Evaluation of indicators of stress in populations of polar bears (*Ursus maritimus*) and grizzly bears (*Ursus arctos*)

by

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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.

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Jason W Hamilton

Abstract

Grizzly and polar bears are both species at the top of the food chain in their respective ecosystems, and as such are indicative of the overall health of the ecosystem. Presently there is little data regarding the stress status of these animals. The development of reliable indicators of stress is important as both species face rapid environmental change. Polar bears from Hudson's Bay (Ontario, Canada) and grizzly bears from Alberta, Canada, were anaesthetized and blood samples retrieved. Samples were assayed for changes in serum-based indicators of stress. Serum cortisol levels, the predominant corticosteroid in mammals and a commonly used indicator of stress, was measured to evaluate its potential as a chronic stress indicator in bears. The induction time of the cortisol response to stressor exposure is rapid and will be influenced by the stress relating to capture. Hence, serum levels of heat shock proteins (hsps), specifically the 60 (hsp60) and 70 kilodalton (hsp70) families of hsps were also measured to evaluate their reliability as a stress indicator in bears. Traditionally, heat shock proteins have been measured in tissues; however recent studies have indicated their presence in serum in response to chronic stress. In addition, the study examined the feasibility of using corticosteroid-binding globulin (CBG), a serum protein that binds cortisol, as a stress indicator in bears. CBG regulates the availability of cortisol to the tissues (only unbound cortisol elicits a response) but unlike cortisol is not rapidly regulated by acute stress. Bear CBG was isolated and a specific *anti-bear CBG* antibody was generated. The development of an enzyme-linked immunoadsorbant assay (ELISA) using this bear anti-CBG has the potential to be a useful tool to determine longer-term stress response in bears. Known

life-history variables were correlated to the observed levels of serum indicators to elucidate which environmental factors impact bears. The length of sea ice coverage was the strongest determinant of serum cortisol and hsp70 levels in polar bears; the longer ice cover reflects more feeding time and this is reduced through climatic warming. This suggests that fasting associated metabolic changes may be impacting serum cortisol response and hsp70 levels in polar bears. For grizzly bears the proportion of protected homerange had the strongest correlation with stress indicators. This suggests that human impact on the environment, including resource extraction and landscape changes, result in altered levels of serum cortisol and hsp70 levels. Hsp60 was not observed to vary significantly in the face of changing environmental variables, and as such no correlation could be made between serum hsp60 levels and environmental variables in bears. Serum hsp70 was observed to change significantly in response to environmental variables in both polar and grizzly bears. These data along with the changes in cortisol and other health based indicators have the potential to make hsp70 a useful indicator of altered health status in bears. This study is the first attempt to integrate the usefulness of a suite of serum indicators of stress as a tool for detecting the health status of bears. The lack of a control group for comparison to wild population limits the utility of the observed variables as a tool to detect stressed states in bears. However, as these serum indicators are also modulated by the animals health life-history, including food limitation, the monitoring of these serum stress indicators, along with other indicators of fed and fasted states, may give a better picture of the health status of the animal related to nutrient availability.

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General Introduction

Field Studies vs Laboratory Studies

The response to stress, both the behavioural and physiological components have been well characterized in laboratory mammals. However, little is known about the physiological responses to stressors in wild populations. One of the main difficulties in assessing stressed states in wild populations stems not only from the myriad of stressors experienced by wild (free-ranging) populations of animals, but also the lack of knowledge about the history of the animal (Reeder and Kramer, 2005). Specifically, in the natural environment there is no control group of animals within (or between) a population(s) by which comparisons of stressed state can be ascertained (Reeder and Kramer, 2005). While recent studies explored the variability in the stress response of wild populations subjected to stressors, these studies required the development of novel techniques to quantify stress (Partecke et al. 2006, Reeder et al, 2004). The timeframe required to observe effects in animals is widely variable depending on the duration and intensity of the stressor(s). First environmental change will be observed as a stress response in individuals, if persistent degraded health in individual bears may become apparent. When enough animals in a population are exhibiting reduced health states, there is a population effect based on reduced reproductive success or a similar measure (Boonstra, 2005). Again the rate at which these changes occur are uncertain, though with environmental change occurring at higher frequencies the time between individual and population effects may be decreasing.

With growing concern regarding environmental change and its influences on wild populations, it is important to quantify the effects of this change on animal function because that will allow managers to make informed decisions about wildlife populations

and subsequently ecosystem health for wildlife management (Cairns et al. 1993; Kutsch et al. 2001).

Stress Physiology

Stress can be defined as a loss of homeostasis in response to external stimuli, commonly termed a stressor. This stressor can be any number (or combination) of physical or psychological occurrences perceived by the organism, which in turn affect the physiology of the animal. In response to stressors, a stress response is elicited, which involves hormonal and metabolic changes, allowing the animal to regain homeostasis (Boonstra, 2005; Reeder and Kramer, 2005). If that is the case, the animal is able to cope with the stressor. However, if the animal is unable to regain homeostasis in response to stressors, that will result in tertiary changes to the animal's systems. This includes affects on immune function, growth and reproduction, leading to population level impact (Reeder and Kramer, 2005). Selye (1956) characterized the stress response in 3 phases; the alarm phase (SNS activation), the resistance phase (HPA activation) and exhaustion phase where chronically elevated glucocorticoid levels begin to harm the animal. These 3 phases make up the General Adaptation Syndrome.

Often overlooked in studies of stress are the psycho-social stressors faced daily by wild animals. These include any stimuli that act to elicit fear, frustration, anxiety or anger (Reeder and Kramer, 2005). Regardless of the stressors, the stress response is highly conserved and encompasses a wide array of physiological and behavioural responses which work to neutralize the effect of the stressor and re-establish homeostasis.

Stress may be divided into two major types based mainly on a temporal scale: i) *acute* stress is usually shorter-term, from minutes to hours, and the animal is allowed to

recover after the stressor insult, and ii) *chronic stress* would persist over a longer time period and the animal is constantly exposed to the stressor leaving little time to recover and is associated with a consistent upregulation of the stress response. In wild populations, animals are faced daily with multiple types and intensities of stressors. Frequently these stimuli comprise day-to-day survival challenges presented to the animal which maintain the stress axis functioning at a basal level (Reeder and Kramer, 2005, Wingfield and Romero, 2001). However when a novel stimulus is sensed in conjunction with these *common* stressors there is an upregulation of the stress axis and a stress response is mounted (Sapolsky 2002). This coupling of common and novel psychological and physical stressors may incur additive effects and may result in chronic activation of the stress axis.

Stress Axis

Composed of the limbic system of the brain (dentate gyrus and hippocampus) and the hypothalamic-pituitary-adrenal (HPA) axis, the stress axis functions primarily to allow the animal to cope with the stressor (Boonstra, 2005) Though much of the research of stress physiology focuses on the stress axis, a myriad of downstream effectors (cytokines, neurotransmitters, hormones) are integral parts to the organism's return to homeostasis (Sapolsky et al. 2000). Within the stress axis, the nervous system plays an integral role in the stress response. The nervous system is involved in the perception of potential stressors and the initiation of the stress response. However, field measurements of nervous system activities are quite challenging.

Catecholamines are amine hormones released from the adrenal medulla in response to many types of stimuli, and work in conjunction with the HPA axis to initiate

the stress response. Epinephrine and norepinephrine are released almost immediately from the sympathetic nervous system in response to a stressor (Sapolsky et al. 2000). Their effects include increased cardiovascular output, immune activation, energy mobilization, and enhanced brain activity (Sapolsky, 2002). Though an important player in stress physiology, catecholamines have a brief half-life in circulation and are present only in minute amounts, which makes assaying them in field samples unpractical and unreliable.

The most common indicator of stress routinely measured is the serum level of corticosteroids, which is considered to be the stress hormone and plays a key role in adapting the animal to stress (Sapolsky et al., 2000).

Role of Cortisol

When faced with a stressor, there is an associated upregulation in hypothalamic-pituitary-adrenal (HPA) axis activity; the ultimate result of this is a rise in plasma glucocorticoid levels (Alexander and Irvine, 1998). Perception of a stressor by the nervous system triggers a cascade of events in the HPA axis beginning with a signal reaching the hypothalamus, which releases corticotropin-releasing factor (CRF). The target site of CRF is the anterior pituitary gland; under CRF influence, the anterior pituitary is stimulated to release adrenocorticotropic hormone (ACTH). The anterior pituitary also releases prolactin, which suppresses reproduction during stress. ACTH enters the general circulation and acts on the adrenal cortex to release cortisol; the predominant glucocorticoid in mammals. Cortisol has a number of target sites in the animal, producing important behavioural, neural and metabolic changes necessary to cope with the stress (Fleshner et al., 1995).

Each step in the HPA axis is governed by a feedback loop which is in place to prevent excessive release of any given hormone. These negative feedback loops are activated when target receptors for a particular hormone reach a threshold level of saturation. Signals are sent to attenuate the release of that hormone; thus regulating the release of cortisol and preventing chronic stimulation of target tissues. Throughout the body 2 types of glucocorticoid receptors are present; mineralcorticoid (type 1) receptors are occupied at basal levels of glucocorticoids. Type 2 (glucocorticoid) receptors have a lower affinity for glucocorticoids and are only occupied at elevated levels; it is these receptors which act as a negative feedback mechanism on the HPA axis (Sapolsky et al. 2000).

Previous studies have shown that serum cortisol levels rise within 2-3 minutes after the imposition of the stressor, and subsequently return to basal levels after 1-2 hours upon termination of the stressor (Fleshner et al., 1995; Boonstra et al., 2001). Due to the rapid nature of the activation and degradation of the cortisol response, it is useful only in ascertaining responses to short-term or acute stressors. Thus, cortisol levels, though an accurate measure of the induction of a stress response will not give sufficient information as to the prevalence of a chronic type stressor.

The exhaustion phase of the General Adaptation Syndrome is of particular interest as prolonged elevations in cortisol levels have been shown to attenuate immune function, induce bone and muscle atrophy, effect wound healing, inhibit growth and induce cellular apoptosis. (Boonstra, 2005) With such deleterious effects in response to elevated cortisol, it is necessary to regulate its release and action, as well as the activity of the entire HPA axis; this is achieved by negative feedback mechanisms in place at each level

of the pathway (Whitnall, 1993). Providing the stressor is acute, the presence of high levels of circulating glucocorticoids acts to downregulate their production by the HPA axis. Glucocorticoid receptors, only saturated at higher than normal cortisol levels are present in the hypothalamus and anterior pituitary and binding of glucocorticoids here act to reduce the levels of CRH and thus ATCH (Fig. 1), resulting in reduced release by the adrenal cortex.

Samples collected from captured mammals in the field will indicate a stressed state as blood taken will show a marked spike in cortisol levels due to stress imposed during capture (Cattet et al. 2003*c*). Due to the nature of capturing and sampling stress imposed on free-ranging mammals in a field study, it is difficult to ascertain basal levels of cortisol for these animals. Hence, incorporating other measures of stress into the studies is imperative to gain further insight into the stress response of these animals as well as to identify more reliable indicators of stress.

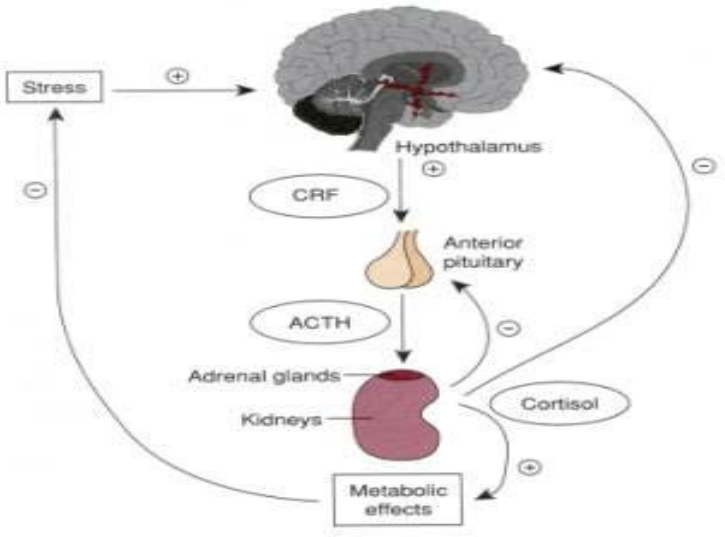


Fig 1. Schematic outlining Hypothalamic-Pituitary-Adrenal (HPA) Axis Activation

Effects of Stress

Although the same event is perceived by two organisms, how each responds is quite variable, one may show little or no effect while the other may begin the processes required to re-establish lost equilibrium. In such a case, the event is only considered a stressor for the organism that mounts a response. Whether an event is perceived as a stressor is dependent on a unique combination of life history, developmental history and reproductive condition (Reeder and Kramer, 2005). The overall response of the animal to stress will include, altered behaviour (avoidance/escape), increased awareness, altered pain and sensory thresholds and physiological changes (von Holst, 1998). This occurs in conjunction with suppression in feeding and reproductive behaviour, yielding a major reallocation of energy to behaviours required for immediate action to cope with the stressor (von Holst, 1998).

Responding to a stressor requires an expenditure of energy. The variation in this energy reallocation is of utmost importance in deciphering the effects of stress on wild populations. The degree of change is immensely variable with different environmental conditions and life-stage of the affected animal (Boonstra, 2005). The previously mentioned behavioural and physiological adaptations required to cope with a stressor are also dependent on the availability of glucose, the major fuel to cope with the increased energy demand of stress. Production of glucose occurs in two stages, first catecholamines, glucagon and growth hormone (GH) initiate this change by mobilizing stored glucose and increasing insulin resistance to prevent glucose storage (Sapolsky et al. 2000). This is achieved through stimulating hepatic gluconeogenesis pathways (Munck et al. 1984), in conjunction with an upregulation in glycogenolytic pathways,

yielding free glucose, (Grascolas & Robin, 2001). Catecholamines, glucagons and GH act quickly to raise glucose levels, while cortisol is slower to react but extends the response over a greater time period (Sapolsky et al. 2000). Cortisol is a key player in maintaining glucose for long period of time after a stressful episode. This is achieved by this steroid stimulating gluconeogenesis as well as glycogenolysis in the liver (Munck et al. 1984). As glycogen stores are exhausted, cortisol initiates lipolysis and proteolysis to provide the necessary glucose to the organism (Sapolsky et al. 2000). While elevated cortisol levels leads to liberation of glucose, it concurrently works to suppress all anabolic pathways (Sapolsky, 2002). During a stress response, when mobilization of energy stores is coupled with inhibited feeding behaviour, the organism quickly can run into energy debt if the stressor persists. This would be particularly evident when animals are below their peak fitness, for instance with aging or pregnancy, leading to extreme energy debt and the subsequent potential to succumb to the effects of the stressor (Spencer et al. 1996).

Role of Binding Proteins

Mammalian plasma contains two high-affinity, steroid-binding glycoproteins, which regulate the bioavailability of steroid hormones (Rosner, 1991). Sex hormone-binding globulin (SHBG) is directed toward the binding of testosterone, dihydrotestosterone and estradiol (Rosner, 1991), while corticosteroid-binding globulin (CBG) binds cortisol and progesterone, reversibly and with a high-affinity (Rosner, 1991). The binding of cortisol is of particular interest as this steroid hormone is released during stress and CBG regulates its cellular availability, thereby its action (Breuner and

Orchinik, 2002). CBG is a glycoprotein of approximately 55 kDa and synthesized in the liver (Rosner, 1991) as early as 10-12 days of fetal life (Scrocchi et al. 1993). Mammalian studies characterised the dissociation constant of CBG with cortisol (37˚C) to be 2 x 10^{-8} M (Rosner, 1991).

