from the gel, purified, and sequenced at the York University Core Molecular Biology and DNA Sequencing facility (Toronto, ON). The complete coding domain nucleotide and amino acid sequences for grizzly bear CBG were submitted to GenBank (accession number EU571738).

3.2.3. Multiple Sequence Alignment and Phylogenetic Tree

CBG protein sequences for grizzly bear, dog (XP_547960), chimpanzee (*Pan troglodytes*, XP_510143), human (AAB59523), pig (*Sus scrofa*, NP_998977), rat (*Rattus norvegicus*, NP_001009663), sheep (Ovis aries, P49920), and gray short-tailed opossum (*Monodelphis domestica*, XP_001370999) were aligned by ClustalX 2.0.12. The positions of the steroid binding residues and conserved cysteine residues of rat CBG [15] were used to determine conserved binding and cysteine residues in bears. N-linked glycosylation sites in the gbCBG amino acid sequence were predicted using NetNGlyc 1.0 [16]. A phylogenetic tree of CBG nucleic acid sequences was constructed with PHYLIP 3.69. The CBG nucleotide sequences

Table 3.1: Primers Used for RT-PCR.
Primers were designed against conserved nucleotide sequences found in dog (*Canis lupis familiaris*) and human (*Homo sapiens*) CBG.

Set 1	Forward Primer	5’ TCCCAGGTCACATAGCCAAT 3’
	Reverse Primer	5’ CAAGTCTACAATTTTCCCTTGTGTC 3’
Set 2	Forward Primer	5’ CCATGGCCTTAGCTATGCTG 3’
	Reverse Primer	5’ TTAGGTCGGATTCACAACCTTT 3’

used for tree construction were (GenBank accession numbers in parentheses): dog (XM_547960), grizzly bear (EU571738), horse (XM_001495734), rhesus macaques isoform 1 (XM_001098039) and isoform 2 (XM_001098128), chimpanzee (XM_510143), orangutan (NM_001132481), squirrel monkey (S68757), human (J02943), mouse (NM_007618), rat (NM_001009663), golden hamster (M74776), pig (NM_213812), sheep (X73615), opossum (XM_001370962), and platypus (XM_001515356). An unidentified cattle sequence whose nucleotide sequence was similar to CBG was also included (XR_028138). Human (NM_000295), cattle (NM_173882), and zebrafish (NM_001077758) alpha-1-antitrypsin (A1AT) were used as the outgroup sequences, with the latter used to root the tree. The tree was constructed using the maximum likelihood method and bootstrap algorithm with 1000 bootstrap trials.

3.2.4. CBG Peptide Antibody Synthesis

From the deduced amino acid sequence for grizzly bear CBG, an affinity purified polyclonal antibody was generated in rabbits for the peptide sequence c-VQAKDPDTDVSPRTPHRDLAPNNVC-n (21st Century Biochemicals, Marlboro, MA, USA).

3.2.5. Antibody Validation

The specificity of the anti-bear CBG antibody for grizzly, polar, and black bear sera was confirmed by: i) comparing western immunoblots of grizzly bear sera that were incubated with pre-immune challenged rabbit sera and diluted anti-bear CBG antibodies as the primary antibody; ii) comparing the western immunoblots of recombinant human CBG and grizzly bear sera that were incubated with anti-bear CBG antibody and anti-human CBG antibody; iii) analyzing the densitometry of serially-diluted bear sera (2.5 – 20 μg protein/lane) with the anti-bear CBG antibody; and iv) analyzing the western immunoblot of recombinant bear CBG incubated with anti-bear CBG antibody as the primary antibody. Bear sera were depleted of albumin and IgG with Aurum Serum Protein Mini-kits (BioRad, Hercules, CA, USA). Total

serum protein concentrations were determined by the bicinchoninic acid (BCA) method using bovine serum albumin as the standard. Serum proteins were separated by SDS-PAGE. Briefly, depleted serum samples in SDS sample buffer (0.06 M Tris-HCl [pH 6.8], 25% (v/v) glycerol, 0.02% (w/v) SDS, 0.001% (w/v) bromophenol blue [Fisher Scientific, Fair Lawn, NJ]) were loaded (2.5 µg protein/sample/lane) onto 10% reducing polyacrylamide gels according to established protocols [17]. A low-range molecular weight marker (BioRad) was also loaded to confirm the molecular mass of the protein detected. Serum proteins were separated (200 V for 50 min; Mini Protean III [BioRad]) using a discontinuous buffer. The separated proteins were transferred to a 0.22 µm pore size nitrocellulose membrane (BioRad) using a Transblot SD Semi-Dry Electrophoretic Transfer Cell (BioRad) and transfer buffer (25 mM Tris [pH 8.3], 192 mM glycine, 20% v/v methanol). Equal protein loading and transfer efficiency were confirmed by Ponceau S (BioRad) staining of the membrane and Coomassie brilliant blue (BioRad) staining of the polyacrylamide gel.

The membranes were rinsed and blocked with 5% skim milk in TTBS (20 mM Tris pH 7.5 [Fisher], 300 mM NaCl [Sigma], 0.1% (v/v) Tween 20 [Biorad]). Blots were probed with either pre-immune rabbit antiserum (diluted to 1:3000) or affinity-purified polyclonal rabbit anti-grizzly bear (gb)CBG for 1 h at room temperature. The blots were washed with TTBS (3 x 15 min) and incubated with anti-rabbit IgG conjugated horseradish peroxidase (HRP) (Biorad) at 1:3000 dilution (secondary antibody) for 1 h at room temperature. The blots were further washed (3 x 5 min in TTBS and 1 x 5 min with TBS) and the proteins detected using ECL Plus western blotting detection reagent (GE Healthcare). The protein bands were scanned using the Typhoon Variable Mode Imager (GE Healthcare). The specificity of the gbCBG antibody was confirmed by western blotting with serially diluted antibody (1:500, 1:1000, 1:2000, 1:4000, 1:8000) and grizzly, polar, and black bear serum samples (2.5 µg, 5 µg, 10 µg, and 20 µg total serum protein). The cross-reactivity of the gbCBG antibody to human CBG was assessed by incubation with different concentrations of purified hCBG (Affiland, Belgium). Also, a commercially available rabbit polyclonal antibody for hCBG (Fitzgerald Industries, Acton, MA, USA) was tested for cross-reactivity with grizzly bear serum CBG.

3.2.6. CBG ELISA development

3.2.6.1. Grizzly Bear CBG ELISA Materials

Costar 96-well EIA/RIA high protein-binding plates were purchased from Corning (Corning, NY). The following reagents were purchased from Sigma-Aldrich: ampicillin, isopropyl-β-D-thiogalactopyranosid (IPTG), protease inhibitor cocktail, dimethyl pimelimidate, sodium carbonate, sodium bicarbonate, and 3,3',5,5'-tetramethylbenzidine (TMB). Bovine serum albumin (BSA, fraction V), dimethylsulfoxide (DMSO), EZ-Link Sulfo-NHS-SS-Biotinylation kit, streptavidin coupled to horseradish peroxidase (streptavidin-HRP), and acetic and sulfuric acid were purchased from Thermo Fisher Scientific (Waltham, MA). Tris base, NaCl, Tween-20, and potassium citrate were purchased from Bioshop. 2YT broth was purchased from Invitrogen (Carlsbad, CA). Sepharose beads coupled to protein A were purchased from Bio-Rad (Hercules, CA). 10,000 MWCO centrifugal filter units were purchased from Millipore (Billerica, MA)

3.2.6.2. Bear CBG Recombinant Protein

We produced recombinant gbCBG protein according to established protocols [18]. Briefly, the cDNA sequence encoding gbCBG (GenBank Accession EU571738) protein was cloned into a pHAT20 vector, and the construct was introduced into a BL21 DE3 *Escherichia coli* strain and plated onto media containing 100 µg/mL ampicillin. A single colony from the selection plates was inoculated into 2YT broth for 16 – 18 h at 37°C. 3 mL of this overnight culture was used to inoculate 100 ml of 2YT broth containing 100 µg/ml of ampicillin. The inoculum was incubated for approximately 4 – 6 h at 37°C until reaching OD_{600} = 0.6. At this point, gbCBG protein expression was induced with 1 mM of IPTG. 18 h post-induction, cells were pelleted by centrifugation (8000 x G for 10 min at 4°C), flash-frozen on dry ice, and stored at –80°C.

Frozen cells were thawed on ice and resuspended at a ratio of 100 mg of pellet to 1 ml of ice-cold lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl with protease inhibitor cocktail, pH 8). The cells were first treated with 1 mg/mL of lysozyme and then sonicated with six x 10 s bursts with a 10 s cooling period between each burst. The lysate was centrifuged at 10,000 rpm for 30 min at 4°C to remove bacterial cellular debris. The recombinant CBG was purified using a

custom affinity binding column. Rabbit polyclonal anti-gbCBG was coupled with dimethyl pimelimidate to protein A–sepharose beads as described before [19]. Bacterial lysate was loaded onto the affinity column and incubated for 4 h at room temperature on a shaker. The column was washed with lysis buffer, and bound protein was eluted with 200 mM glycine (pH 4.0). The purified CBG was confirmed by immunodetection with a rabbit polyclonal anti-gbCBG antibody [20] before desalting and concentration in a 10,000 molecular weight cutoff centrifugal filter unit. This purified recombinant protein was used as standards for the CBG ELISA.

3.2.6.3. CBG ELISA Protocol

A direct ELISA was developed to measure CBG concentration in grizzly bear sera. Serum was diluted 1:1000 in 10 mM Tris (pH 7.5), and 2.0 µL of this diluted serum in 200 µL of carbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) directly in duplicate wells of an EIA plate. The recombinant gbCBG was diluted with 10 mM Tris (pH 7.5) to 0 – 4 nmol gbCBG/L, and diluted 10 fold with carbonate coating buffer directly in wells of the ELISA plate in triplicate. Proteins were allowed to coat the wells for 2 h (all incubations were performed in a shaker-incubator set to 30°C). Plates were then washed in an automatic plate washer (Immunowash 1575, Bio-Rad) with a custom washing protocol [4 repetitions of aspiration, washing with 400 µL TTBS (20 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.4), and agitation for 5 s; and one final aspiration]. Plates were blocked with 5% BSA in TTBS for 1 h and washed again. The primary detection antibody (polyclonal rabbit anti-gbCBG) was diluted 1:1500 in 1% BSA in TTBS, and 200 µL was added to each well, except for non-specific binding wells where 200 µL 1% BSA in TTBS was added instead, and incubated for 2 h. After washing, bound biotinylated anti-CBG antibody was detected and amplified with 200 µL per well of streptavidin-HRP diluted 1:3000 in 1% BSA in TTBS, incubated for 1 h. After a final wash, the detection solution was freshly prepared [500 µL 41 mM TMB in DMSO into 19.5 mL 200 mM potassium citrate, 3.075 mM H_2O_2 (pH 4.0)], and 200 µL was added to each well and incubated for 1 h. The reaction was stopped with 100 µL stopping solution (8.5 M acetic acid, 0.5 M sulfuric acid). Plates were read at 450 nm excitation wavelength on a VersaMax microplate reader using SoftMax Pro 3.1 software (Molecular Devices, Sunnyvale, CA).

3.2.6.4. CBG ELISA Validation

The CBG ELISA was validated by: i) testing its cross-reactivity with BSA and rabbit and bear sera, ii) by measuring CBG concentration in serial dilutions of serum, and iii) and the recovery of recombinant gbCBG at various dilutions. BSA was diluted to 80 µg/mL (the approximate mean concentration of 1:1000 diluted bear sera) and rabbit serum was diluted 1:1000 in 10 mM Tris (pH 7.5). These samples were then loaded (0.25 to 5 µL) into triplicate wells into carbonate coating buffer. For the serial dilution test, grizzly, polar, and black bear sera were diluted 1:1000 in 10 mM Tris (pH 7.5), and 0.25 – 10 µL of this diluted serum was diluted 10 fold in duplicate wells into carbonate coating buffer. For the recovery test, 0 – 373 pM recombinant gbCBG in 10 mM Tris (pH 7.5) was added to rabbit sera (1:1000 total dilution) prior to loading in triplicate wells. The sensitivity of the assay was measured as the mean CBG concentration + 2 standard deviations of the duplicate 0 ng/mL standard in four separate assays. The inter-assay variability of the ELISA was determined by measuring the percent coefficient of variation (%CV) between standard curves in triplicate from 4 different assays. The intra-assay variability of the ELISA was measured by determining the %CV of the representative grizzly bear serum sample measured 32 times in a single assay.

3.2.7. Serum Total Cortisol Assay and Free Cortisol Estimates

Serum total cortisol was measured by radioimmunoassay (RIA) using a commercial ^{125}I kit (MP Biomedicals, Orangeburg, NY) as described by Hamilton [21]. Samples collected prior to 2008 were published as part of a MSc thesis [21].

Free cortisol concentrations were calculated by the equation of Barsano and Baumann [22]:

$$fCORT = \frac{-\left(CBG - tCORT + \frac{1}{K_a}\right) \pm \sqrt{\left(CBG - tCORT + \frac{1}{K_a}\right)^2 - 4\left(\frac{-tCORT}{K_a}\right)}}{2}$$

Where *fCORT* is free cortisol concentration (mol/L), CBG is CBG concentration (mol/L), tCORT is total cortisol concentration (mol/L), and $K_a = 1/K_d$ = equilibrium association constant (L/mol). Values for K_d were determined in Chapter 2.

3.2.8. Serum Glucose Assay

Glucose concentrations in bear sera were determined by the glucose oxidase-peroxidase method as described previously [23], using glucose (Sigma) as the standard. Absorbance was measured at 500 nm on a VersaMax microplate reader.

3.2.9. Cross-species Comparison of Serum CBG Concentrations

Serum CBG and total and free corticosteroid concentrations from placental mammalian species were obtained from the literature and converted to nmol/L (nM). These data were compiled from studies that reported CBG and total and free corticosteroids from control animals that were not subjected to experimental treatments (i.e. control groups). For publications that presented data only in figures, data were extracted from these figures using the "digitize" (version 0.0.1-07) package in R.

3.2.10. Data Analyses

Four-parameter non-linear regression analysis was employed to fit standard curves for the CBG ELISA. Data were fit to the equation:

$$\mathrm{OD} = \frac{\mathrm{A} - \mathrm{D}}{1 + \left(\frac{L}{C}\right)^{B}} + D$$

Where OD is the optical density of the standard read at 450 nm; L is the concentration of recombinant grizzly bear CBG; A is the minimum asymptote; B is the slope factor; C is the

inflection point; and D is the maximum asymptote. A, B, C, and D were determined by regression.

Data are presented as mean ± standard error of the means (SEM). One-way ANOVAs were employed to test for significant differences between the means of CBG, total and free cortisol, and glucose concentrations in black, grizzly, and polar bears. *Post hoc* significance testing was performed using Tukey's Honest Significant Difference test. p-values < 0.05 were considered statistically significant.

3.3. Results

3.3.1. Corticosteroid Binding Globulin Sequencing and Phylogeny

CBG primers strongly amplified a product from cDNA samples isolated from the grizzly bear testicle (**Figure 3.1B**, lane 2), even though the RNA gel did not indicate good quality rRNA (**Figure 3.1A**, lane 4). Products were not amplified from other tissue samples that did not have good quality rRNA (e.g. **Figure 3.1A**, lane 3 and **Figure 3.1B**, lane 1). Therefore, the testicle sample was used to amplify and sequence CBG. PCR reactions were fractionated in 1.5% agarose gels along with DNA molecular weight standards and stained with ethidium bromide. Bands were excised, purified, and sequenced at the York University Core Molecular Biology and DNA Sequencing facility (Toronto, ON). The complete coding domain sequence for Grizzly bear CBG was submitted to GenBank (accession number EU571738).

The grizzly bear CBG cDNA nucleotide sequence was 1218 base pairs (bp) in length, and coded for 405 amino acids (**Figure 3.2**) with a predicted molecular mass of 45,619 Daltons. The multiple sequence alignment (**Figure 3.3**) revealed that the nucleotide and amino acid sequences of gbCBG were 90% and 83% identical, respectively, to dog CBG; 79% and 68% identical to human CBG; and 68% and 58% identical to rat CBG. The phylogenetic tree of selected mammalian CBG showed that grizzly bear and dog CBG were clustered in the same

clade (**Figure 3.4**). Four N-linked glycosylation sites were present in gbCBG and the steroid binding residues were well conserved across a wide variety of mammalian species (**Figure 3.3**). There were two cysteine residues in gbCBG outside of the export signal sequence, and the residue at position 250 (**Figure 3.3**) corresponded to the conserved cysteine residue found in all species. An inserted glutamic acid (glu-118) in gbCBG was found that is not present in any of the other compared species (**Figure 3.3**).

3.3.2. Anti-bear CBG Antibody Validation

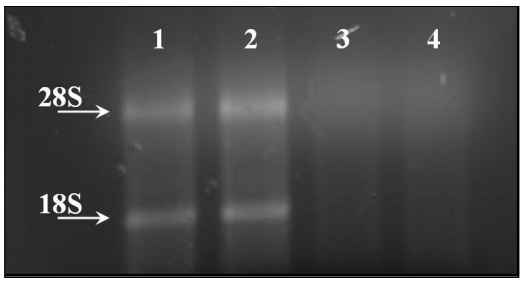
The specificity of the antibody, generated in rabbits from a gbCBG peptide sequence, to grizzly bear CBG was confirmed by western blotting. The affinity-purified antibody, but not the pre-immune challenged rabbit serum cross-reacted with grizzly bear serum (**Figure 3.5A**). Bands appeared at approximately 55 kDa in sera and 42 kDa for recombinant gbCBG (**Figure 3.5D**). The higher molecular weight for the former bands was likely due to glycosylation of the protein, and the apparent molecular weight of the recombinant gbCBG was near its predicted, unmodified weight of 45.6 kDa. A commercially available anti-human CBG did not immunodetect grizzly bear CBG (**Figure 3.5B**). The gbCBG antibody was specific for bear sera and did not cross-react with purified human CBG (**Figure 3.5C**). This anti-bear CBG antibody cross-reacted with grizzly (**Figure 3.6A**), polar (**Figure 3.6B**), and black bear (**Figure 3.6C**) sera. The anti-bear CBG antiserum detected doublets of around 55 kDa in sera of all three bear species; the heavier band was usually more prominent and the lighter one was not always seen.

3.3.3. Corticosteroid Binding Globulin ELISA Validation

A typical ELISA standard curve covering its linear range from 19 – 373 pmol gbCBG/L and typical dilution curves for grizzly, polar and black bear sera are shown in **Figure 3.7**. Rabbit sera and BSA effectively did not exhibit cross-reactivity in the ELISA. The average recovery of recombinant gbCBG, diluted from 18.6 – 373 pM in untreated, 1:1000 diluted rabbit serum with the ELISA was 94% (**Table 3.2**). Bear serum was dilutionally linear from 0.25 to 5.0 µL

Figure 3.1: Validation of cDNA Synthesis and gbCBG Transcript Amplification.
A) RNA denaturing gel with total RNA samples. Lanes 1 and 2: polar bear muscles, lane 3: grizzly bear liver, and lane 4: grizzly bear testicle. Bands are 28S and 18S rRNA. B) RT-PCR amplification of a 650 bp section of corticosteroid binding globulin (CBG) with cDNA. Lane 1: grizzly bear liver, lanes 2 and 3: grizzly bear testicle, and lanes 4 and 5: polar bear muscles.

A



B

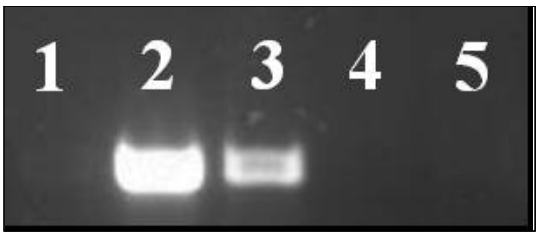
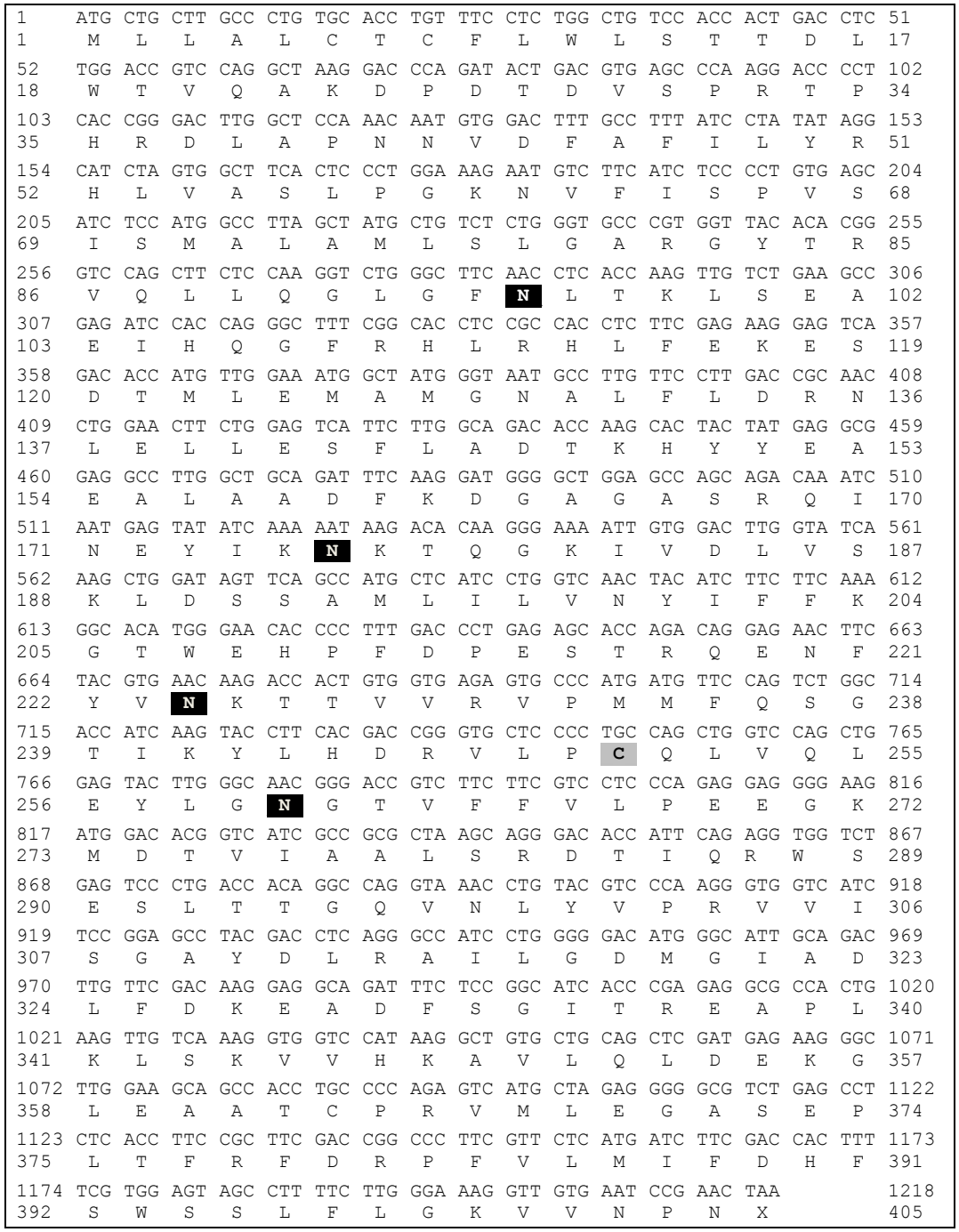
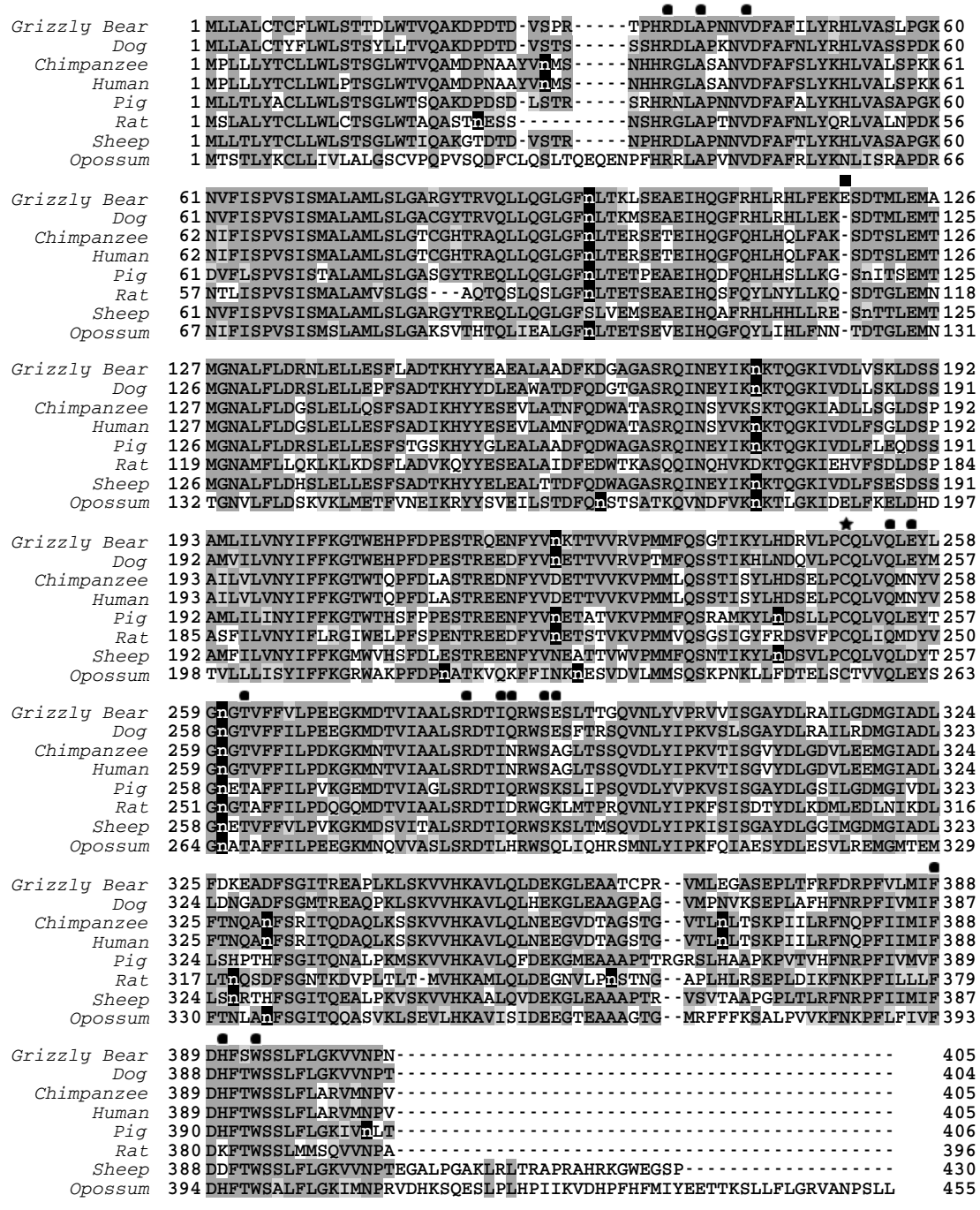


Figure 3.2: Grizzly Bear CBG Complete Nucleotide Coding Domain (Top) and Predicted Amino Acid Sequence (Bottom).
Putative N-linked glycosylation sites are highlighted in black, and the conserved cysteine residue is highlighted in grey.



```
1    ATG CTG CTT GCC CTG TGC ACC TGT TTC CTC TGG CTG TCC ACC ACT GAC CTC 51
1     M   L   L   A   L   C   T   C   F   L   W   L   S   T   T   D   L 17

52   TGG ACC GTC CAG GCT AAG GAC CCA GAT ACT GAC GTG AGC CCA AGG ACC CCT 102
18    W   T   V   Q   A   K   D   P   D   T   D   V   S   P   R   T   P 34

103  CAC CGG GAC TTG GCT CCA AAC AAT GTG GAC TTT GCC TTT ATC CTA TAT AGG 153
35    H   R   D   L   A   P   N   N   V   D   F   A   F   I   L   Y   R 51

154  CAT CTA GTG GCT TCA CTC CCT GGA AAG AAT GTC TTC ATC TCC CCT GTG AGC 204
52    H   L   V   A   S   L   P   G   K   N   V   F   I   S   P   V   S 68

205  ATC TCC ATG GCC TTA GCT ATG CTG TCT CTG GGT GCC CGT GGT TAC ACA CGG 255
69    I   S   M   A   L   A   M   L   S   L   G   A   R   G   Y   T   R 85

256  GTC CAG CTT CTC CAA GGT CTG GGC TTC AAC CTC ACC AAG TTG TCT GAA GCC 306
86    V   Q   L   L   Q   G   L   G   F   N   L   T   K   L   S   E   A 102

307  GAG ATC CAC CAG GGC TTT CGG CAC CTC CGC CAC CTC TTC GAG AAG GAG TCA 357
103   E   I   H   Q   G   F   R   H   L   R   H   L   F   E   K   E   S 119

358  GAC ACC ATG TTG GAA ATG GCT ATG GGT AAT GCC TTG TTC CTT GAC CGC AAC 408
120   D   T   M   L   E   M   A   M   G   N   A   L   F   L   D   R   N 136

409  CTG GAA CTT CTG GAG TCA TTC TTG GCA GAC ACC AAG CAC TAC TAT GAG GCG 459
137   L   E   L   L   E   S   F   L   A   D   T   K   H   Y   Y   E   A 153

460  GAG GCC TTG GCT GCA GAT TTC AAG GAT GGG GCT GGA GCC AGC AGA CAA ATC 510
154   E   A   L   A   A   D   F   K   D   G   A   G   A   S   R   Q   I 170

511  AAT GAG TAT ATC AAA AAT AAG ACA CAA GGG AAA ATT GTG GAC TTG GTA TCA 561
171   N   E   Y   I   K   N   K   T   Q   G   K   I   V   D   L   V   S 187

562  AAG CTG GAT AGT TCA GCC ATG CTC ATC CTG GTC AAC TAC ATC TTC TTC AAA 612
188   K   L   D   S   S   A   M   L   I   L   V   N   Y   I   F   F   K 204

613  GGC ACA TGG GAA CAC CCC TTT GAC CCT GAG AGC ACC AGA CAG GAG AAC TTC 663
205   G   T   W   E   H   P   F   D   P   E   S   T   R   Q   E   N   F 221

664  TAC GTG AAC AAG ACC ACT GTG GTG AGA GTG CCC ATG ATG TTC CAG TCT GGC 714
222   Y   V   N   K   T   T   V   V   R   V   P   M   M   F   Q   S   G 238

715  ACC ATC AAG TAC CTT CAC GAC CGG GTG CTC CCC TGC CAG CTG GTC CAG CTG 765
239   T   I   K   Y   L   H   D   R   V   L   P   C   Q   L   V   Q   L 255

766  GAG TAC TTG GGC AAC GGG ACC GTC TTC TTC GTC CTC CCA GAG GAG GGG AAG 816
256   E   Y   L   G   N   G   T   V   F   F   V   L   P   E   E   G   K 272

817  ATG GAC ACG GTC ATC GCC GCG CTA AGC AGG GAC ACC ATT CAG AGG TGG TCT 867
273   M   D   T   V   I   A   A   L   S   R   D   T   I   Q   R   W   S 289

868  GAG TCC CTG ACC ACA GGC CAG GTA AAC CTG TAC GTC CCA AGG GTG GTC ATC 918
290   E   S   L   T   T   G   Q   V   N   L   Y   V   P   R   V   V   I 306

919  TCC GGA GCC TAC GAC CTC AGG GCC ATC CTG GGG GAC ATG GGC ATT GCA GAC 969
307   S   G   A   Y   D   L   R   A   I   L   G   D   M   G   I   A   D 323

970  TTG TTC GAC AAG GAG GCA GAT TTC TCC GGC ATC ACC CGA GAG GCG CCA CTG 1020
324   L   F   D   K   E   A   D   F   S   G   I   T   R   E   A   P   L 340

1021 AAG TTG TCA AAG GTG GTC CAT AAG GCT GTG CTG CAG CTC GAT GAG AAG GGC 1071
341   K   L   S   K   V   V   H   K   A   V   L   Q   L   D   E   K   G 357

1072 TTG GAA GCA GCC ACC TGC CCC AGA GTC ATG CTA GAG GGG GCG TCT GAG CCT 1122
358   L   E   A   A   T   C   P   R   V   M   L   E   G   A   S   E   P 374

1123 CTC ACC TTC CGC TTC GAC CGG CCC TTC GTT CTC ATG ATC TTC GAC CAC TTT 1173
375   L   T   F   R   F   D   R   P   F   V   L   M   I   F   D   H   F 391

1174 TCG TGG AGT AGC CTT TTC TTG GGA AAG GTT GTG AAT CCG AAC TAA         1218
392   S   W   S   S   L   F   L   G   K   V   V   N   P   N   X         405
```

Figure 3.3: Multiple Sequence Alignment of CBG Amino Acid Sequences.
Sequences were used from grizzly bear, dog (*Canis familiaris*), chimpanzee (*Pan troglodytes*), human (*Homo sapiens*), pig (*Sus scrofa*), rat (*Rattus norvegicus*), sheep (*Ovis aries*) and opossum (*Monodelphis domestica*) CBG. Residues shaded with dark grey match the consensus sequence residue of the compared sequences. Lighter grey shading indicates lesser conservation. Residues highlighted in black are consensus N-linked glycosylation sites. Residues indicated with a black dot (•) are conserved steroid binding residues. Conserved cysteine residues are indicated with a star (*). The inserted glutamic acid in grizzly bear CBG at position 118 is indicated with a solid square (■).