Steroids persist within mammalian plasma in two states, free or bound to a protein carrier; it is the free steroids that are able to diffuse through capillary membranes and into the cell to initiate the action of the hormone (Mendel, 1989). Despite this, the greatest proportion of cortisol in mammalian plasma persists in the bound state, with percentages of CBG-bound cortisol ranging from 67-87% in domestic species (Gayrard et al. 1996); other studies (Rosner, 1990) suggest this number is as high as 90-95%. CBG serves as a reservoir of cortisol, as the unbound fractions are quickly excreted by the kidneys. (Boonstra, 2005) There have been studies proposing that CBG acts to bind excess cortisol in circulation, thereby protecting tissues from over-stimulation by cortisol, this is referred to as the *Buffer hypothesis* (Romero, 2002). Contrarily, the *Carrier hypothesis* suggests CBG act only as a vessel to transport cortisol to distal target tissues (Breuner and Orchinik, 2002.) Whichever hypothesis is correct, they both share the fact that fluctuations in plasma CBG concentrations would extrapolate into substantial changes in free cortisol concentrations.

Circulating levels of CBG are controlled by both the stress axis and the gonadal axis through negative feedback systems (Boonstra 2005). Life-history variables such as age and reproductive state have marked effect on cortisol and subsequently CBG levels. Mature males show much greater free cortisol levels in conjunction with reduced CBG levels (Boonstra et al. 2001) and this is thought to be due to testosterones inhibition of

CBG synthesis (Mataradze et al. 1992). Females conversely show much higher CBG levels coupled with reduced free cortisol levels especially while pregnant (Boonstra et al. 2001). This would suggest that CBG acts to *buffer* the females and the offspring from prenatal and postnatal cortisol excess, which may affect survival (Sachser and Kaiser, 1996). Though CBG levels are controlled by a number of variables, of interest here is the effect of stress, both acute and more specifically chronic stress, on its circulating levels.

The imposition of a stressor starts a response that yields an increase in the amount of free cortisol in circulation, a fraction of which is excreted from the adrenal cortex and another fraction is liberated from its CBG-bound state to exact an affect. A number of studies have illustrated the link between stressors and a reduction in CBG binding capacity. Restraint, tail-shock stress, social stress and food-deprivation have been shown to reduce the capacity of CBG to bind cortisol (Tinnikov, 1993, Fleshner et al. 1995, Spencer et al. 1996, Marti et al. 1997, Alexander and Irvine 1998). It seems the rapid induction of free cortisol comes from adrenal excretion while this change in CBG capacity offers a way to maintain higher cortisol levels without constant excretion (Breuner and Orchinik, 2002). While acute shock does in fact alter the capacity of CBG to bind cortisol, this observation is not apparent for a few hours after stressor exposure (Breuner and Orchinik, 2002).

Of particular interest is the effect of chronic stress on CBG and its potential as a biomarker in wild populations. Previous studies have shown chronic stress to yield sustained high levels of cortisol, which led to an observable reduction in CBG levels (Boonstra et al. 1998, 2001, Frairia et al. 1988, Schlechte and Hamilton, 1987). Citing this relationship, it should be possible to determine the presence/absence of chronic-term

stress on an individual, if some pertinent life-history variables are known. It is probable that both a reduced binding capacity and a reduction in circulating levels of CBG is responsible for maintaining elevated cortisol levels during a situation of chronic stress. As previously stated, many types of stimuli can be perceived as stressors by the animals and, due to the conserved nature of the stress response, CBG show similar reactions to both psychological and physical stressors (Alexander and Irvine, 1998). As Selye (1956) pointed out, the *exhaustion* stage of the stress response is characterized by sustained elevated corticosteroid levels which potentially produce a deleterious effect. When the animal is under a state of stress for this extended time frame it requires this stress response to be sustained to adapt with the situation and this can lead to observable degradation of overall health and survivorship (Boonstra, 2005). Having a tool to predict long term effects of stress, such as CBG, is important to wildlife managers, to better understand and protect bear populations.

Role of Heat Shock Proteins

Heat shock proteins (hsps) or stress proteins are inducible proteins synthesized in the face of a wide range of environmental stressors (Lewis et al. 1999). Hsps are present within the cells during normal conditions to promote proper folding and aggregation of cellular proteins (Hartl, 1996). Hsp60, Hsp70 and Hsp90 are widely expressed and highly conserved across taxa, while the lower molecular weight proteins are much less conserved (Lewis et al. 1999). A number of studies have shown the utility of hsp60 and hsp70 as environmental biomarkers of stress following exposure to organic pollutants (Sanders et al. 1991, Bradley, 1993). During a bout of cellular stress, degraded proteins

are detected which sets in motion a series of events leading to the increase of hsps in tissue. In the cytosol hsps are bound to heat shock factors (hsfs), and maintained in an inactive state (Kregel, 2002). A broad array of stressors induces the disassociation of hsf and the bound hsps. Protein kinases phosphorylate hsfs, which subsequently form trimers in the cytosol (Kregel, 2002). These hsf trimer complexes enter the nucleus and bind to heat shock elements (HSE) in the promoter region of the hsp genes. Hsp mRNA is then transcribed and leaves the nucleus for the cytosol, where new hsps are synthesized as required to combat proteotoxicity (Kregel, 2002).

Hsp levels in tissue are elevated in response to a number of environmental stressors including thermal stress, hypoxia and protein degradation (Walsh et al. 2001) Also a reduction of glucose availability, such as in a state of general stress, initiates the production of hsp70 (Sciandra and Subjeck, 1983). Sanders et al. (1992) observed that hsp60 and hsp70 were induced and remained elevated during persistent thermal stress, giving potential to hsps as a biomarker of chronic term stress. Similar to the constituents of the HPA axis, hsp expression gives rise to stress tolerance during persistent exposure of environmental stressors. Animals living in the wild seldom undergo a single stressor and are usually coping concurrently with a wide variety of stressors; hence, it will be difficult to attribute the induction of hsps to a single variable. However, for the purposes of this study it is possible to use the known life-history variables to predict the level of environmental disturbance faced by a particular animal or population. This information is important to put findings into context and discount to natural factors as a possible reason for the observed change in hsps level. For instance, as mammals age, there is an accumulation of protein damage coupled with a reduced capacity to express hsps and

other stress responses (Holbrook and Udelsman, 1994, Lee et al. 1996) allowing the stressor impact to be much greater.

Though traditionally the hsps were detected intracellularly, there has been some evidence of hsps entering into circulation through stress induced apoptosis (Wright et al. 2000). It has been shown that hsps are released in response to chronic diseased states and play a role in immune activation in circulation (Wright et al. 2000). Also Walsh et al. (2001) have indicated strenuous exercise has the potential to increase serum levels of hsp70 approximately 1h after the initiation of exercise. This may have the potential to determine the state of stress due to both acute and chronic term stressors. A further benefit of including hsp70 as a biomarker of stress is it has been shown that minor daily disturbances will not alter hsp70 levels in serum (Kristensen et al. 2004). The exact mechanism of release of hsps into serum is at present, unknown, however it is postulated that cells undergoing stress-induced apoptosis release hsp into circulation. There is evidence indicating recovery from trauma by patients with elevated serum levels of hsps (Pittet et al. 2002). Findings from Walsh et al. (2001) would imply that serum levels of hsp70 are elevated in response to stressful states. This agrees with the study by Pockley et al. (2000), which indicates serum levels of hsp60 to increase in response to a chronic stress (disease) state. There appears to be evidence for fasting based differences in tissue levels of hsp in response to stress, which is of particular interest as the bears in the study are sampled near the end of their yearly fasting period. Gasbarrini et al. (1998) determined a reduced level of tissue hsp70 gene expression in stressed rats previously subjected to a period of fasting. Though the precise mechanism of release of hsps into

serum is unknown, they are well proven to be apart of the stress response and as such are included in this study as an indicator of persistent stress.

Stress in Wildlife

Detecting stress in free-ranging mammals is a difficult undertaking due to the variability in the environmental pressures and perceptions of the individuals. In order to measure stress in free ranging wildlife it is necessary to develop a suite of indicators or biomarkers of stress. Certain attributes are required of such biomarkers to be useful in free-ranging animals. Couple this with the lack of a control group to use as a basal level of biomarkers it is only possible to compare animals and populations with respect to each other. Also, the problem of natural factors such as reproductive status, life stage and diet work to push these indicators from basal levels while not being directly attributed to stress. This however, is the reason for utilizing multiple biomarkers in conjunction with a known life-history to focus in on which variables are altered due to imposed environmental stress. Due to the nature of capturing wild mammals for research there is an induction of the stress response. The use of biomarkers which exhibit a lag time of action are preferred to separate the acute capture stress from persistent environmental pressures. Using serum based indicators gives a minimally invasive biomarker which can illustrate the animal's state of stress in both the acute and chronic terms. Also a single serum sample can supply a number of different markers of stress. Alternatively, there are totally non-invasive methods to collect stress data on a population level. Through the use of scat-sniffing dogs and animal feces it is possible to ascertain a great deal about wildlife. DNA from scat gives insight to distribution and sex ratios of a given population,

particularly if coupled with GPS or radiotelemetry data (Woods et al. 1999, Mills et al. 2000). Fecal glucocorticoids provide a non-invasive window to the health of individuals and the population (Wasser, 1996). Furthermore, Graczyk et al. (2001) were able to determine the animal's diet and confirm the presence/absence of pathogens or parasites within the animals scat sample.

By observing the effects of anthropogenic disturbance on these animals it may be possible to change land use strategies and other human activities to reduce the impact on the ecosystems involved.

There have not been many attempts to categorize the stress of bears in the wild. As a large, top order mammal in their ecosystem, bears (both polar and grizzly) act as a barometer of ecosystem health. In association with mark-recapture studies on both species it has been possible to collect large data sets in many cases spanning several years. A number of life-history variables have been observed for these bears and by utilizing these variables conjunction with stress markers, we hope to determine the effect of biological (sex, age, condition) and environmental (habitat based) variables on the health status of bears.

Research Objectives

The primary objective of this study was to evaluate the utility of corticosteroid-binding globulin (CBG) as an indicator of chronic stress in bears. To accomplish this CBG was purified and a bear specific antibody generated to develop a bear specific CBG ELISA. Measured in conjunction with CBG were serum levels of cortisol and heat shock proteins, well established indicators of stress in mammals, to determine if they are reliable indicators of chronic stress in wild populations of polar and grizzly bears.

Materials and Methods

Study Animals-General Data

Certain common measures and descriptions are utilized throughout this project for both grizzly (*Ursus arctos*) and polar bears (*Ursus maritimus*). The reproductive classification of both species are as follows; 1-solitary adult female, 2-adult female with dependent offspring, 3-juvenile/subadult female, 4-adult male, 5-juvenile/subadult male. The basis of these classifications is tooth aging techniques as well as observation in the field.

The Body Condition Index (BCI), based on residuals from the regression of total body mass (TBM) and straight-line body length (SLBL), was found to be an accurate measure of body condition for both species independent of body size (Cattet et al. 2002). The following equations were used to determine BCI in sampled bears:

Polar bears $BCI=(\ln TBM - 3.07\cdot \ln SLBL + 10.76) \div (0.17 + 0.009\cdot \ln SLBL)$

Grizzly Bears $BCI=(\ln TBM - 3.21\cdot \ln SLBL + 11.64) \div (0.29 - 0.17\cdot \ln SLBL)$

The complete derivation and explanation of the equations is found in Cattet et al. (2002).

Polar Bear Biology

Polar bears from three Canadian populations were sampled and used to determine the utility of various stress biomarkers in wild populations. The data came from the Southern Hudsons Bay (SH) Western Hudsons Bay (WH) and Lancaster Sound (LS) populations as shown below (Fig. 2)

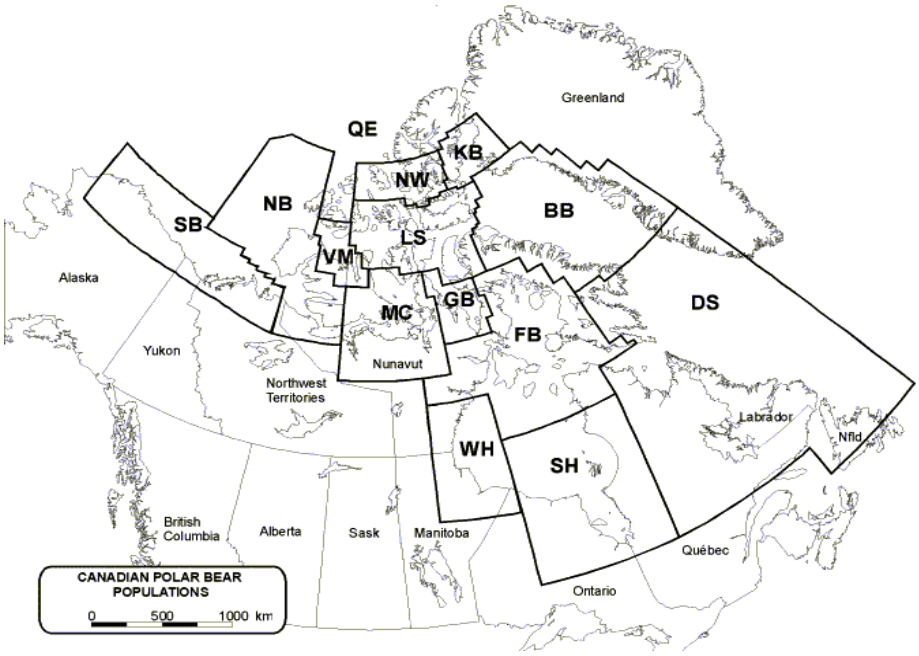


Fig. 2. Map defining polar bear populations in Canada. 14 discrete populations exist in Canada. South Beaufort Sea (SB), North Beaufort Sea (NB), Viscount Melville (VM), Lancaster Sound (LS), McClintock Channel (MC), Gulf of Boothia (GB), Foxe Basin (FB), West Hudson's Bay (WH), South Hudson's Bay (SH), Davis Strait (DS), Baffin Bay (BB), Norwegian Bay (NW), Kane Basin (KB), Queen Elizabeth Islands (QE). Courtesy: Canadian Wildlife Service. See appendix 1 for copyright information.

The SHB population is of particular interest as it represents the southernmost population of polar bears in the world. Since the study subjects are from free-ranging populations, there is no *control* group in this study and will be a comparison of population levels of different stress indicators. Serum samples were collected from WHB polar bears by the University of Saskatchewan in 1995-96 and from SHB polar bears by the Ontario Ministry of Natural Resources in 2003-05 (M. Cattet, personal communication). Bears denoted WHB/SHB had been captured in both study areas and considered as part of the SHB population for this study. The captures were carried out near the end of the open-water, fasting season for these animals. The LS population sampled in 1995-1996 (Dr. Marc Cattet) were captured from March (bears were still feeding) through to August (after a period of fasting) allowing for a comparison of stress indicators between the fed

and fasted state in bears. The amount of time the bears went without food was also determined by the date of breakup of the sea ice for that year; it was assumed that without ice the bears were unable to hunt seals and aside from opportunistic kills, they fasted from breakup to freezeup (Stirling and Derocher, 1993; Stirling and Parkinson, 2006). The data regarding sea ice cover was obtained from Gough et al. (2004), Stirling and Parkinson (2006) and the Canadian Ice Service (http://ice.ec.gc.ca).

Polar Bear Capture Methods

During the months of September and October 1995-1996 and 2003-2005, polar bears were captured on the Southern and Western coasts of Hudson's Bay, Ontario Canada. The SHB study area (Fig. 3) included offshore islands and areas up to 40 km inland of the coast. The capture and handling of the animals was annually approved by the Animal Care Committee of the Ontario Ministry of Natural Resources (OMNR) and followed the guidelines of the American Society of Mammalogists (Committee for Field Methods in Mammalogy, 1987).