Grizzly Bear
Dog
Chimpanzee
Human
Pig
Rat
Sheep
Opossum

loading of 1:1000 diluted serum (**Table 3.3**). The amount of CBG quantified at the 10 µL loading was lower than at lower loadings, suggesting that 10 µL of 1:1000 diluted serum exceeds the detection capacity of the ELISA. Inter-assay variability for CBG using the ELISA was 16%, while the intra-assay variability was 9.7%. The sensitivity of the assay was 9.3 pM.

3.3.4. Between-species Variations in CBG, Cortisol, and Glucose

Serum CBG concentrations in the sera of black, grizzly, and polar bears were quantified by the homologous grizzly bear CBG ELISA. Mean CBG concentrations were significantly higher in grizzly bears relative to black and polar bears (**Figure 3.8A**; $F_{2, 341} = 56.8$, $p < 0.001$). Mean serum total cortisol levels were significantly elevated in polar bears relative to black and grizzly bears (**Figure 3.8B**; $F_{2, 401} = 19.5$, $p < 0.001$), while mean cortisol levels were marginally different between the latter two species.

Mean serum and/or plasma CBG and total and free cortisol concentrations for selected placental mammals and grizzly bears are presented in Table 3.4. In mammals (grizzly bear ranges in parentheses), CBG concentrations ranged from 34 to 6078 nM (9.0 to 490 nM), total corticosteroid from 4 to 6240 nM (9.7 to 1781 nM), and free corticosteroid from 1 to 3120 nM (0.39 to 1616 nM). The serum CBG and total and free corticosteroid concentrations of ursids were similar to most mammals except the new world primates, which had lower CBG concentrations.

Free cortisol concentrations were estimated using these CBG and total cortisol values. There were significant differences in mean CBG and total and free cortisol concentrations between species. Free cortisol levels were significantly (**Figure 3.8C**; $F_{2,380} = 47.5$, $p < 0.001$) elevated in polar bears over both grizzly and black bears. Mean serum glucose concentrations were significantly (**Figure 3.8D**; $F_{2,216} = 7.51$, $p < 0.001$) elevated in black bears relative to grizzly and polar bears.

CBG concentrations were measured in the animals for which maximum corticosteroid binding capacity (MCBC) levels were determined in Chapter 2. There appeared to be a discrepancy between the patterns of mean MCBC and mean CBG concentrations between bear species.

Mean MCBC levels were significantly elevated in polar bears relative to black and grizzly bears, but mean CBG concentrations were elevated in grizzly bears relative to black and polar bears. Overall, mean MCBC levels were 5.0, 2.6, and 8.8-fold higher than mean CBG levels in black, grizzly, and polar bears, respectively. As a theoretical exercise, the relative difference between the mean MCBC and CBG values for grizzly bears was used to normalize black and polar bear CBG concentrations. By this method, serum CBG levels in polar bear sera were adjusted upwards by 3.4-fold and by 2.0-fold in black bear sera. These bias-corrected CBG concentrations (bCBG) are shown in **Figure 3.8E**. bCBG concentrations were significantly different between the three species ($F_{2,380} = 16.0$, $p < 0.001$), and were elevated in polar bears relative to grizzly and black bears. The free cortisol concentrations were recalculated using bCBG (**Figure 3.8F**), and the mean bias-corrected free cortisol levels were elevated in polar bears relative to grizzly and black bears ($F_{2,380} = 9.96$, $p < 0.001$). These bias-corrected data were not used elsewhere in this thesis.

3.4. Discussion

In this chapter, the cloning of grizzly bear CBG and the development of a homologous grizzly bear CBG ELISA that also cross-reacts with black and polar bear sera was described. This CBG ELISA represents the first development of a homologous assay for this protein in a free-ranging mammalian species. This ELISA will allow for the rapid and reliable quantification of this protein that plays important roles in modulating glucocorticoid action in animals, and which may provide wildlife managers and conservationists with an additional tool to monitor and manage free-ranging species.

3.4.1. Grizzly Bear CBG Cloning and Sequencing

We cloned and sequenced a grizzly bear CBG cDNA, and the deduced nucleotide and amino acid sequences suggested that this protein was well conserved in mammals. The species whose

Figure 3.4: Phylogeny of Mammalian CBG.
This rooted phylogenetic tree of CBG nucleotide sequences constructed by the maximum likelihood method. A1AT sequences (denoted with 'A1AT') from zebrafish, cattle, and humans were used as outgroup sequences, and zebrafish A1AT was used to root the tree. Nodes indicated with an asterisk (*) are supported by > 90% bootstrap values for 1000 bootstrap simulations.

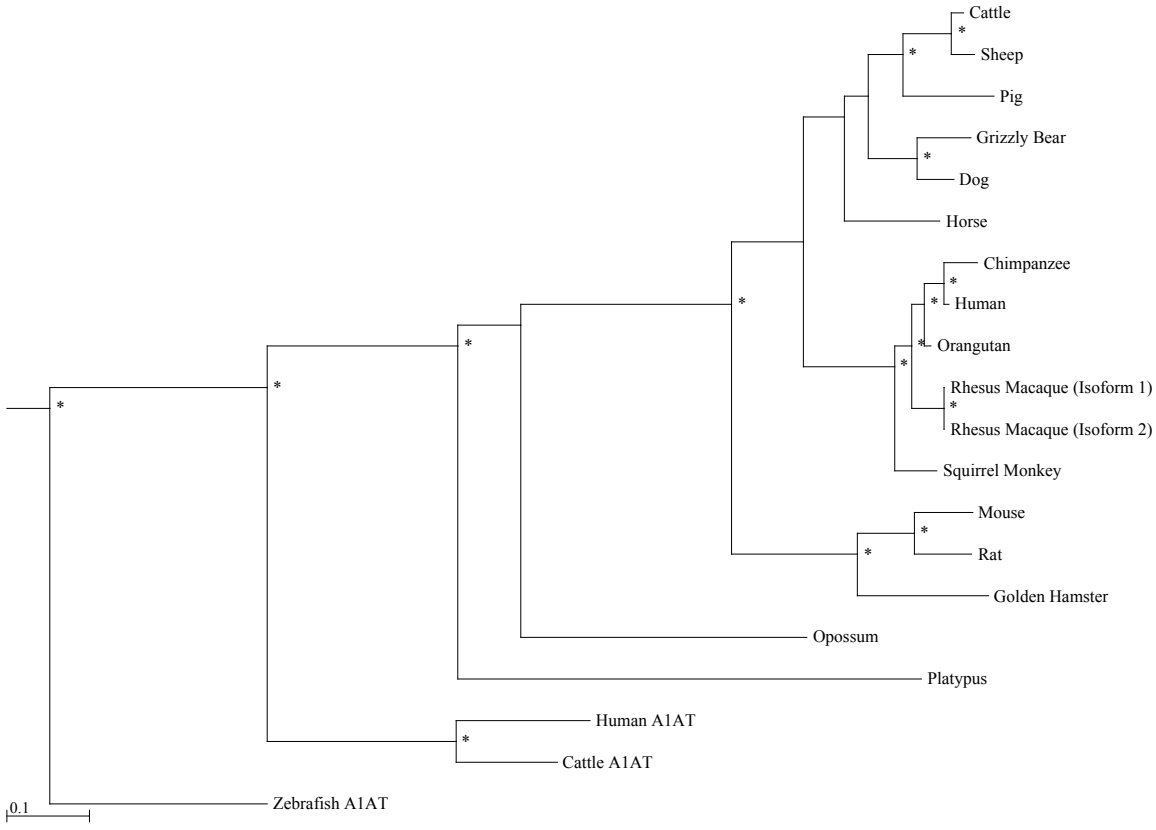

Cattle
Sheep
Pig
Grizzly Bear
Dog
Horse
Chimpanzee
Human
Orangutan
Rhesus Macaque (Isoform 1)
Rhesus Macaque (Isoform 2)
Squirrel Monkey
Mouse
Rat
Golden Hamster
Opossum
Platypus
Human A1AT
Cattle A1AT
Zebrafish A1AT
0.1

Figure 3.5: Anti-bear CBG Antibody Validation: Species Cross Reactivity
A) Western Blot of grizzly bear serum incubated with pre-immune rabbit serum and affinity-purified anti-bear CBG antibody. 2.5 µg grizzly bear serum protein was loaded in each lane. B) Western blot immunodetected using an anti-human CBG antiserum (1:3000 dilution) as the primary antibody. Lane 1: grizzly bear reference serum (2.5 µg total serum protein), Lanes 2-4: serially diluted human CBG (20 ng, 200 ng and 2000 ng hCBG, respectively). The hCBG antibody does not detect gbCBG in grizzly bear serum. C) Western blot using nthe affinity-purified anti-bear CBG antibody (1:3000 dilution) as the primary antibody. Lane 1: grizzly bear reference serum (2.5 µg total serum protein), Lanes 2-4: serially diluted human CBG (20 ng, 200 ng and 2000 ng hCBG, respectively). Only the grizzly bear reference serum CBG is detected using this antibody. D) 270 ng/lane recombinant gbCBG immunodetected using the affinity-purified anti-bear CBG antibody (1:3000 dilution) as the primary antibody.

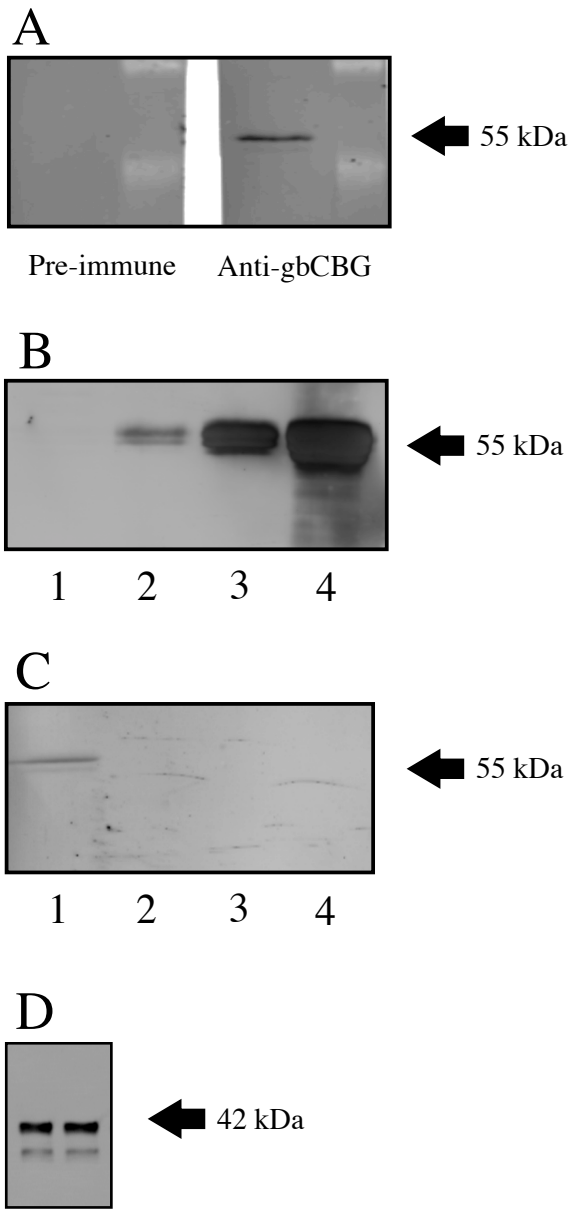

A
55 kDa
Pre-immune
Anti-gbCBG
B
55 kDa
1
2
3
4
C
55 kDa
1
2
3
4
D
42 kDa

Figure 3.6: Anti-bear CBG Antibody Validation: Serial Dilution
Serially diluted serum from A) grizzly, B) polar, and C) black bears were immunodetected using 1:3000 diluted anti-bear CBG antibody. Total serum protein (μg) loaded per lane is indicated inset.

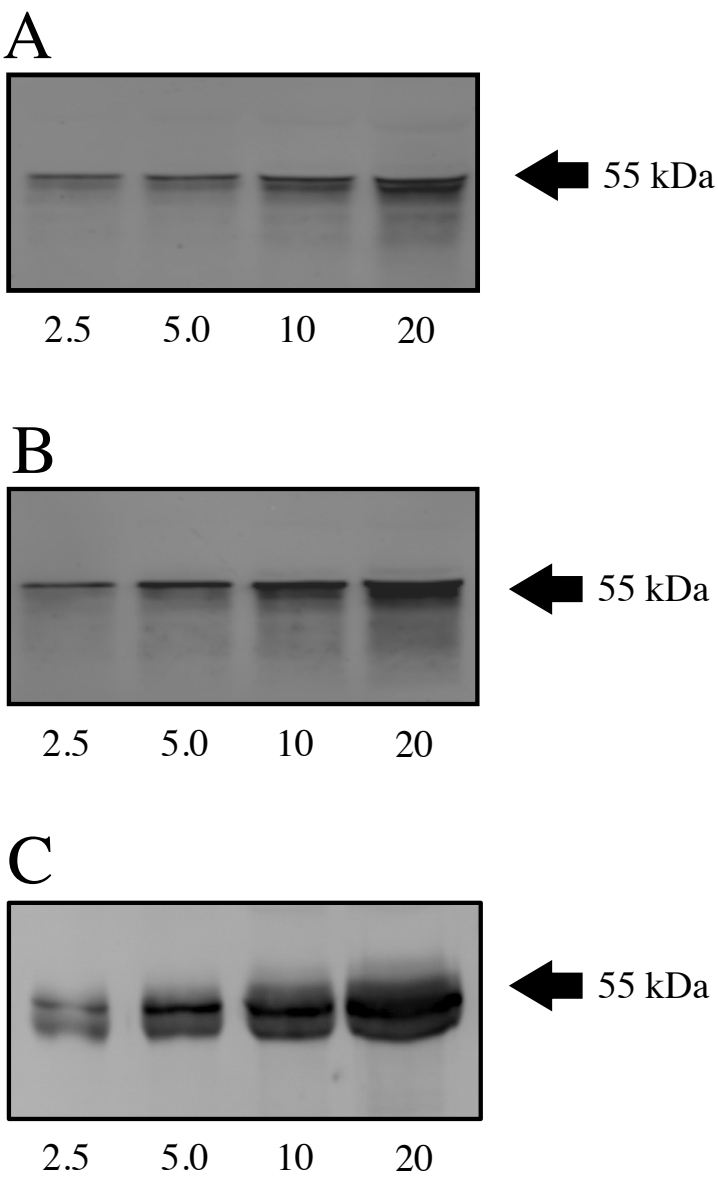

A
55 kDa
2.5
5.0
10
20
B
55 kDa
2.5
5.0
10
20
C
55 kDa
2.5
5.0
10
20

Figure 3.7: CBG ELISA Standard Curve and Bear Sera Dilution Series.
The standard curve used recombinant grizzly bear CBG from 18.6 – 373 pM (closed black circles, ●). Grizzly bear serum (red squares, ■), polar bear serum (blue squares, ■), black bear serum (green squares, ■), fish plasma (open diamonds, ◇), and bovine serum album (open triangles, △) were diluted, and 0.25 – 5 µL of this diluted serum was loaded into duplicate wells. X-axes are plotted on log-scale.

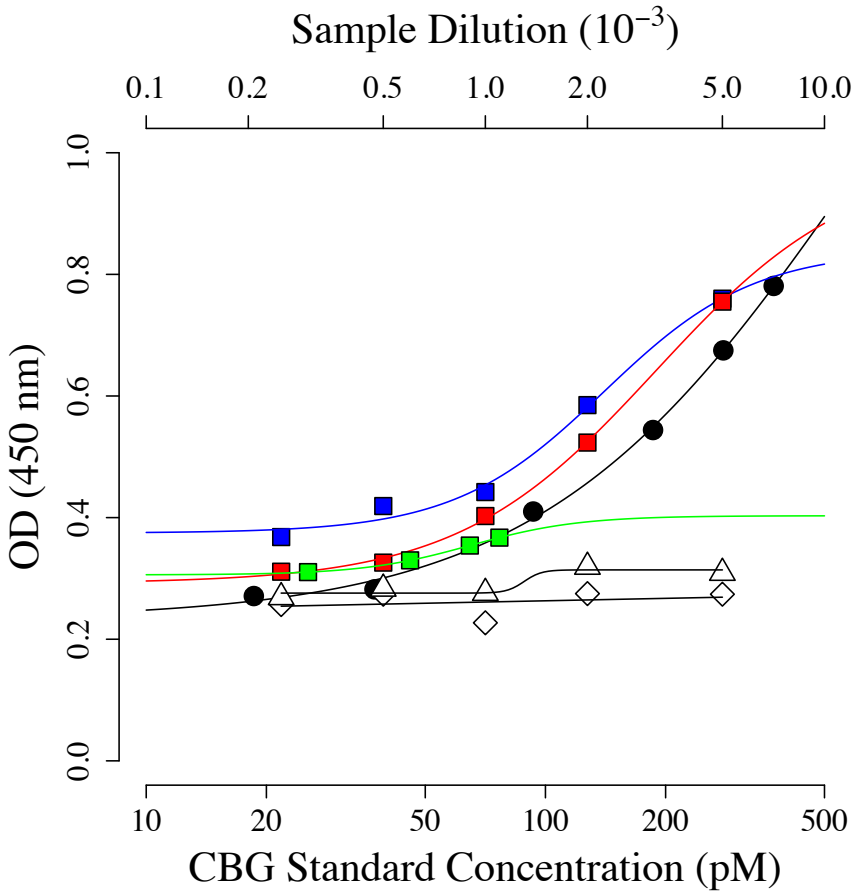

Sample Dilution (10^{-3})
0.1
0.2
0.5
1.0
2.0
5.0
10.0
OD (450 nm)
0.0
0.2
0.4
0.6
0.8
1.0
10
20
50
100
200
500
CBG Standard Concentration (pM)

Table 3.2: Percent Recovery of Recombinant Grizzly Bear Corticosteroid Binding Globulin Added to Rabbit Serum.
Corticosteroid binding globulin concentrations are expressed as mean ± standard error of the means (n = 6). The expected CBG concentration is the added recombinant grizzly bear CBG plus the observed CBG concentration of the rabbit serum with no recombinant CBG added.

Added CBG (nM)	Measured CBG (nM)	Background Subtracted CBG (nM)	Recovered CBG (%)
0.373	0.400 ± 0.0386	0.391	105%
0.279	0.288 ± 0.0305	0.279	100%
0.186	0.229 ± 0.0140	0.220	118%
0.0932	0.0821 ± 0.00482	0.0732	78.6%
0.0373	0.0439 ± 0.00324	0.0350	93.9%
0.0186	0.0221 ± 0.00434	0.0131	70.6%
0.000	0.00894 ± 0.00179	0.000	NA
		Average Recovery	94.3%

Table 3.3: Specificity (Dilutional Linearity) of the Corticosteroid Binding Globulin ELISA.
0.25 – 10.0 μL of 1:1000 diluted grizzly bear serum was coated to duplicate wells. Corticosteroid binding globulin concentrations are dilution corrected and expressed as mean ± standard error of the means (n = 2).

Volume of Sample Loaded (µL)	Dilution Corrected CBG Concentration (nM)
10	254 ± 10.3
7.5	366 ± 2.84
5.0	438 ± 6.92
2.0	478 ± 8.43
1.0	508 ± 7.99
0.5	463 ± 23.1
0.25	426 ± 49.7

Figure 3.8: Serum Cortisol Dynamics in Black, Grizzly, and Polar Bears.
Mean + SEM concentrations between black, grizzly, and polar bears of A) CBG, B) total cortisol, C) free cortisol, and D) glucose. The bias-corrected E) CBG and F) free cortisol means + SEM are also shown. Statistically significant differences between means are indicated by different letters.

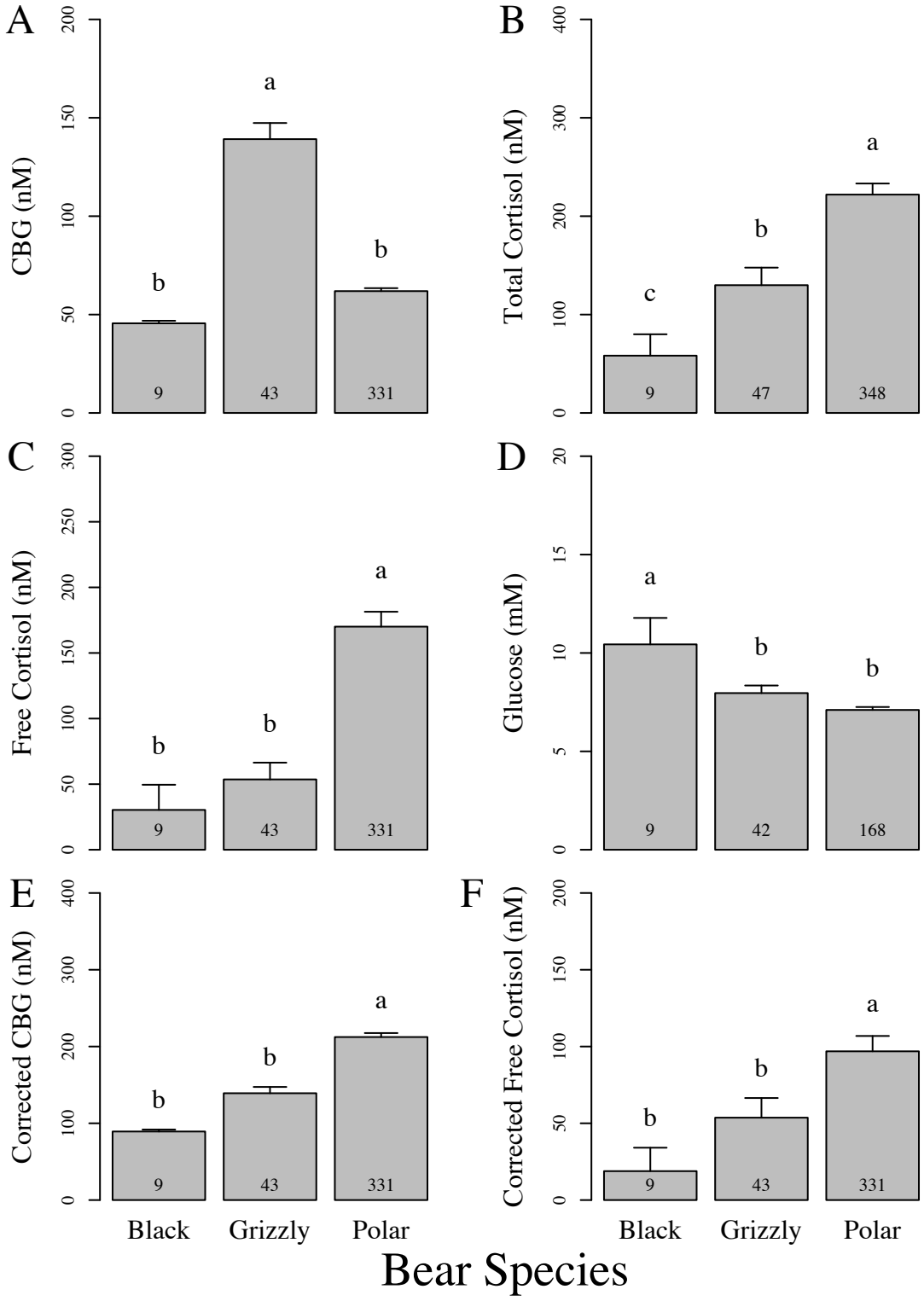

A
CBG (nM)
0
50
100
150
200
b
a
b
9
43
331
B
Total Cortisol (nM)
0
100
200
300
400
c
b
a
9
47
348
C
Free Cortisol (nM)
0
50
100
150
200
250
300
b
b
a
9
43
331
D
Glucose (mM)
0
5
10
15
20
a
b
b
9
42
168
E
Corrected CBG (nM)
0
100
200
300
400
b
b
a
9
43
331
F
Corrected Free Cortisol (nM)
0
50
100
150
200
b
b
a
9
43
331
Black
Grizzly
Polar
Bear Species

Table 3.4: Serum or Plasma Glucocorticoid Dynamics in Selected Placental Mammals. CBG, total (tCORT) and free glucocorticoid (fCORT) levels were extracted from publications. Data were converted to nM if they were reported in other units. % fCORT is calculated as the percentage of free to total glucocorticoid concentrations.

CBG (nM)	tCORT (nM)	fCORT (nM)	fCORT (%)	Animal	Sex	Reference
45.6	58.2	30.4	52%	Black Bear	F	This study
139	130	53.6	41%	Grizzly Bear	Both	This study
61.9	222	172	77%	Polar Bear	Both	This study
60	187	144	77%	Arctic Ground Squirrel	M	[56]
230	4	1	25%	Arctic Ground Squirrel	F	[56]
242	363	157	43%	Arctic Ground Squirrel	M	[57]
570	595	36	6%	Baboon	M	[58]
524	490	34	7%	Baboon	F	[58]
193	687	485	71%	Brown Lemmings	M	[39]
5195	2900	144	5%	Brown Lemmings	F	[39]
185	54	10	19%	Brown Lemur	M	[58]
398	51	7	14%	Brown Lemur	F	[58]
34	284	111	39%	Capuchin	M	[58]
134	395	182	46%	Capuchin	F	[58]
110	21	2	10%	Cattle	F	[4]
831	660	46	7%	Cynomolgus Macaque	F	[58]
409	647	78	12%	Cynomolgus Macaque	M	[58]
1180	560	2	0.4%	Cynomolgus Monkey	Both	[4]
82	23	2.3	6%	Dog	M	[4]
220	130	15	6%	Horse	Both	[4]
633	352	13	12%	Human	Both	[59]
1330	1251	125	4%	Japanese Macaque	F	[58]
853	847	68	10%	Japanese Macaque	M	[58]
5406	1624	45	8%	Meadow Vole	M	[60]
6078	3016	29	3%	Meadow Vole	F	[60]
770	130	8	1%	Mouse	M	[61]
1553	1821	107	6%	Prairie Vole	M	[62]
799	205	26	13%	Rat	M	[62]
1913	2025	312	6%	Red squirrel	M	[57]
569	715	79	15%	Rhesus Macaque	F	[58]
370	519	67	11%	Rhesus Macaque	M	[58]
289	348	90	13%	Richardson's Ground Squirrel	M	[63]
472	254	33	26%	Ring-tail Lemur	F	[58]
346	182	24	13%	Ring-tail Lemur	M	[58]
78	50	7	13%	Sheep	F	[4]
66	6240	3120	14%	Silver Marmoset	M	[58]
101	4483	2152	50%	Silver Marmoset	F	[58]
0	1600	777	48%	Squirrel Monkey	M	[4]
38	698	294	42%	Titi Monkey	M	[58]
132	1277	396	31%	Titi Monkey	F	[58]

CBG primary sequence was most closely related to gbCBG was the dog, where the nucleotide and amino acid sequences were 90% and 83% identical, respectively. Many residues in the ligand-binding domain of grizzly bear CBG and other mammalian species were identical to previously reported steroid-binding residues in rat CBG [15], including arg-36, ala-39, val-43, gln-254, thr-262, arg-282, ile-285, gln-286, phe-388, and trp-393. However, ile-285 of gbCBG was not conserved in opossum, and ser-289 and his-390 of gbCBG were not conserved in rat [15, 24]. The binding residues glu-256, gln-286, and glu-290 were less conserved across the mammalian species compared. The conserved cysteine residue that was observed in all CBGs was also present in gbCBG and may be playing a key role in steroid binding to this protein [24].

Also, four N-linked glycosylation consensus sites were present in gbCBG, which is two fewer than in human [25] and rat [26], and one less than sheep [27]. However, the fifth consensus glycosylation site in hCBG has been reported to be non-essential for protein secretion [28] and was not conserved in gbCBG and dog CBG. The N-glycosylation site at asn-260, which is essential for the formation of a high-affinity steroid binding site in humans [28], aligned with identical residues in CBG of other species, including grizzly bear. Likewise, the glycosylation site at asn-95 was also conserved among mammals, the only exception being the sheep, and the removal of this residue along with asn-260 by site-directed mutagenesis significantly reduce recombinant human CBG expression in CHO cells [28]. Together the sequence characteristics of gbCBG confirmed that this protein is well conserved, and support a critical role in corticosteroid transport and tissue availability in mammals.

3.4.2. Grizzly Bear CBG Antibody and ELISA Validation

Using the deduced amino acid sequence of gbCBG, we developed a peptide-based polyclonal antibody to detect serum CBG content in bears. The differences between the predicted molecular mass of gbCBG, the recombinant gbCBG, and the observed molecular mass of immunodetected bear CBG in sera on reducing SDS-PAGE gels (45,619 Da, ~42,000 Da, ~55,000 Da, respectively) were likely due to post-translational modifications at the N-glycosylation consensus sites for the latter. The addition of carbohydrate chains at these sites is

the source of variation between predicted and observed molecular mass in other species [28]. The doublets seen in bear CBG were similar to observations in rat [29], rabbit [30], human [31], and sheep [27], and are likely the result of glycosylation variants or isoforms, as shown previously in rat CBG [29]. While the lack of consistency in seeing a CBG doublets in bear sera is unclear in the present study, it is known that glycosylation patterns of CBG can be altered by hormones, including dexamethasone, insulin, thyroxine, and estradiol [27, 32]. It remains to be seen if such hormonal modulation is involved in the differential expression of CBG doublets in bear sera.

This CBG ELISA represents the first report of an assay for this protein specific for grizzly bears that also cross-reacts with black and polar bear CBG. Generally, CBG concentrations in free-ranging animals are indirectly measured by saturation binding experiments or maximum corticosteroid binding capacity assays because antibodies that cross-react with CBG in these species are in general not available. This ELISA represents a method to quantify this protein directly in bear sera and without the use of radioisotope-labeled tracer. The performance characteristics of the grizzly bear CBG ELISA compared favorably with other CBG ELISAs [32, 33, 34, 35, 36]. This ELISA was capable of detecting CBG at picomolar concentrations and exhibited good recovery of recombinant CBG.

3.4.3. CBG and Cortisol in Black, Grizzly, and Polar Bears

CBG, total cortisol, and glucose concentrations were measured in bear sera, and clear differences in these serum components were observed between black, grizzly, and polar bears. CBG and cortisol concentrations in these bear species may be modulated by biological and environmental factors, as seen in other species [37, 38, 39], and will be analyzed in greater detail in subsequent chapters.

3.4.3.1. CBG

The mean serum CBG concentrations in bears were in general in the lower portion of the range of values in the literature for other mammals (Table 3.4; see also other compilations in references [40, 41]). CBG levels in dogs (*Canis lupis familiaris*) and cats (*Felis domesticus*)

were similar to levels in bears (~80–180 nM). Information on CBG concentrations in other carnivorans are scant, although at least one study [42] reported serum binding capacities for corticosteroids in foxes (*Vulpe fulva*) and ferrets (*Mustela nigripes*) that were comparable to levels in dogs and cats.

As a measure of validation for the CBG ELISA, mean CBG concentrations were compared to the mean MCBC levels (reported in Chapter 2) of black, grizzly, and polar bears. However, the results of this comparison suggested that the MCBC assay may be overestimating CBG concentrations in bear sera or vice versa. There were large discrepancies between the mean MCBC and CBG concentrations for all three species, with MCBC levels higher than CBG levels. The MCBC assay measured the amount of cortisol bound to the protein in serum, and CBG concentrations were estimated based on the assumption that one mole of cortisol is bound per mole CBG. In contrast, the ELISA directly measures CBG protein concentrations. The underlying causes of the differences between the results of these assays are unclear at this time, but we suggest that the elevated MCBC levels may reflect non-specific binding that was not accounted for in the assays. Altogether, the validation of the antibody and ELISA presented in this study suggests that the ELISA is suitable for comparing CBG levels between individual samples within the species with which the assay was validated.

3.4.3.2. Cortisol

It is generally accepted that to obtain baseline measurements of cortisol, sampling must occur within 3 min of the initiation of capture [43]. Blood sampling within 3 min of capture was not feasible for free-ranging bears, so the total cortisol levels reported in this investigation were likely elevated above baseline, unstressed levels because blood sampling generally occurred > 30 min after the initiation of capture. Additionally, the action of anesthetics may themselves induce a glucocorticoid response [44]. Our results support these inferences because the mean proportion of free to total cortisol in black, grizzly, and polar bears were 52, 41, and 78%, respectively. These proportions were elevated over the 10% unbound to total proportion that are reported in unstressed animals (Table 3.4; also see [4, 40]).

Despite the lack of physical exertion associated with capture in free-ranging ursids, the captive black bears used in this study had similar total and free cortisol levels as free-ranging grizzly

bears captured by helicopter darting, although the *post hoc* p-value was marginal ($p < 0.10$). These levels of cortisol in black bears were similar to those in other reports with captive black bears [45]. In contrast, Palumbo and coworkers [9] reported that total corticosteroids (the sum of cortisol, corticosterone, and 11-deoxycortisol) in captive black bears in the fall season to be over 7.8 µg/dL (approximately 200 nM when converted using the molar mass of cortisol, 364.42 g/mol). Other studies with free-ranging black bears reported higher cortisol concentrations, likely due to the increased acute stress of capture by leg-hold snaring or culvert trapping [8, 46].

Moreover, both grizzly and polar bears used in the comparisons in Figure 3.8 were captured by helicopter darting, which has been shown to induce a lower cortisol response than leg-hold snaring in grizzly bears [10]. Indeed, total cortisol levels for black and grizzly bears captured by leg-hold snaring and culvert trapping were higher than the mean levels reported here [8, 10, 46]. In comparison, the total cortisol levels after helicopter darting was higher in polar bears relative to the terrestrial bears, and these values in the former were comparable to those in reported for animals captured from Svalbard, Norway (ranges from 109 to 557 nM [47, 48, 49]).

The factors underlying variations in species-specific total cortisol concentrations are largely unknown. There are several hypotheses to explain this variation in house sparrows, which exhibit seasonally elevated total cortisol and CBG levels during the breeding season [40]. Breuner and coworkers posit that the increased pool may allow for cellular access to cortisol without a concomitant elevation in adrenal activity or after the adrenal glands are inactive. CBG is related to the serpin protease inhibitor superfamily of proteins [2], and specific cleavage of CBG by proteases causes a decrease in the binding affinity of CBG for cortisol, leading to the localized elevation of cortisol concentrations [5]. Furthermore, decreases in serum CBG have been observed after exposure to acute stressors [50], which may lead to sustained increases in free cortisol levels without concomitant increased adrenal activity. CBG-bound cortisol has also been proposed to have biological activity via binding to specific cell membrane receptors and activating adenylyl cyclase activity [51]. This may allow for the specific delivery of cortisol to tissues, as opposed to the non-specific deliver of free cortisol to

all tissues. However, the contribution of these alternative pathways for cortisol delivery in bears is currently unknown.

3.4.3.3. Glucose

Glucose levels were significantly elevated in black bears relative to the other two species, which did not corroborate with the patterns of unadjusted and the bias-corrected mean CBG and free cortisol values between the bear species. However, the anaesthetic combination used to sedate black bears (ketamine-xylazine, "KX") was different from that used in grizzly and polar bears (xylazine-zolazepam-tiletamine, "XZT"). To my knowledge, the physiological and metabolic effects of these anaesthetic combinations have never been directly compared in the literature, but these anaesthetics may be acting on adrenergic receptors to induce hyperglycemia [52]. In particular, ketamine may induce hyper- and hypoglycemia at low and high doses, respectively, in rabbits [53]. Xylaxine causes hyperglycemia by inhibiting insulin secretion without affecting glucagon [54]. XZT is known to induce a greater glucose response in polar bears relative to ZT alone (8.6 versus 6.2 mM) [12], and KX induced hyperglycemia to a maximum of 16.2 mM from baseline values of 5.8 mM in fed rats [55].

Furthermore, there were differences in the sampling methodology between the captive black bears and the free-ranging grizzly and polar bears. For the latter samples, the separation of liquid serum from the solid cell and clotting factor fractions did not occur until up to 8 h after collection. In contrast, serum was collected and frozen more rapidly for black bears because of the proximity of the lab equipment to the sampling locations. This difference in processing time may have contributed to the elevated glucose concentrations in black bear sera because glucose is metabolized by the cellular fraction of blood until separation. Thus, it may not be valid to directly compare serum glucose values of black to grizzly and polar bears because of the different methodologies employed, but this remains to be tested.

3.4.4. Conclusions

In conclusion, we have developed for the first time an ELISA that quantitatively measures serum CBG in the sera of grizzly and polar bears, and using this data, we reported differences

in serum CBG and total and free cortisol concentrations between black, grizzly, and polar bears. This CBG ELISA will be employed for subsequent studies to determine the effect of life-history variables, including age, sex, and reproductive and nutritional statuses, on cortisol dynamics in ursids.

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Chow, B. A., Hamilton, J., Cattet, M. R. L., Stenhouse, G. B., Obbard, M. E., Vijayan, M. M. (2010) Serum corticosteroid binding globulin expression is modulated by fasting in polar bears (*Ursus maritimus*). Comp Biochem Physiol A Physiol 158: 111-115.

CHAPTER 4:
MODULATION OF CORTISOL DYNAMICS BY CAPTURE METHODS, LIFE HISTORY TRAITS, AND SEASONAL DIETARY PATTERNS IN GRIZZLY AND POLAR BEARS

4.1. Introduction

Glucocorticoids play important adaptive roles in energy metabolism and reestablishment of homeostasis in animals [1]. The dynamics of cellular glucocorticoid hormone action are dependent, in part, on the bioavailability of this hormone to tissues. Corticosteroid binding globulin (CBG) binds to glucocorticoids reversibly and with high affinity, and bound steroid is unable to traverse plasma membranes to act on its intracellular receptors to mediate changes in gene transcription that underlie the major physiological actions of this steroid hormone. [2]. Thus, CBG is a major regulator of glucocorticoid dynamics in animals [3]. Moreover, its characterization is important to fully understand the dynamics of glucocorticoids because CBG expression may vary independently of total glucocorticoid secretion such that the variation in the free fraction does not necessarily covary with total glucocorticoid levels [2]. Thus, free glucocorticoid levels are more indicative of the biological effects of the hormone.

Anthropogenic-related environmental changes and activity are thought to be major factors contributing to decreased survival in some grizzly [4, 5] and polar bear populations [6, 7, 8, 9]. Widespread landscape changes in Alberta, Canada have resulted in the fragmentation of and increased human access to core grizzly bear habitats, which are linked to the unsustainable mortality rates of grizzly bears in this province [10]. Grizzly bears slowly reproduce due to late sexual maturity, small litter sizes, and long reproductive intervals [11], and the reproductive rates of some populations within Alberta, Canada are lower than populations elsewhere [12, 13]. These factors are thought to play roles in the poor performance and, combined with the small population sizes [10], the uncertain persistence of the grizzly bear populations in this

province [4, 10, 13, 14, 15]. Similarly, polar bears are sensitive to changes in sea ice conditions, and declines in the extent and duration of seasonal ice cover during the peak hunting season (spring and early summer) that are associated with climatic warming may negatively impact the reproductive success and health of this species [6, 7, 16, 17]. Indeed, long-term declines in the body condition of polar bears in the Western [16] and Southern Hudson Bay regions [18] are linked to climatic warming and declining sea ice conditions [6].

These environmental changes may constitute chronic stressors acting on these animals, but this has not been tested in bears. In other mammals, chronic stressors have been associated with changes in glucocorticoid dynamics, including elevated baseline glucocorticoid levels [19] and decreased CBG concentrations [20], leading to elevated free levels of glucocorticoids and downstream detrimental effects on reproduction [21], growth [22], and immunity [23]. The development of chronic stress in a population may lead to negative impacts on population health and persistence, including increased mortality and decreased reproductive performance [24]. Thus, the monitoring of glucocorticoid and CBG levels in free ranging animals may be an important tool that wildlife managers may utilize to assess the health of animals [25].

However, circulating glucocorticoid and CBG levels may be modulated by various endogenous and exogenous factors, and the characterization of these factors are an important step in the development of any physiologic marker to be used for wildlife monitoring in bears. During periods of high metabolic demand and/or low nutrient availability, including reproduction and seasonal fasting, the secretion, response to stress, bioavailability, and/or tissue sensitivity of glucocorticoids may be altered to help the animal adapt to changing environmental conditions [20, 26, 27]. Additionally, glucocorticoid dynamics may also be modulated by age and sex (e.g. [28, 29]), reproductive status (e.g. [30]), nutritional status (e.g. fat [31] and/or protein content of diet [32]), fasting status (e.g. [33]), and body condition (e.g. [34]). Another important consideration is that the act of capture and sampling itself initiates a stress response that may obscure the baseline, pre-capture levels of glucocorticoids [35], and the magnitude of this response is associated with the severity of the stress [36]. Thus, the factors that modulate glucocorticoid dynamics must be identified and characterized to establish normal ranges to which future data may be compared.

The objectives of this study were to characterize the variation in serum cortisol dynamics, including CBG, cortisol, and glucose concentrations, in free-ranging grizzly and polar bears in response to capture stress in the former species, and reproductive status and variations in nutritional and dietary patterns in both. We wanted to test the hypotheses: 1) serum CBG levels do not respond to capture stress within the handling time frame, and 2) variations in cortisol dynamics may be playing adaptive roles in the life history and dietary patterns of these animals.

4.2. Methods and Materials

4.2.1. Animals

For the serum CBG and total and free cortisol determinations in grizzly and polar bears, samples from 174 grizzly bears and 355 polar bears were used. Grizzly bears were captured by one of three methods: 29 by culvert trapping (CT), 47 by remote injection of anesthetic from a helicopter (HD), and 98 by leg-hold snaring (LGS) [37]. All animals were anesthetized by remote drug delivery prior to handling using a combination of xylazine and zolazepam–tiletamine (XZT) administered intramuscularly as xylazine (Cervizine 300; Wildlife Pharmaceuticals, Inc., Fort Collins, Colorado) at 2 mg/kg and Telazol (Fort Dodge Laboratories, Inc., Fort Dodge, Iowa) at 3 mg/kg estimated body weight. Blood was collected by venipuncture from the jugular vein into sterile tubes, and the sample was centrifuged within 8 h of collection to extract serum (and this was stored frozen at –20°C) for analysis. At the conclusion of handling, atipamezole (Antisedan; Novartis Animal Health Canada Inc., Mississauga, Ontario, Canada) was administered at 0.15 – 0.20 mg/kg, half-volume intramuscularly and half-volume intravenously, to reverse the effects of xylazine. The capture and sampling protocol was reviewed and approved by the University of Saskatchewan's Committee on Animal Care and Supply, and was in accordance with guidelines provided by the American Society of Mammalogists' Animal Care and Use Committee [38] and the Canadian Council on Animal Care [39].

Sera were collected from polar bears that were captured and sampled in conjunction with a long-term research project in the Canadian Arctic conducted by the Ontario Ministry of Natural Resources. The samples were collected and processed similarly to grizzly bears captured by heli-darting (above), with sera frozen within 8 h of collection and maintained frozen (–20°C) in long-term storage at the University of Saskatchewan. The research protocols for both projects were approved annually by animal care committees at the University of Saskatchewan and Ontario Ministry of Natural Resources and was in accordance with guidelines provided by the American Society of Mammalogists' Animal Care and Use Committee [38] and the Canadian Council on Animal Care [39] for the safe handling of wildlife.

We assessed the impact of some biological variations on cortisol dynamics in these two bear species, including capture method, reproductive status and season, age, and nutritional status. The effect of capture method (culvert trapping [CT], helicopter darting [HD], and leg-hold snaring [LGS]) on cortisol dynamics will be tested. Polar bears, on the other hand, were captured only by helicopter darting and, thus, the effects of capture method on serum cortisol dynamics were not analyzed for this species.

Reproductive and age classes in grizzly and polar bears were analyzed as factors: solitary adult females (SF), adult females with dependent cubs or yearlings (AF), adult males (AM), juvenile females (JF), and juvenile males (JM). Furthermore, adults and juveniles were aggregated and analyzed separately.

Grizzly bears were grouped into nutritional seasons, which were defined by seasonal food habits as per Munro [40]. Animals captured prior to the 166th Julian day (June 15th) were in the hypophagic season (Season 1), prior to 220th Julian day (August 16th) were in the early hyperphagic season (Season 2), and after that in the late hyperphagic season (Season 3). No sampling occurred during the winter denning period.

The interactions between some of these factors were also considered. The interaction between reproductive class and nutritional season was included in these analyses because the pattern of weight changes during different nutritional seasons varies between reproductive classes in grizzly bears [41]. Also, we were interested in investigating the interaction between capture

method and reproductive class. We also investigated the possible effect of urea to creatinine (UC) ratios and body condition indices on cortisol dynamics. UC ratios were used as a surrogate for dietary protein intake in grizzly bears (UC < 10 indicates low dietary protein), and as an indicator of fasting status in polar bears (UC < 10 indicates fasting). The body condition index is an indicator of the true body condition of bears, and is based on residuals from the regression of total body mass against straight-line body length [42].

4.2.2. Serum Assays

Serum CBG concentrations were determined using the homologous grizzly bear CBG ELISA as detailed in Chapter 3. Total cortisol concentrations were determined by a commercial ^{125}I radioimmunoassay kit (MP Biomedicals, Orangeburg, NY), as described in previous chapters. Total cortisol values for grizzly and polar bears sampled prior to 2008 were taken from Hamilton [43]. Serum glucose concentration was determined by the glucose oxidase-peroxidase method as described previously [44], using glucose (Sigma) as the standard. Absorbance was measured at 500 nm on a VersaMax microplate reader. Urea and creatinine concentrations were determined in bear sera using an Abbott Spectrum H Series II biochemistry analyzer (Abbott Laboratories Diagnostic Division, Abbott Park, IL, USA).

4.2.3. Calculations and Statistics

4.2.3.1. Free Cortisol Calculations

Calculations and statistical significance testing was conducted in R 2.14.0. Free cortisol was calculated using the equation of Barsano and Baumann [45]:

$$fCORT = \frac{-\left(CBG - tCORT + \frac{1}{K_a}\right) \pm \sqrt{\left(CBG - tCORT + \frac{1}{K_a}\right)^2 - 4\left(\frac{-tCORT}{K_a}\right)}}{2}$$

Where *fCORT* is free cortisol concentration (mol/L), *CBG* is CBG concentration (mol/L), *tCORT* is total cortisol concentration (mol/L), and $K_a = 1/K_d$ = equilibrium association constant (L/mol).

4.2.3.2. Body Condition Index Calculations

The body condition index (BCI) of bears is a morphometric-based measure of the true body condition (fat plus lean body mass) of these animals [42]. In other species, poor body condition is associated with altered HPA function (e.g. [21, 31, 46]). BCI can be used to compare the body condition of grizzly and polar bears within species, and is thought to be independent from some of the other factors used in these analyses [42]. The formula used to calculate BCI in grizzly bears is:

$$BCI = \frac{\ln(TBM) - 3.07 \times \ln(SLBL) + 10.76}{0.17 + 0.009 \times \ln(SLBL)}$$

Where *TBM* is the total body mass as determined by suspending the bear from a spring-loaded or electronic load scale, and *SLBL* is the straight line body length from the tip of the nose to the last vertebra of the tail. The BCI formula for polar bears is:

$$BCI = \frac{\ln(TBM) - 3.21 \times \ln(SLBL) + 11.64}{0.29 - 0.017 \times \ln(SLBL)}$$

4.2.3.3. Statistics

Values are shown as mean + standard error of the means (SEM). To determine the relationships between cortisol dynamics and the biological variables that were outlined above, we analyzed the variation of the dependent variables (CBG, total and free cortisol, and glucose concentrations) using univariate ANCOVAs with capture method, reproductive class, and nutritional season as categorical variables, and urea to creatinine ratio and body condition index as continuous predictor variables for grizzly bears. In polar bears, only reproductive class was used as a factor (**Table 4.1**). Due to the limiting sample size, the three-way interactions between the categorical factors in the grizzly bear analyses were not included, but the interactions between nutritional season and reproductive class, and between capture method and reproductive class were considered. Variables were natural log transformed as required to

meet the parametric assumption of normality. *Post-hoc* significance testing was performed using Tukey's Honest Significant Differences test.

4.3. Results

4.3.1. Cortisol Dynamics in Grizzly Bears

Sera were collected from 174 grizzly bears throughout their range in Alberta, Canada. The means of CBG, total and free cortisol, and glucose concentrations in grizzly bears within each categorical variable analyzed in the ANOVA models are presented in **Figure 4.1**, and the associated statistics are presented in **Table 4.2**. The associations between the dependent variables and continuous predictor variables (body condition index [BCI] and urea to creatinine [UC] ratio) are presented as scatterplots in **Figure 4.2** and **4.3**, respectively.

There were differences in mean total and free cortisol levels between capture methods (**Figure 4.1**). Total cortisol levels were elevated in animals captured by leg-hold snaring (LGS) relative to animals captured by both culvert trapping (CT) and helicopter darting (HD). Similarly, free cortisol levels were elevated in bears captured by LGS relative to HD, but grizzlies captured by CT had levels that were not statistically different from animals captured by either HD or LGS. There were no significant differences in CBG and glucose concentrations between the capture methods (**Figure 4.1**).

There were no effects of reproductive class on total and free cortisol and glucose concentrations (**Figure 4.1**). However, mean CBG concentrations were different between these classes, with levels in juvenile females (JF) being significantly lower than in solitary adult females (SF) and adult males (AM). Juvenile males (JM) and adult females with dependent cubs (AF) had intermediate mean CBG concentrations, but levels in JM were not significantly different ($p < 0.10$) compared to the AM group. Overall, CBG concentrations were lower in juveniles than adults ($F_{1,156} = 5.63$, $p < 0.05$).

No effect of nutritional season was found on CBG, cortisol, or glucose concentrations (**Figure 4.1**). The BCI of grizzly bears was not significantly associated with any of the dependent variables. Total and free cortisol levels were positively associated with urea to creatinine (UC) ratios (**Figure 4.3**; $p < 0.001$, adjusted $R^2 = 0.165$ and 0.132 for total and free cortisol, respectively).

4.3.2. Cortisol Dynamics in Polar Bears

Sera were collected from 355 polar bears from the Lancaster Sound, and Western and Southern Hudson Bay populations. Similar to grizzly bears, the CBG, total and free cortisol, and glucose concentration in these sera were determined, and the effects of reproductive class, UC ratio, and body condition index on the variation in these serum constituents were examined. The associated statistics are shown in **Table 4.2**.

CBG, free cortisol, and glucose concentrations were different between polar bear reproductive classes (**Figure 4.4**). JF animals had significantly lower CBG levels relative to AF and AM classes, and levels in JM were also lower than AM. Free cortisol levels were significantly elevated in JF relative to AF and AM. Glucose concentrations were significantly lower in AM relative to JM. CBG levels in juveniles were significantly lower than in adults ($F_{1,329} = 16.1$, $p < 0.001$).

BCI were not significantly associated with CBG, cortisol, or glucose concentrations in polar bears (**Figure 4.5**). Total ($p < 0.05$, adjusted $R^2 = 0.043$) and free cortisol concentrations ($p < 0.05$, adjusted $R^2 = 0.039$) were significantly negatively associated with UC ratios (**Figure 4.6**). There was no association between UC ratio and CBG and glucose concentrations.

Table 4.1: Factors Included in the ANOVA Models for Grizzly and Polar Bears.

Factor	Grizzly Bears		Polar Bears	
	Levels	n	Levels	n
Capture Method	Culvert trapping (CT)	29		
	Heli-darting (HD)	47		
	Leg-hold snaring (LGS)	98		
Reproductive Class	Adult Female (SF)	36	Adult Female	35
	Female with Dependents (AF)	26	Female with Dependents	82
	Adult Male (AM)	51	Adult Male	103
	Juvenile Female (JF)	17	Juvenile Female	55
	Juvenile Male (JM)	44	Juvenile Male	80
Nutritional Season	1: Hypophagic	119		
	2: Early hyperphagic	26		
	3: Late hyperphagic	29		

Table 4.2: Summary of Statistical Analyses on Serum Cortisol Dynamics in Grizzly Bears.

This table summarizes the effects of factors (Table 4.1) and covariates on the variation in A) CBG, B) total and C) free cortisol, and D) glucose levels. Capture method, nutritional season, and reproductive class were treated as factors. Body condition index and urea to creatinine (UC) ratio were treated as covariates in the ANCOVA models to analyze their effects on the dependent variables. Factors that had significant effects on the dependent variables were included in the respective ANCOVA models. Asterisks ('*') indicate statistical significance ($p < 0.05$), while periods ('.') indicate marginal significance ($p < 0.10$).

	Factor	df Effect	df Error	SS	F	p	
A) CBG	Capture Method	2	129	0.095	1.841	0.163	
	Reproductive Class	4	129	0.318	0.084	0.018	*
	Nutritional Season	2	129	0.049	0.949	0.390	
	Capture Method x Reproductive Class	8	129	0.399	1.933	0.060	.
	Reproductive Class x Nutritional Season	8	129	0.325	1.576	0.138	
	BCI	1	85	0.007	0.247	0.620	
	UC Ratio	1	85	0.005	0.168	0.683	
B) Total Cortisol	Capture Method	2	140	4.031	14.17	0.000	*
	Reproductive Class	4	140	0.850	1.494	0.207	
	Nutritional Season	2	140	0.222	0.780	0.460	
	Capture Method x Reproductive Class	8	140	0.572	0.590	0.785	
	Reproductive Class x Nutritional Season	8	140	0.853	0.749	0.648	
	BCI	1	89	0.095	0.665	0.417	
	UC Ratio	1	89	1.092	7.613	0.007	*
C) Free Cortisol	Capture Method	2	129	13.46	13.87	0.000	*
	Reproductive Class	4	129	3.218	1.659	0.164	
	Nutritional Season	2	129	0.807	0.832	0.437	
	Capture Method x Reproductive Class	8	129	4.639	1.196	0.307	
	Reproductive Class x Nutritional Season	8	129	4.649	1.198	0.305	
	BCI	1	81	0.563	1.108	0.296	
	UC Ratio	1	81	3.742	7.362	0.008	*
D) log(Glucose)	Capture Method	2	109	5.710	0.425	0.655	
	Reproductive Class	4	109	36.08	1.343	0.259	
	Nutritional Season	2	109	2.800	0.208	0.812	
	Capture Method x Reproductive Class	8	109	31.09	0.579	0.794	
	Reproductive Class x Nutritional Season	8	109	56.25	1.047	0.406	
	BCI	1	77	1.920	0.367	0.547	
	UC Ratio	1	77	14.34	2.736	0.102	

Figure 4.1: The Effect of Capture Method, Reproductive Class, and Nutritional Season on Serum Cortisol Dynamics in Grizzly Bears.
Means + SEM of dependent variables (CBG, total and free cortisol, and glucose) plotted against the factor levels of the ANOVA models used to determine the biological variation of these stress axis parameters in grizzly bears captured by all capture methods. Significantly different means, where applicable, are indicated by different letters and sample sizes are provided as inset.

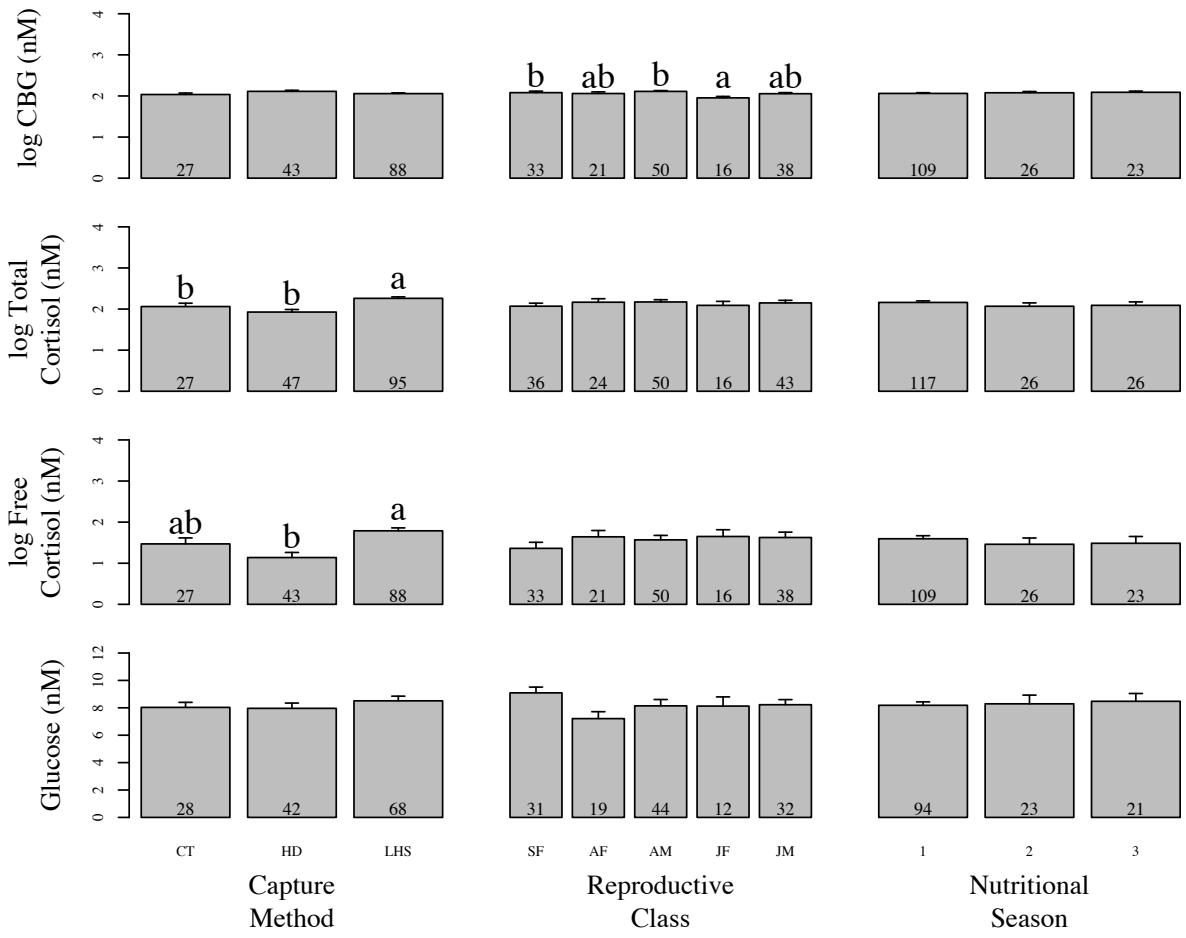

log CBG (nM)
log Total Cortisol (nM)
log Free Cortisol (nM)
Glucose (nM)
0 1 2 3 4
0 2 4 6 8 10 12
b ab b a ab
b b a
ab b a
27 43 88 33 21 50 16 38 109 26 23
27 47 95 36 24 50 16 43 117 26 26
27 43 88 33 21 50 16 38 109 26 23
28 42 68 31 19 44 12 32 94 23 21
CT HD LHS
Capture Method
SF AF AM JF JM
Reproductive Class
1 2 3
Nutritional Season

Figure 4.2: The Effect of Body Condition Index on Serum Cortisol Dynamics in Grizzly Bears.
Scatterplots of A) CBG, B) total and C) free cortisol, and D) glucose concentrations against the body condition index of grizzly bears. The trend lines were calculated by linear least squares regression. BCI was not significantly correlated with any of these dependent variables.