Polar Bear Anaesthesia

Polar bears were visually located from a helicopter and the weight estimated for drug dosage by knowledgeable personnel. The animals were immobilised by remote darting from a Bell 206L helicopter using either zolazepam-tiletamine (Telazol®) at a dose of 8-8.5 mg/kg body weight as described by Stirling et al. (1989) or a mixture of Telazol® and xylazine hydrochloride (Cervizine 300®) at a dose of 5mg/kg body weight following the procedures outlined by Cattet et al. (2003*b*). Throughout the handling of animals, vital signs, respiratory rate, rectal temperature, and oxygen saturation (SPO_2) were monitored every 10 minutes to ensure animal safety.

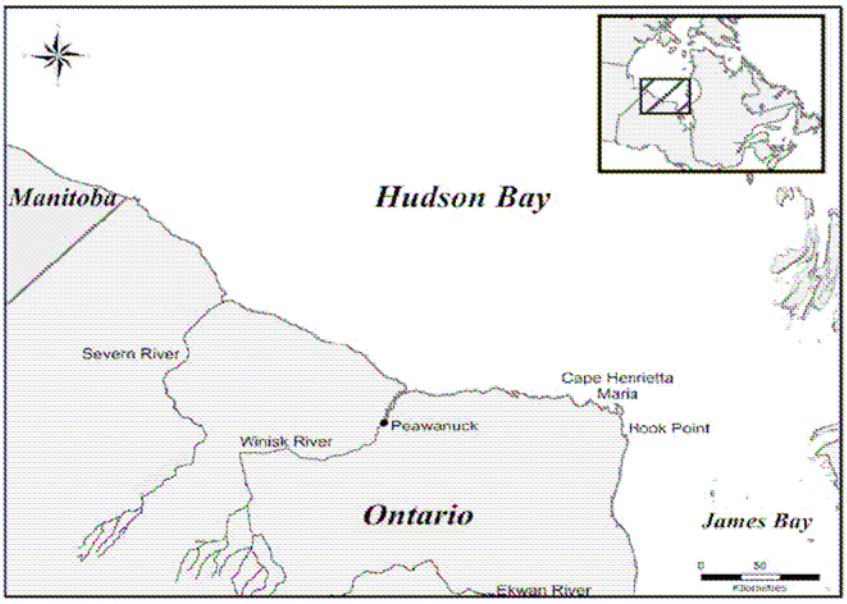


Fig. 3. Study area ranging from Hudson Bay coast at the Ontario–Manitoba border (ca. 56°50'N 89° W) to Hook Point (ca. 54° 50'N 82° 15'W) on north-western James Bay.

Polar Bear Sampling

Once safely immobilised, a series of morphometric measurements were taken, including straight line body length (SLBL), chest circumference and skull size. Body mass was measured by suspending a sling from a load scale. The condition of the animal was assessed to estimate the fat stores and overall condition. The bears were given tattoos on the upper lip and ear tags (earplug samples were taken at this time) to aid in identification and for keeping record of the life history of the animal. Bears captured for the first time had a tooth removed (1st premolar) to age the animal. Samples of claw and hair were taken in addition to a fat biopsy from the hindquarters of the animal for use in contaminant assays. A blood sample (30 ml) was taken by needle and syringe from the jugular or femoral vein of the animal and stored on ice until processing. Following sampling, a reversal drug Atipamezole (Antisedan®; 5 mg/ml) was administered (at 0.2 mg/kg) to metabolise the aesthetic, with half the volume given by intravenous route and

the other half by intramuscular route (Cattet et al. 2003*b*). The bear was visually observed from a safe distance for signs of recovery (lifting of head) and capture sites were checked a few hours later for further confirmation.

Grizzly Bear Biology

Grizzly bears were sampled by the Foothills Model Forest (FMF) Grizzly Bear Project from 2000-2006, yielding 219 bears (including recaptures). The study area included the Eastern Slopes of the Rocky Mountains in Alberta, Canada. The study areas are shown in Fig. 4. The populations being compared in this study are designated on Fig. 4 and defined below. The field sampling for grizzly bears was carried out mostly from April through June, though samples were collected throughout the spring, summer and fall, allowing for comparison of fed and fasted animals. Since most of the samples were from bears recently emerged from hibernation, they were in the fasted state, similar to the polar bears sampled in the fall. Once again, since this is a field study striving to determine stress levels in response to environmental change there is no control group/population of grizzly bears. For this reason, it was necessary to compare populations and relate the stress indicator responses to habitat attributes. The proportion of protected home range is a measured percentage of the animal's home range, which is land protected from development by a National Park or other wildlife refuge. In theory, these animals would be less impacted from habitat alteration by humans (McLoughlin et al., 2003). A survey by Banci et al. (1994) indicates that only 37% of the some 3.5 million km^2 of grizzly bear habitat in Canada is protected.

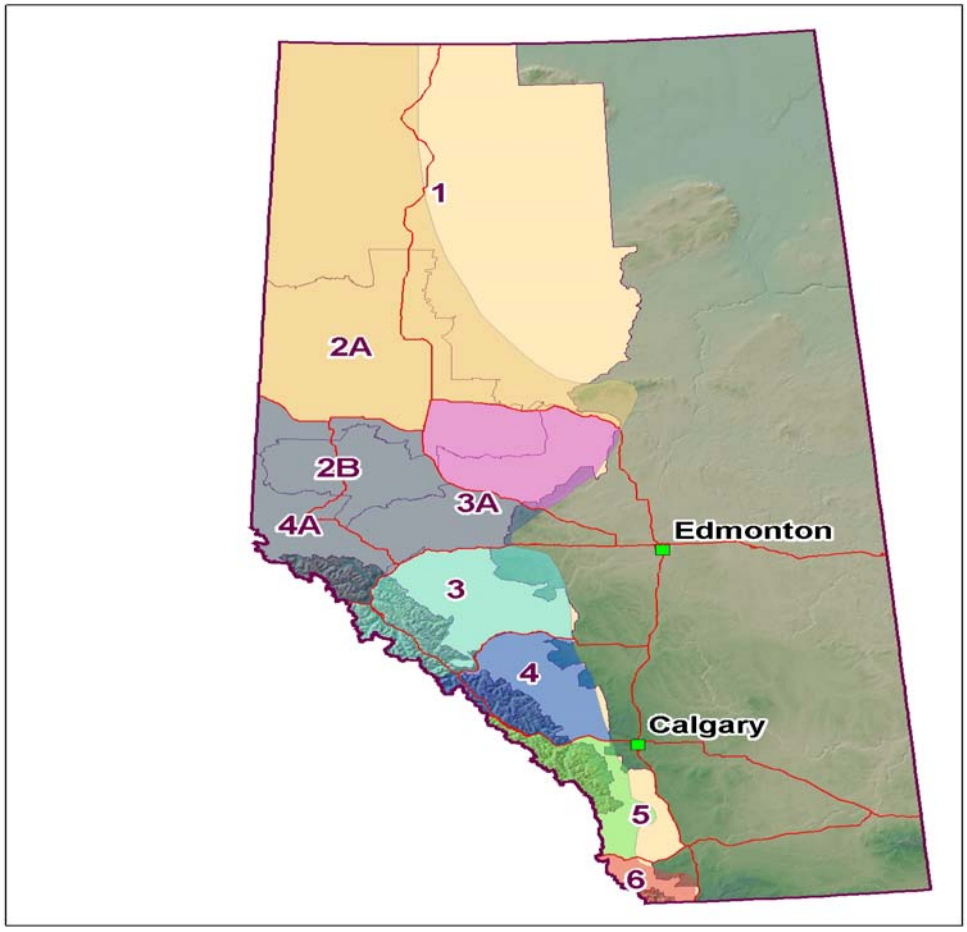


Fig. 4. Map of Foothills Model Forest Grizzly Bear Project study Areas. Population designations are outlined as follows. *Population 1* includes areas 1 and 2A. *Population 2A* includes areas 2B, 3A and 4A and is north of Hinton, AB. *Population 2B* is designated by the pink area of the map and is east of Hinton, AB. *Population 3* outlined by area 3 lies within Jasper National Park. *Population 4* outlined by area 4 is adjacent to Banff National Park. *Population 5* outlined by area 5 is also adjacent to Banff National Park. *Population 6* outlined by area 6 is the southernmost population living south of Calgary, AB

Road density in home range (km/km^2) gives another measure of the level of fragmentation of an animals' habitat and the probability that an animal will encounter humans will usually increase with a higher road density. McLellan (1989) has shown that bear mortalities increase in correlation with an increase in the number of roads in that animal's homerange.

The mean resource selection function (RSF) score is a measure of the probability that a bear will select a particular habitat or area. In this project, the area is a pixel of land as defined by geographic information system (GIS) mapping of the study area. The RSF score is based on telemetry data acquired and analyzed against GIS data. The presence or absence of bears in an area, or the presence of bears in the available habitat, indicates which types of habitat are selected for most frequently using GPS collar data in conjunction with GIS mapping of the bears homerange (Boyce et al. 2002). Since resource (food) availability changes from early-hyperphagia (summer) to late-hyperphagia (fall) (Boyce et al. 2002), it is necessary to understand the seasonal differences in diet of the bears and how this will effect the selection of habitat. RSF modelling can be a powerful management tool as it can predict the presence of bears in a given landscape, which can lead to better informed land-use decisions.

Mean mortality risk is a model that predicts the risk of mortality based on landscape attributes. Nielsen et al. (2004) have found that mortality risk increases with human access, water and edge features, while the risk decreases with terrain ruggedness and an increased green index. Increased human access is positively associated with road density and provides fragmentation to the bears' habitat. Human alteration of habitat, forestry, resource extraction or settlement creates edges and fragments usable habitat, funnelling animals along routes, which may take them away from preferred areas or into contact with humans. Both scenarios have the potential to reduce the survivorship of the bears. Data regarding GIS and landscape features was provided by the Foothills Model Forest Grizzly Bear Research Program.

Grizzly Bear Capture Methods

Between spring 2001 and 2006 grizzly bears of all age classes were captured in the Alberta, Canada, study area using leg-hold snare traps, culvert traps and remote darting from helicopters. All procedures were performed in accordance with the recommendations of the Animal Care Committee of the University of Saskatchewan and the Western College of Veterinary Medicine in Saskatoon, Saskatchewan. These procedures were consistent with the guidelines set forth by the American Society of Mammologists (Animal Care and Use Committee 1998) and the recommendations of the Canadian Council on Animal Care (2003).

The method of capture is dependant on the local habitat attributes, open terrain is suited best for helicopter darting, while heavily treed habitat requires the implementation of culvert traps or leg-hold snares. Bears remotely darted from a helicopter were chased for a brief period (<1 minute) and the anaesthetic effect was observed usually within 15 minutes after darting (Cattet et al. 2003*c*). Culvert traps have a single open door and were baited with an attractant to lure bears into them. Once the animal attempted to consume the bait, a mechanism is triggered closing the door, thus preventing escape. Traps were checked daily and in most cases, the bears were trapped for less than 12 h prior to handling and release. Leg-hold snares were employed in treed areas where road accessibility is insufficient to use a culvert trap, allowing animals to be sampled in more remote areas. The snare was set in an area frequented by grizzly bears, and anchored to a large tree. Once triggered, the snare tightens around the forelimb of the bear and a locking mechanism prevents loosening. All snares were checked once daily and the time of entrapment to release is usually under 12 h (Cattet et al. 2003*c*).

Grizzly Bear Anaesthesia

A combination of intramuscularly administered xylazine and zolazepam-tiletamine was used to anaesthetize grizzly bears in the field. The drugs used were xylazine (Cervizine 300®, Wildlife Pharmaceuticals, Inc., Fort Collins, Colorado, USA) at 3 mg/kg and Telazol® (Fort Dodge Laboratories, Inc., Fort Dodge, Iowa, USA) at 4.5 mg/kg estimated body weight (Cattet et al. 2003*a*). The darts used were lightweight, moderate velocity, slow-injection darts (Paxarms NZ Ltd., Timaru, New Zealand and Dan-Inject Canada, Edmonton, Canada) shot from a dart rifle. Studies have suggested the use of this type of drug delivery system to minimize trauma to the bears (Cattet et al. 2006). During handling, the animal is monitored every 10-15 minutes by a wildlife veterinarian to maintain the safety of the animal during the sampling procedure.

Grizzly Bear Sampling

Basic morphometric measurements including, straight-line body length (SLBL) from the nose to the tip of the last tail vertebrae, skull size and chest circumference were taken to describe the body size. Body mass (BM) was measured by weighing bears on a climbing portaledge (Black Diamond Equipment, Ltd., Salt Lake City, Utah, USA) suspended beneath a load scale (MSI-7200 Dynalink, Precision Giant Systems Inc., Edmonton, Alberta, Canada). A tooth (second premolar) was taken from bears on their first capture for use in aging. In addition, samples of hair and skin were acquired for use in stress monitoring. A blood sample (60 ml) was taken by syringe and needle from the jugular or femoral vein for determining serum indicators of animal health. Also, the bear is fitted with a radio-telemetry collar (Televilt, Lindesberg, Sweden) to monitor movement and provide necessary data regarding the life history of the animal.

Prior to release a reversal drug of Atipamezole (Antisedan®, 5 mg/ml) was administered at 0.2 mg/kg with half the volume given by intravenous route and the other half by intramuscular route. The bear was then monitored from a safe distance until it showed signs of recovery. Within a day of capture, bears are located (using the fitted radio-collar and fixed wing aircraft) to visually assess their recovery from the sampling protocol.

Serum stress indicators

Serum cortisol levels in bear samples were determined using a commercially available ^{125} I cortisol radioimmunoassay (RIA) kit (#07-221102 MP Biomedicals, Irvine, California, USA). The details of the kit are provided in the appendix 2.1.

Serum hsp60 and hsp70 levels were measured using commercially available enzyme-linked immunoadsorbent assay (ELISA) kits (#EKS-600, #EKS-700 StressGen Biotechnologies, Victoria, British Colombia, Canada). Details of the hsp ELISA kit protocols are found in appendix 2.3 (hsp60) and appendix 2.4 (hsp70).

Protein Purification-Dexamethosone-agarose column

In this step, CBG was purified from bear serum samples in order to identify its crossreactivity with commercial antibodies. Since serum samples contain a large amount of total protein, a number of steps were utilized to isolate the protein of interest. First, the sera was passed through a dexamethasone-agarose column (D9285 Sigma; discontinued) under the following protocol. The serum (2 millilitres) of 4 adult, female grizzly bears was combined (0.5 millilitres each) with 1 millilitre of Buffer A (50 mM sodium phosphate pH 7, 0.5 M potassium chloride) and incubated overnight at 4°C with gentle shaking. The CBG was bound to the dexamethosone, a cortisol mimic, and non-target

proteins washed through the column. Column was drained and washed with 3 volumes (2 millilitres) of buffer A and 3 column volumes of buffer B (50 mM sodium phosphate pH 7, 0.2 M sodium chloride), to yield the unbound fraction.. One column volume of buffer B + 0.2 mM cortisol was incubated for 2 hours at room temperature, drained and washed two with two further volumes of buffer B + cortisol, to yield the bound fractions. The high salt buffer was used to disassociate CBG from the dexamethosone and CBG bound cortisol, and was then collected as an eluate. The eluates were concentrated by centrifugation in YM-30 2ml centricon tubes (Millipore) at 5000g for 10 minutes.

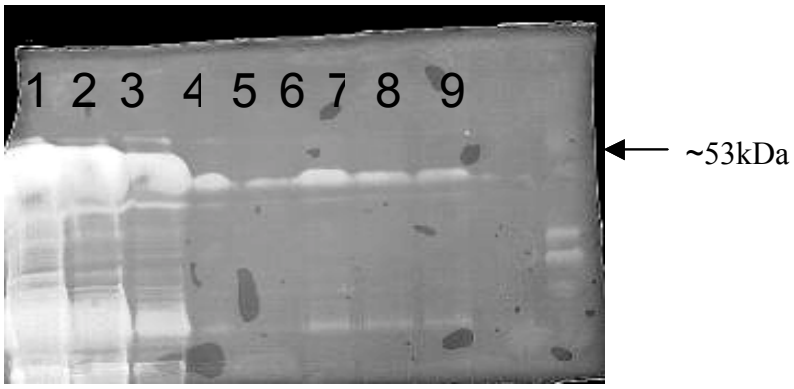


Fig 5. SDS-PAGE gel outlining protein purification using dexamethasone-agaronse column. Left to right lanes represent: bear whole serum, 1st column eluate (buffer A), 2nd-3rd column eluate (buffer B), 4th-9th column eluates (buffer C); Gel was visualized using Coomassie staining.