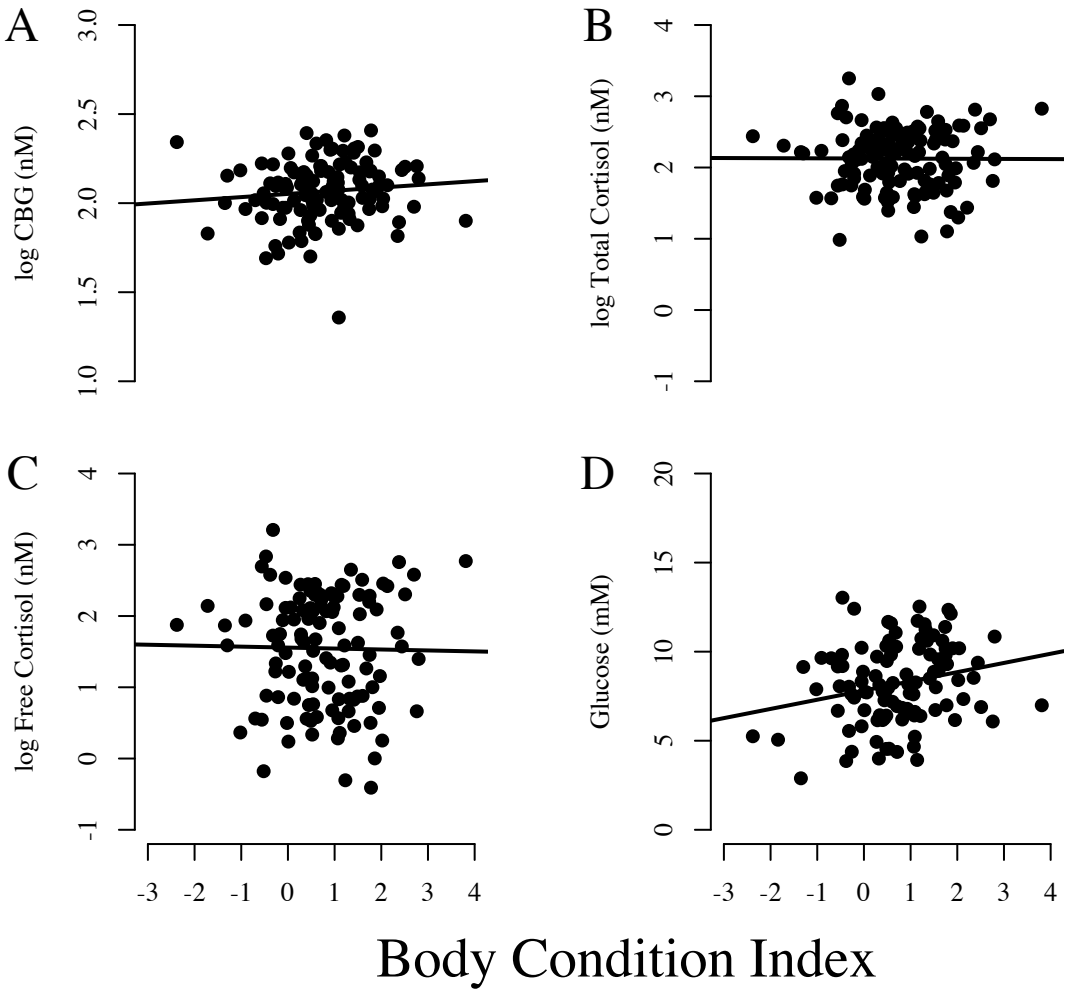

A
log CBG (nM)
1.0
1.5
2.0
2.5
3.0
B
log Total Cortisol (nM)
-1
0
1
2
3
4
C
log Free Cortisol (nM)
-1
0
1
2
3
4
D
Glucose (mM)
0
5
10
15
20
-3
-2
-1
0
1
2
3
4
Body Condition Index

Figure 4.3: The Effect of Urea to Creatinine Ratio on Serum Cortisol Dynamics in Grizzly Bears.
Scatterplots of A) CBG, B) total and C) free cortisol, and D) glucose concentrations against the urea to creatinine (UC) ratio in grizzly bears. The trend lines were calculated by linear least squares regression. Total cortisol was significantly negatively associated with UC ratio (ANOVA, $p < 0.05$, adjusted $R^2 = 0.129$), and free cortisol was marginally associated with UC ratio ($p < 0.10$).

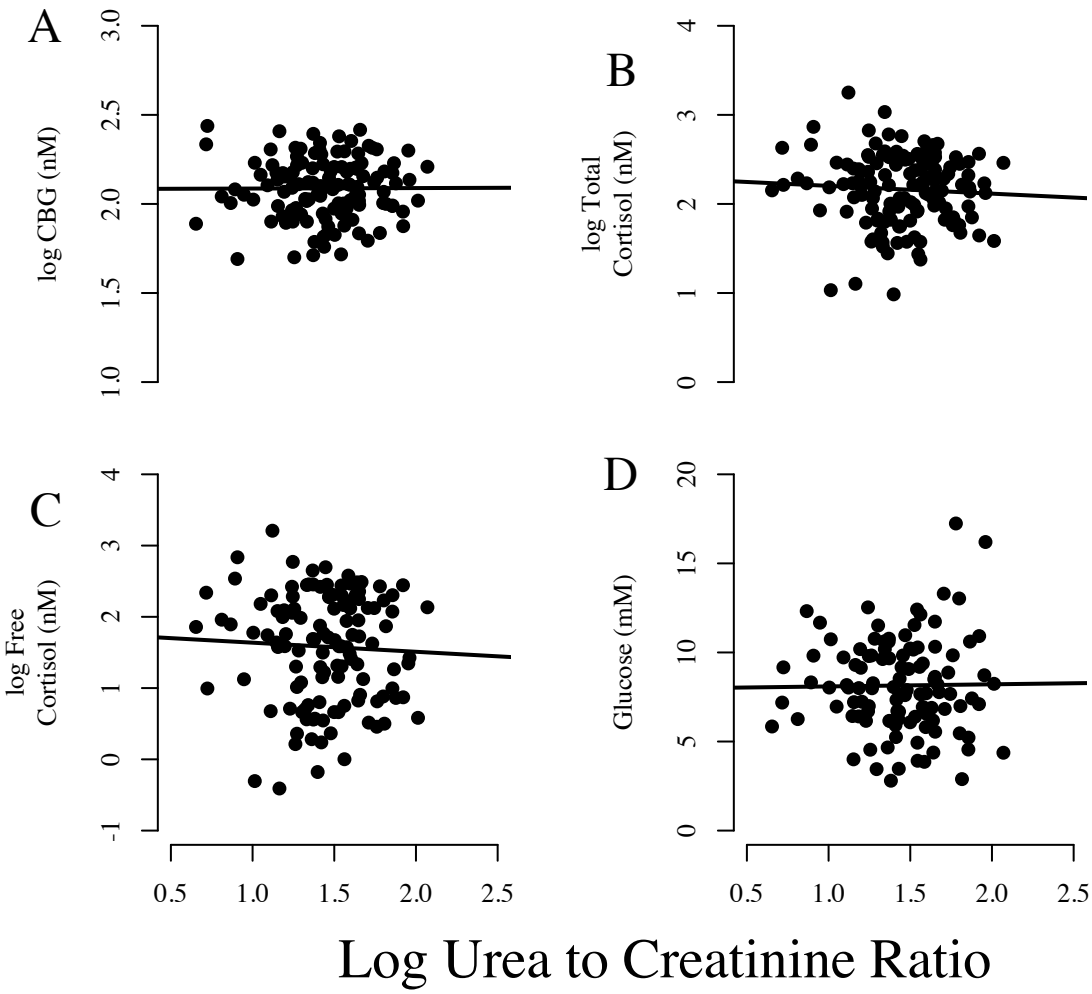

A
log CBG (nM)
1.0
1.5
2.0
2.5
3.0
B
log Total
Cortisol (nM)
0
1
2
3
4
C
log Free
Cortisol (nM)
-1
0
1
2
3
4
D
Glucose (mM)
0
5
10
15
20
0.5
1.0
1.5
2.0
2.5
Log Urea to Creatinine Ratio

Table 4.3: Summary of Statistical Analyses on Serum Cortisol Dynamics in Polar Bears. This table summarizes the effects of factors (Table 4.1) and covariates on the variation in A) CBG, B) total and C) free cortisol, and D) glucose variation in polar bears. Reproductive class was treated as a factor. Body condition index and urea to creatinine (UC) ratio were treated as covariates in the ANCOVA models to analyze their effects on the dependent variables. Reproductive class was included in the ANCOVA models for CBG and free cortisol analysis. Asterisks ('*') indicate statistical significance.

	Factor	df Effect	df Error	SS	F	p	
A) log(CBG)	Reproductive Class	4	326	1.121	4.711	0.001	*
	Body Condition Index	1	90	0.063	0.635	0.428	
	UC Ratio	1	90	0.094	0.948	0.333	
B) log(Total Cortisol)	Reproductive Class	4	343	1.089	1.912	0.108	
	Body Condition Index	1	97	0.044	0.270	0.605	
	UC Ratio	1	97	1.041	6.412	0.013	*
C) log(Free Cortisol)	Reproductive Class	4	326	3.432	2.951	0.020	*
	Body Condition Index	1	90	0.042	0.124	0.726	
	UC Ratio	1	90	1.782	5.220	0.025	*
D) Glucose	Reproductive Class	4	163	46.53	3.269	0.013	*
	Body Condition Index	1	24	1.395	0.931	0.344	
	UC Ratio	1	24	0.475	0.317	0.579	

Figure 4.4: The Effect of Reproductive Class on Serum Cortisol Dynamics in Polar Bears. Means + SEM of dependent variables (CBG, total and free cortisol, and glucose) plotted against the factor levels of the ANOVA models used to determine the biological variation of these stress axis parameters in polar bears. Statistically significant differences in means are indicated by different letters and sample sizes are provided as inset in each figure.

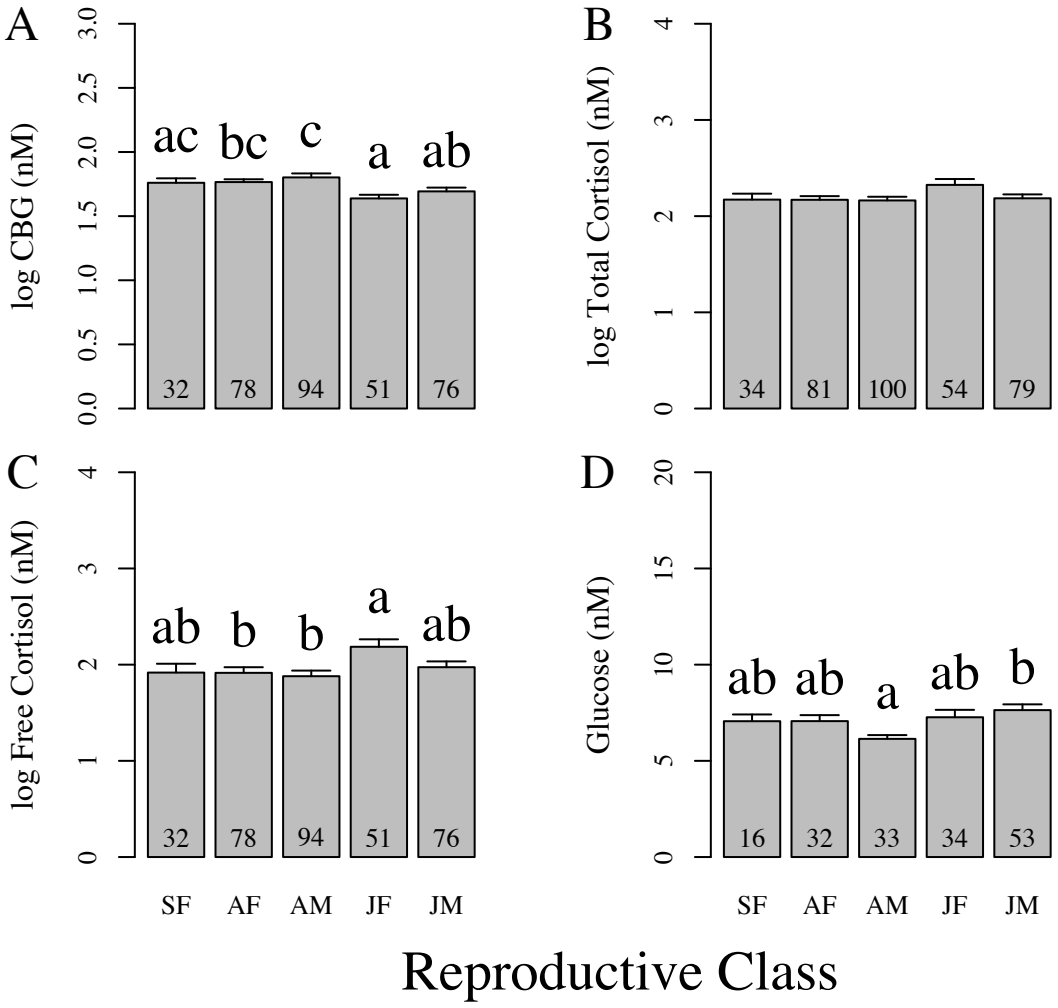

A
log CBG (nM)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
ac
bc
c
a
ab
32
78
94
51
76
B
log Total Cortisol (nM)
0
1
2
3
4
34
81
100
54
79
C
log Free Cortisol (nM)
0
1
2
3
4
ab
b
b
a
ab
32
78
94
51
76
D
Glucose (nM)
0
5
10
15
20
ab
ab
a
ab
b
16
32
33
34
53
SF
AF
AM
JF
JM
Reproductive Class

Figure 4.5: The Effect of Body Condition Index on Serum Cortisol Dynamics in Polar Bears.
Scatterplots of A) CBG, B) total and C) free cortisol, and D) glucose concentrations against the body condition index of polar bears. The trend lines were calculated by linear least squares regression. None of dependent variables were significantly associated with the body condition index, but free cortisol levels were marginally associated with BCI ($p < 0.10$).

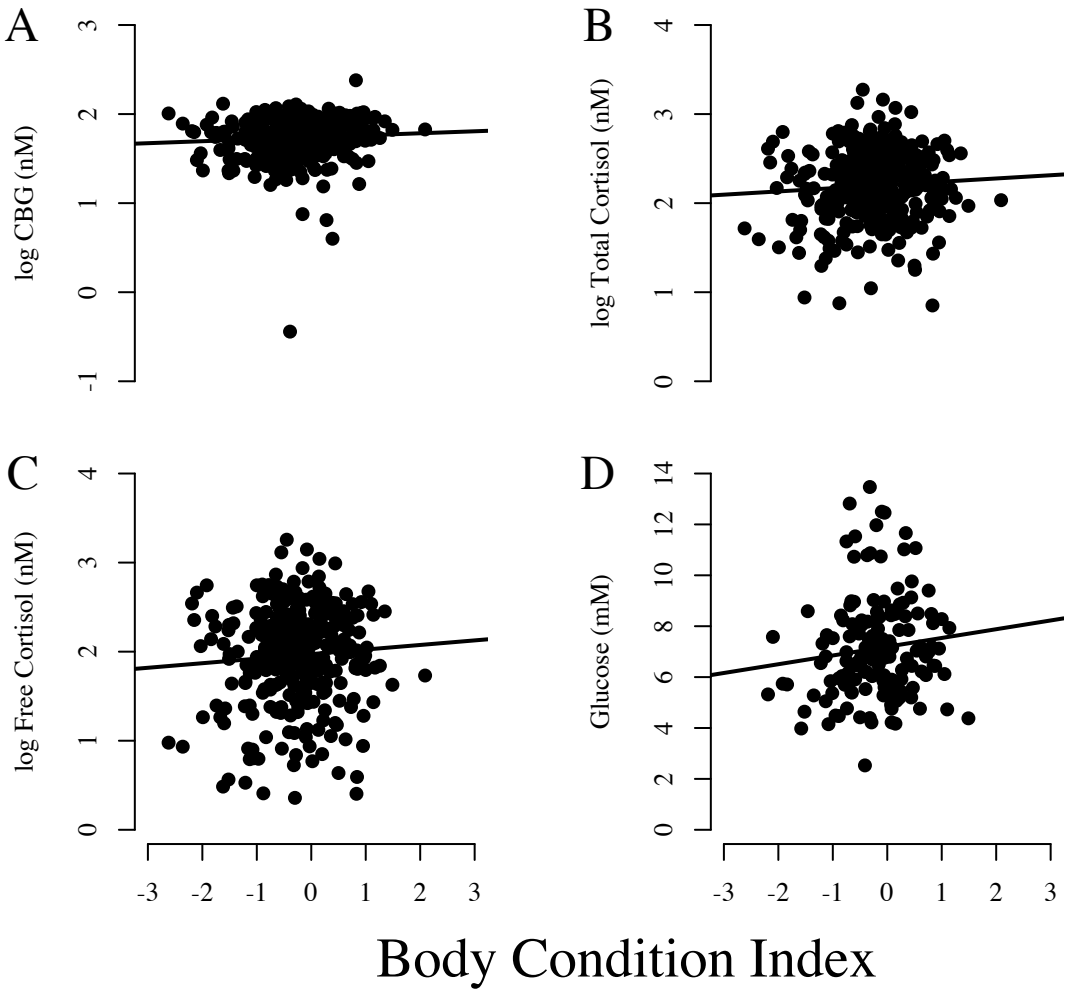
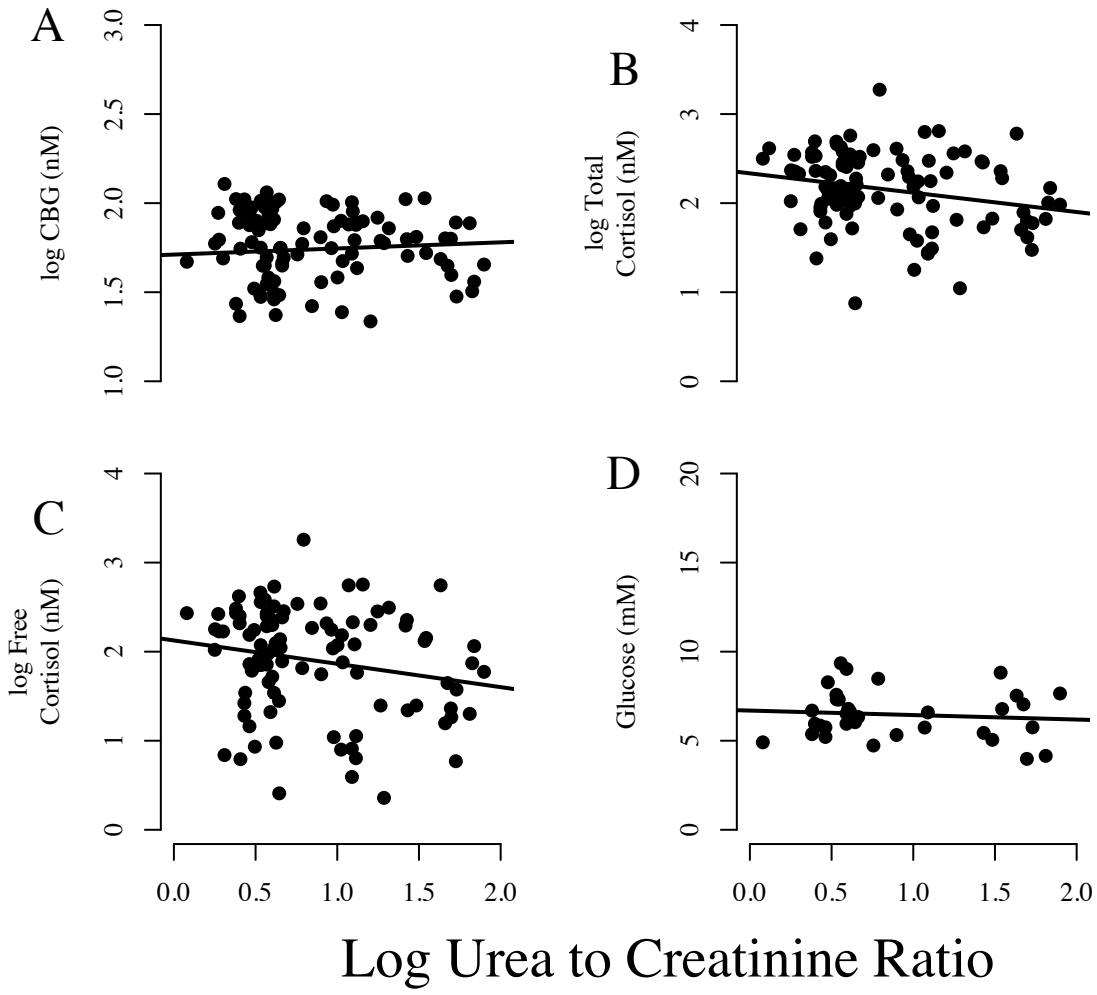

A
log CBG (nM)
-1
0
1
2
3
B
log Total Cortisol (nM)
0
1
2
3
4
C
log Free Cortisol (nM)
0
1
2
3
4
-3
-2
-1
0
1
2
3
D
Glucose (mM)
0
2
4
6
8
10
12
14
-3
-2
-1
0
1
2
3
Body Condition Index

Figure 4.6: The Effect of Urea to Creatinine Ratio on Serum Cortisol Dynamics in Polar Bears.
Scatterplots of A) CBG, B) total and C) free cortisol, and D) glucose concentrations against the urea to creatinine (UC) ratio of polar bears. The trend lines were calculated by linear least squares regression. Total (ANOVA, $p < 0.05$, adjusted $R^2 = 0.049$) and free ($p < 0.05$, adjusted $R^2 = 0.031$) cortisol was significantly negatively associated with UC ratio.


A
log CBG (nM)
1.0
1.5
2.0
2.5
3.0
B
log Total
Cortisol (nM)
0
1
2
3
4
C
log Free
Cortisol (nM)
0
1
2
3
4
D
Glucose (mM)
0
5
10
15
20
0.0
0.5
1.0
1.5
2.0
Log Urea to Creatinine Ratio

4.4. Discussion

The results of this investigation represent the first characterization of cortisol dynamics, including serum CBG and free cortisol concentrations in free-ranging grizzly and polar bears. The results suggested that cortisol dynamics is modulated by capture method, reproductive class, and energy substrate status of the animal suggesting a key role for this hormone in the metabolic adjustments associated with seasonal changes and life history traits in bears.

4.4.1. Effect of Capture Method in Grizzly Bears

The effect of capture stress on CBG concentration and free cortisol levels were measured for the first time in grizzly bears. Serum CBG levels were not modulated by capture stress suggesting that free cortisol concentrations are largely determined by the adrenal response to capture. Our results confirmed the activation of the HPA axis by capture stress in grizzly bears [36, 47], and leg-hold snaring elicited greater increases in serum cortisol levels compared to culvert trapping and heli-darting. This may be related to the potential for longer holding times and greater physical injury associated with leg-hold snaring [36] compared to the other capture methods employed in this study. In particular, grizzly bears captured by leg-hold snaring may be held for up to 24 h because some traps were set in remote locations and were checked only once a day, and markers of muscle injury and dehydration suggest prolonged physiological disturbance [36]. In contrast, helicopter darting was associated with acute, strenuous physical activity, but blood sampling generally occurred sooner than with leg-hold snaring. However, the intense physical activity preceding immobilization is associated with delayed drug induction times and/or decreased susceptibility to immobilizing drugs [36]. While culvert trapping also employed physical immobilization like leg-snaring, the intensity of the stress and physiological disturbance associated with this capture method is thought to be comparatively less in bears [48] and other animals [49]. This notion was supported by the lower total cortisol levels with culvert traps compared to leg-hold snares (Figure 4.1). Altogether, helicopter darting and sampling evoked the least free cortisol stress response in grizzly bears, which adds

further evidence to the adverse short-term physiological effects of leg-hold snaring in comparison to other capture methods employed in the immobilization of bears, which includes the increased probability of muscle injury and dehydration [36, 37]. Moreover, there are also long-term effects of capture on grizzly bears, including 3 – 6 weeks of lower movement rates post-capture and lower age-specific body condition in bears captured more than once [37], but it is unclear if the greater capture stress and cortisol response induced by leg-hold snaring plays a role in modulating these long-term effects.

The lack of change in serum CBG concentrations with capture stress was in agreement with other studies showing a delayed response in the changes in expression of this protein. For instance, significant changes in serum CBG levels manifested only several hours after capture stress in Richardson's ground squirrels [50] and Japanese quail [51], restraint and tail shock stress in rats [52, 53] and short-term starvation in birds [54]. While grizzly bears captured by leg-hold snaring were immobilized up to 24 hours prior to sampling [36], CBG concentrations were not significantly different between capture methods, suggesting that this was not the case in the present study and that these animals were likely sampled prior to the induction of significant changes in CBG expression. It is also possible that CBG biosynthesis and secretion may be less sensitive to acute stressors in grizzly bears than in other animals, but this remains to be tested. Our results demonstrate that serum CBG concentration was unaffected by the different stress profiles and duration of the capture methods employed. Consequently, the differences in free cortisol levels between capture methods reflect different profiles of adrenal cortisol secretion, which is associated with the severity of stress induced by the capture method and the patterns of physiological disturbance that are associated with each method.

The cortisol response seen with capture was not associated with a similar glucose response in grizzly bears, suggesting that this metabolite is tightly regulated in bear blood. One possible explanation for this discrepancy was that the shorter duration of the sampling protocols may mask the cortisol-induced glucose response as it may take longer for the effects of the steroid to manifest [55]. Secondly, the rapid release of catecholamines and the activation of β-adrenoceptors associated with the acute stress of capture masked any further changes in serum glucose levels due to steroid stimulation. The high serum glucose levels observed in our captured animals (~8 mM) compared to "unstressed" levels reported in animals (<5 mM in

dogs [56]) support the latter notion and may have obscured any increases in glucose levels to capture. Additionally, serum may have been in contact with the cellular fraction of blood for a prolonged period of time in grizzly and polar bears compared to black bears due to the remote location of sampling sites relative to the processing facilities. Blood cells actively metabolize glucose, which may obscure the pre-sampling levels of this metabolite in grizzly and polar bears leading to lower levels relative to the black bears, but this remains to be tested. Altogether, capture stress was stressful to the animal, and our results supported heli-darting as the capture method that induced the lowest increase in cortisol concentrations in grizzly bears [36, 47]. However, the results suggest that the animals are exposed to elevated biologically active cortisol levels in response to capture stress given the absence of any changes in CBG levels after capture. The long-term effects of this hypercortisolemia post-recovery from sampling episodes in grizzly bears remain to be determined.

4.4.2. Cortisol Dynamics between Reproductive Classes

The modulation of cortisol dynamics due to reproductive class followed similar trends in both grizzly and polar bears. CBG levels were modulated by age class in both species, with mean levels in juveniles lower than in adults. Sex steroids modulate CBG expression [2], and the lower levels in juveniles may reflect their prepubescent state of development and low circulating levels of sex steroids. Similarly, juveniles tend to have lower CBG levels than adults in Arctic ground squirrels [57] and rats [58]. However, in these species free glucocorticoid levels are regulated at levels that are not different between age classes, suggesting that glucocorticoid dynamics is altered by other factors in addition to CBG modulation. Studies have reported that the levels of cortisol and its metabolites in grizzly and polar bear hair [59, 60] and feces [61] are similar between age groups. Since the rate of incorporation of cortisol into hair and its excretion via feces is dependent on the free fraction of this hormone in circulation [62, 63], our results suggest that free serum cortisol levels must also be similar between age classes, and that the secretion of cortisol is regulated to maintain bioavailable levels. Thus, we hypothesize that the differences in CBG levels between juvenile

and adult bears are unlikely to have metabolic consequences for the younger age classes as the free cortisol levels are maintained.

We found that stress-induced total cortisol levels were also similar between age classes. Given our hypothesis (above) that baseline total cortisol levels may be lower in juvenile bears, these results suggest that the magnitude of the cortisol response to capture stress (the change from baseline to stress-induced levels) is greater in juvenile relative to adult bears. If this notion holds true, then the finding may have implications for future studies involving the capture and handling of juvenile bears. For instance, it has been shown that captures have negative, long-term effects on age-specific body condition index in black and grizzly bears [37]. However, the long-term physiological and health effects associated with repeated captures in juvenile bears are currently unknown.

There were no differences in cortisol dynamics between reproductive classes in adult grizzly or polar bears, suggesting that expression of CBG and the cortisol response to capture stress is not sexually dimorphic. We hypothesized differences in cortisol dynamics between reproductive classes of bears because: 1) in other species, sexually dimorphic CBG expression and HPA axis activity was associated with the actions of sex steroids, including androgens (downregulation) and estrogens (upregulation) [2, 64], and 2) lactation is metabolically expensive in bears [65, 66], and lactation while fasting has been associated with elevated cortisol levels in other species, including some phocid seals [46, 67] to facilitate energy substrate mobilization.

Reproductive status in grizzly and polar bears did not modulate cortisol dynamics, which is consistent with similar findings in long-lived species that have multiple opportunities to reproduce [20, 68]. The serum cortisol buffering capacity and glucocorticoid response to stressors in these species are generally maintained to prevent the detrimental effects of elevated free cortisol levels in the former case and to promote long-term survival over short-term reproductive success in the latter. This contrasts to species where, for example, intense intraspecific competition between males for mates is associated with decreased CBG expression and elevated free cortisol levels [20]. The changes in glucocorticoid dynamics in these species play a role in mobilizing energy stores to meet the energetic demands of

reproduction, and in some species, may contribute to the loss of long-term health for short-term reproductive success. Seasonal variations in sex hormones in animals may modulate cortisol dynamics and, thereby, impact reproductive status [64]. We did not measure sex steroids in this study, but previous reports found that the level of testosterone in males tend to be elevated around the respective reproductive seasons of bears [69, 70]. In female bears, estrogen levels are elevated around the estrous period in black bears [71], but peak 3 – 4 months after the breeding season for polar bear females [69]. However, we cannot definitely rule out the seasonal modulation of cortisol dynamics in grizzly and polar bears because the small sample sizes prevented the analysis of temporal variations in CBG, cortisol, and glucose levels. Further research may elucidate the roles that seasonal variations in sex hormone levels may play in modulating cortisol dynamics in bears.