Protein Purification-Fast performance Liquid Chromatography (FPLC)

Following the primary purification step using the dexamethosone-agarose column, it was necessary to employ a second purification step to eliminate non-target proteins. A FPLC DuoFlow apparatus (BioRad) and Column were employed to selectively bind CBG in the partially purified samples. The samples (bound fractions from dexamethasone-agarose step) were first incubated overnight at 4°C in dialysis

tubing in 10 mM sodium phosphate; pH 7, to remove the high salt buffer from the previous purification step and to facilitate binding to the column. The experiment was carried out with a flow rate of 0.5 millilitres per minute. All solutions were filtered (0.2 μm) and degassed prior to use. First the column was rinsed with 3 column volumes (5 millilitres) of low salt buffer (10mM sodium phosphate pH 7) then the sample was added (3 millilitres), followed by 2 column volumes of low salt buffer. A gradient was created slowly to transfer flow to a high salt buffer (400 mM potassium phosphate pH7) to elude the bound fractions. The column was rinsed with two volumes of high salt buffer followed by two volumes of low salt buffer. Samples were collected, fractions showing high protein elution were pooled, and protein identity was verified using SDS-PAGE and western blotting.

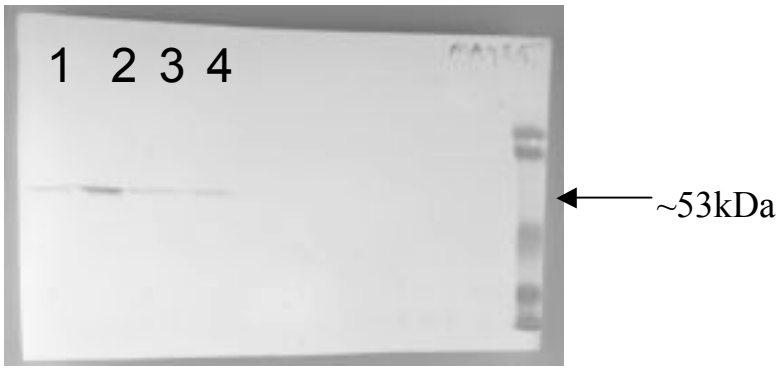


Fig. 6. Western blot outlining crossreactivity of rabbit anti-human CBG antibody with bear CBG. Left to right, lanes 1 and 2 polar bear serum; lanes 3 and 4 grizzly bear serum. Remaining lanes where negative control. *Rabbit-anti human CBG* polyclonal antibody 1:3000 dilution.

Antibody Generation

Two rabbits were requisitioned from Charles River Laboratories (St. Constant, Quebec) and housed according to University of Waterloo Animal Utilization Project Proposal (AUPP) #05-09. This followed all standard operating procedures set out by the

University of Waterloo, Office of Research Ethics. Purified protein was injected into rabbits in order to generate a *rabbit anti-bear CBG* polyclonal antibody. The protein was suspended in 0.85% (vol/vol) sterile saline solution. The concentration of the protein in solution was 150µg/ml. Equal parts of the protein/saline solution and Freund's complete adjuvant and vortexed until emulsified. The rabbits were given four subcutaneous injections (0.25 millilitres each) following UWSOP457. Booster injections were given three weeks later using the same protein/saline solution (150µg/ml) and Freund's incomplete adjuvant. Again, four injections of 0.25 millilitres were given. Two more booster injections were administered at three-week intervals. Prior to each set of injections a blood sample was taken via needle and syringe to check for a titre of the antibody. Three weeks following the final booster the rabbits underwent and terminal cannulation following UWSOP451 and UWSOP453. All procedures were carried out by Martin Ryan, the animal care technician for the Department of Biology.

Antibody Purification

The antibody was tested using SDS-PAGE and western blotting for crossreactivity and purity. The results suggested that purification steps need be employed to remove any anti-albumin IgG from the rabbit sera. Using an albumin-agarose column (A3790 Sigma) to bind any anti-albumin IgG present in the rabbit serum, the anti-bear CBG IgG should pass through the column leaving a purified antibody. Approximately, 10 milligrams (4 millilitres) of rabbit sera was added to the column and incubated with rocking at room temperature for 1 hour. The unbound fraction was collected and retained for analysis. The column was washed with three column volumes of buffer (0.1 M

sodium phosphate, pH 7). Then the bound IgG was eluted with three column volumes of elution buffer (0.1 M sodium phosphate + 2 M sodium chloride, pH 7), samples were collected and stored for analysis. SDS-PAGE and western blotting was employed to determine the purity of the antibody.

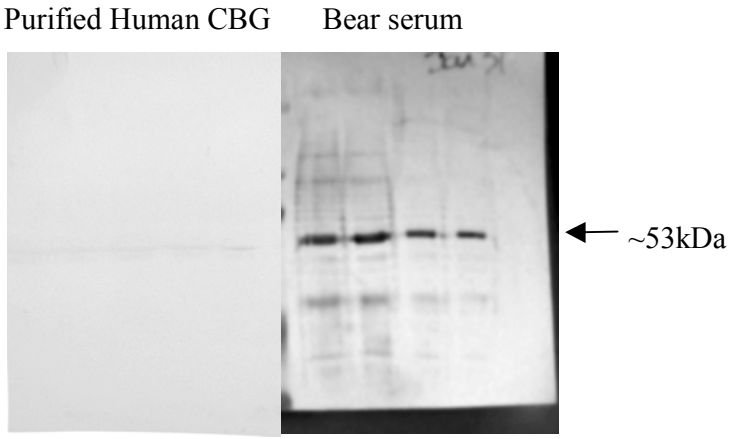


Fig. 7. Membrane representing binding of *rabbit-anti bear CBG* anti body to bear serum and purified human CBG. Left side is purified human CBG. Right side (L to R) Polar bear serum, grizzly bear serum, polar bear serum diluted 100x, grizzly bear serum diluted 100x. Antibody dilution 1:20000

CBG Radioimmunoassay (RIA)

Since it was determined the commercial *rabbit-anti human CBG* polyclonal antibody (#20-CR82 Cedarlane Laboratories, Burlington, ON, Canada) would cross react with bear CBG, a commercially available CBG RIA was tested.

Serum samples were stored at -80˚C prior to use and the assay was performed at room temperature. The principal of the RIA is based on competitive binding of unlabelled CBG (in serum samples) and radiolabelled ^{125} I-CBG to a CBG specific antibody. In serum samples with more CBG there will be less binding of radiolabelled CBG with antibody. A standard curve was developed by using known amounts of

unlabelled CBG bound to constant amounts of radiolabelled CBG and antibody. The standards use were; 0, 0.56, 0.93, 1.4, 2.1, 4.0, 7.7μg/ml. The samples are centrifuged to separate bound fractions from unbound and radioactivity is measured. The curve is created by graphing B/B0% against concentration of CBG (μg/ml); using the equation for the trendline of this curve the unknown concentrations of CBG in the bear serum samples can be interpolated.

Bear serum samples were diluted 25 times with the provided dilution buffer (phosphate buffer with bovine serum albumin and azide; <0.1%). To begin the assay one hundred microlitres of controls (25.6±6.4, 111±16 μg/ml), standard and samples were added to polystyrene tubes in duplicate. A non-specific binding (NSB) sample comprised of two hundred microlitres of zero standard and a total count sample with one hundred microlitres of ^{125}I-CBG were included in the assay. To each tube (except total count) one hundred microlitres of mouse-monoclonal CBG antiserum (in phosphate buffer with bovine serum albumin and azide; <0.1%) was added. Following this, one hundred microlitres of ^{125}I-CBG (in phosphate buffer with bovine serum albumin and azide; <0.1%) was added to all tubes. The tubes were shaken and covered, then incubated for two hours at room temperature. At this point one hundred microlitres of immunoadsorbent (anti-mouse gamma globulin antiserum covalently linked to microcrystalline cellulose) was added to all tubes (except total counts). Samples were shaken for 20 seconds and incubated for 20 minutes at room temperature. Three millilitres of wash buffer (TRIS-HCl, diluted 70 times with distilled water) added to all tubes (except total counts) then tubes were centrifuged for 15 minutes at 1500g. The supernatant was decanted without disturbing the pellet and the tubes were allowed to dry

for 20 minutes. The radioactivity of the pellet was read for 60 seconds in a gamma counter. The standard was developed from the standards (0, 0.56, 0.93, 1.4, 2.1, 4.0, 7.7μg/ml) and B/B0% was calculated for each sample as follows:

B/B0%={(counts standard/sample)-(countsNSB)/(counts 0 standard)-(countsNSB)}x100

B/B0% was graphed against the log CBG concentrations and a logarithmic trendline was added. Using the equation of the trendline, bear serum CBG concentrations were determined. The performance of the assayed was checked by confirming the concentrations of the provided positive controls, these fell well within the provided standard deviations.

In order to check the cross reactivity of the RIA with bear CBG it was necessary to create serial dilutions of bear sera to observe if the assay produced a dilution curve mirroring that of the standard curve. Bear serum samples were diluted with the provided dilution buffer as follows: 10, 25, 50, 100, 200, 300, 400, 500, 1000 times. Using the B/B0% as calculated from the standard curve the dilution curve was calculated and checked to see if the dilution of the serum samples yielded lower amounts of CBG from the assay.

Western Blotting

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting was used to check for the specificity of the commercial CBG antibody with both grizzly bear and polar bear serum samples. Also, this technique was used to determine the specificity of the bear-specific purified CBG protein as well as the anti-bear CBG antibody. The western blotting procedure is very well established in the laboratory (Boone et al. 2002). Samples (commercial human CBG, bear-specific

CBG, bear serum samples) were loaded on to the polyacrylamide gels (8%) and were separated using the discontinuous buffer system of Laemmli (Laemmli 1970). The separated proteins were electrophoretically transferred (20 V for 20 minutes) onto nitrocellulose membranes [SemiDry Transfer Unit (BioRad) using transfer buffer consisting of 25 mM Tris pH 8.3, 192 mM glycine, and 20% (vol/vol) methanol]. The membranes were blocked for 1 hour with 5% skim milk in TTBS [20 mM Tris pH 7.5, 300 mM NaCl, 0.1% (vol/vol) Tween 20] with 0.02% sodium azide. Antibody dilutions (either the commercial antibody or the anti-bear CBG) were made with the same blocking solution to the appropriate concentrations. The membranes were incubated for 1 hour at room temperature with either the rabbit anti-human CBG polyclonal antibody (#20-CR82 Cedarlane Laboratories, Burlington, ON, Canada) at a 1:500 dilution or the anti-bear CBG at a 1:3000 dilution. The membranes were then washed (3 x 5 minutes) with TTBS, after which they were incubated for 1 hour with an alkaline phosphatase conjugated goat anti-rabbit IgG antibody (1:3000; BioRad). The membranes were washed (3 x 5 minutes) with TTBS and (1 x 10 minutes) with TBS. The bands were visualized using chromogenic substrates, nitroblue tetrazolium (0.033% wt/vol) and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (0.017% wt/vol) (Fisher Scientific, Burlington, Ontario, Canada).

Statistical analyses

Since no evidence was observed when bears captured multiple times were observed all captures were used in comparison of serum indicators. The bear results were compared for statistical significance using analysis of covariance (ANCOVA), followed

by Sidak *post-hoc* test to determine statistical difference between groups. Statistical significance was set at $p<0.05$.

There were a number of covariates which needed to be included in the calculations as they were found to affect the level of stress indicators in polar bears (Table 1) and grizzly bears (Table 2). In polar bears, age was a covariate with respect to cortisol, while serum hsp60 and hsp70 did not have any covariates. Unless otherwise noted the covariates were used for all statistical computations. In grizzly bears, cortisol was shown to be effected by the proportion of protected homerange; however, since the available data set for this attribute was very small this was not included as a covariate. BCI was included as a covariate for serum hsp60, while body mass was a covariate for serum hsp70. Unless otherwise noted, the covariates were used for all statistical computations. Due to the significant effect of capture methods on serum cortisol and hsp70 levels, capture method was used as a fixed factor when computing these results in grizzly bears.

Results

Corticosteroid-binding globulin (CBG)

Despite two protein purification steps the solution injected to the rabbits evidently contained non-target proteins, thus generating an antibody which reacted with other serum constituents. Since CBG was present in bear serum in very small amounts its required large volumes of serum to retrieve sufficient levels of protein to inject into the rabbits. This coupled with the time consuming procedure of generating the antibodies in the rabbits prevented the repetition of this procedure. Following attempts to clean up the generated antibody it was determined that the generated antibody lacked the specificity required to move forward in developing a bear specific CBG ELISA.

As previously determined, a commercially available mammalian antibody positively reacted with bear CBG, thus the attempt to use a commercial mammalian CBG RIA. The concentrations outlined in the protocol failed to produce accurate results. There were a number of dilutions of both the samples and the detection reagents attempted but it was not possible to accurately assay changes in CBG within bear serum samples. The kit did not have sufficient detection limits to assay CBG at *in vivo* concentrations in bear serum samples. Thus it is further necessary to develop a bear specific ELISA which would provide the required detection limits and ability to assay a large number of samples at one time.

Polar Bears:

Correlations and Covariates:

There were a number of biological and environmental variables present for polar bears which held the potential to influence the serum indicators studied. Prior to statistical comparison, it was necessary to determine which variables were correlated

strongly with the indicators. Using partial correlation analysis, it was possible to determine whether a variable was significantly correlated to the indicators. In polar bears age was determined to be a covariate for cortisol. Body length was found to have significant effects on serum hsp60 in polar bears. The environmental variables were treated as categorical due to their restriction to a few years or ice information pertaining to this study. Julian day of capture was also found to have a significant effect on the serum hsp70. The elapsed time since breakup to sampling was determined to directly correlate to both cortisol and serum hsp70, when controlling for population in partial correlation analysis. This was considered to be indicative of duration of fasting.

Table 1. Determination of Covariates to be used in statistical analysis of polar bear stress indicators.

Covariate	Cortisol	Serum Hsp60	Serum Hsp70
Body mass	*ns*	*ns*	*ns*
Body length	*ns*	r= 0.13 P= 0.03 n= 264	*ns*
Age	r= -0.139 P= 0.045 n= 206	*ns*	*ns*
Julian day of capture	*ns*	*ns*	r= -0.23 P= 0.03 n= 262
Elapsed time since ice breakup to sampling	r= 0.112 P= 0.049 n= 217	*ns*	r= 0.173 P= 0.005 n= 214

Partial correlation analysis was used to determine if any continuous variables had a significant effect on the measured stress indicators. Between body mass, body length and age one variable was compared with cortisol while the other two variables were the controlling factors and it was determined only age was a significant covariate for serum cortisol levels. Hsp60 was shown to be directly correlated to body length. Julian day of capture was inversely correlated to serum hsp70 levels. Elapsed time since breakup was

directly correlated to cortisol and serum hsp70 when controlling for population (SHB and WHB). The biological covariate age and body length, for cortisol and hsp60 respectively have weak associations and are not included in statistical analyses.

There were a number of biological factors which were analysed to ascertain their effect on polar bear serum indicators. They were observed to not have any significant effect on serum indicators. As such these figures and statistical analyses can be found in Appendix 3.