Our results suggested that the provisioning of parental care, including lactation, did not modulate cortisol dynamics in female grizzly and polar bears. While lactation is associated with increased metabolic demand in bears [65, 66], this is not always associated with altered glucocorticoid dynamics in other species. In lactating rats, the energetic demands of lactation were not associated with changes in glucocorticoid dynamics if caloric intake was increased to compensate [72]. However, lactation supported by endogenous energy stores may be associated with increased glucocorticoid secretion. In other species that fast while lactating, including subantarctic fur seals, total glucocorticoid levels increase as adipose stores are depleted, which may act to mobilize protein stores for lactogenesis [46]. Thus, our results suggested that lactating female bears are in a metabolic state that is similar to other adult reproductive classes, resulting in similar CBG, cortisol, and glucose levels across these classes. However, we cannot rule out the possibility that other factors may interact with lactation state to modulate cortisol dynamics.

We lacked sufficient data to analyze temporal and seasonal changes in glucocorticoid dynamics. For example, the quantity and energy content of milk decreases as cubs age [65, 73], which likely affects the metabolic load on the lactating female. Also, the decline in body condition as endogenous energy stores are depleted during denning in grizzly bears and fasting in polar bears may also be exacerbated by the additional demands of lactation. However, our

analyses lead us to propose a metabolic hypothesis, including depleted adipose stores, as a key factor modulating cortisol dynamics in the bears, but this remains to be determined.

4.4.3. Effect of Nutrition and Body Condition

Serum cortisol dynamics were not modulated by nutritional season in grizzly bears. The nutritional seasons were defined by the predominant food types in their diets, which is dominated by high carbohydrate berries in the late hyperphagic season in the foothills of Alberta, Canada [40]. We hypothesized elevated cortisol levels in grizzly bears during the hyperphagic season because studies have shown that other species, including little brown Myotis (*Myotis lucifugus*) [74], yellow-bellied marmots (*Marmota flaviventris*) [75], and black bears (*Ursus americanus*) [76], have elevated serum glucocorticoid levels during the pre-hibernation, hyperphagic period. It is thought that hypercortisolemia during hyperphagic periods may interact with elevated insulin levels to promote lipid deposition in adipose stores [64, 77]. Also, low protein, calorie sufficient/excess diets, similar to the diets that are adopted by grizzly and other bear species during the late hyperphagic season [40, 41], are found to be associated with elevated baseline glucocorticoid and decreased CBG levels in other species, including humans [78], rodents [32], and birds [79]. Moreover, protein malnutrition in domesticated cockrels is associated with greater adrenal weight, steroidogenic capacity, and cellular sensitivity to ACTH, supporting elevated glucocorticoid levels [80]. Clearly, glucocorticoid dynamics are modulated by nutritional quality and predictable, seasonal variations in food availability in other species, but that does appear to be the case in grizzly bears.

Cortisol dynamics in grizzly bears were not modulated by seasonal shifts in the major components of their diets. It should be noted that the dietary composition of grizzly bears are variable and highly dependent on the types and availability of forage located in each individual's home range [81]. For example, there are clear differences in the diet of grizzly bears that have home ranges in the foothills versus the mountains of Alberta, Canada, with animals in the former having greater access to ungulates and, therefore, dietary protein [40]. There may be population or subpopulation level differences in the diets of the animals that we

did not include in our analysis, but we suggest that other factors, including the protein composition of their diet may be a key nutritional modulator of cortisol dynamics in this species.

Urea is a product of amino acid catabolism in animals [82], while creatinine is the product of creatine degradation [83]. Low UC ratios observed in active black bears is associated with low protein diets, which in other animals leads to a protein sparing state that includes decreased urea biosynthesis [84]. However, elevated creatinine levels are not affected by diet in bears, and are associated instead with hibernation in terrestrial bears and fasting in polar bears [85, 86]. Moreover, it should be noted that blood urea concentrations may be affected by other factors other than dietary protein in other animals, including the energy content of the diet and hydration status [87], but these potential confounders on blood urea levels have not been fully characterized in bears, to our knowledge. However, the significant negative association of total and free cortisol levels with UC ratios in grizzly and polar bears leads us to propose that factors causing low UC ratios may lead to changes in adrenal physiology in grizzly bears, including increased steroidogenic capacity and ACTH sensitivity [80]. This is supported by the greater capture-induced cortisol levels in low UC ratio animals in the present study and may be a consequence of increased steroidogenic capacity and sensitivity to central HPA axis stimulation. These changes in adrenal physiology in grizzly bears may play a role in the adoption of foraging strategies that aim to meet protein requirements in the face of diets deficient in this macronutrient, resulting in hyperphagia and the deposition of substantial adipose stores [41, 88, 89], but further research is required to confirm the associations between dietary protein, UC ratios, and cortisol dynamics in grizzly bears.

While both grizzly and polar bears with low UC ratios exhibit elevated capture-induced cortisol levels, it is likely that the mechanisms underlying these changes in these species are different. The biochemical and metabolic state of fasting polar bears are comparable in some ways to hibernating terrestrial bears, including low UC ratios that arise from decreased urea synthesis and renal clearance of creatinine [90]. Hibernation in black bears is associated with elevated total cortisol levels relative to summer active bears [86, 91], which is similar to our findings of elevated cortisol levels in polar bears with low relative to higher UC ratios. This finding suggested that the function of the HPA axis may adapt to fasting in polar bears much in

the same way as to hibernation in terrestrial bears. However, it remains unclear how fasting polar bears and, by extension, hibernating terrestrial bears reduce the catabolic effects of cortisol on lean tissues. Hibernation is characterized by remarkable protein sparing [86], but some protein is lost in fasting polar bears in proportion with the relative fatness of the animal prior to the initiation of the fast [92]. In other animals that fast for prolonged periods and rely on the oxidation of fat to supply energy for metabolism, circulating glucocorticoid levels are depressed to prevent the catabolic effects of this hormone on protein stores [93]. Only when adipose stores are depleted beyond a species-specific critical point do glucocorticoid levels increase to mobilize amino acids from protein stores to supply energy. However, the cortisol response to capture stress in fasting polar bears appeared to be hyperactive, leading to greater cortisol levels in fasting relative to feeding animals. Furthermore, there were no changes in CBG levels between fasting and feeding polar bears, suggesting that changes in cortisol buffering capacity does not play a role in modulating the catabolic effects of this steroid hormone on lean tissues. It appears that the reduction of bioavailable cortisol was not associated with protein sparing in fasting polar bears, and other factors not considered in our analyses may be playing roles in modulating the catabolic effects of cortisol on tissues during fasting in polar bears.

The body condition of grizzly and polar bears was not associated with CBG or cortisol levels, which may contradict our above hypothesis that the state of endogenous energy stores modulates cortisol dynamics in bears. However, these results may be intertwined with the effects of fasting and protein sparing on cortisol dynamics as discussed above. For example, the lack of an association between cortisol levels and BCI in polar bears could indicate that none of the animals sampled had depleted their adipose reserves to the point that protein catabolism was necessary to support their metabolic demands [93], but this critical adipose depletion level is currently unknown in bears. In contrast, we expected that there would be no association between BCI and cortisol dynamics in the grizzly bears sampled in this study because they were sampled during the active season, where they are likely to be feeding and adding to their endogenous energy reserves throughout this period [41]. Under these conditions, cortisol dynamics may be driven instead by the quality of nutrition, including the dietary protein composition.

4.4.4. Conclusions

The characterization of the factors that modulate glucocorticoid dynamics in free-ranging grizzly and polar bears is a key initial step in the development of CBG and cortisol levels as markers of health status in these species. We found that CBG levels were not modulated by different capture methods, despite the large differences in the magnitude of acute cortisol responses that were induced by these methods, suggesting that CBG levels may reflect the chronic health and stress status of bears. There were no differences in CBG and cortisol levels between reproductive classes in adult grizzly and polar bears, which is consistent with the hypothesis that long-lived species will maintain CBG levels and HPA axis reactivity to stressors throughout reproduction. Instead, environmental factors may be the key modulators of cortisol dynamics. However, CBG levels were not associated with markers of nutritional status, but serum total and free cortisol concentrations were negatively associated with UC ratio in both grizzly and polar bears. We propose that this HPA hyperactivity may be indicative of enhanced muscle protein breakdown with low-protein diets in grizzly bears, and in polar bears may reflect the physiologic and biochemical adaptations that are similar to those found in hibernating black bears.

4.5. Acknowledgements

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CHAPTER 5:
CORTISOL DYNAMICS IN THE HIBERNATING AMERICAN BLACK BEAR

5.1. Introduction

Hibernation is an adaptation to conserve energy in the face of extreme environmental conditions and low food availability in several animal phyla [1]. This process is characterized by changes in the homeostatic set points of the organism, leading to the depression of the metabolic rate and a corresponding decrease in energy demand. Some of the striking adaptations of ursid hibernation include the near complete conservation of nitrogen [2] and the marked reduction of muscle atrophy during this period of disuse [3]. Since glucocorticoids play an important role in modulating the catabolism of proteins [4], we hypothesize that changes in cortisol dynamics, including reduced free cortisol concentrations, may be a key player in the modulation of protein catabolism during hibernation in bears. Corticosteroid binding globulin (CBG) reversibly binds a large proportion of circulating glucocorticoids with high affinity in most species [5]. CBG-bound glucocorticoids are generally not available to tissues because only the unbound glucocorticoid fraction may cross plasma membranes to act on the intracellular receptors that transduce the actions of this hormone on tissues [6]. Thus, CBG buffers lean tissues against the catabolic effects of glucocorticoids. While studies have characterized serum total cortisol levels in hibernating bears [7, 8, 9, 10], none of these reports have examined the bioavailable pool of corticosteroid during hibernation. Generally, total cortisol levels remain elevated during hibernation in black bears relative to the seasonal nadir in the summer [8, 9, 10], but changes in the bioavailable fraction of this hormone are not known.

Ursids exhibit delayed implantation, where the fertilized egg is in embryonic diapause at the blastocyst stage for several months. Implantation occurs approximately 1 – 2 months prior to

den entry in late November to early December [11], and parturition occurs in late January to early February [12]. The first 2 – 3 months of lactation occurs while denning [13], which places an astonishingly high metabolic demand on the mother relative to non-reproductive females [14, 15, 16]. Cortisol levels are elevated in other species that fast while lactating, including the subantarctic fur seal [17] and the northern elephant seal [18]. While cortisol levels have been measured in hibernating female black bears [7, 8, 9], the effect of reproductive status on cortisol dynamics has not been investigated.

Thus, the objective of this study was to characterize the bioavailability of cortisol in the sera of American black bears (*Ursus americanus*) during three time periods: prior to, during, and after hibernation. We hypothesize that CBG levels and/or its binding affinity for cortisol may change in synchrony with total cortisol levels to maintain low free levels of this hormone to limit its catabolic effects on tissues during hibernation. Furthermore, the potential differences in cortisol dynamics between reproductive and non-reproductive animals were examined to test the hypothesis that cortisol levels were elevated to supply substrates for the increased energy demands in the former. The levels of serum CBG were quantified directly by the grizzly bear CBG ELISA. Total and free cortisol levels were determined, and serum glucose and protein levels were quantified as metabolic end points.

5.2. Methods and Materials

5.2.1. Animals

Serum samples were taken from nine captive American black bears at three time points: pre-hibernation (PRE), during hibernation (HIB), and post hibernation (POST; **Table 5.1**). Animals typically entered hibernation in early January and left their dens by early April. Animals were anesthetized with a 2:1 mixture of ketamine (100 mg/mL):xylazine (100 mg/mL) at a dosage of 1 cc of the mixture per 45.5 kg of body mass. Blood samples were drawn from the femoral vein while the animal was anesthetized, and the samples were

transported to the laboratory in an ice- packed cooler. Immediately on return to the laboratory, the blood was spun to isolate the serum and was frozen at –20°C. The Virginia Polytechnic Institute and State University Animal Care Committee approved all bear handling protocols (#98-069-F&WS).

5.2.2. Saturation Binding Assays

The dissociation constant (K_d) for gbCBG was determined by saturation binding experiments with serum as described previously [19]. Serum was stripped of steroids by incubation with washed activated charcoal for 4 h at 37°C. 100 µL of stripped serum diluted 1:100 in phosgel (100 mM phosphate, 0.1% gelatin, pH 7.4) was incubated with 1.56 – 100 nM 1,2,6,7-^{3}H(N) hydrocortisone (^{3}H-F, Perkin-Elmer, Waltham, Massachusetts, USA) for 4 h at 37°C in glass tubes in duplicate. A parallel set of tubes with 2000 nM unlabeled cortisol along with the tritiated hydrocortisone and diluted bear serum were incubated to determine non-specific binding (NSB). Additionally, another set of tubes with no serum was incubated to determine total counts (TC). After the incubation, all tubes were cooled in an ethanol-ice-water bath, and unbound hydrocortisone was separated by the addition of 700 µL dextran T-70 (Sigma) coated activated charcoal (Sigma) in phosgel. Tubes were incubated for 20 min, centrifuged at 2800 rpm for 12 min at 4°C. 350 µL of the supernatant was transferred and mixed with 2 mL scintillation fluid. Samples were counted in a Beckmann LS-9600 liquid scintillation counter. Specific binding (SB) was calculated as the difference between the TB and NSB counts. K_d and B_{max} were first estimated by Scatchard analysis [20] and Rosenthal correction [21]. These estimates were then refined by non-linear least squares (NLS) regression in R 2.14.0 to the equation:

$$B_s = \frac{B_{max} * L}{K_d + L}$$

Where B_s is specific binding (cpm), B_{max} is the maximum binding capacity of cortisol to serum (cpm), and L is the free tritiated hydrocortisone (nM). The K_d and B_{max} from the Scatchard analyses were used as seed values for NLS analysis. To compare K_d and B_{max} changes between

hibernation states, saturation assays were performed on sera from 6 animals, 3 of which were reproductive and 3 were non-reproductive, between the PRE and HIB time points.

5.2.3. CBG ELISA

To quantify CBG concentrations in black bear sera, serum samples were diluted 1:1000 in 10 mM Tris (pH 7.5), and 2.0 µL of this diluted serum in 200 µL of carbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) directly in duplicate wells of an EIA plate. The recombinant gbCBG was diluted with 10 mM Tris (pH 7.5) to 0 – 4 nmol gbCBG/L, and diluted 10 fold with carbonate coating buffer directly in wells of the ELISA plate in triplicate. Proteins were allowed to coat the wells for 2 h (all incubations were performed in a shaker-incubator set to 30°C). Plates were then washed in an automatic plate washer (Immunowash 1575, Bio-Rad) with a custom washing protocol [4 repetitions of aspiration, washing with 400 µL TTBS (20 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.4), and agitation for 5 seconds; and one final aspiration]. Plates were blocked with 5% BSA in TTBS for 1 h and washed again. The primary detection antibody (polyclonal rabbit anti-gbCBG) was diluted 1:1500 in 1% BSA in TTBS, and 200 µL was added to each well, except for non-specific binding wells where 200 µL 1% BSA in TTBS was added instead, and incubated for 2 h. After washing, bound biotinylated anti-CBG antibody was detected and amplified with 200 µL per well of streptavidin-HRP diluted 1:3000 in 1% BSA in TTBS, incubated for 1 h. After a final wash, the detection solution was freshly prepared [500 µL 41 mM TMB in DMSO into 19.5 mL 200 mM potassium citrate, 3.075 mM H_2O_2 (pH 4.0)], and 200 µL was added to each well and incubated for 1 h. The reaction was stopped with 100 µL stopping solution (8.5 M acetic acid, 0.5 M sulfuric acid). Plates were read at 450 nm excitation wavelength on a VersaMax microplate reader using SoftMax Pro 3.1 software (Molecular Devices, Sunnyvale, CA).

5.2.4. Total Cortisol, Glucose, and Protein Assays

Total cortisol concentrations were determined by a commercial ^{125}I radioimmunoassay kit (MP Biomedicals, Orangeburg, NY). Serum glucose concentration was determined by the glucose

oxidase-peroxidase method as described previously [22], using glucose (Sigma) as the standard. Absorbance was measured at 500 nm on a VersaMax microplate reader. The protein concentration of black bear sera was determined before and after depletion of albumin and immunoglobulin G by the bicinchoninic acid method [23] using bovine serum albumin as the standard. Bear sera was depleted of albumin and IgG with Aurum Serum Protein Mini-kits (BioRad, Hercules, CA, USA).

Free cortisol was calculated using the equation of Barsano and Baumann [24] :

$$fCORT = \frac{-\left(CBG - tCORT + \frac{1}{K_a}\right) \pm \sqrt{\left(CBG - tCORT + \frac{1}{K_a}\right)^2 - 4\left(\frac{-tCORT}{K_a}\right)}}{2}$$

Where *fCORT* is free cortisol concentration (mol/L), *CBG* is CBG concentration (mol/L), *tCORT* is total cortisol concentration (mol/L), and $K_a = 1/K_d$ = equilibrium association constant (L/mol).

5.2.5. Statistics

To compare significant differences in the means of K_d, B_{max}, and CBG, cortisol, and glucose concentrations, repeated measures ANOVAs were constructed using hibernation status (levels: PRE, HIB, and POST, if applicable) as a within-subjects main categorical effect and reproductive status as a between-subjects main categorical effect in R 2.14.0. Interactions between the two effects were tested for. Variables were log-transformed as required to meet the parametric assumption of normality. Post-hoc significance testing was performed using Tukey's Honest Significant Differences (HSD) test, and $p < 0.05$ was considered to be significant.

5.3. Results

Nine female American black bears were sampled prior to ("Pre-hibernation" or "PRE"), during ("HIB"), and after arousal from hibernation ("Post-hibernation" or "POST"). Sample data are shown in **Table 5.1**. Six animals were pregnant entering hibernation: five gave birth to litters of two cubs, and one gave birth to three cubs. One set of twin cubs died shortly after birth. For this analysis, this animal (bear #107, **Table 5.1**) was considered to be reproductive at PRE and non-reproductive (i.e. non-lactating) for HIB and POST periods.

Saturation binding assays were performed on serum samples from 6 animals in the PRE and HIB periods to characterize the binding affinity of CBG to cortisol in these two time periods. K_d was calculated by fitting a curve through the specific binding versus unbound labeled cortisol plots by non-linear regression. Mean K_d (**Figure 5.1**) was not significantly different in reproductive animals relative to non-reproductive animals (**Table 5.2**), nor between PRE and HIB periods.

CBG and glucose concentrations were quantified in nine black bears in the PRE, HIB, and POST periods, and in non-reproductive and reproductive animals (**Table 5.1** and **Figures 5.2B** and **D**, respectively). There were no statistically significant differences in mean CBG and glucose levels between these hibernation periods, nor were there differences between non-reproductive and reproductive animals. Total cortisol concentrations were similarly quantified in black bears (**Table 5.1C** and **Figure 5.2C**). Levels of this hormone were significantly elevated during the HIB period relative to the PRE and POST periods. There were no significant differences between reproductive classes. There was a weakly significant interaction effect ($p < 0.10$). Post-hoc significance testing suggested that there was a significant increase (Tukey's HSD $p < 0.05$) in total cortisol levels from PRE to HIB in reproductive animals. Free cortisol concentrations followed these trends exactly (**Table 5.1C** and **Figure 5.2C**).

Total protein concentrations (**Table 5.1E** and **Figure 5.2E**) were lower during the POST period relative to PRE and HIB. There were no differences between reproductive classes. In

contrast, the protein concentration of sera depleted of albumin and immunoglobulin G (**Table 5.1F** and **Figure 5.2F**) showed no statistically significant differences between hibernation periods or reproductive classes.

5.4. Discussion

The results of this investigation represent the first characterization of cortisol dynamics in the hibernating American black bear. While seasonal changes in total cortisol concentrations have been previously reported in this species [7, 8, 9, 10], seasonal changes in CBG and free cortisol concentrations have not been studied. Hibernation in black bears is associated with the remarkable lack of lean tissue atrophy relative to other animals that experience prolonged unloading, including bone [7, 25] and skeletal muscle [26], which we hypothesized was partly due to changes in cortisol dynamics to limit the catabolic effects of this hormone on lean tissues. Our results show that there were no changes in CBG and free cortisol levels in non-reproductive black bears between hibernation states, suggesting that the remarkable reduction in disuse atrophy during this period in this species may not involve the downregulation of bioavailable cortisol. However, we report the novel finding that cortisol concentrations were elevated in reproductive female black bears, and this may represent an adaptation to mobilize energy substrate resources to cope with the increased metabolic demands of lactation during denning.

There were no differences in the binding affinity of CBG for cortisol (K_d) between active (PRE) and hibernating (HIB) black bears as determined by the saturation binding experiments, which shows that changes in the K_d do not play a role in modulating cortisol dynamics during hibernation. The K_d of CBG is not known to change appreciably within an individual due to endogenous or exogenous factors [6], and K_d is considered to be a constant within species for the most part.

Table 5.1: Black Bear Animal and Sampling Data.

Bear #	Sampling Days for PRE, HIB, and POST samples, respectively (Julian Day)	Pregnant at Time of Den Entry	Parturition Day (Julian Day)	Initial Weight (lbs)	Weight Change during Hibernation (% of Initial)
102	324, 59, 119	Y	14	235	-20.9%
103	334, 49, 99	N	NA	234	-20.5%
104	283, 69, 109	Y	28	241	-24.9%
105	324, 69, 119	Y	11	321	-20.2%
107	294, 49, 109	Y	2	111	-34.3%
108	285, 49, 119	N	NA	156	-40.4%
109	276, 59, 119	Y	36	214	-27.1%
110	285, 59, 109	Y	35	241	-26.0%
111	284, 59, 131	N	NA	244	-23.8%

Figure 5.1: Binding Affinity of CBG for Cortisol in Active and Hibernating American Black Bears.
Mean + SEM dissociation binding constant (K_d) of American black bear CBG for cortisol of black bear serum between pre-hibernation (PRE) and hibernation (HIB) states and non-reproductive (dark grey bars) and reproductive animals (light grey bars). Sample sizes are shown as inset. There were no significant differences between groups.

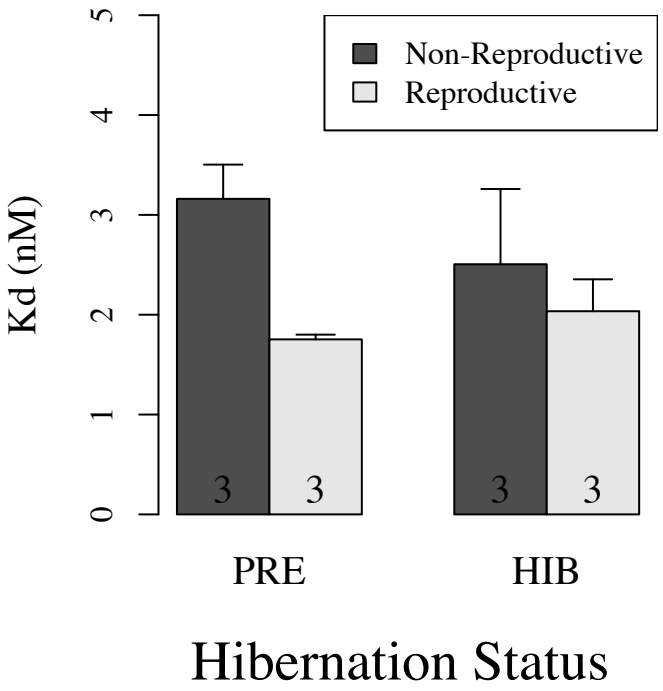
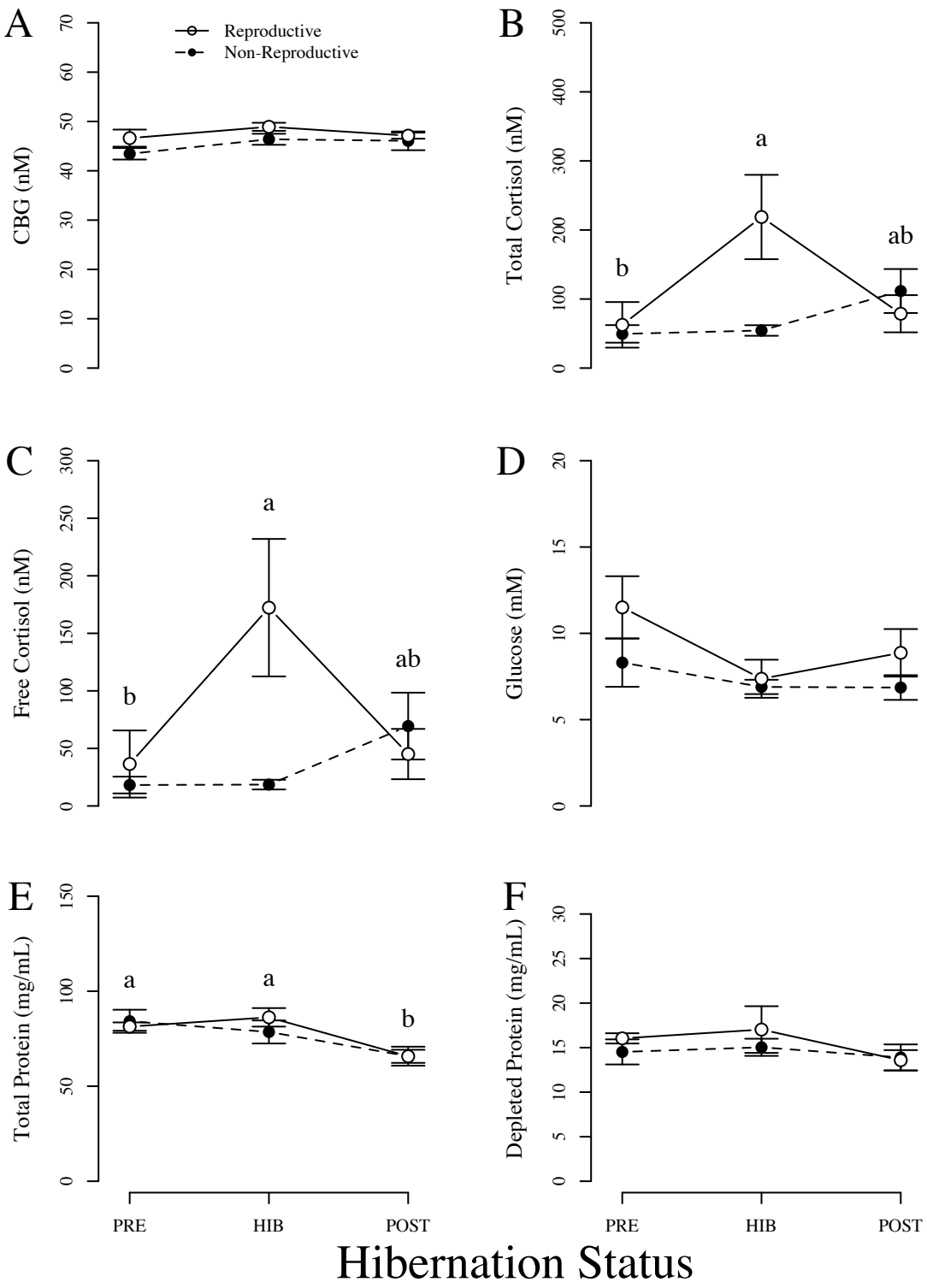

Non-Reproductive
Reproductive
Kd (nM)
0
1
2
3
4
5
3
3
3
3
PRE
HIB
Hibernation Status

Table 5.2: Summary of Statistical Analyses of Cortisol Dynamics in Hibernating Black Bears.
Effects of hibernation status (HIB), reproductive status (REPRO), their interactions (HIB X REPRO), and residuals on A) K_d, B) CBG, C) total cortisol, D) free cortisol, E) glucose, F) total serum protein, and G) depleted serum protein concentrations. Marginal statistical significance ($p < 0.10$) is indicated by '.', $p < 0.05$ by '*'.

	Factor	Sum of Squares	df	F	p	
A) Kd	Hibernation Status	0.104	1	0.176	0.686	
	Reproductive Status	2.645	1	4.466	0.068	.
	HIB X REPRO	0.659	1	1.112	0.323	
	Error	4.738	8			
B) CBG	Hibernation Status	27.418	2	1.618	0.222	
	Reproductive Status	31.744	1	3.747	0.066	.
	HIB X REPRO	5.082	2	0.300	0.744	
	Error	177.905	21			
C) Total Cortisol	Hibernation Status	4.476	2	3.813	0.039	*
	Reproductive Status	0.218	1	0.371	0.549	
	HIB X REPRO	4.028	2	3.431	0.051	.
	Error	12.326	21			
D) Free Cortisol	Hibernation Status	12.795	2	3.544	0.047	*
	Reproductive Status	0.201	1	0.111	0.742	
	HIB X REPRO	12.088	2	3.348	0.055	.
	Error	37.912	21			
E) Glucose	Hibernation Status	0.464	2	2.292	0.126	
	Reproductive Status	0.202	1	1.998	0.172	
	HIB X REPRO	0.078	2	0.387	0.684	
	Error	2.125	21			
F) Total Serum Protein	Hibernation Status	1672.730	2	9.663	0.001	*
	Reproductive Status	19.970	1	0.231	0.636	
	HIB X REPRO	127.490	2	0.737	0.491	
	Error	1817.560	21			
G) Depleted Serum Protein	Hibernation Status	25.425	2	1.476	0.254	
	Reproductive Status	6.207	1	0.721	0.407	
	HIB X REPRO	6.284	2	0.365	0.699	
	Error	163.641	19			

Figure 5.2: Changes in Serum Cortisol Dynamics, and Glucose and Serum Protein Concentrations Between Hibernation States in Black Bears.
Mean + SEM A) CBG, B) total cortisol, C) free cortisol, D) glucose, and E) total serum protein concentrations between PRE, HIB, and POST hibernation states, and non-reproductive (dark grey bars) and reproductive animals (light grey bars). Sample sizes are shown as inset. Statistically significant differences in means between hibernation states are indicated by different letters (2-way RMANOVA $p < 0.05$). There were statistically significant elevations in total and free cortisol levels between PRE and HIB in reproductive animals (Tukey HSD $p < 0.05$).