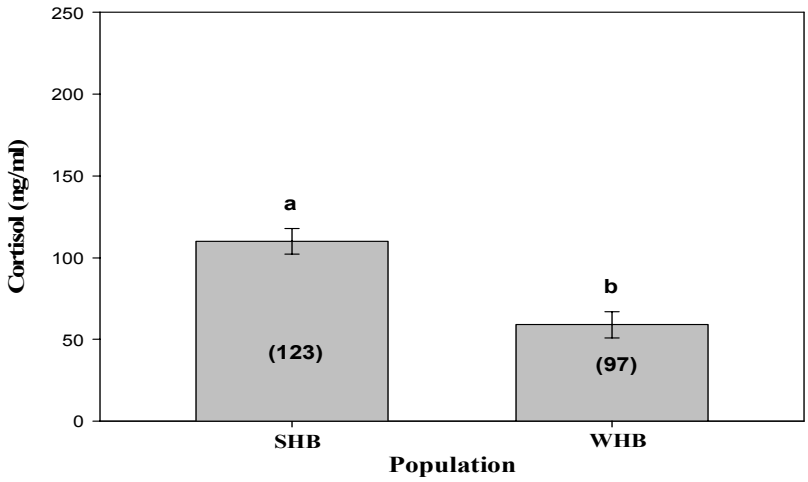
Population Based Differences in Polar Bear Stress Indicators

Comparison was limited to the SHB and WHB populations, with the LS population omitted. This was done because there was no data outlining the time on land prior to capture available for the LS population and this factors was found to significantly influence serum levels of the indicators. Bears captured in both SHB and WHB were treated as SHB as they came onto land under the influence of climatic conditions governing the ice cover for Southern Hudson's Bay for that particular year.

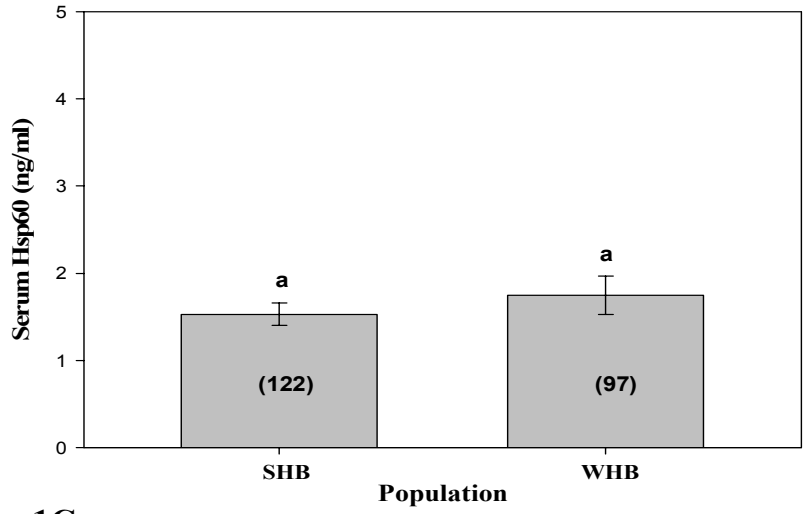
Figure 1. Population Based Differences in Polar Bear Stress Indicators. Serum levels of cortisol (Fig. 1A), hsp60 (1B) and hsp70 (1C) were analysed for difference based on comparison between SHB and WHB populations. Values represent mean ± SEM. Sample size shown in parentheses; bars with the same letters are not statistically different ($p<0.05$). Analyses performed as follows:

Fig.1A: ANCOVA: $F=23.125$, $P\leq0.001$, $N_{WHB}=97$, $N_{SHB}=123$; with time since breakup as a covariate.
Fig. 1B: ANCOVA: $F=1.473$, $P>0.05$, $N_{WHB}=97$, $N_{SHB}=123$; with time since breakup as a covariate.
Fig. 1C: ANCOVA: $F=12.035$, $P\leq0.001$, $N_{WHB}=97$, $N_{SHB}=123$; with time since breakup and julian day of capture as covariates.

1A


Cortisol (ng/ml)
250
200
150
100
50
0
a
b
(123)
(97)
SHB
WHB
Population

1B


Serum Hsp60 (ng/ml)
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(122)
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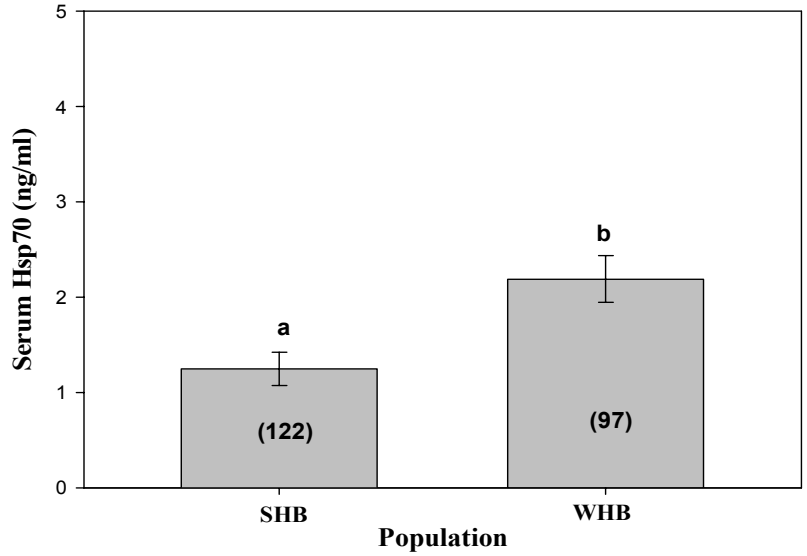
SHB
WHB
Population

1C


Serum Hsp70 (ng/ml)
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(122)
(97)
SHB
WHB
Population

Grizzly Bears:

Grizzly Bear Correlations and Covariates

There were a number of biological and environmental variables present for grizzly bears which held the potential to influence the serum indicators studied. Prior to statistical comparison, it was necessary to determine which variables were correlated strongly with the indicators. By using partial correlation analysis, it was possible to determine whether a variable was significantly correlated to the indicators. For grizzly bears cortisol showed no significant biological covariates. For serum hsp60, there were no significant biological covariates. For serum hsp70 body mass was a significant covariate when using partial correlation analysis. The environmental variables were no included as covariates as they are only available for a limited subset of the available bears.

Table 2. Determination of Covariates to be used in statistical analysis of grizzly bear stress indicators

Covariate	Cortisol	Serum hsp60	Serum hsp70
Body mass	*ns*	*ns*	r = 0.16 P = 0.04 n = 170
Body length	*ns*	*ns*	*ns*
BCI	*ns*	*ns*	*ns*

Partial correlation was used to determine potential covariates. R is the correlation coefficient, P is the significance of the correlation and n represents the sample size, while *ns* denote no significance found for the particular covariate.

As with polar bears there were biological and environmental variables investigated which had no effect of serum indicators, these figures and analyses can be found in Appendix 4.

Effect of Capture Method on Grizzly Bear Stress Indicators

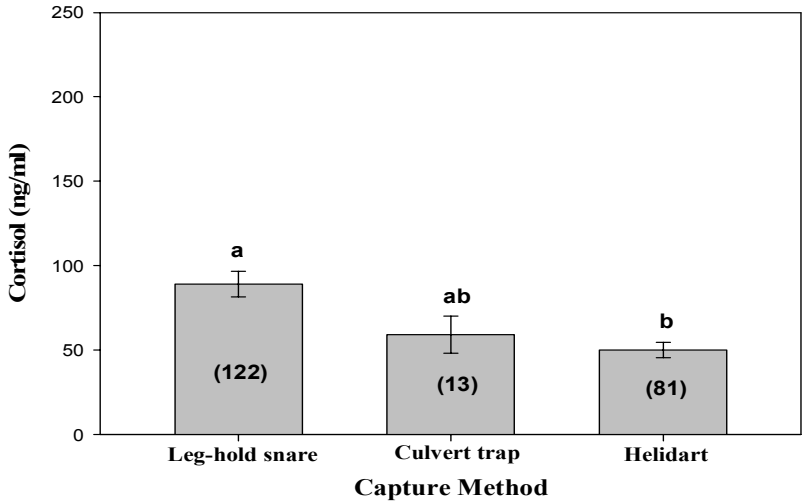
Grizzly Bears were captured by 3 main methods: helidart, culvert trap and leg-hold snare. Ground dart had a limited data set (n=2) and thus was omitted from analysis. Bears captured by leg-hold snare exhibited significantly higher serum cortisol levels (Fig. 2A) compared with animals captured by helidart. Bears captured by culvert trap showed levels intermediate to the other methods. Levels of serum hsp60 were not determined to be affected by capture method. (Fig. 2B). While serum hsp70 (Fig. 2C) levels were again significantly elevated in bears captured by leg-hold snare versus bears captured by helidarting.

Figure 2. Effects of capture method on grizzly bear stress indicators. Grizzly bears captured by different methods were analysed for serum levels of cortisol (Fig. 2A), hsp60 (2B) and hsp70 (2C) were analysed for difference based on comparison between SHB and WHB populations. Values represent mean ± SEM. Sample size shown in parentheses; bars with the same letters are not statistically different ($p<0.05$).

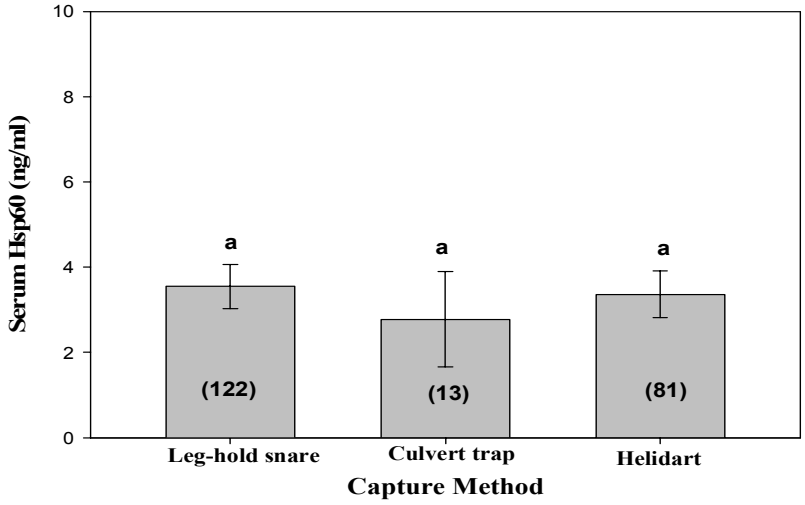
Analyses performed as follows:

2A: ANCOVA; $F=7.8$, $P\leq0.001$, $N_{\text{leg-hold snare}}=122$, $N_{\text{culvert trap}}=13$, $N_{\text{helidart}}=81$
2B: ANCOVA: $F=0.132$, $P>0.05$, $N_{\text{leg-hold snare}}=122$, $N_{\text{culvert trap}}=13$, $N_{\text{helidart}}=81$
2C: ANCOVA: $F=4.77$, $P<0.01$, $N_{\text{leg-hold snare}}=122$, $N_{\text{culvert trap}}=13$, $N_{\text{helidart}}=81$ with body mass as a covariate.

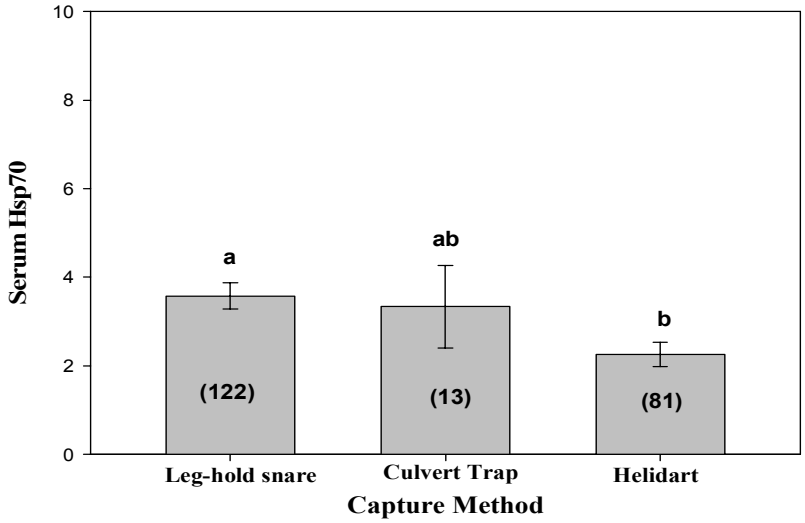
2A


250
200
150
100
50
0
Cortisol (ng/ml)
a
ab
b
(122)
(13)
(81)
Leg-hold snare
Culvert trap
Helidart
Capture Method

2B


10
8
6
4
2
0
Serum Hsp60 (ng/ml)
a
a
a
(122)
(13)
(81)
Leg-hold snare
Culvert trap
Helidart
Capture Method

2C


10
8
6
4
2
0
Serum Hsp70
a
ab
b
(122)
(13)
(81)
Leg-hold snare
Culvert Trap
Helidart
Capture Method

Effect of Proportion of Protected Homerange on Grizzly Bear Stress Indicators

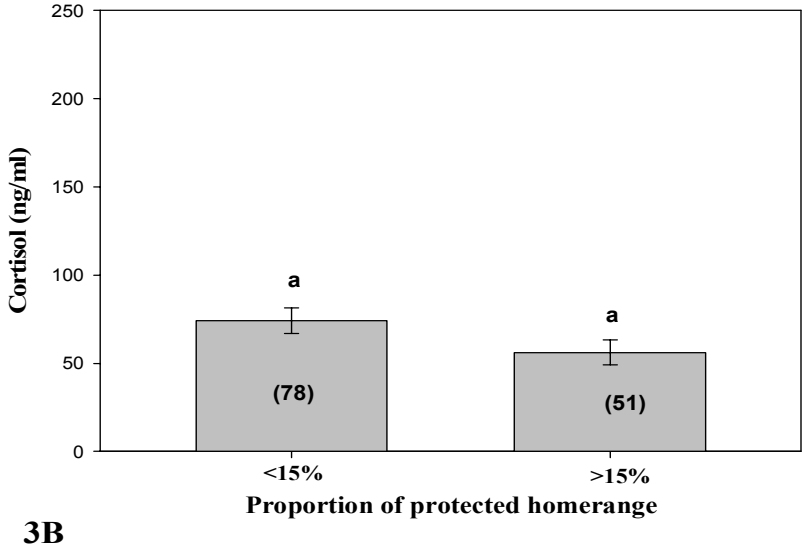
Environmental variables were only available for populations 3 and 4 and, therefore, not used as covariates despite the fact the some variables showed a significant effect on stress indicators. The home ranges are based on the 95% kernel home range as observed from GPS collar or radiotelemetry data for that particular year, this information was provided by FMF Grizzly Bear Project. Protection is in the form of national or provincial parks or wildlife refuges. Groups of grizzly bears were separated in amount of protected area within their 95% homerange as follows: ≤15% homerange protected from human activity and ≥15% homerange protected from human activity.

Data analysis indicates no significant difference in serum cortisol (Fig. 3A), or hsp60 (Fig. 3B). However there was a significant increase in serum hsp70 (Fig. 3C) levels in grizzly bears living in homeranges with ≤15% homerange protected from human activity. Capture method was not included in this analysis, though it is known to affect both cortisol and hsp70.

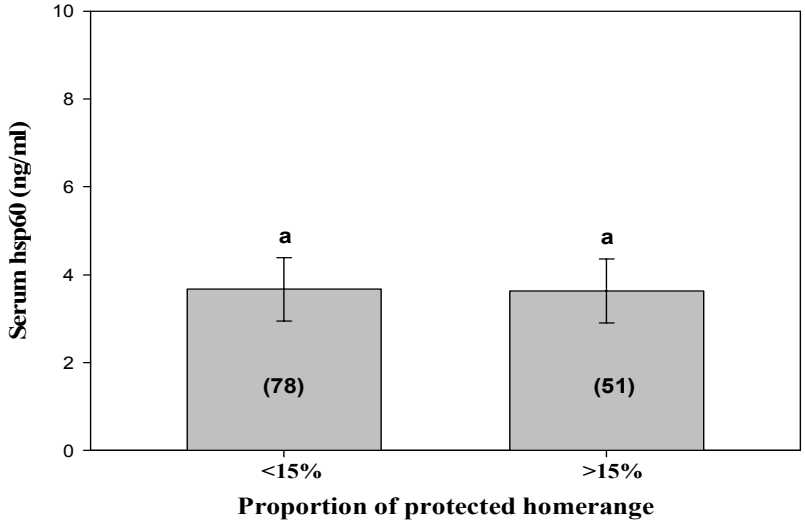
Figure 3. Effect of protected homerange on grizzly bear stress indicators. Grizzly bear serum was analyzed for difference in cortisol (A), hsp60 (B), and hsp70 (C) levels on the basis of level of homerange protection. Values represent mean ± SEM. Sample size shown in parentheses; bars with the same letters are not statistically different. Analyses performed as follows:

3A: ANCOVA: $F=2.919$, $P>0.05$, $N_{\leq 15\%}=78$, $N_{\geq 15\%}=51$
3B: ANCOVA: $F=0.002$, $P>0.05$, $N_{\leq 15\%}=78$, $N_{\geq 15\%}=51$
3C: ANCOVA: $F=9.938$, $P<0.01$, $N_{\leq 15\%}=78$, $N_{\geq 15\%}=51$ with body mass as a covariate.

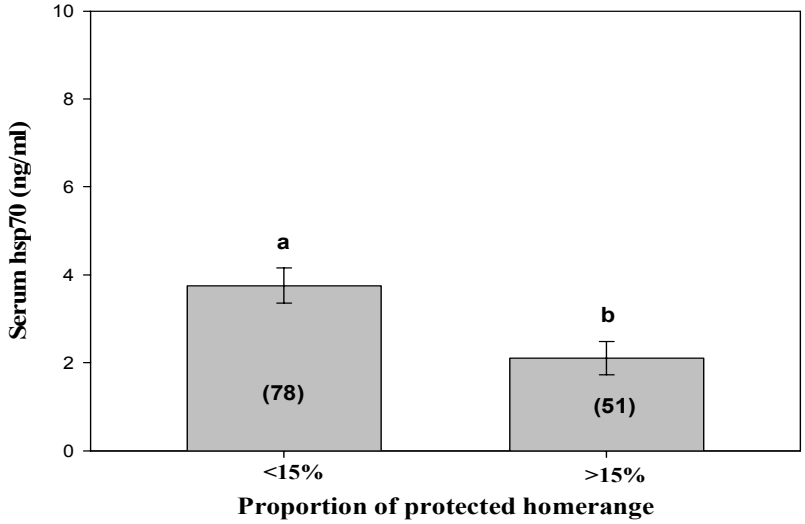
3A


250
200
150
100
50
0
Cortisol (ng/ml)
a
a
(78)
(51)
<15%
>15%
Proportion of protected homerange

3B


10
8
6
4
2
0
Serum hsp60 (ng/ml)
a
a
(78)
(51)
<15%
>15%
Proportion of protected homerange

3C


10
8
6
4
2
0
Serum hsp70 (ng/ml)
a
b
(78)
(51)
<15%
>15%
Proportion of protected homerange

Effect of Proportion of Protected Homerange on Serum hsp70 in Bears Captured by Helicopter Darting Only

Since capture method was determined to affect serum indicators in grizzly bears it was necessary to analyze the affect of proportion of protected homerange separate from capture stress. Homerange protection was previously determined to only significantly affect hsp70 levels. Bears living in areas with ≤15% homerange protected from human activity were captured more often by leg-hold snare, while bears living in areas with ≥15% homerange protected from human activity were mostly captured by helicopter darting. This difference was mostly a function of terrain attributes preventing the use of helicopter darting in certain areas. This has the potential to skew the effects of protected homerange on the stress indicators. Since capture by helicopter darting has been determined to increase the levels of serum indicators the least it and was present in both ≤15% and ≥15% protected homeranges in sufficient sample sizes, it was used as a basis to evaluate the effect of homerange protection.

Partial correlation analysis using only bears captured by helidarting were analysed controlling for body mass. It was determined serum hsp70 was significantly inversely correlated to proportion of protected homerange. ($r= -0.36$, $P= 0.012$, $N= 45$).

Discussion

CBG Implications

CBG was isolated and purified from the sera of both polar and grizzly bear sampled in the field. An *anti-bear CBG* polyclonal antibody was generated and checked for positive cross-reaction with bear and human CBG isolates. A commercially available mammalian CBG-RIA kit was employed to assay CBG levels in bear serum samples and it was deduced that the levels present in the samples were at the extreme low end of the assay's detection limits. Thus it was not possible to ascertain with any certainty or reproducibility any changes in the levels of CBG between samples. One possible explanation is that the commercial antibody recognizes a part of CBG which it can bind only in the denatured state the protein is found in for western blotting techniques. In serum samples bear CBG would be found in its native, folded state and the commercial antibodies may not bind it in this state.

This limited our ability to use CBG as an indicator of stress for this study; however this in no way diminishes the possibilities for the use of CBG as a biomarker of stress/health status in wild mammals. Future recommendations include determining the protein sequence of bear CBG and using recombinant vector techniques to produce large quantities of pure bear CBG. With this purified bear CBG the generation of a specific *rabbit anti-bear CBG* antibody would be possible. Using this bear-specific CBG antibody to develop an ELISA to detect this serum protein in bears. The homologous assay should provide the sensitivity and detection limits required to accurately measure serum levels of CBG in bears. The potential for CBG as a biomarker of chronic stress in wildlife deserves further investigation.

Polar Bear General Observations

This study is the first to measure serum hsps as an indicator of stress in wildlife. While hsps have been commonly used as an indicator of cellular stress response, recent studies suggest a strong correlation between stressor exposure and serum hsps levels, both in chronic disease state (Pockley et al. 2000) and more acute stressors (Walsh et al. 2001). Consequently, serum hsps were measured in this study to evaluate its potential as an indicator of stress in bears. Overall, in polar bears, both hsp60 and hsp70 did not appear to change very much with animal characteristics, including sex and reproductive status, which is contrary with findings by Kristensen et al. (2004). However, there was a weak inverse correlation between serum hsp70 levels and ice breakup date and previous winter's ice coverage. This suggests that food availability and fasting associated metabolic responses may affect serum hsp70 levels in polar bears.

Cortisol, as a biomarker of stress, must always be used cautiously especially considering the field capture methods employed to sample large, free-ranging mammals such as bears. The tendency to interpret observed cortisol levels as an indicator of chronic stress state may be masked by the elevation in cortisol levels attributed to capture stress (Cattet et al. 2003*c*). Given that all polar bears were sampled in a similar fashion (Cattet et al. 2003*c*; Obbard et al. 2006), it is prudent to assume similar cortisol responses among bears and groups of bears to this capture stress. Indeed, each animal captured will show elevated cortisol levels (Cattet et al. 2003*c*), but depending on the stress state of the animal prior to capture this elevation may be modulated (Alexander and Irvine, 1998). For instance, it has been proposed that a chronically stressed bear may have a reduced activation of the cortisol stress axis in response to the acute capture stress compared to an

"unstressed animal" (Boonstra, 2005). This attenuated response to capture stress may be due to the long term adjustments to the hypothalamus-pituitary-adrenal axis, including exhaustion of the corticotrophin and steroid production, leading to a lower plasma cortisol response (Romero, 2002). In the present study it was observed that cortisol showed an inverse correlation with age (Table 1) suggesting that either older or larger animals are less able to activate the stress axis during capture. However, whether this lower response to capture stress is related to either a lack of trophic hormone stimulation or adrenal exhaustion remains to be determined.

Evaluation of cortisol as a stress indicator in polar bears

There were no significant differences in serum cortisol levels between sexes and reproductive classes of bears. Age did show a slight inverse correlation with cortisol which has been observed in other mammalian species (Boonstra, 2005).

Polar bears undergo yearly cycles of fasting once sea ice melts in the spring until it freezes again in the fall, denying them access to hunting seals effectively (Stirling and Parkinson, 2006). Recent reports suggest reduced periods of suitable ice cover in Hudson's Bay (Gough et al. 2004), resulting in reduced food for bears and prolonging the fasting period. Environmental change perpetuated by climatic warming is working to increase the fasting period of polar bears especially in the Southern Hudson's Bay (SHB) population (Obbard et al. 2006) with an overall decrease in sea ice cover by approximately 25 days per year, since 1971 (Gough et al. 2004).

For analysis of the ice cover data for Hudson's Bay from Gough et al. (2004) and the Canadian Ice Service website (http://ice.ec.gc.ca) it was necessary to separate the WHB and SHB populations as the ice cover data were not available for common years.

The data were analyzed for each population separately then comparisons were made afterwards. The LS population was excluded from this analysis as there were no ice cover data available for the study years, however due to its latitude there it would be assumed to have ice cover for an longer period than both the WHB and SHB populations. Since the breakup date and freezeup date data available was only for a few years and none of these years overlapped for SHB and WHB it was not possible to compare between these years with certainty. The best measure was the elapsed time from sea ice breakup to capture; this was determined to be indicative of the duration the animal was fasting. Again the use of environmental variables is indicative of feeding status, because the earlier the breakup of the sea ice the longer the bears will be without food (Obbard et al. 2006; Stirling and Parkinson, 2006).

It was observed that there was a significant positive correlation between cortisol levels and time spent on land prior to capture. This indicates that cortisol levels increase throughout the period of fasting in polar bears. Comparison of cortisol levels between the sampled populations indicate that the WHB had significantly lower cortisol levels than did the SHB population, when controlling for time spent on land prior to capture. Studies in other mammalian species have indicated an initial increase in cortisol levels in response to fasting. When the lack of food first begins to effect the animal there is an elevation in cortisol levels which is required to affect changes in metabolic pathways to conserved energy (Boonstra 2005) and increase the activity of lipolytic pathways (Fleshner et al. 1995). As the fasting persists and the cortisol levels remain elevated there must be a long-term adjustment in CBG to prevent over stimulation of the target tissues and atrophy of the HPA axis (Alexander and Irvine, 1998). Though cortisol is important

for metabolic adaptations in mammals (Sapolsky et al. 2000) it is quite imperative to the long term health of the animal that the HPA axis is not chronically activated which has been proven to yield deleterious effects to the animals (Boonstra, 2005).

The polar bears from the various populations were subjected to similar capture stress (Cattet et al. 2003*b*) which would presumably give rise to comparable elevations in cortisol levels. However, polar bears from the WHB population were on average captured more times than SHB bears (WHB 4.97 times and 1.59 times for SHB); this may be a source of difference in the cortisol increase with response to capture stress.

This reduced cortisol response to an acute stressor during the fasting period would suggest that there was a long-term adaptation to cortisol regulation in these polar bears. Alexander and Irvine (1998) observed a reduced response to stress as a stressor was imposed multiple times. This was measured in magnitude of cortisol response (Alexander and Irvine, 1998). Potentially there may be a learned response to capture or a long-term depression in the HPA axis to prevent over stimulation. A change on this temporal scale would imply CBG levels were altered to attenuate chronic release of cortisol (Fleshner et al. 1995).

Aside from differences in capture frequencies the elevated cortisol levels in the SHB population may result of another factor other than fasting. Since the analysis compared the 2 populations while controlling for time on land (or duration of fasting) there are other factors at play which are affecting the bears. It is difficult to pinpoint the cause but it has been shown that the SHB population had a degraded BCI since the last survey in the 1980s (Obbard et al. 2006).

Observing a known variable such as prolonged fasting in polar bears, may provide insights into the long-term adjustments in cortisol and CBG. How the serum levels of these indicators react to this long-term stressor may provide insight into how their levels would fluctuate in response to other chronic stressors. Though cortisol is influenced greatly by capture stress, it may have some utility as an indicator of fasting in polar bears. When employed with other, slower reacting, measures of stress cortisol has the potential to provide insight into the health status of the bears, and how this is changing with reduced sea ice cover as a function of global warming.

Evaluation of heat shock proteins as a stress indicator in polar bears

Sex and reproductive class were observed to have no significant effect on serum levels of either hsp60 or hsp70 in polar bears. These observations counter those found by Kristensen et al. (2004), who found reproductive status in female cattle to significantly effect serum hsp70 levels. The lack of response between sex and reproductive classes on the stress indicators in polar bears is not known but may be a species difference and/or related to their habitats.

Serum levels of hsp60 were observed to show no significant change in polar bears based on duration of fasting or time on land prior to capture. Previous studies have observed reduced level of serum hsp60 with lowered levels of stress and metabolism (Pockley et al. 2000; Wiersma et al. 2004). However, in the present study there was no significant change in serum hsp60 levels with any of the measured variables.

Serum hsp70 was observed to have a direct correlation with duration of fasting, interpreted as higher levels of serum hsp70 with greater number of days on land prior to

capture, which translates into an increase of hsp70 levels in response to fasting. Though polar bears undergo yearly fasting periods (Stirling and Parkinson, 2006), if this period is extended past a timeframe for which they have fat stores, it may illicit a response which gives rise to altered serum hsp70 levels to cope with this demand. Serum hsp70 levels are significantly lower in SHB population compared to the WHB population, when controlling for the duration of fasting. There are a wide array of factors which could lead to the SHB population having lowered levels of hsp70, as stated, it is this southernmost population predicted to be first affect by global warming and thus changes in fasting periods as a result (Derocher et al. 2004; Obbard et al. 2006). Kristensen et al. (2004) determined a reduction in the release of hsp70 into the serum during stressful periods citing that periods of higher stress required more of the protein intracellularly, thus less hsp70 entered the serum under these conditions. Previous studies have also observed the reduced levels of serum hsp70 in response to the reduced metabolism of the fasting period (Pockley et al. 2000; Rea et al. 2001; Kristensen et al. 2004). Therefore, the possibility that SHB are faced with more survival challenges than the WHB bears, this is translated into higher intracellular requirements of hsp70, WHB bears with lower intracellular demands have greater levels in the serum.

Polar bears undergo a yearly fast, and if this period continues to increase, the lack of food may become a chronic stressor on these animals as body condition reaches a critical threshold. Though the mechanism of release is unknown to date, increased levels of serum hsp70 have been previously shown to coincide with periods of elevated metabolism (Walsh et al 2001; Kristensen et al. 2004). The longer-time required for serum hsp70 change (Kristensen et al. 2004) suggests that capture stress (from helidart

capture) will not modulate this protein level in bear serum. Consequently, serum hsp70 levels show promise as a predictor of altered health status in polar bears, which may be stressor related.

Grizzly Bear General Observations

As previously discussed, the use of cortisol has the potential to create erroneous interpretations as its rapid induction time is drastically effected by capture stress (Cattet et al. 2003*a*). When analyzing the data on stress indicators for grizzly bears we first measured the effect of the various capture methods of the stress indicators to eliminate changes resulting from stressful capture methods. When analyzing the results, capture method was used as a fixed factor in the statistics and thus changes relating to cortisol and serum hsp70 (serum hsp60 showed no effect with capture method) based on capture were accounted for. Body mass was determined to be a significant covariate for hsp70 levels in grizzly bears. The homerange (environmental) variables were only available for populations 3 and 4, so despite the correlation between these variables and the indicators they could not be included for the whole study set. The strongest correlation was observed to be with proportion protected homerange, the levels of the serum indicators decreased as the proportion of homerange protected increased.

Evaluation of cortisol as a stress indicator in grizzly bears

Cortisol levels differed significantly between the helidart and leghold snare methods indicating that leghold snare is more stressful on the bears and causes a greater elevation in cortisol, which corresponds with results observed by Cattet et al. (2003*c*).

Grizzly bears captured by helidart certainly showed elevated serum cortisol levels (Cattet et al. 2003*c*) compared to levels reported in unstressed domestic animals. This response is more consistent with an acute stress event resulting in the activation of the brain-pituitary-adrenal axis (Fleshner et al. 1995; Boonstra 2005). Bears captured by leghold snare are exposed to the stressor for a greater time period and thus both physical and psychological responses are imposed, causing a secondary suite of responses, including metabolic changes to occur (Fleshner et al. 1995; Alexander and Irvine, 1998). Therefore plasma cortisol levels in grizzly bears may not be a good indicator of long-term health status as it is so strongly effected by capture stress, since there is no control group with which to compare basal cortisol levels.