A
Reproductive
Non-Reproductive
CBG (nM)
0
10
20
30
40
50
60
70
B
Total Cortisol (nM)
0
100
200
300
400
500
b
a
ab
C
Free Cortisol (nM)
0
50
100
150
200
250
300
b
a
ab
D
Glucose (mM)
0
5
10
15
20
E
Total Protein (mg/mL)
0
50
100
150
a
a
b
F
Depleted Protein (mg/mL)
0
5
10
15
20
25
30
PRE
HIB
POST
PRE
HIB
POST
Hibernation Status

Although serum CBG levels did not vary significantly during hibernation, total and free cortisol levels were elevated in the reproductive relative to non-reproductive animals, suggesting that the HPA axis is hyperactive in the former group. The elevated cortisol levels in hibernating reproductive female black bears reflected the energetic demands and consequences of lactation, which includes greater mass loss and energy consumption relative to non-reproductive animals [16, 27]. Our results parallel similar observations of elevated cortisol levels in other species that similarly lactate while fasting, including phocids, otariids, and cetaceans [17, 18]. These elevated cortisol levels may be playing a role in the mobilization of substrates for lactogenesis, including amino acids and lipids [17, 18]. Furthermore, bear #107, who lost her cubs shortly after parturition and thus was unlikely to be lactating, had low total and free cortisol concentrations during the HIB period comparable to non-lactating females, which lends support to the hypothesis that increased energy demand modulates serum cortisol levels in hibernating black bears.

The return of cortisol levels in reproductive black bears in the POST period to PRE levels may suggest that the resumption of feeding after leaving the den lessens the metabolic burden of lactation on the endogenous energy stores of the lactating animal. These findings in black bears may also provide some context to our observations in Chapter 4, where we found that female grizzly and polar bears with dependent cubs had mean total and free cortisol levels that were no different from solitary females that are not lactating. We hypothesized in Chapter 4 that the metabolic status of lactating grizzly and polar bears may be a key modulator of cortisol dynamics, but we lacked supporting data for these species. Our results in this investigation provides evidence towards this hypothesis that the energy demand of lactation modulates HPA activity in bears during periods of fasting, including denning.

Serum glucose concentrations were not associated with the changes in cortisol dynamics between hibernation periods. These observations of a lack of association between cortisol and glucose levels are similar to those made in grizzly and polar bears (Chapter 4), and may be due to the effects of the immobilizing drugs (e.g. xylazine) that induce hyperglycemia [28] and masks any possible glucose dysregulation prior to sampling. However, the measurements revealed that despite the varying body conditions and metabolic demands, including lactation, of these animals, the glucose response to sampling is tightly regulated.

Serum total protein concentrations were similar between PRE and HIB periods, but decreased in the POST period. Other studies found small increases in total protein concentrations during hibernation [14, 29, 30], which are likely due to the incorporation of amino acids into serum proteins rather than degradation via the urea cycle [13]. This protein sparing mechanism may be active only during hibernation because we found that total protein levels are decreased during the POST period. This decrease in total protein levels in the POST period may suggest that serum protein dynamics were altered, and may be related to the altered turnover of albumin and/or IgG concentrations. In support of this argument, we found that protein concentration of albumin- and immunoglobulin G-depleted samples do not change between seasons. Decreases in serum albumin concentrations are an indicator of poor body condition in bears [29], and it is likely that the body condition of the animals in this investigation were lower at arousal [14] but body length measurements were not taken, so body condition could not be estimated.

How do hibernating or fasting ursids avoid lean body mass catabolism by cortisol if the bioavailability of cortisol is not reduced by upregulating CBG levels? The dynamics of cortisol action is dependent on the rates of biosynthesis and secretion, cellular uptake, and catabolism [31], and this investigation only characterized the bioavailability of cortisol. The catabolic actions of glucocorticoids on tissues may also be suppressed via changes in the sensitivity of the tissue to glucocorticoids, which may include changes to glucocorticoid receptor (GR) and 11β-hydroxysteroid dehydrogenase (11β-HSD) expression or activity [32], but tissue sensitivity to glucocorticoids has not been investigated in any ursid species. GR is responsible for mediating the actions of cortisol on cells by modulating gene expression, and decreased expression of this protein may reduce the sensitivity to glucocorticoids in tissues. GR activity may also be downregulated by altered phosphorylation status, altered interactions with its chaperones (e.g. heat shock protein 90 and immunophilins), by the upregulation of GR inhibitors, including AP-1 and calreticulin. 11β-HSD modulates tissue glucocorticoid sensitivity by the conversion of active glucocorticoids to inactive forms (e.g. cortisol to cortisone) or vice versa. 11β-HSD isoform 1 catalyzes the reverse reaction (cortisone to cortisol) and has been implicated to increase the intracellular concentration of active cortisol, while 11β-HSD isoform 2 produces cortisone from cortisol and plays a role in limiting the

activation of the high glucocorticoid affinity mineralocorticoid receptor by cortisol. The downregulation of the former and upregulation of the latter protein may reduce tissue sensitivity to glucocorticoids. It is currently unclear if and how GR and 11β-HSD expression and activity are modulated by hibernation, but studies in other hibernators show the wide spread, reversible inhibition of a wide range of transcription factors [33]. These proteins may be interesting targets for future studies to further investigate the mechanisms behind the changes in cortisol dynamics in the hibernating black bear.

Other hormones may also be interacting with glucocorticoids to reduce tissue catabolism during hibernation, including the growth hormone (GH)/insulin-like growth factor 1 (IGF1) axis, which plays a role in modulating protein catabolism during fasting [34]. Recent studies in hibernating black bears found evidence that the GH/IGF1 axis in hibernating animals is suppressed in the late hyperphagic season and early denning (December – January) and returns to summer active levels in late denning (February – March) [35, 36]. These findings suggest that protein catabolism is not suppressed by the GH/IGF1 axis consistently throughout hibernation, and the extent of protein sparing during late hyperphagia and early hibernation may be distinct from mechanisms involved during late hibernation. For instance blood urea nitrogen was not different during the former two periods, but decreases during late hibernation [9], suggesting different modes of action. Moreover, studies in fasting northern elephant seals suggest that elevated growth hormone concentrations may promote protein sparing in spite of elevated total cortisol concentrations, and the two hormones may act synergistically to increase lipolytic activity [37]. Thus, it appears that cortisol may be playing a role in the regulation of energy substrate mobilization along with other metabolic hormones, including GH and IGF1, in the hibernating black bear.

In conclusion, the novel finding was that serum free cortisol levels, the bioavailable fraction, was upregulated during hibernation only in lactating American black bears. This increased HPA activity was likely due to the enhanced metabolic demand associated with lactation during a period of total fasting in bears. Our results suggested that changes in serum CBG levels and binding affinity for cortisol were not playing a role in modulating cortisol dynamics in hibernating black bears. Overall, the reduction of bioavailable cortisol is not likely a contributor to the limited disuse atrophy of lean tissues in denning bears. We hypothesize that

cortisol is primarily mediating the mobilization of energy substrates in response to increased energy demand, including lactation, while other metabolic hormones, including GH and IGF1, may have an important role to play in sparing lean tissues during hibernation.

5.5. Acknowledgements

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CHAPTER 6:
HIBERNATION-ASSOCIATED SERUM PROTEOME CHANGES IN THE AMERICAN BLACK BEAR

6.1. Introduction

Hibernation is an adaptation to cope with extreme environmental conditions and low food availability [1]. This process is characterized by changes in the homeostatic set points of the organism, including body temperature, leading to the depression of the metabolic rate and a corresponding decrease in energy demand. While the molecular bases of hibernation in small mammals and ectotherms are beginning to be understood (see review [1]), fewer studies have been carried out on such molecular adaptations to hibernation in ursids [2].

Ursids, including the American black bear (*Ursus americanus*), are among the largest animals that hibernate, and exhibit some of the typical physiological and biochemical changes common amongst hibernating animals, including lowered body temperature and metabolic rate [3, 4], slowed heart rate [3], altered serum composition [5], and the catabolism of lipids as a primary energy source [6]. In contrast to small, "deep" hibernating mammals, including sciurids, the core body temperature of hibernating ursids is only decreased by a few degrees Celsius and there is a lack of frequent arousals [1]. Also, the metabolic rate, as a percentage of the active basal metabolic rate, is depressed to ~25% in hibernating black bear compared to ~2-5% in deep hibernators [4]. There are striking and unique changes in ursid metabolism during hibernation, including the near complete conservation of nitrogen [6] and the maintenance of wound healing [7]. As well, hibernating bears prevent disuse osteoporosis by maintaining balanced bone resorption and formation [8, 9, 10]. In contrast, some small hibernators exhibit decreased protein translation [11], suppression of wound healing [12, 13], and an imbalance in bone remodeling, leading to loss of bone mass [14, 15]. Research into the regulatory

mechanisms allowing for such unique hibernation phenotype in bears may yield insights into treatments for human diseases.

A recent study demonstrated that broad changes in gene expression patterns, rather than specific hibernation-related genes, reflect changes in metabolism during hibernation in black bears [16], similar to that seen in small mammals [11, 17]. The gene expression changes may give rise to the remodeling of tissue proteome that may be essential for hibernation in bears. Indeed protein turnover is elevated in black bear serum during hibernation [16, 18], and changes in specific serum proteins, including acute phase proteins [19, 20], aminotransferases [21] and corticosteroid binding globulin (CBG), have been reported in hibernating bears. However, systemic changes in the serum proteomes of large hibernators, including bears, have not been characterized to our knowledge. The objective of this study was to examine the changes in the serum proteome of the active and hibernating black bear to identify differentially expressed proteins in order to provide novel insights into the biochemical adaptation to hibernation in bears. The serum proteome changes were assessed using a two-dimensional difference gel electrophoresis (DIGE) approach [22] from the same animals prior to and during hibernation. Some of the differentially regulated proteins identified by DIGE were also confirmed using SDS PAGE followed by immunodetection.

6.2. Methods and Materials

6.2.1. Animals

6.2.1.1. Proteomics Studies

For the comparison of serum proteomes between active and hibernating black bears, paired serum samples from 8 animals (animals 1–8 in **Table 6.1**) were taken from two time points: prior to (PRE) and during hibernation (HIB). For the follow up western immunoblotting studies, paired serum samples were used from an additional four animals (**Table 6.1**). Animals were anesthetized with a 2:1 mixture of ketamine (100 mg/ml):xylazine (100 mg/ml) at a

Table 6.1: Black Bear Animal and Sampling Data.

Bear #	Sampling Days for PRE and HIB samples, respectively (Julian Day)	Pregnant at Time of Den Entry	Parturition Day (Julian Day)	Initial Weight (lbs)	Weight Change during Hibernation (% of Initial)
1	324, 59	Y	14	186	-20.9%
2	334, 49	N	NA	186	-20.5%
3	283, 69	Y	28	181	-24.9%
4	324, 69	Y	11	256	-20.2%
5	294, 49	Y	2	111	-34.3%
6	285, 49	N	NA	93	-40.4%
7	276, 59	Y	36	156	-27.1%
8	284, 59	N	NA	186	-23.8%

dosage of 1 cc of the mixture per 45.5 kg of body mass. Blood samples were drawn from the femoral vein while the animal was anesthetized, and the samples were transported to the laboratory in an ice- packed cooler. Immediately on return to the laboratory, the blood was spun to isolate the serum and was frozen at –20°C. The Virginia Polytechnic Institute and State University Animal Care Committee approved all bear handling protocols (#98-069- F&WS).

6.2.2. Difference Gel Electrophoresis (DIGE)

Black bear sera were processed prior to proteome analysis exactly as described previously for grizzly bear sera [23]. Briefly, serum samples were depleted of albumin and IgG with Aurum Serum Protein Mini-kits (BioRad, Hercules, CA, USA), and total serum protein concentration was determined by the bicinchoninic method [24] using bovine serum albumin (Thermofisher Scientific, Waltham, MA, USA) as the standard. After processing, proteins were separated by a modified DIGE method [22]. Proteins were precipitated using a 2-D Clean-Up kit (GE Healthcare, Piscataway, NJ, USA) and resuspended to a final concentration of 5 µg protein / µL in lysis buffer [7 M urea, 2 M thiourea, 5 mM magnesium acetate, 30 mM TRIS, 4% (v/v) CHAPS; all reagents purchased from Thermofisher Scientific]. Samples were labeled with Cy Dye DIGE Fluor minimal dyes (GE Healthcare) according to manufacturer's instructions and to minimize dye bias. A pooled internal standard consisting of 25 µL of each sample was labeled with Cy2 dye. 50 µL of labeled pre-hibernation serum samples were combined with 50 µL of hibernation samples and 50 µL of the internal standard. Each of these combined samples were diluted in rehydration buffer [7 M urea, 2 M thiourea, 4% (v/v) CHAPS, 40 mM DTT, 0.5% (v/v) pH 4-7 IPG buffer] and rehydration-loaded onto 24 cm, pH 4-7 Immobiline DryStrip IPG strips (GE Healthcare) in a reswelling tray for 12 h. Isoelectric focusing was done on a IPGphor II (GE Healthcare) under the following conditions: step 100 V, 1 h; step 500 V, 2 h; gradient to 1000 V, 2 h; gradient to 3000 V, 3 h; step 3000 V, 2 h; gradient to 8000 V, 3 h; step 8000 V, 9 h; step to 500 V, 13 h; total 109600 Vh. For the second dimension separation, IPG strips were equilibrated [6 M urea, 30% (v/v) glycerol, 50 mM TRIS, bromophenol blue (Thermofisher Scientific)] with first 1% (w/v) DTT for 30 min, then 2.5% (w/v) IAA for an additional 30 min. Equilibrated strips were placed on top of 12% SDS-PAGE

gels and sealed with 1% agarose. 2nd dimension electrophoresis was done using an Ettan DALTsix electrophoresis unit (GE Healthcare) at 1.5 W per gel for 30 min, then 17.5 W per gel until the dye front reached the bottom of the gels. Gels were scanned on a Typhoon Variable Mode Imager (GE Healthcare) at excitation/emission wavelengths of 457/520 nm (Cy2), 532/580 nm (Cy3), and 633/670 nm (Cy5). Protein spot expression was analyzed with DyCyder 7 software (GE Healthcare). After spot expression analysis, a preparatory gel was run to isolate proteins for identification by mass spectrometry. 1st dimension separation was the same as described above, except proteins were not labeled with Cy Dyes, and 500 μg unlabeled, pooled serum protein was loaded onto a single IPG gel strip. 2nd dimension separation was the same as described above. After 2D separation, the gel was stained with colloidal Coomassie G-250 [0.12% (w/v) Coomassie G-250, 10% (w/v) ammonium sulfate, 10% (v/v) phosphoric acid, 20% (v/v) methanol] and destained with 10% (v/v) phosphoric acid and 20% (v/v) methanol, then dH_2O. Protein spots of interest were excised manually and stored in microcentrifuge tubes at 4°C with deionized water until mass spectrometric analysis.

6.2.3. Tandem Mass Spectrometry (MS/MS)

Protein spots were identified by tandem mass spectrometry. A gel piece containing 1 pmol bovine serum albumin (BSA) stained with colloidal Coomassie stain was digested in parallel with protein spots as a control. Excised gel plugs were diced into approximately 1 mm^3 portions, and the Coomassie stain was removed by washing three times with ddH_2O, three times with 50 mM NH_4CO_3 in 50% acetonitrile (ACN), and a final wash with 100% ACN for 5 min each (Thermofisher Scientific). After washing, samples were reduced with 10 mM DTT in 100 mM NH_4CO_3 for 30 min at 50°C. After another 5 min 100% ACN wash, samples were alkylated with 55 mM IAA in 100 mM NH_4CO_3 for 30 min at room temperature in the dark. Samples were washed again with 100 mM NH_4CO_3 for 15 min, then 100% ACN for 5 min. Samples were dried down on a SpeedVac (Thermofisher) at 4°C for 20 min. 10 ng trypsin in 100 mM NH_4CO_3 was added to the samples and incubated for 16 h in a 37°C water bath. Samples were diluted with ddH_2O and bath sonicated for 10 min. After centrifugation at 1000 rpm for 30 s, the supernatant was transferred to a collection tube with 5 μL of 5% formic acid

(FA) in 50% ACN. Digested peptides remaining in the digested gel plugs were extracted with 5% formic acid (FA) in 50% ACN. The volume of the collected supernatant was reduced to 10 to 15 µL in a SpeedVac. Samples were cleaned using C18 ZipTips (Millipore, Billerica, MA, USA). Samples were acidified with 1% FA, and peptides were bound to equilibrated ZipTips (wetting with 50% ACN 3 times, then 0.1% FA 3 times) by 20 cycles of drawing and expelling of the sample. ZipTips were washed twice with 0.1% FA, and peptides were eluted by 10 cycles of drawing and expelling of 5 µL 50% ACN. MS/MS was performed on an Qtrap 2000 LC/MS/MS (Applied Biosystems, Foster City, CA). 60 fmol of a BSA digest was used as a LC/MS/MS control. Peptides were identified using MASCOT MS/MS Ion Search (Matrix Science, Boston, Massachusetts, USA) against the NCBI non-redundant protein database. Mass spectra with fewer than 30 peaks were discarded, except for very dilute samples where we discarded spectra with fewer than 10 peaks. The following parameters were used for MASCOT searches: trypsin digestion, carbamindomethyl fixed modifications, methionine oxidation variable modifications, monoisotropic mass values, ± 1.2 Da peptide and ± 0.8 Da fragment mass tolerances, and up to one missed cleavage. GOMiner was used to determine significantly enriched Gene Ontology (GO) pathways [25]. UniProt gene identifiers for homologous human genes were obtained for each unique, differentially expressed black bear protein (n = 15) that was identified by MS/MS. A list of the N-terminal serum proteome of human blood (n = 213) was used as the background total gene list [26] because similar data does not exist for the American black bear, to our knowledge. The enrichment of each GO category was calculated as the proportion of changed to total proteins in the category relative to the expected proportion. GO categories with high enrichment (> 1.5) and one-tailed Fisher's exact test p-values < 0.05 were considered categories of interest for the generation of hypotheses.

6.2.4. Western Blotting

Commercial antibodies against several proteins were obtained, including sheep anti-human α_2-macroglobulin (Affinity BioReagents, Golden, Colorado, USA), goat anti-dog transferrin (Tf, Bethyl Laboratories, Montgomery, Texas, USA), sheep anti-human apolipoprotein A1 (ApoA1, Abcam, Cambridge, Massachusetts, USA), rabbit anti-human haptoglobin (Hp,

Sigma, St. Louis, Missouri, USA), anti-rabbit α1-antitrypsin (A1AT, Sigma), and anti-rabbit kininogen 1 (KNG, Sigma). Secondary antibodies were also obtained, including donkey anti-sheep IgG and rabbit anti-goat IgG (Bethyl), and goat anti-rabbit IgG (BioRad), all conjugated to horseradish peroxidase (HRP). Serum proteins were detected by western immunoblotting exactly as described previously [23]. Total protein concentrations were determined by the BCA method [24], and samples were adjusted to 125 µg protein/mL with Laemmli's buffer [27]. 2.5 µg of total protein was loaded into wells on polyacrylamide gels along with a broad range molecular weight protein standard (BioRad), and proteins were separated at 200 V for 45 min using a discontinuous buffer. Proteins were transferred to a 0.22 µm pore size membrane (BioRad) using a TransBlot SD semi-dry electrophoretic transfer cell (BioRad). Transfer efficiency was checked by Ponceau S staining of the membrane. Membranes were then washed with Tris buffered saline with Tween-20 [TTBS; 20 mM Tris, 300 mM NaCl, 0.1% Tween-20 (BioShop, Burlington, ON, Canada), pH 7.4] and blocked with 5% skim milk powder in TTBS (SM) for 1 h at room temperature. Blots were rinsed with TTBS, and 10 mL primary detection antibody diluted 1:1500 in SM was added. After 1 h incubation, blots were washed 3x with TTBS, and appropriate secondary antibody diluted 1:3000 in SM was added. After another 1 h incubation, membranes were washed 3x with TTBS and 1x with TTBS without Tween-20. ECL Plus (GE Healthcare) detection solution was freshly prepared, and 1 mL was applied to the membrane. After 5 min, membranes were scanned on a Pharos scanner using the QuanityOne software (BioRad). Protein band densitometry was performed using ImageJ 1.45s [28] and expression shown as arbitrary units.

6.2.5. Statistics

Differential protein expression was compared using repeated measures one-way analysis of variance (1-way RMANOVA) in the Biological Variation Analysis module in DeCyder 7 (GE Healthcare). p-values were corrected for multiple comparisons by the method of Benjamini and Hechberg [29] in R 2.14.0 [30] to yield false discovery rates (FDR) for the DIGE experiments. Significant differences in protein expression using immunodetection were compared using 1-way RMANOVA in R 2.14.0.

6.3. Results

The average Julian day of sampling were 301 and 58, respectively for PRE and HIB samples. All animals were adult females (mean age: 8.3 years), and five were pregnant at the time of den entry. All five gave birth during hibernation, and the average Julian day of parturition was 18. One cohort of cubs was lost shortly after birth (bear #5). A mean of 57.4 lbs or 27% of initial body weight was lost during hibernation, and no correlation was observed in this weight loss between pregnant and non-pregnant animals.

6.3.1. DIGE Analysis

The DIGE analysis identified a maximum of 2230 total protein spots per sample, of which 70 spots were differentially expressed in the hibernating bears. In total 36 and 34 protein spots were significantly down and up regulated, respectively, in the HIB compared to the PRE samples (**Figure 6.1** and **Table 6.2**). Many of these differentially expressed spots formed electrophoretic trains, suggesting multiple isoforms. The differentially expressed protein spots were ranked based on their p-values and the top 29 spots were chosen for identification by mass spectrometry. We obtained 23 protein IDs (**Table 6.2** and highlighted spots in **Figure 6.1**); all but two of the identified proteins were matched to giant panda (*Ailuropoda melanoleuca*) predicted proteins in the NCBI non-redundant protein database, and the remaining two were matched to dog (*Canis familiaris*) proteins. The identified proteins that were significantly upregulated during hibernation (abbreviated gene names and accession numbers in parentheses) included α_2-macroglobulin (A2M; EFB20759), α_1B-glycoprotein (A1BG; EFB23492), complement components C1s (C1S; EFB13954) and C4 (C4; EFB21208), immunoglobulin μ heavy (IGHM; AAX73309) and J chains (IGJ; EFB23253), α_1-antitrypsin (A1AT; XP_002920519), clusterin (CLU; EFB22766), and haptoglobin (HP; EFB23129). Significantly downregulated proteins included C4b binding protein α chain (C4BPA; EFB13508), transferrin (TF; EFB18586), kininogen 1 (KNG; XP_002914859), α_2-

HS-glycoprotein (AHSG; XP_002914863), and apolipoproteins A-I (APOA1; XP_002919539) and A-IV (APOA4; XP_546510).

These identified proteins were grouped into Gene Ontology categories of biological function in GoMiner, and selected significantly enriched categories are presented in Table 6.3. Many of the proteins are multifunctional and involved in several pathways, including immune system processes (GO:002376: CLU, AHSG, KNG, C4, C1S, C4BPA, A2M, APOA1, IGHM, APOA4, and IGJ). These proteins can also be subcategorized as proteins involved in adaptive (GO:0002250) and innate immunity (GO:0045087), complement activation (GO:0006956), and the acute phase response (GO:0006953). Three other significantly enriched GO categories included digestion (GO:0007686: APOA1 and APOA4), platelet degranulation (GO:0002576: A2M, APOA1, CLU, KNG, A1AT, and TF) and the response to wounding (GO:0009611: CLU, AHSG, A1AT, APOA1, C4, KNG, A2M, APOA4, HP, and TF).

6.3.2. Immunodetection

The validity of the proteomics results was confirmed by immunodetection of select serum proteins in the bears prior to and during hibernation. We performed western immunoblotting on an expanded set of black bear serum samples collected during the PRE and HIB periods using antibodies against 6 of the proteins identified in our MS/MS analysis (**Figure 6.2**). We found that pregnancy status had no effect on the expression of any of the tested proteins, so this factor was omitted from the models. KNG ($F = 10.7$, $p < 0.05$) and TF ($F = 10.4$, $p < 0.05$) were significantly reduced in HIB compared to PRE bears and this is in agreement with our DIGE results (**Table 6.2** and **Figure 6.2E/F**). Also, A2M expression was higher ($F = 3.22$, $p = 0.07$) in the HIB bears compared to PRE animals (**Figure 6.2B**). There were no significant differences in A1AT, APOA1, and HP expression in HIB compared to PRE bears (**Figure 6.2A**, **C**, and **D**).

Figure 6.1: Representative Images of a Cy2-stained Black Bear Serum Proteins Separated by Difference Gel Electrophoresis.
Proteins were separated utilizing a pH 4 – 7 (left to right) immobilized pH gradient isoelectric focusing gel strip for the 1st, horizontal separation and a 12% SDS-PAGE gel in the 2nd, vertical separation. Significantly ($p < 0.05$) up (yellow) and down regulated (green) proteins are indicated by colour. Protein spots that were subsequently identified by tandem mass spectrometry are indicated by numbers, which correspond to spot IDs in table 2.

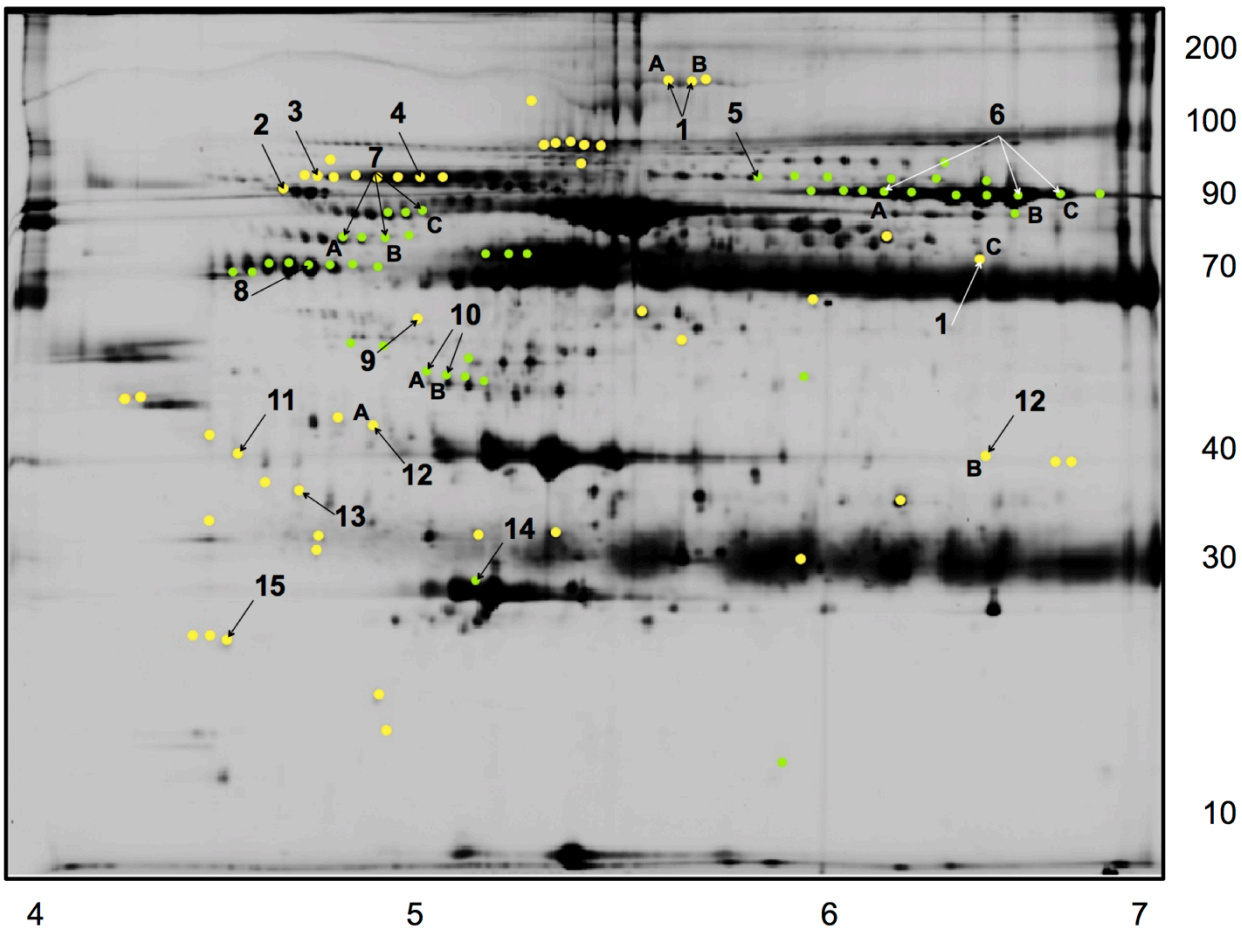
200
100
90
70
40
30
10
4
5
6
7
A
B
1
2
3
4
5
6
7
A
B
C
8
9
10
A
B
11
12
13
14
15
12
B
1
C

Table 6.2: Protein Spots Identified by Tandem Mass Spectrometric Analysis and MASCOT Database Searching.
Spot IDs correspond to labeled spots in Figure 1. p-values from the 1-way RMANOVA and False Discovery Rate (FDR) for each protein are shown. Fold changes are relative change in protein spot volume from pre-hibernation to hibernation.

Spot ID	Protein ID	Accession	Fold Change	p	FDR
1A	α_2-macroglobulin	EFB20759	2.1	0.003	0.128
1B	α_2-macroglobulin	EFB20759	1.7	0.004	0.130
1C	α_2-macroglobulin	EFB20759	1.8	0.013	0.205
2	α_1B-glycoprotein	EFB23492	1.5	0.021	0.263
3	Complement C1s subcomponent	EFB13954	1.6	0.001	0.128
4	Immunoglobulin μ Heavy Chain	AAX73309	1.6	0.002	0.128
5	C4b binding protein α chain precursor	EFB13508	-1.5	0.007	0.165
6A	Transferrin precursor	EFB18586	-1.8	0.011	0.192
6B	Transferrin precursor	EFB18586	-1.9	0.006	0.164
6C	Transferrin precursor	EFB18586	-2.1	0.004	0.130
7A	Kininogen 1	XP_002914859	-1.7	0.011	0.192
7B	Kininogen 1	XP_002914859	-2.5	0.005	0.147
7C	Kininogen 1	XP_002914859	-1.8	0.022	0.268
8	α_2-HS-glycoprotein	XP_002914863	-1.9	0.024	0.195
9	Complement component C4	EFB21208	1.5	0.003	0.128
10A	Apolipoprotein A-IV	XP_546510	-5.7	0.003	0.128
10B	Apolipoprotein A-IV	XP_546510	-8.1	0.004	0.128
11	α_1-antitrypsin	XP_002920519	1.6	0.024	0.268
12A	Clusterin	EFB22766	1.5	0.032	0.303
12B	Clusterin	EFB22766	2.0	0.004	0.139
13	Haptoglobin	EFB23129	2.0	0.007	0.168
14	Apolipoprotein A-I	XP_002919539	-1.7	0.042	0.323
15	Immunoglobulin J chain	EFB23253	1.5	0.044	0.328

Figure 6.2: Protein Expression with Immunoblots.
Data are presented as histograms of the mean + standard error of the mean (n = 8) protein expression (arbitrary units as a proportion of pooled reference black bear serum) between pre-hibernating ("Pre") and hibernating ("Hib") black bears using antibodies against A) α_1-antitrypsin, B) α_2-macroglobulin, C) apolipoprotein A1, D) haptoglobin, E) kininogen 1, and F) transferrin. Changes in protein expression between hibernation states of individual bears are overlaid on top of the histogram. A representative western blot is shown inset, and the left and right wells are loaded with pre-hibernation and hibernation serum samples, respectively. Statistically significant differences ($p < 0.05$, paired 1-way ANOVA) between hibernation states are indicated with asterisks. A2M was marginally significant ($p = 0.07$, indicated inset).