Similar to polar bears there was no significant difference in cortisol levels between males and females of grizzly bears. This, however, differs from previous studies which indicate sex-based variation in cortisol levels (Boonstra et al. 2001; Boonstra, 2005). The capture-induced elevation in cortisol may be masking any baseline differences present between the sexes or reproductive classes in grizzly bears.

In general, the analyzed environmental variables did not significantly effect cortisol levels nor show any observable trends in grizzly bears. Previous studies indicate that road development (Banci et al. 1994), mean RSF score of the bear's homerange (Boyce et al. 2002) and mean mortality risk (Neilson et al. 2004) all play a significant role in the survivorship of grizzly bears. However, these variables were not observed to induce changes in the serum indicators studied. The proportion of protected homerange had no significant effect on cortisol levels in grizzly bears. Woodruffe (2000) found that habitat loss through increased human land use is a threat to survival of large carnivores.

Fragmentation of prime habitat and reduction in food availability with land use changes forces bears to travel further to access food which has been shown to impact survival and reproduction (Pease and Mattson, 1999). In homeranges with little or no protection from (human perpetuated) environmental change, grizzly bears are being faced with increased challenges to survival which may be indicative of increased stress.

The different populations of grizzly bears were not observed to have different cortisol levels. Although this may be a function of very small data sets for some populations. As research on this project progresses, the data set will become more robust and provide further answers to the changes in analyzed serum indicators.

Due to the effect of capture on sampled grizzly bears the levels of serum cortisol are highly variable; this capture stress may be masking environmental induced changes. This study was not able to ascertain any trends in cortisol levels among grizzly bears in response to changing environmental variables. The changes in cortisol in grizzly bears must be validated with changes in serum indicators with a slower induction time and thus separation from the capture stress.

Evaluation of heat shock proteins as a stress indicator in grizzly bears

Serum levels of hsp60 showed no significant difference between capture methods in grizzly bears, indicating physical restraint has no effect on hsp60 in circulation to the 24 hour upper limit of trap time. Earlier studies have shown serum hsp60 to be affected more by chronic or systemic stressors and not show fluctuations in acute disturbances (Pockley et al. 2000; Merino et al. 2006).

Serum hsp70 levels were significantly elevated in bears captured by leghold snare in comparison with those capture via helidart, suggesting that the extended physical

restraint plays a role in altering serum levels of hsp70. Cattet et al. (2003b) have previously shown leghold snares are associated with a longer period of stress and physical exertion. The great deal of physical output in fighting the snare has been shown to lead to muscle injury (Cattet et al. 2003b) which would explain elevated levels of serum hsp70 (Kristensen et al. 2004). Research by Walsh et al. (2001) has shown strenuous exercise to increase circulating levels of hsp70 in as little as 30 minutes following the commencement of exertion.

There was no statistically significant difference observed in serum heat shock proteins when differentiated by sex or reproductive class, despite that both hsp60 and hsp70 show a great deal of variation. When compared between sex and reproductive class there is no definite trend observed here. This is contrary to the findings of Kristensen et al. (2004).

As with cortisol, other than protected homerange, the environmental variables of road development mean homerange RSF score, and mean mortality risk show no effect on either hsp60 or hsp70. Despite the association between these variables and survival of grizzly bears in previous studies (Banci et al. 1994; Boyce et al. 2002; Neilson et al. 2004) there were no strong correlations were found in this study.

Bears whose 95% kernel homerange was ≤15% protected from human activity was determined to have elevated serum hsp70 levels, irrespective of capture method. This suggests some factor of human activity in the bear's homerange elicits a physiological response of increased hsp70 in the serum to cope with the survival challenges presented. When only bears captured by helidarting, the method of capture proven to have the least effect on the serum indicators studied were correlated similar

results were observed. There was a significant inverse correlation between proportion of protected homerange and serum hsp70 levels. Kristensen et al. (2004) determined that hsp70 in serum is not affected by minor daily disturbances and as such it may be hypothesized that a chronic stressor is acting to alter these hsp70 levels over a long term.

Both hsp60 (Pockley et al. 2000) and hsp70 (Walsh et al. 2001) have been determined to be elevated with increased metabolic rate and physical demand. Grizzly bears living in areas that have been subject to extensive environmental change (lower levels of homerange protection) have been determined to work harder to find sufficient food and resources (Banci et al. 1994; Pease and Mattson, 1999).

The studied grizzly bear populations were widely variable in levels of both hsp60 one consistent trend was observed with hsp70 levels elevated in grizzly bears in populations inhabiting the Southern regions of Alberta.

Presently it cannot be certain whether a reduced amount of protected homerange for a particular bear or group or bears mean implicitly there is more stress acting on these animals. However, serum hsp70 in particular is strongly influenced by this environmental variable. Studies by Banci et al. (1994) and Hamer and Herrero (1987) have suggested that changes brought about by human development affected grizzly bear survival. Although hsp70 is affected by capture, the changes observed in this relationship with protected homerange give hsp70 the potential to indicate of chronic stress in grizzly bears.

Conclusions

A field study of large mammals such as bears, on the basis of individual or population stress levels is difficult since there is no baseline or control group to compare the observations to. The use of multiple biomarkers of stress, whose induction times vary, may help to work around sampling related stress and include persistent stressors acting on these animals.

Both polar and grizzly bears exhibited no significant change in stress indicators on the basis of sex or reproductive class, which was interesting as previous studies have shown the upregulation cortisol (Boonstra et al. 2001) and heat shock proteins (Pockley et al. 2000; Kristensen et al. 2004) in response to stress to be strongly effected by both sex and reproductive class. This may be a species specific response, or an adaptation to their particular habitat.

Serum levels of hsp60 overall did not to show much variation in the areas investigated here. Earlier research suggests serum levels of hsp60 are altered by more systemic disease states and not as much by environmental stressors (Merino et al. 2006; Pockley et al. 2000). This study it was determined that assaying serum levels of hsp60 is not a useful tool for correlating with environmental change in bears.

Cortisol, as a measure of chronic stress, must always be used with caution, due to the strong influence of capture stress on cortisol levels involved with sampling wild bears. Despite this, there were persistent changes in polar bears in response to fasting, cortisol levels were observed to be elevated in direct correlation with duration of fasting in polar bears. The WHB population had significantly lower cortisol levels than did the SHB population. Ice cover data suggest that the WHB population of polar bears begin fasting approximately 25 days prior to the SHB bears. However, by analyzing the data

controlling for duration of fasting this implies other factors leading to the elevated levels of cortisol in the SHB polar bears. Number of captures may be one determinate as WHB bears were sampled more and thus may not show a cortisol response to the same degree as the less-sampled SHB bears.

Capture by leghold snare was determined to significantly elevate cortisol levels in grizzly bears, compared to other methods of capture. There was no observable trend in cortisol levels in grizzly bears in response to the environmental variables.

Serum levels of hsp70 proved to be significantly affected by environmental variables in this study. In grizzly bears a strong correlation between protected homerange and hsp70 was observed, which indicate that human activity in an animal's homerange acts to evoke adaptations to survive, potentially reducing the health status. Though it may not be due to of increased stress, some aspect of living in a homerange with increased anthropogenic change to evokes changes in their stress axis.

Fasting in polar bears was observed to correlate with increased levels of serum hsp70. As such, hsp70 may be a good indicator of the nutritional status of the animal. As climate change works to reduce the amount of sea ice cover on which polar bears hunt, the period of fasting for these animals will be increased. There is link between nutritional state and environmental change in polar bears. The observed changes in serum hsp70 with fasting give this serum indicator the potential to gauge the affect of environmental change in these animals.

There is no single indicator of stress which can be assayed for a wild population as there is no control group to provide baseline levels of these indicators. By analyzing a suite of indicators, which have been proven in other studies to be indicative of stress, the

attempt was made to uncover correlations between changing serum indicators and shifting biological and environmental variables known for these animals. Both cortisol and serum hsp70 proved to be significantly affected by environmental variables. Due to the method of capture and its influence on cortisol, further work must be done using CBG as an indicator of changes within the HPA axis over a longer time period. The observations that cortisol is changed in different populations of polar bear apart from a fasting response suggests another factor working to degrade these animals health state. By using changes in CBG it would be possible to determine completely separate from capture what the changes in the HPA axis would be. Also since there may be an attenuated cortisol response in WHB which have been captured more frequently, it may be prudent to test for adrenal atrophy in these animals. ACTH stimulation of captured bears will provide insight into the activity of the animal's HPA axis.

The tools are in place to develop a bear-specific ELISA that would allow the rapid measurement of large volumes of bear samples. Using data regarding changes in these indicators in response to habitat alteration may assist wildlife managers in making decisions regarding habitat use and conservation of these bears.

Literature Cited

Alexander SL and Irvine CHG. 1998. The effect of social stress on adrenal axis activity in horses: the importance of monitoring corticosteroid-binding globulin capacity. Journal of Endocrinology. 157: 425-432.

Animal Care and Use Committee. 1998. Guidelines for the capture, handling and care of mammals as approved by the American Society of Mammologists. Journal of Mammology. 79:1416-1431.

Banci V, Demarchi DA, Archibald WR. 1994. Evaluation of the population status of grizzly bears in Canada. International Conference on Bear Research and Management. 9: 129–142.

Boone AN, Ducouret B, Vijayan MM. (2002). Glucocorticoid-induced glucose release is abolished in trout hepatocytes with elevated hsp70 content. *J. Endocrinol.* 172, R1–R5

Boonstra R, Hik D, Singleton GR, Tinnikov A. 1998. The impact of predator-induced stress on the snowshoe hare cycle. Ecological Monographs. 68:371-394.

Boonstra R. Hubbs AH, Lacey EA, McColl CJ. 2001. Seasonal changers in glucocorticoid and testosterone concentrations in free-living arctic ground squirrels from the boreal forest of the Yukon. Canadian Journal of Zoology. 79:49-58.

Boonstra R. 2005. Equipped for life: Adaptive role of stress axis in male mammals. Journal of Mammology. 86:236-247.

Boyce MS, Vernier PR, Nielsen SE, Schmiegelow FKA. 2002. Evaluating resource selection functions. Ecological Modelling. 157:281-300.

Bradley BP. 1993. Are stress proteins indicators of exposure or effect? Marine Environmental Research. 35:85-88.

Breuner CW and Orchinik M. 2002. Beyond carrier proteins. Plasma binding proteins as mediators of corticosteroid action in vertebrates. Journal of Endocrinology. 175:99-112.

Cairns J, McCormick PV, Niederlehner BR. 1993. A proposed framework for developing indicators of ecosystem health. Hydrobiologia. 263(1):1-44.

Canadian Council on Animal Care. 2003. CCAC guidelines on: the care and use of wildlife. Canadian Council on Animal Care, Ottawa, Ontario, Canada.

Canadian Ice Service. Environment Canada. Government of Canada. http://ice.ec.gc.ca/

Cattet MRL, Bourque A, Elkin BT, Powley KD, Dahlstrom DB, Caulkett NA. 2006. Evaluation of the potential for injury with remote drug-delivery systems. Wildlife Society Bulletin 34: 741–749.

Cattet MRL, Caulkett NA, Obbard ME, Stenhouse GB. 2002. A body condition index for ursids. Canadian Journal of Zoology. 80:1156-1161.

Cattet MRL, Nelson, Ramsay, Leighton, Vijayan MM. In preparation. Carbohydrate metabolism and regulation in polar bears.

Cattet MRL, Caulkett NA, Stenhouse GB. 2003*a*. Anaesthesia of grizzly bears using xylazine-zolazepam-tiletamine. Ursus 14: 88–93.

Cattet MRL, Caulkett NA, Lunn NJ. 2003*b*. Anaesthesia of polar bears using xylazine-zolazepam-tiletamine or zolazepam-tiletamine. Journal of Wildlife Diseases. 39(9):655-664.

Cattet MRL, Christison K, Caulkett NA, Stenhouse GB. 2003*c*. Physiologic responses of grizzly bears to different methods of capture. Journal of Wildlife Diseases. 39(3):649-654.

Committee for Field Methods in Mammalogy. 1987. Acceptable field methods in mammalogy: preliminary guidelines approved by the American Society of Mammalogists. Journal of Mammalogy 68:1-18.

Derocher, A. E., N. J. Lunn, and I. Stirling. 2004. Polar bears in a warming climate. Integrative and Comparative Biology 44: 163-176.

Fleshner M, Deak T, Spencer RL, Laudenslager ML, Watkins LR, Maier SF. 1995. A long term increase in basal levels of corticosterone and a decrease in corticosteroid-binding globulin after acute stressor exposure. Journal of Endocrinology. 136(12): 5336-5342.

Frairia R, Agrimonti F, Fortunati N, Fazzari A,Gennari P,Berta L. 1988. Influence of naturally occurring and synthetic glucocorticoids on corticosteroid-binding globulin–steroid interaction in human peripheral plasma. Annals of the New York Academy of Sciences. 538:287–303

Gasbarrini A, Esposti SD, Di Campli C, De Notariis S, Loffredo S, Abraham A, Simoncini M, Pola R, Colantoni A, Trevisani F, Bernardi M, Gasbarrini G. 1998. Effect of ischemia-reperfusion on heat shock protein 70 and 90 gene expression in rat liver relation to nutritional status. Digestive Diseases and Sciences. 43(12):2601-2605.

Gayrard, V, Alvinerie, M, Toutain, PL. 1996. Interspecies variations of corticosteroid-binding globulin parameters. Domestic Animal Endocrinology. 13(1): 35-45.

Graczyk, TK, DaSilva AJ, Cranfield MR, Nizeyi JB, Kalema GR, Pieniazek NJ. 2001. Cryptosporidium parvum genotype 2 infections in free-ranging mountain gorillas (Gorilla gorilla beringei) of the Bwindi Impenetrable National Park, Uganda. Parasitology Research. 87: 368–370.

Grascolas R and Robin JP. 2001. Long term fasting and refeeding in penguins. Journal of Comparative Biochemistry and Physiology. 128:645-655.

Gough WA, Cornwell A R,Tsuji LJ. 2004. Trends in seasonal sea ice duration in southwestern Hudson Bay. Arctic 57: 299-305.

Hamer D and Herrero S. 1987. Grizzly bear food and habitat in the front ranges of Banff National Park Alberta. International. Conference on Bear Research and Management. 7:199–213.

Hartl FU. 1996. Molecular chaperones in cellular protein folding. Nature. 381:571-580.

Holbrook NJ, Udelsman R. 1994. Heat shock protein gene expression in response to physiological stress and aging. In *Heat Shock Proteins: Structure, Function and Regulation.* Cold Springs Harbor Laboratory Press, Cold Springs Harbor, NY. Pp 577-593.

Kregel KC. 2002. Heat shock proteins: Modifying factors in physiological stress responses and acquired thermotolerance. Journal of Applied Physiology. 92:2177-2186.

Kristensen TN, Løvendahl P, Berg P, Loeschcke V. 2004. Hsp72 is present in plasma from Holstein-Friesian dairy cattle, and the concentration level is repeatable across days and age classes. Cell Stress and Chaperones. 9(2):143-149

Kutsch WL, Steinborn W, Herbst M, Baumann R, Barkmann J, Kappen L. 2001. Environmental Indication: A Field Test of an Ecosystem Approach to Quantify Biological Self-Organization. Ecosystems. 4(1):49-66.

Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** 680–685.

Lee YK, Manalo D, Liu AY. 1996. Heat shock response, heat shock transcription factor and cell aging. Biological Signals. 5:180-191.

Lewis s, Handy RD, Cordi B, Billinghurst Z, Depledge MH. 1999. Stress Proteins (HSP's): Methods of Detection and their use as an environmental biomarker. Ecotoxicology. 8:351-368.