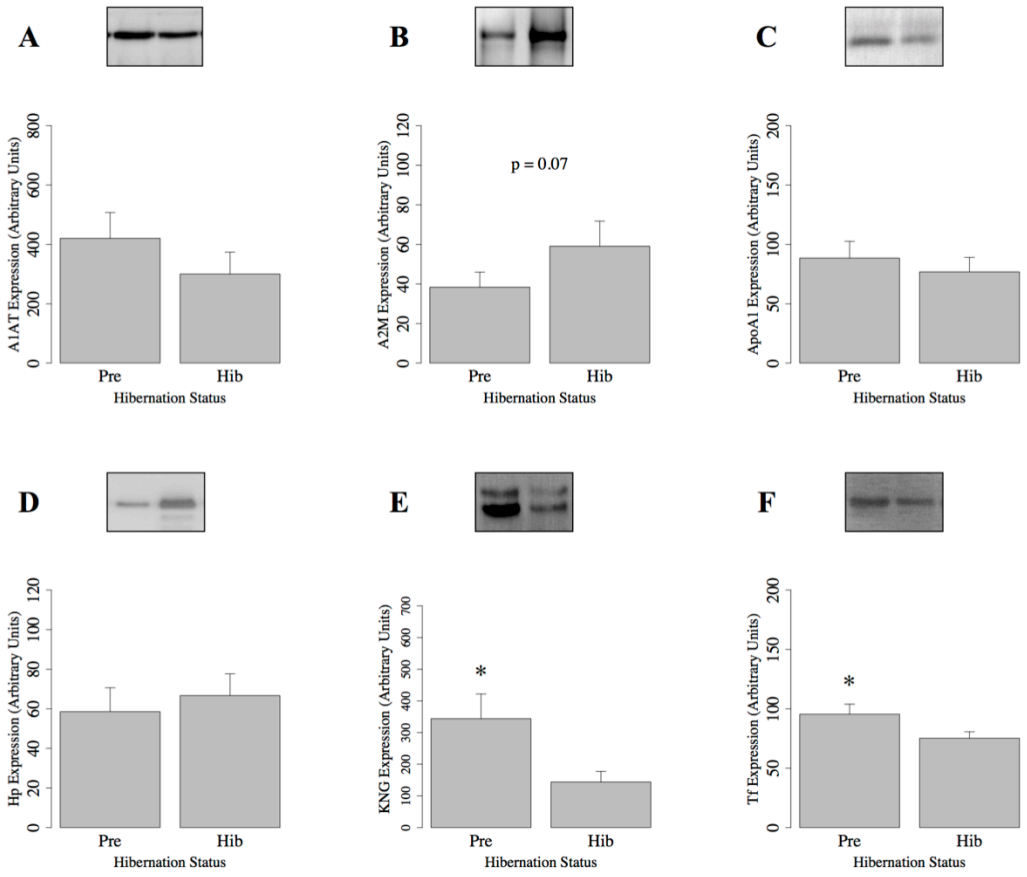
A
A1AT Expression (Arbitrary Units)
0
200
400
600
800
Pre
Hib
Hibernation Status
B
A2M Expression (Arbitrary Units)
p = 0.07
0
20
40
60
80
100
120
Pre
Hib
Hibernation Status
C
ApoA1 Expression (Arbitrary Units)
0
50
100
150
200
Pre
Hib
Hibernation Status
D
Hp Expression (Arbitrary Units)
0
20
40
60
80
100
120
Pre
Hib
Hibernation Status
E
KNG Expression (Arbitrary Units)
*
0
100
200
300
400
500
600
700
Pre
Hib
Hibernation Status
F
Tf Expression (Arbitrary Units)
*
0
50
100
150
200
Pre
Hib
Hibernation Status

Table 6.3: Significantly Enriched Selected Gene Ontology Categories of Serum Proteins in Hibernating Black Bears.

Category Name	GO Category ID	p-value	FDR	Enrichment	Changed Proteins	Total Proteins in Category
Immune System Process	002376	0.0036	0.0000	1.98	11	79
Adaptive Immune Response	0002250	0.0111	0.0562	3.23	5	22
Innate Immune Response	0045087	0.0212	0.1018	2.43	6	35
Acute Phase Response	0006953	0.0001	0.0000	6.09	6	14
Complement Activation	0006956	0.0275	0.2000	2.63	5	27
Response to Wounding	0009611	0.0083	0.0556	1.95	10	73
Digestion	0007586	0.0257	0.1791	7.10	2	4
Platelet Degranulation	0002576	0.0035	0.0000	3.41	6	25

6.4. Discussion

Our results demonstrated for the first time the changes in the serum proteome that occur during hibernation in the American black bear. Only a small proportion (< 5%) of serum protein spots were differentially regulated according to our selection criteria during hibernation compared to active black bears, and comparable numbers of proteins were up or downregulated. In comparison, studies on gene expression changes in the liver of black bears found that approximately 7% of all genes were differentially expressed (using our selection criteria), and 67% of these differentially expressed genes were upregulated during hibernation [16]. Our results lead us to suggest that specific changes in protein expression are associated with hibernation in black bears, including proteins involved in immunity, coagulation, and bone metabolism.

Immunity-related processes were significantly enriched with differentially expressed proteins in hibernating black bears. Prolonged fasting and hypothermia in animals generally has suppressive effects on immunity, including the atrophy of lymphoid tissues, suppressed reactions to antigens, and leukopenia [31, 32, 33]. In other hibernators, including sciurids, both the adaptive and innate immune systems exhibit reduced functionality during bouts of torpor [34]. The differences in core body temperature between hibernating sciurids (near freezing) and black bears (30-36°C) may be a reason for this difference as the suppression of immune function is associated with the degree of hypothermia [31]. During the hibernation season, squirrels frequently arouse from torpor to warm up to normothermic body temperature for 5 – 24 h before re-entering torpor [1, 35]. However, despite the large energetic costs associated with arousals in sciurids, the frequent episodes of near normothermia are associated with bursts of immune function essential to clear the pathogen loads that may have accumulated during the torpor bout [34, 36].

Bears have a prolonged continuous hibernation physiology, unlike the 10-14 day bouts of hypometabolism in sciurids that are interrupted by brief (5 – 24 h) interbout arousal periods. Thus, ursids may have adopted different strategies to cope with this different physiological state of metabolic depression. The differential regulation of immunity-related proteins during

hibernation may be one such adaptation that allows bears to remain in their hypometabolic and mildly hypothermic state, while aiding in the maintenance of immune competence and resistance against infection and disease. However, the increased expression of immunity-related proteins may come at the cost of reduced efficiency of energy conservation [33]. Moreover, a recent report demonstrated that the healing of cutaneous wounds is maintained during hibernation [7], suggesting that the mechanisms underlying wound healing and immune function are active during hibernation in bears. This is a unique adaptation because hypothermia and metabolic depression suppresses wound healing in other animals [12, 13]. Since the initial response to wounding includes the participation of the innate immune system in restoring hemostasis and preventing the development of infections [37], our results suggest that the differential expression of immune-related proteins may be playing a key role in this unique healing ability of black bears during hibernation. Moreover, it is unlikely that the differential expression of these immune proteins were induced by an immune response since A2M and HP have been shown to be upregulated during hibernation independent of inflammatory acute phase reaction in brown bears [19]. It has been shown that handling stress may induce an acute phase reaction in other animals [38], but it is currently unclear if handling stress similarly modulates the expression of acute phase proteins in captive black bears. In general, however, the immune system of ursids is presently poorly characterized, and it remains to be determined how the changes in protein expression that were observed in this investigation translate to the functioning of the immune system during hibernation.

Other proteins that were modulated by hibernation, including A2M and KNG, appear to play roles in the regulation of blood coagulation. The blood of hibernating animals have been observed to be in a hypocoagulable state, including in squirrels and hedgehogs [39, 40], and platelet aggregation has been shown to be reduced in brown bears several days after arousal from hibernation [41]. This decrease in coagulation activity may contribute to the prevention of blood clotting in the face of low cardiac output during hibernation [2]. Elevations in serum A2M levels during hibernation in squirrels are linked to increased clotting times [42]. The downregulation of KNG expression during hibernation may also contribute to this state of hypocoagulation as this protein is a coagulation cascade cofactor that increases the rate of some enzymatic reactions, including prekallikein to kallikein and factor XI and XII to XIa and XIIa, respectively [43, 44]. Moreover, decreased body temperatures during hibernation and in

hypothermic states may be playing a role by reducing coagulation enzyme activity and platelet adhesion, which may also contribute to increased clotting times [39, 45]. Based on our serum proteome data, we propose that coagulation activity and blood clotting may be reduced in hibernating bears.

Hibernating black bears preserve bone mass despite several months of inactivity and anuria [15], and the mechanisms underlying this phenomenon has been the subject of intense research due to its possible implications for human medicine. Bone remodeling during hibernation in bears decreases to approximately 25% of summer active levels [8] and bone formation and resorption are balanced as suggested by changes in some markers of bone metabolism, including carboxy-terminal telopeptide of type I collagen [46, 47], osteocalcin [46], and parathyroid hormone [48]. Other hibernators exhibit increased osteoclastic and decreased osteoblastic activity [14, 49], resulting in overall decrease in bone mass during torpor. We did not detect these markers of bone metabolism in this study, but we suggest that these low molecular weight peptides are poorly resolved in 2D gels and changes in the levels of these markers were not detected in our analysis.

However, we found that serum AHSG levels were downregulated during hibernation in bears. AHSG is secreted by the liver into circulation, where it is a major carrier protein of calcium phosphate and carbonate, and is a major non-collagen protein constituent of bone [50]. This protein has been implicated as an inhibitor of osteogenesis [51] and may regulate bone remodeling by binding to and blocking the action of cytokines that modulate bone marrow cell proliferation and mineralization by the transforming growth factor (TGF)-β family of cytokines [52]. The targeted deletion of one copy of the AHSG gene in mice resulted in a two-fold decrease in serum AHSG levels and abnormal bone development, including decreased mineral formation rate and increased mineral content [52], which may suggest a role for this protein in modulating the rate of bone formation. Thus, we propose that the downregulation of AHSG in hibernating black bears may lead to a reduction of TGF-β antagonist activity and the modulation bone formation rates. Furthermore, the immune system plays an important role in bone remodeling [53], and the changes in serum immune-related proteins in black bear serum during hibernation lead us to hypothesize a role for these proteome changes in bone

remodeling. However, further research is warranted to elucidate the specific proteins and processes involved and their modes of action.

We found that A1BG is upregulated during hibernation in bears. A1BG is a homologue of the woodchuck hibernation induction trigger (HIT), which has been proposed as a putative cross-species hibernation inducer by acting on opioid receptors [54]. There have been reports of successful induction of hibernation in active animals by injection with purified serum containing HIT [55], but other attempts to reproduce this effect in other species have not been successful [56]. HIT possesses properties similar to that of the delta opoid receptor agonist [D-Ala^2, D-Leu^5]-enkephalin (DADLE) [57] and this opioid activity can protect organs against ischemia-reperfusion injury [58, 59]. However, the mechanisms underlying the actions of this protein remain unclear, but the upregulation of A1BG during hibernation in black bears suggests that this opioid activity may be an adaptation to protect tissues and organs during hibernation, including hypothermia and reduced blood flow.

There were other protein changes during hibernation, including possible markers of nutritional status, but their functions are poorly characterized or have never been studied in bears. For instance, APOA1 [60], APOA4 [60, 61], and TF [62] are implicated as markers of fasting in other species, and their downregulation in the serum of hibernating, anoretic black bears supports these proteins as markers of fasting status in animals. Additionally, these proteins are involved in various facets of peripheral nutrient transport [63], and increases in serum APOA4 levels has also been implicated as a putative satiety signal [64]. Together, the downregulation of these proteins may reflect a lower metabolic capacity and nutrient transport during hibernation in black bear.

Some potential confounders may affect the analysis of our data. Recently, Seger and coworkers found that levels of some markers of bone metabolism were different between lactating and non-lactating hibernating black bears [47], and suggested that the metabolic demands of lactogenesis were associated with increased bone resorption. We tested whether reproductive status of our experimental animals had an effect on protein expression in our immunoblot experiments, but no effect of reproduction was observed (data not shown) and this was subsequently dropped from the analysis. We lacked sufficient statistical power in the present

study to analyze the effect of lactation on the hibernating serum proteome of black bears, but this would be an interesting avenue of research to pursue in the future. Furthermore, the mechanisms underlying the changes in the hibernating serum proteome of bears are largely unclear. Some changes in protein levels may be due to decreased elimination or increased expression of proteins from serum. For example, anuria may contribute to the increase in the levels of some serum proteins because many serum proteins may be excreted in normal urine [65]. Moreover, the differential expression of some serum protein genes in the liver of hibernating black bears have been reported, including α_2-HS-glycoprotein, clusterin, and α_2-macroglobulin [16, 66]. We suggest that future studies should be designed to elucidate the mechanisms underlying these serum proteome changes in hibernating black bears.

In conclusion, for the first time we demonstrated that hibernation in black bears is associated with differential expression of serum proteins involved in immunity, coagulation, and bone metabolism. These results suggest novel mechanisms for some of the unique and remarkable metabolic and physiologic attributes of hibernating black bears, including the maintenance of wound healing [7], which contrasts with the compromisation of immune function in other species during fasting and hibernation [34]. The differential expression of immunity-related proteins during hibernation conceivably confers a survival advantage by enhancing processes that prevent the development of infections and diseases that may tax the limited resources of the fasting and prolonged denning in black bear. Furthermore, we identified differentially expressed proteins that were associated with fasting, coagulation, and bone remodeling, but further research is warranted to test the hypotheses that were generated by our proteomics experiments and to further understand the mechanisms that underlie the changes in the serum proteome of hibernating black bears.

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CHAPTER 7:
GENERAL CONCLUSIONS

The experiments described in this thesis aimed to investigate the roles that corticosteroid-binding globulin (CBG), bioavailable cortisol, and serum proteome changes play in the physiologic adaptation to stress associated with the major life history traits of and environmental challenges faced by ursids, including American black (*Ursus americanus*), grizzly (*U. arctos*), and polar bear (*U. maritimus*). This work has importance for the management of free-ranging animals because this represents the groundwork for the potential monitoring of the health and stress status of individuals and populations of bears. A series of studies were performed to this end, and the following conclusions were derived:

A CBG-like molecule was found in the sera of bears, and its binding affinities for cortisol were characterized (**Chapter 2**).

The amino acid sequence of grizzly bear CBG (gbCBG) shared 83 and 68% identity with dog (*Canis lupus familiaris*) and human (*Homo sapiens*) CBG, respectively. With this gbCBG sequence, a gbCBG-specific antibody and ELISA was developed. This ELISA cross-reacted with and quantified CBG concentrations in black, grizzly, and polar bears, and we showed that there are differences in mean CBG levels between these species (**Chapter 3**).

Mean serum free cortisol but not CBG concentrations were significantly affected by the stress profiles of different methods of capture, and mean CBG levels were lower in juvenile grizzly and polar bears relative to adults. Neither CBG nor cortisol levels varied with adult reproductive class in these species. However, adrenal function, as measured as total and free cortisol concentrations after exposure to the stress of capture and handling, was negatively associated with serum urea to creatinine (UC) ratios, which are surrogate measures for dietary protein in grizzly bears and fasting status in polar bears (**Chapter 4**).

There was an overall increase in bioavailable cortisol during hibernation in female black bears, which argues against the hypothesis that increased CBG expression buffers tissues against the

deleterious catabolic effects of this hormone during this period. Instead, we suggest that elevated free cortisol levels may be playing a role in supplying substrates to meet the metabolic demands of lactation in hibernating bears (**Chapter 5**).

The upregulation of innate immunity may be a key adaptation of hibernating black bears. Immunity-related proteins were significantly altered in response to hibernation and may contribute to maintaining immune competence during this period of hypothermic body temperature and hypometabolism (**Chapter 6**).

One of the major challenges in studying stress in free-ranging species is that the act of capture and handling constitutes an acute stressor that may obscure the pre-capture levels of pertinent indicators of the physiologic status of the animal, including glucocorticoids. However, stress-induced cortisol levels may still provide useful information regarding the health status of an animal. We found that stress-induced total and free cortisol levels are negatively associated with UC ratio in grizzly and polar bears, and positively associated with lactation in hibernating black bears, suggesting that the variation in adrenal function may be playing an important role in the adaptation to adverse environmental conditions and/or metabolic stress in bears. The measurement of stress-induced cortisol levels only tell a part of the story; however, the quantification of baseline levels of this hormone is required to make definitive conclusions regarding the health and stress status of animals [1]. Thus, the development of a bear CBG ELISA and the finding that CBG levels are not modulated by capture stress is an important advance towards a tool that may be employed to monitor the health status of wildlife. The biological factors that potentially modulate CBG expression in bears were then investigated to characterize the variation in the levels of this protein.

Altogether, we infer that there are few changes in CBG levels throughout much of the annual life cycle of bears, assuming that the variation of this protein by life history factors is similar between the bear species investigated in this thesis. This implies that CBG does not play a major adaptive role in the life history traits of bears, which is consistent with the hypothesis that long-lived species do not trade off long-term survival for short-term gains [2]. For example, in species that are short-lived or have limited opportunities to reproduce, CBG concentrations generally decrease during the reproductive season, leading to elevated free

cortisol levels that are linked to pathologies associated with chronic stress, including suppressed immunity and organ failure [2]. In long-lived species, including bears, environmental and nutritional factors may be the major driver of variation in CBG levels (e.g. in long-lived seabirds [3]). However, CBG was not significantly associated with our measures of dietary patterns and nutrition, including body condition, seasonal dietary patterns, and fasting, and the vast majority of the observed variation in the levels of this protein and free cortisol in bears remain unexplained. This thesis did not investigate the effect of other environmental variables on CBG levels, and we suggest that future studies should further examine the variation of CBG expression by factors that may impact the chronic health and stress status in free-ranging bears, including the temporal and spatial variability in food availability and habitat quality.

The physiologic state of fasting in polar bears has been suggested to be comparable to the state of hibernation in terrestrial bears, including hypometabolism and protein sparing [4]. We found some parallels between hibernating black bears and fasting polar bears, including elevated adrenal function and a lack of change in CBG concentrations relative to active and feeding animals, respectively. These parallels may raise the question as to whether the serum proteomic adaptations in hibernating black bears also occur in fasting polar bears. We suggest that since fasting polar bears are not necessarily protected from the elements by denning, some of the adaptations may be relatively more important, including the maintenance of immune competence and wound healing, but this remains to be determined. It may also be important to understand how chronic stressors, including anthropogenic activities and climate change, may impact the performance of individual and populations of free-ranging bears during these periods of low food availability. Overall, monitoring of chronic health and stress status of bears by noninvasive and serum markers, including levels of CBG, free cortisol and novel proteins, identified in the serum proteome of hibernating bears, may provide a tool-kit for use by managers and conservationists for effective management of bear population.

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Chapter 2

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APPENDICES

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Appendix 1: CBG, Cortisol, and Glucose Concentrations in Grizzly and Polar Bears

Figure S1: Cortisol Dynamics between Grizzly Bear Reproductive Classes Captured During the Reproductive Season.
Cortisol dynamics between reproductive classes in grizzly bears sampled during the reproductive season (prior to June 15th). Reproductive classes are: solitary adult females (age ≥ 5 years; SF), adult females with dependent cubs (AF), adult males (AM), juvenile females (age < 5 years; JF), and juvenile males (JM). Statistically different means (1-way ANOVA $p < 0.05$) are indicated with different letters.

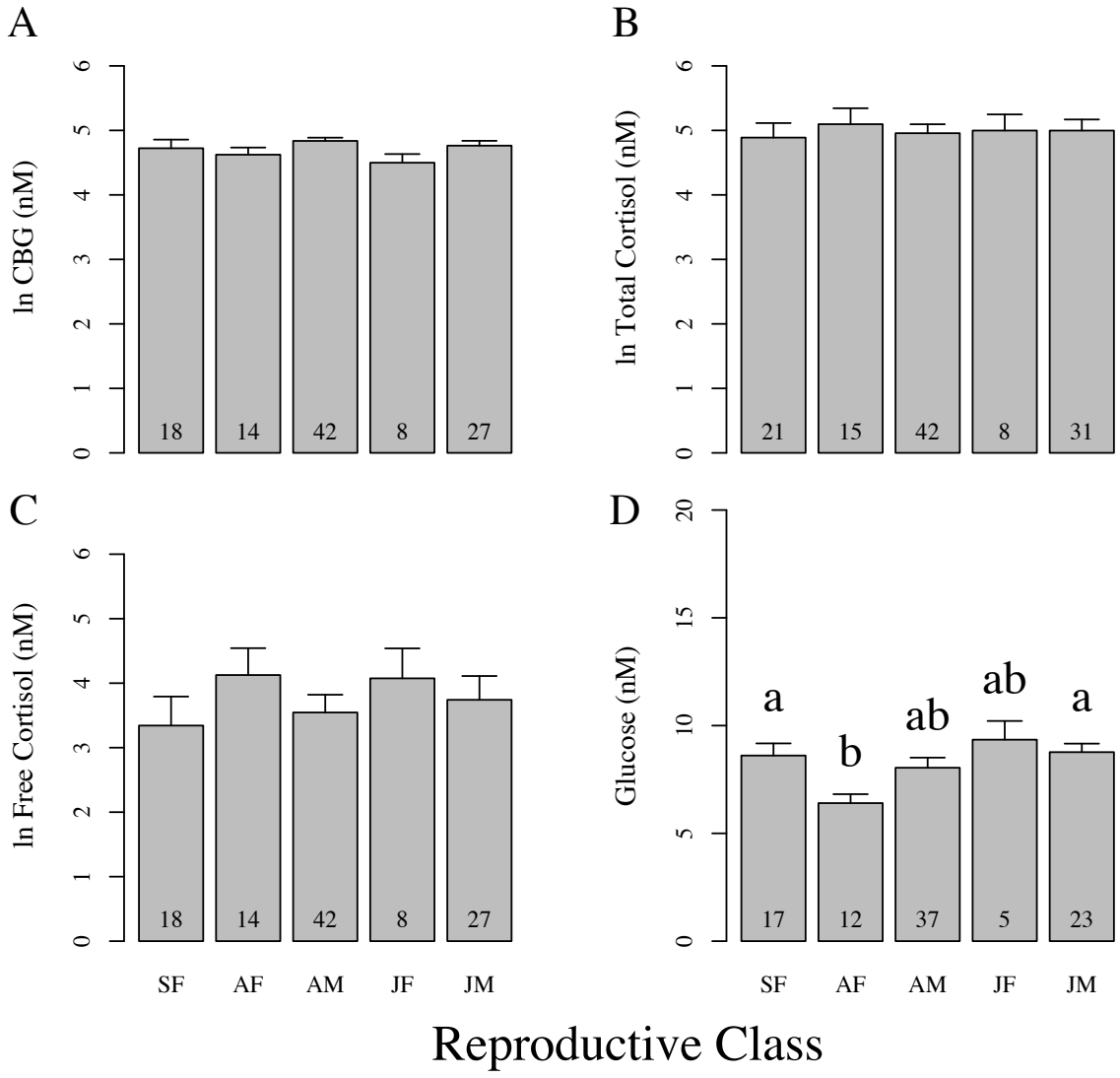

A
ln CBG (nM)
0 1 2 3 4 5 6
18 14 42 8 27
B
ln Total Cortisol (nM)
0 1 2 3 4 5 6
21 15 42 8 31
C
ln Free Cortisol (nM)
0 1 2 3 4 5 6
18 14 42 8 27
D
Glucose (nM)
0 5 10 15 20
a b ab ab a
17 12 37 5 23
SF AF AM JF JM
SF AF AM JF JM
Reproductive Class

Figure S2: Cortisol Dynamics in Grizzly Bears Captured in Different Years.
Mean + SEM concentrations of A) corticosteroid binding globulin (CBG), B) total cortisol, C) free cortisol, and D) glucose in grizzly bears captured in different years (1999 – 2009). None of the dependent variables were significantly different between years, taking into account the effect of age class on CBG and capture method on total and free cortisol levels. Sample sizes are inset.

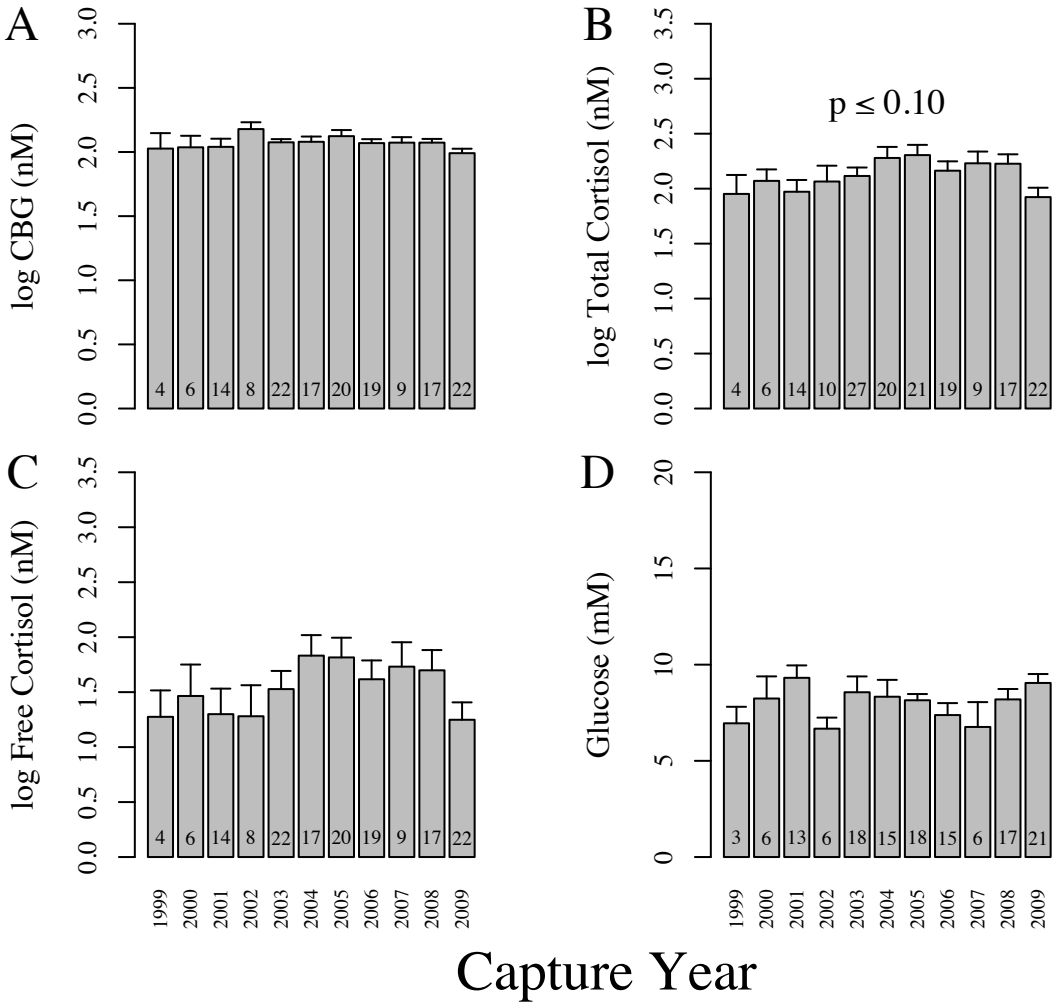

A
log CBG (nM)
0.0 0.5 1.0 1.5 2.0 2.5 3.0
4 6 14 8 22 17 20 19 9 17 22
B
log Total Cortisol (nM)
p ≤ 0.10
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
4 6 14 10 27 20 21 19 9 17 22
C
log Free Cortisol (nM)
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
4 6 14 8 22 17 20 19 9 17 22
D
Glucose (mM)
0 5 10 15 20
3 6 13 6 18 15 18 15 6 17 21
1999 2000 2001 2002 2003 2004 2005 2006 2007 2008 2009
Capture Year

Figure S3: Cortisol Dynamics in Grizzly Bears Captured in Different Months.
Mean + SEM concentrations of A) corticosteroid binding globulin (CBG), B) total cortisol, C) free cortisol, and D) glucose in grizzly bears captured in different months (April – October). None of the dependent variables were significantly different between months, taking into account the effect of age class on CBG and capture method on total and free cortisol levels. Sample sizes are inset.

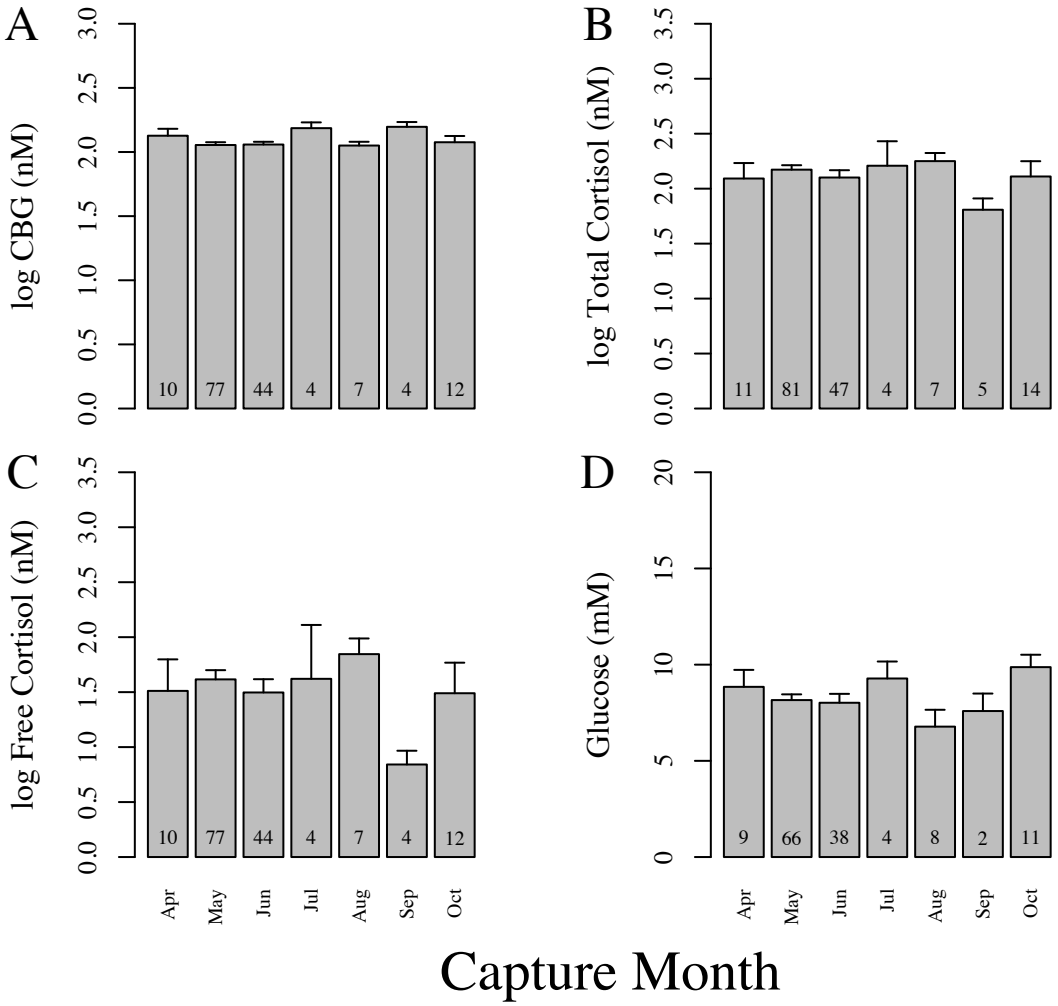

A
log CBG (nM)
0.0 0.5 1.0 1.5 2.0 2.5 3.0
10 77 44 4 7 4 12
B
log Total Cortisol (nM)
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
11 81 47 4 7 5 14
C
log Free Cortisol (nM)
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
10 77 44 4 7 4 12
D
Glucose (mM)
0 5 10 15 20
9 66 38 4 8 2 11
Apr May Jun Jul Aug Sep Oct
Capture Month

Figure S4: Cortisol Dynamics in Grizzly Bears Captured in Different Populations. Mean + SEM concentrations of A) corticosteroid binding globulin (CBG), B) total cortisol, C) free cortisol, and D) glucose in grizzly bears captured in different populations. Populations are defined as: Alberta North (AN), Castle (Cas), Clearwater (CW), Grande Cache (GC), Livingstone (LS), Swan Hills (SH), and Yellowhead (YH). None of the dependent variables were significantly different between years, taking into account the effect of age class on CBG and capture method on total and free cortisol levels. Sample sizes are inset.

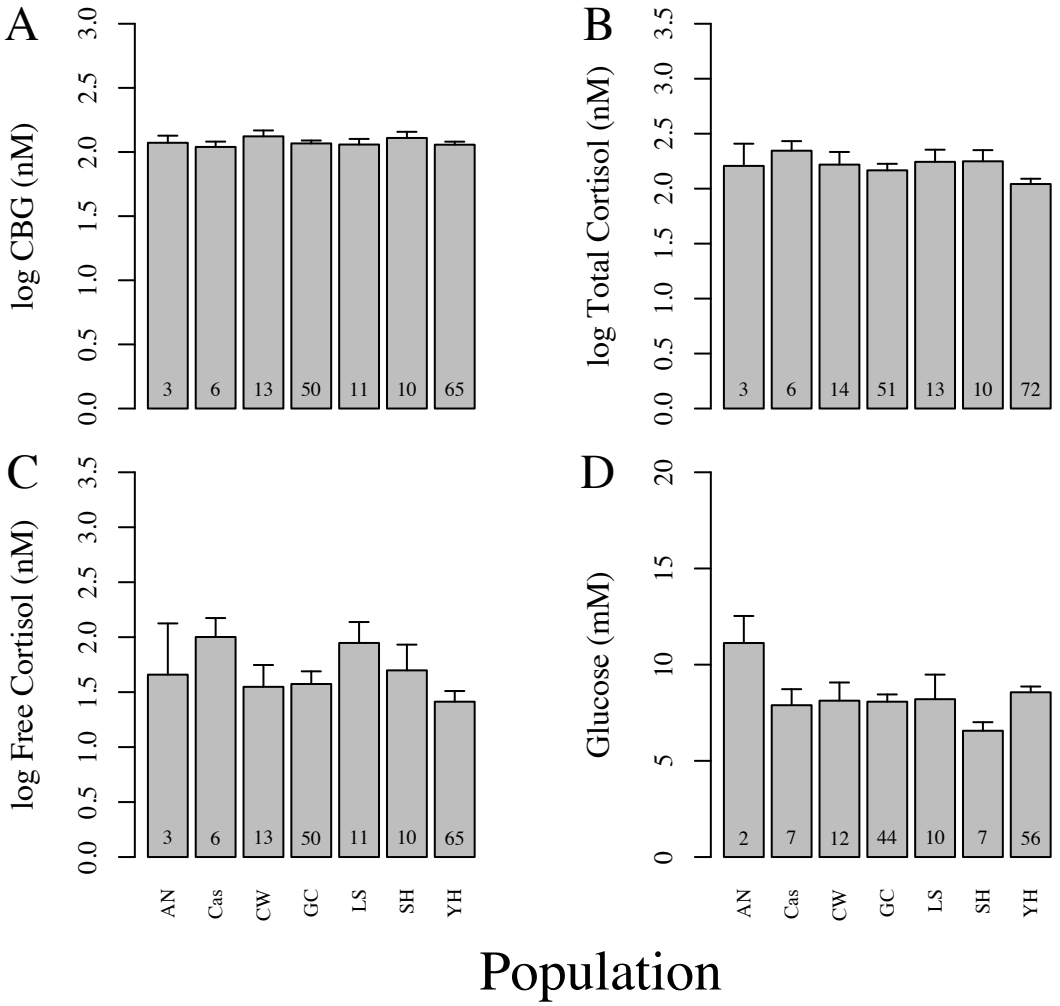

A
log CBG (nM)
0.0 0.5 1.0 1.5 2.0 2.5 3.0
3 6 13 50 11 10 65
B
log Total Cortisol (nM)
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
3 6 14 51 13 10 72
C
log Free Cortisol (nM)
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
3 6 13 50 11 10 65
D
Glucose (mM)
0 5 10 15 20
2 7 12 44 10 7 56
AN Cas CW GC LS SH YH
Population

Figure S5: Cortisol Dynamics in Polar Bears Captured in Different Years.
Mean + SEM concentrations of A) corticosteroid binding globulin (CBG), B) total cortisol, C) free cortisol, and D) glucose in polar bears captured in different years (1995 – 2009). There were significant differences in all of the dependent variables between years, taking into account the effect of age class on CBG and free cortisol, and are indicated by different letters (ANOVA $p < 0.05$). Sample sizes are inset.

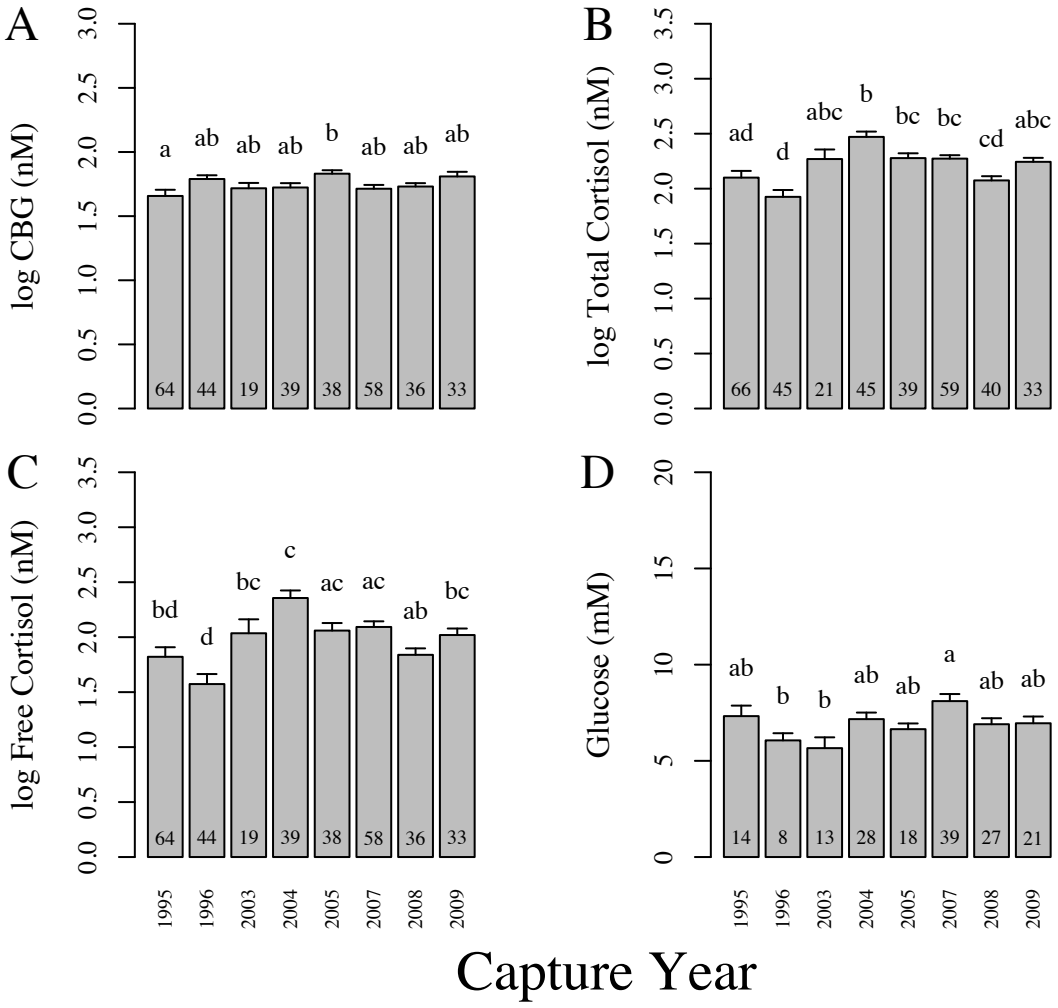

A
log CBG (nM)
0.0 0.5 1.0 1.5 2.0 2.5 3.0
a ab ab ab b ab ab ab
64 44 19 39 38 58 36 33
B
log Total Cortisol (nM)
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
ad d abc b bc bc cd abc
66 45 21 45 39 59 40 33
C
log Free Cortisol (nM)
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
bd d bc c ac ac ab bc
64 44 19 39 38 58 36 33
D
Glucose (mM)
0 5 10 15 20
ab b b ab ab a ab ab
14 8 13 28 18 39 27 21
1995 1996 2003 2004 2005 2007 2008 2009
Capture Year

Figure S6: Cortisol Dynamics in Polar Bears Captured in Different Populations.
Mean + SEM concentrations of A) corticosteroid binding globulin (CBG), B) total cortisol, C) free cortisol, and D) glucose in polar bears captured in different populations. Populations are defined as: Lancaster Sound (LS), Southern Hudson Bay (SHB), and Western Hudson Bay (WHB). There were significant differences in CBG and total and free cortisol levels between populations, taking into account the effect of age class on CBG and free cortisol, and are indicated by different letters (ANOVA $p < 0.05$). Sample sizes are inset. All LS and WHB animals were sampled in the years 1995 – 1996 and SHB between 2003 – 2009.

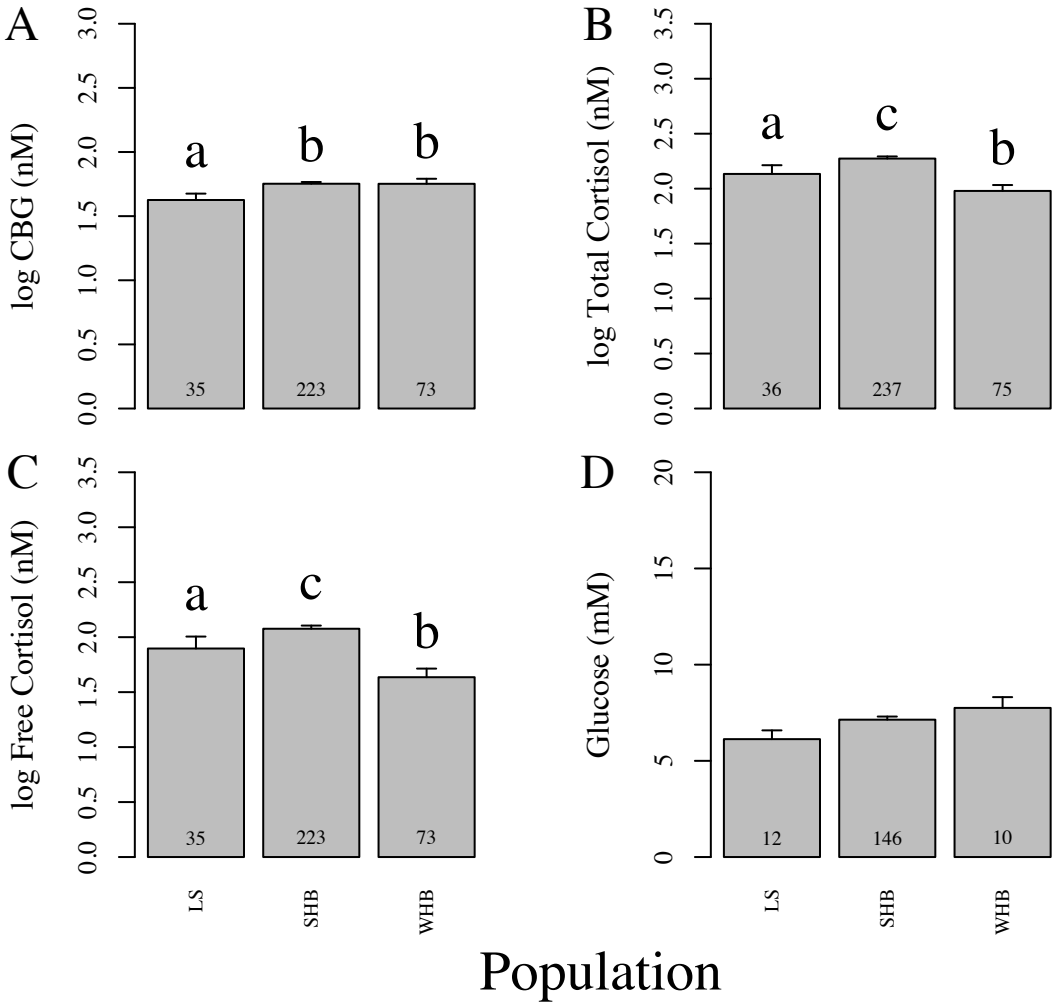

A
log CBG (nM)
0.0 0.5 1.0 1.5 2.0 2.5 3.0
a
b
b
35
223
73
B
log Total Cortisol (nM)
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
a
c
b
36
237
75
C
log Free Cortisol (nM)
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
a
c
b
35
223
73
D
Glucose (mM)
0 5 10 15 20
12
146
10
LS
SHB
WHB
Population

Figure S7: Cortisol Dynamics in Polar Bears Captured in Different Months.
Mean + SEM concentrations of A) corticosteroid binding globulin (CBG), B) total cortisol, C) free cortisol, and D) glucose in polar bears captured in different months (March – May, July – November). There were significant differences in CBG and total and free cortisol levels between months of capture, taking into account the effect of age class on CBG and capture method on total and free cortisol levels, and are indicated by different letters (ANOVA $p < 0.05$). Sample sizes are inset. Animals captured between March – August were from the Lancaster Sound and Western Hudson Bay subpopulations, and those captured after August were from the Western and Southern Hudson Bay subpopulations.

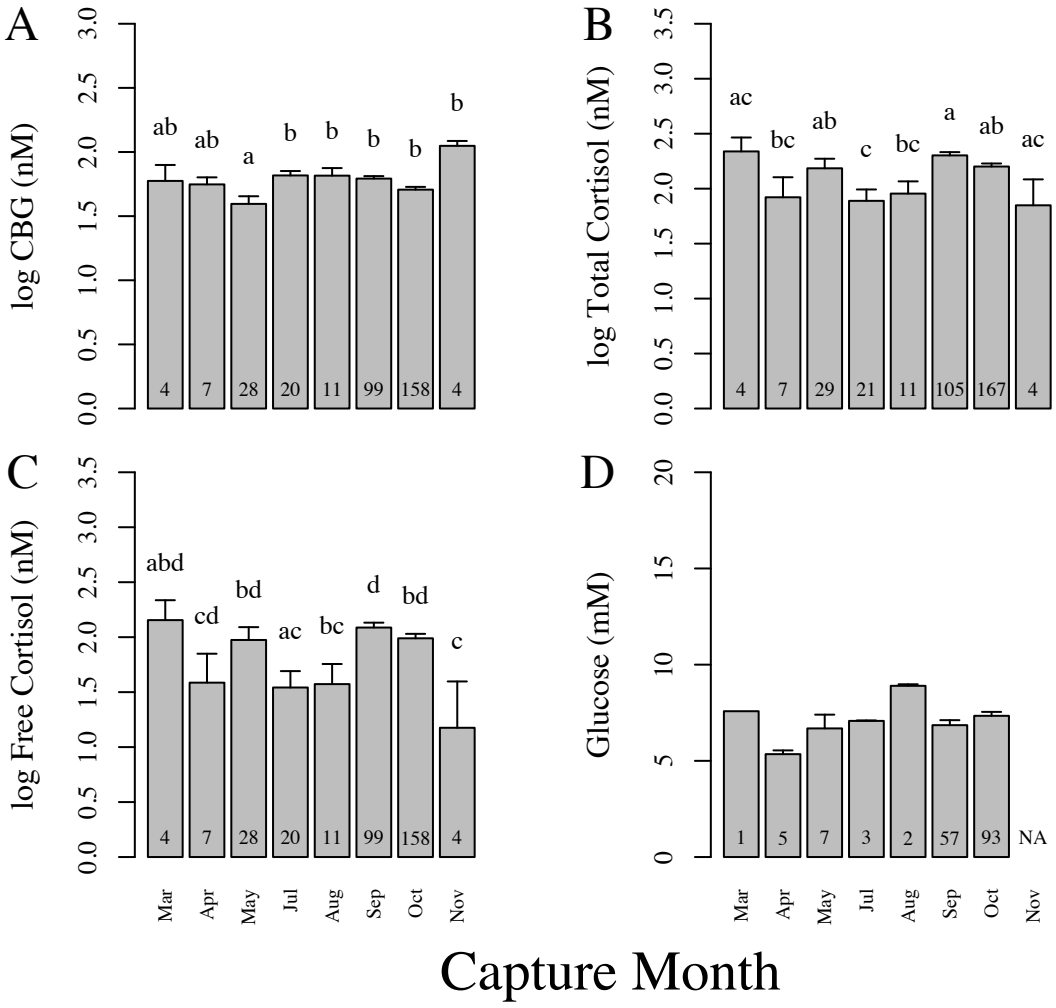
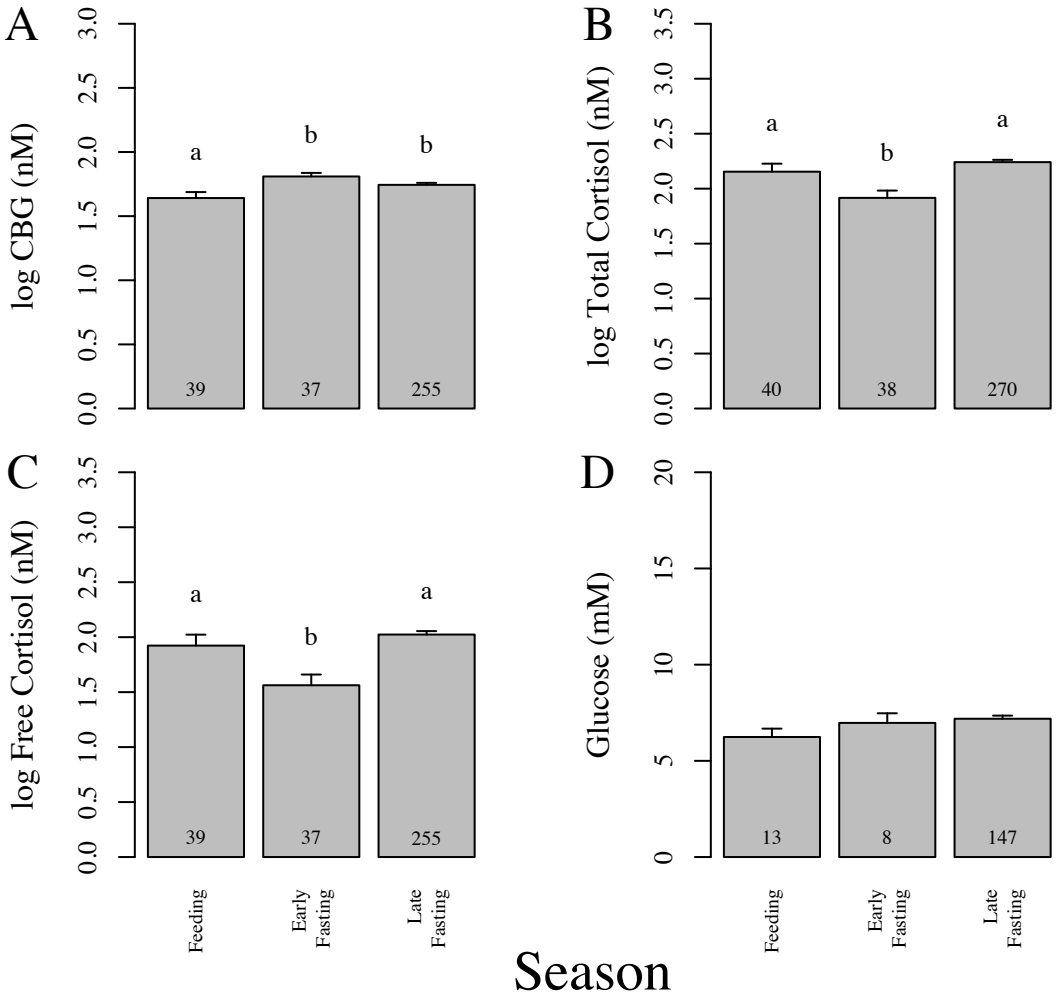

A
log CBG (nM)
0.0 0.5 1.0 1.5 2.0 2.5 3.0
ab ab a b b b b b
4 7 28 20 11 99 158 4
B
log Total Cortisol (nM)
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
ac bc ab c bc a ab ac
4 7 29 21 11 105 167 4
C
log Free Cortisol (nM)
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
abd cd bd ac bc d bd c
4 7 28 20 11 99 158 4
D
Glucose (mM)
0 5 10 15 20
1 5 7 3 2 57 93 NA
Mar Apr May Jul Aug Sep Oct Nov
Capture Month

Figure S8: Cortisol Dynamics in Polar Bears Captured in Different Seasons.
Mean + SEM concentrations of A) corticosteroid binding globulin (CBG), B) total cortisol, C) free cortisol, and D) glucose in polar bears captured in different seasons. Seasons are defined by Julian day of capture: Feeding (prior to the 150^{th} Julian Day), early fasting (between 150 – 225), and late fasting (after 225). There were significant differences in CBG and total and free cortisol levels between populations, taking into account the effect of age class on CBG and free cortisol, and are indicated by different letters (ANOVA $p < 0.05$). Sample sizes are inset.


A
log CBG (nM)
0.0 0.5 1.0 1.5 2.0 2.5 3.0
a
b
b
39
37
255
B
log Total Cortisol (nM)
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
a
b
a
40
38
270
C
log Free Cortisol (nM)
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
a
b
a
39
37
255
D
Glucose (mM)
0 5 10 15 20
13
8
147
Feeding
Early Fasting
Late Fasting
Season

Appendix 2: Supplementary Data for Black Bear Hibernation Proteomics Experiments

Table S1: Gene Ontology Categories that were Significantly Enriched.
This table contains the Gene Ontology categories that were significantly ($p < 0.05$) enriched (Enrichment > 1.5) in the proteins that were identified as differentially expressed during hibernation in the American black bear.

GO ID	Total Genes	Changed Genes	Enrichment	p-value	FDR	Term
6953	14	6	6.09	0.000	0.010	acute-phase response
6952	68	11	2.30	0.001	0.155	defense response
6959	30	7	3.31	0.002	0.178	humoral immune response
50790	30	7	3.31	0.002	0.178	regulation of catalytic activity
51336	23	6	3.70	0.002	0.170	regulation of hydrolase activity
6955	63	10	2.25	0.002	0.142	immune response
10033	43	8	2.64	0.003	0.184	response to organic substance
2526	25	6	3.41	0.004	0.156	acute inflammatory response
2576	25	6	3.41	0.004	0.156	platelet degranulation
2376	79	11	1.98	0.004	0.141	immune system process
2252	34	7	2.92	0.004	0.129	immune effector process
65009	35	7	2.84	0.004	0.133	regulation of molecular function
35270	2	2	14.20	0.005	0.359	endocrine system development
10288	2	2	14.20	0.005	0.359	response to lead ion
19538	96	12	1.78	0.005	0.335	protein metabolic process
2682	46	8	2.47	0.005	0.341	regulation of immune system process
6887	29	6	2.94	0.008	0.361	exocytosis
9611	73	10	1.95	0.008	0.343	response to wounding
31100	7	3	6.09	0.008	0.328	organ regeneration
43691	7	3	6.09	0.008	0.328	reverse cholesterol transport
6954	39	7	2.55	0.009	0.301	inflammatory response
50776	39	7	2.55	0.009	0.301	regulation of immune response
2253	30	6	2.84	0.010	0.299	activation of immune response
16192	51	8	2.23	0.010	0.291	vesicle-mediated transport
52547	14	4	4.06	0.011	0.291	regulation of peptidase activity
48583	63	9	2.03	0.011	0.282	regulation of response to stimulus
19724	22	5	3.23	0.011	0.239	B cell mediated immunity
2250	22	5	3.23	0.011	0.239	adaptive immune response
2460	22	5	3.23	0.011	0.239	adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains
2455	22	5	3.23	0.011	0.239	humoral immune response mediated by circulating immunoglobulin
16064	22	5	3.23	0.011	0.239	immunoglobulin mediated immune response
2449	22	5	3.23	0.011	0.239	lymphocyte mediated immunity
51338	8	3	5.33	0.013	0.260	regulation of transferase activity
30168	32	6	2.66	0.013	0.297	platelet activation

50778	32	6	2.66	0.013	0.297	positive regulation of immune response
50892	3	2	9.47	0.013	0.310	intestinal absorption
30299	3	2	9.47	0.013	0.310	intestinal cholesterol absorption
44241	3	2	9.47	0.013	0.310	lipid digestion
48232	3	2	9.47	0.013	0.310	male gamete generation
44058	3	2	9.47	0.013	0.310	regulation of digestive system process
30300	3	2	9.47	0.013	0.310	regulation of intestinal cholesterol absorption
7283	3	2	9.47	0.013	0.310	spermatogenesis
9719	23	5	3.09	0.014	0.280	response to endogenous stimulus
9725	23	5	3.09	0.014	0.280	response to hormone stimulus
1775	43	7	2.31	0.015	0.280	cell activation
51246	33	6	2.58	0.016	0.276	regulation of protein metabolic process
2443	24	5	2.96	0.017	0.274	leukocyte mediated immunity
43086	16	4	3.55	0.018	0.269	negative regulation of catalytic activity
43085	16	4	3.55	0.018	0.269	positive regulation of catalytic activity
7399	34	6	2.51	0.018	0.266	nervous system development
52548	9	3	4.73	0.019	0.269	regulation of endopeptidase activity
44057	9	3	4.73	0.019	0.269	regulation of system process
45087	35	6	2.43	0.021	0.265	innate immune response
72376	35	6	2.43	0.021	0.265	protein activation cascade
6950	111	12	1.54	0.022	0.262	response to stress
44092	17	4	3.34	0.022	0.262	negative regulation of molecular function
48545	17	4	3.34	0.022	0.262	response to steroid hormone stimulus
32940	36	6	2.37	0.024	0.261	secretion by cell
30301	10	3	4.26	0.025	0.256	cholesterol transport
1935	10	3	4.26	0.025	0.256	endothelial cell proliferation
14070	10	3	4.26	0.025	0.256	response to organic cyclic substance
15918	10	3	4.26	0.025	0.256	sterol transport
7586	4	2	7.10	0.026	0.303	digestion
22600	4	2	7.10	0.026	0.303	digestive system process
14012	4	2	7.10	0.026	0.303	peripheral nervous system axon regeneration
43410	4	2	7.10	0.026	0.303	positive regulation of MAPKKK cascade
6956	27	5	2.63	0.028	0.306	complement activation
42592	37	6	2.30	0.028	0.299	homeostatic process
46903	37	6	2.30	0.028	0.299	secretion
48518	99	11	1.58	0.029	0.295	positive regulation of biological process
42221	73	9	1.75	0.032	0.294	response to chemical stimulus
51649	49	7	2.03	0.032	0.290	establishment of localization in cell
44093	19	4	2.99	0.033	0.289	positive regulation of molecular function
31099	19	4	2.99	0.033	0.289	regeneration
51345	11	3	3.87	0.033	0.293	positive regulation of hydrolase activity
10740	11	3	3.87	0.033	0.293	positive regulation of intracellular protein kinase cascade

48878	29	5	2.45	0.037	0.292	chemical homeostasis
32268	29	5	2.45	0.037	0.292	regulation of cellular protein metabolic process
35466	29	5	2.45	0.037	0.292	regulation of signaling pathway
6958	20	4	2.84	0.039	0.288	complement activation, classical pathway
10035	20	4	2.84	0.039	0.288	response to inorganic substance
51641	51	7	1.95	0.040	0.286	cellular localization
2684	40	6	2.13	0.041	0.281	positive regulation of immune system process
48584	40	6	2.13	0.041	0.281	positive regulation of response to stimulus
31103	5	2	5.68	0.041	0.293	axon regeneration
6576	5	2	5.68	0.041	0.293	cellular biogenic amine metabolic process
6879	5	2	5.68	0.041	0.293	cellular iron ion homeostasis
42439	5	2	5.68	0.041	0.293	ethanolamine-containing compound metabolic process
46651	5	2	5.68	0.041	0.293	lymphocyte proliferation
31102	5	2	5.68	0.041	0.293	neuron projection regeneration
46470	5	2	5.68	0.041	0.293	phosphatidylcholine metabolic process
1938	5	2	5.68	0.041	0.293	positive regulation of endothelial cell proliferation
51347	5	2	5.68	0.041	0.293	positive regulation of transferase activity
32844	5	2	5.68	0.041	0.293	regulation of homeostatic process
50670	5	2	5.68	0.041	0.293	regulation of lymphocyte proliferation
32269	12	3	3.55	0.043	0.294	negative regulation of cellular protein metabolic process
51346	12	3	3.55	0.043	0.294	negative regulation of hydrolase activity
1666	12	3	3.55	0.043	0.294	response to hypoxia
70482	12	3	3.55	0.043	0.294	response to oxygen levels
35556	30	5	2.37	0.043	0.294	intracellular signal transduction
31667	21	4	2.70	0.047	0.299	response to nutrient levels