Marti O, Martin M, Gavalda A, Giralt M, Hidalgo J, Hsu BRS, Kuhn, Armario A. 1997. Inhibition of corticosteroid-binding globulin caused by a severe stressor is apparently mediated by the adrenal but not the glucocorticoid receptors. Endocrine. 6:159-164

Mataradze GD, Kurabekova RM, Rozen VB. 1992. The role of sex steroids in the formation of sex-differentiated concentrations of corticosteroid-binding globulin in rats. Journal of Endocrinology. 132:235–240

McLellan BN. 1989. Dynamics of a grizzly bear population during a period of industrial resource extraction. II. Mortality rates and causes of death. Canadian Journal of Zoology. 67: 1861–1864.

McLoughlin PD, Taylor MK, Cluff HD, Gau RJ, Mulders R, Case RL, Messier F, 2003. Population viability of barrenground grizzly bears in Nunavut and the Northwest Territories. Arctic 56: 185–190.

Mendel CM. 1989. The free hormone hypothesis: A physiologically based mathematical model. Endocrine Reviews. 10:232-274.

Merino S, Moreno J, Tomas G, Martinez J, Morales J, Martinez-De La Puente J,Osorno JL. 2006. Effects of parental effort on blood stress protein HSP60 and immunoglobulins in female blue tits: a brood size manipulation experiment. Journal of Animal Ecology. 75:1147–1153.

Mills LS, Citta JJ, Lair KP, Schwarz MK, Tallman DA. 2000. Estimating animal abundance using noninvasive DNA sampling: promise and pitfalls. Ecological Applications 10:283–294.

Munck A, Guyre P, Holbrook N. 1984. Physiological functions of glucocorticoids during stress and their relation to pharmacological actions. Endocrinology Review. 5:25-44.

Nielsen SE, Herrero S, Boyce MS, Mace RD, Benn B, Gibeau ML, Jevons S. 2004. Modelling the spatial distribution of human-caused grizzly bear mortalities in the Central Rockies ecosystem of Canada. Biological Conservation. 120:101-113.

Obbard ME, Cattet MRL, Moody T, Walton LR, Potter D, Inglis J, Chenier C. 2006. Temporal Trends in the Body Condition of Southern Hudson Bay Polar Bears. Ontario Ministry of Natural Resources Climate Change Research Information Note. 3.

Partecke J, Schwabl I, Gwinner E. 2006. Stress and the city: urbanization and its effects on the stress physiology in European blackbirds. Ecological Society of America. 87(8):1945-1952.

Pease CM and Mattson DJ. 1999. Demography of the Yellowstone grizzly bears. Ecology. 80:957-975.

Pittet JF, Lee H, Morabito D, Howard MB, Welch WJ, Mackersie RC. 2002. Serum levels of Hsp 72 measured early after trauma correlate with survival. J Trauma. 52:611–617

Pockley GA, Wu R, Lemne C, Kiessling W, de Faire U, Frostegård J. 2000. Circulating heat shock protein 60 is associated with early cardiovascular disease. Hypertension. 36:303-307.

Rea IM, McNerlan S, Pockley AG. 2001. Serum heat shock protein and anti-heat shock protein antibody levels in aging. Experimental Gerontology 36:341-352.

Reeder DM and Kramer KM. 2005. Stress in free-ranging mammals: Integrating physiology, ecology, and natural history. Journal of Mammology. 86(2):225-235.

Reeder DM, Osteczko NSK, Unz THZ, Idmaier EPW. 2004. Changes in baseline and stress-induced glucocorticoid levels during the active period in free-ranging male and female little brown myotis, Myotis lucifugus (Chiroptera: Vespertilionidae). General and Comparative Endocrinology 136:260–269.

Romero LM. 2002. Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. Endocrinology. 128:1-24.

Rosner W. 1990. The functions of corticosteroid-binding globulin and sex hormone–binding globulin: recent advances. Endocrine Reviews. 11:80–91

Rosner W. 1991. Plasma Steroid-Binding Proteins. Endocrinology and Metabolism Clinics of North America. 20(4): 697-720.

Sanders BM, Martin LS, Nelson WG, Phelps DK, Welch, W. 1991. Relationships between accumulation of a 60 kDa stress protein and scope for growth in *Mytilus edulis* exposed to a range of copper concentrations. Marine Environmental Research. 31:81-97.

Sanders BM, Pascoe VM, Nakagawa PA, Marin LS. 1992. Persistence of the heat-shock response over time in a common Mytilus mussel. Molecular Marine Biology. 1(2):147-154.

Sachser N and Kaiser L. 1996. Prenatal social stress masculinizes the females' behaviour in guinea pigs. Physiological Behaviour . 60:589-594.

Sapolsky RM 2002. Endocrinology of the stress-response. In: Becker J, Breedlove S, Crews D, McCarthy M (eds), Behavioral Endocrinology, 2nd edition. MIT Press. Pp. 409-450.

Sapolsky, RM, Romero LM, Munck AU. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory and preparatory actions. Endocrine Reviews. 21(1): 55-89.

Sciandra JJ and Subjeck JR. 1983. The effects of glucose on protein synthesis and thermosensitivity in Chinese hamster ovary cells. Journal of Biological Chemistry. 258:12091–12093.

Schlechte JA, Hamilton D. 1987. The effect of glucocorticoids on corticosteroid binding globulin. Clinical Endrocrinology. 27:197–203.

Scrocchi LA, Orava M, Smith CL, Han VKM, Hammond GL. 1993. Spatial and temporal distribution of corticosteroid-binding globulin and its messenger ribonucleic acid in embryonic and fetal mice. Journal of Endocrinology. 132: 903-909.

Selye H. 1956. The stress of life. McGraw-Hill, New York

Spencer RL, Miller AH, Moday H, McEwen BS, Blanchard RJ, Blanchard DC, Saki RR. 1996. Chronic social stress produces reductions in available splenic type II corticosteroid receptor binding and plasma corticosteroid-binding globulin levels. Psychoneuroendocrinology. 21: 95-109.

Stirling I, Spencer C, Andriashek D. 1989. Immobilization of polar bears (*Ursus maritimus*) with Telazol® in the Canadian Arctic. Journal of Wildlife Diseases 25:159-168.

Stirling I and Derocher A. 1993. Possible impacts of climatic warming on polar bears. Arctic. 46(3):240-245.

Stirling I and Parkinson CL. 2006. Possible effects of climate warming on selected populations of polar bears (*Ursus maritimus*) in the Canadian Arctic. Arctic. 59(3):261-275.

Tinnikov AA. 1993. Corticosteroid-binding globulin levels in the rat serum under conditions of starvation and restriction of motions. Hormone and Metabolic Research. 25:88-89.

Von Holst D. 1998. The concept of stress and its relevance for animal behaviour. Advances in the Study of Behaviour. 27:1-131.

Walsh RC, Koukoulas I, Garnham A, Moseley PL, Hargreaves M, Febbraio MA. 2001. Exercise increases serum Hsp72 in humans. Cell Stress and Chaperones. 6(4):386-393.

Wasser SK. 1996. Reproductive control in wild baboons measured by fecal steroids. Biology of Reproduction. 55:393–399.

Whitnall MH. 1993. Regulation of the hypothalamic corticotropin-releasing hormone neurosecretory system. Progress in Neurobiology. 40:573-629.

Wiersma, P., Selman, C. Speakman, J.R. & Verhulst, S. (2004) Birds sacrifice oxidative protection for reproduction. Proceedings of the Royal Society of London B Supplement. 271: S360–S363.

Wingfield JC and Romero LM. 2001. Adrenocortical responses to stress and their modulation in free-living vertebrates. In: McEwen BS, Goodman HM (Eds.), Handbook of Physiology; Section 7: The Endocrine System; Volume IV: Coping with the Environment: Neural and Endocrine Mechanisms. Oxford University Press, New York, pp. 211–234.

Woodruffe R. 2000. Predators and people: using human densities to interpret declines of large carnivores. Animal Conservation. 3:165-173.

Woods JG, Paetkau D, Lewis D, McLellan BN, Proctor M, Strobeck C. 1999. Genetic tagging free ranging black and brown bears. Wildlife Society Bulletin. 27:616–627.

Wright BH, Corten JM, El-Nahas AM, Wood RAM, Pockly AG. 2000. Elevated levels of circulating heat shock protein 70 (hsp70) in peripheral and renal vascular disease. Hearts and Vessels. 15(1):18-22.

Appendix 1

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Cc: "'Enviroinfo [NCR]'" <Enviroinfo@ec.gc.ca>
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Subject: FW: FW: ON-COPYRIGHT- NATURE-SEPT 180-LM/01 Forward: Polar Bear Populations Map
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Appendix 2.1 Serum Cortisol Radioimmunoassay (RIA)

Quantification of serum cortisol in grizzly and polar bear samples was achieved be using a radioimmunoassay (#07-221102 MP Biomedicals, Irvine, California, USA). This RIA uses ^{125} I cortisol to measure the prevalence of cortisol in serum samples. The quantification of cortisol in the samples comes from the competition between the ^{125} I cortisol and nonradioactive cortisol to bind the antibody. As more nonradioactive cortisol is added, the less radioactive cortisol remains bound to the antibody, until equilibrium between the bound and unbound antigens occur. In this RIA kit, the anti-cortisol antibody is covalently bound to the provided polypropylene tubes. Thus, the bound antigen is also bound to the tube. Samples were stored at -80°C; the assay was performed at room temperature. Cortisol standards (0, 1, 3, 10, 30, 100 µg/ml) and samples were added (25µl) to the tubes. One millilitre of ^{125} I cortisol was added to each tube and mixed by vortexing for 5 seconds. The tubes were covered and incubated in a 37°C water bath for 45 minutes. The contents were decanted and the tubes were inverted and allowed to dry on filter paper for 30 minutes. The tubes were counted in a gamma counter for 60 seconds each giving a result in counts per minute (cpm). The mean of each standard was used to determine the percent bound (B/B0%), by dividing the cpm of the sample by the cpm of the 0 standard. Plotting B/B0% against concentration a standard curve was constructed; by using the equation of the curve's logarithmic trendline, it was possible to interpolate the concentration of the samples.

Appendix 2.2 Hsp60 enzyme-linked immunoadsorbant assay (ELISA)

EKS-600

The ELISA is of a quantitative sandwich type, in which the provided immunoassay plate is precoated with a mouse monoclonal antibody specific for Hsp60. This antibody shows no reactivity with other members of the Hsp60 family such as Hsp65 or GroEL. Hsp60 is bound by the immobilized antigen and detected by an Hsp60 specific, goat polyclonal antibody. Subsequently, the goat polyclonal antibody is bound by a horseradish peroxidase (HRP) conjugated anti-goat IgG antibody. A development solution of tetramethylbenzidine (TMB) produces a blue colour proportional to the amount of bound Hsp60. The reaction is stopped by the addition of an acid stop solution, which changes the color to yellow. The absorbance is read at 450 nm and a standard curve constructed from samples of known concentration. Unknown sample concentrations are interpolated from the standard curve.

One hundred microlitres of prepared Hsp60 standards (0.195-50 ng/ml), serum and/or buffy coat samples were added to the immunoassay plate. The plate was covered and incubated at room temperature, with gentle shaking for 1 hour. The liquid was decanted from the wells and the wells washed 6 times with Wash Buffer provided in the kit. One hundred microlitres of goat polyclonal Anti-Hsp60 antibody (diluted 500x) was added to the wells and incubated for 1 hour. The liquid again was removed and the wells washed 6 times prior to the addition of 100µl of HRP conjugated anti-goat IgG antibody (diluted 500x), this was followed by a 30 minute incubation. The wells were washed as previously described and 100µl TMB substrate was added and incubated for 15 minutes at which point Acid Stop Solution 2 was added to the wells. Absorbance was read at

450nm (VersaMax, Molecular Devices). The absorbance of the blank (0 ng/ml) was subtracted from all other readings to account for absorbance of the reagents. The sample concentrations were interpolated from a standard curve and expressed as amount of Hsp60 in ng/ml.

Appendix 2.3 Hsp70 enzyme-linked immunoadsorbant assay (ELISA)

EKS-700

Similarly, the Hsp70 assay kit employs a sandwich ELISA. The provided immunoassay plate is precoated with a mouse monoclonal antibody specific to inducible to both recombinant and native Hsp72, and reacts with a number of mammalian species. The antibody shows no reactivity with Hsp73, GRP78, *E. coli* DnaK, *M. tuberculosis* Hsp71 or Hsp60. Hsp72 is captured by the antibody and detected with Hsp72 specific, biotinylated rabbit polyclonal antibody. A secondary antibody, avidin/HRP conjugate binds the biotinylated antibody and colour development is achieved by TMB substrate, yielding a blue colour proportional to the amount of bound Hsp72. Acid stop solution halts the colour development and converts the colour to yellow. Absorbance is read at 450nm and unknown sample concentrations were interpolated from a standard curve.

Prepared Hsp72 standards (0.195-50 ng/ml) and serum/buffy coat samples (100µl) were added to the wells of the immunoassay plate, which was covered and incubated at room temperature for 2 hours with gentle rocking. The liquid was decanted and the wells washed 6 times with the provided Wash Buffer. One hundred microlitres of Anti-Hsp70 Biotin antibody (diluted 500x) were added and incubated at room temperature for 1 hour. The wells were emptied and washed 6 times prior to the addition of 100µl of Avidin-HRP conjugate (diluted 500x). Following a 1 hour incubation, the

liquid was removed and wells were washed 6 times, TMB substrate was added and incubated for 10 minutes. Acid Stop Solution was added to all the wells and the absorbance read at 450nm (VersaMax, Molecular Devices). The absorbance of the assay blank (0 ng/ml) was subtracted to negate the background absorbance of the reagents from the results. A standard curve was constructed from which sample concentrations were interpolated and expressed and amount Hsp70 in ng/ml.

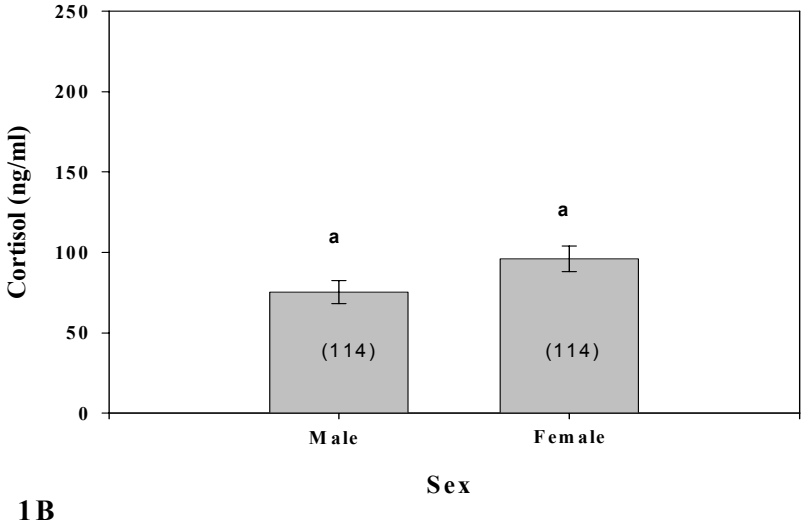
Appendix 3 Polar Bear Biological Variable Analysis

Effects of sex on polar bear stress indicators

Polar bears were separated by sex to determine if any difference in serum based stress indicators existed between male and female bears. Statistical comparison between males and females, with body length as a covariate, showed no significant difference in serum cortisol (Fig. 1A), hsp60 (Fig. 1B) or hsp70 levels (Fig. 1C) in polar bears.

Figure 1. Effects of sex on polar bear serum stress indicators. Serum from male and female polar bears was compared for cortisol (A), hsp60 (B) and hsp70 (C) levels. Values represent mean ± SEM. Sample size is shown in parentheses; bars with the same letters are not statistically different (ANCOVA with age as a covariate (A); $P<0.05$).

1A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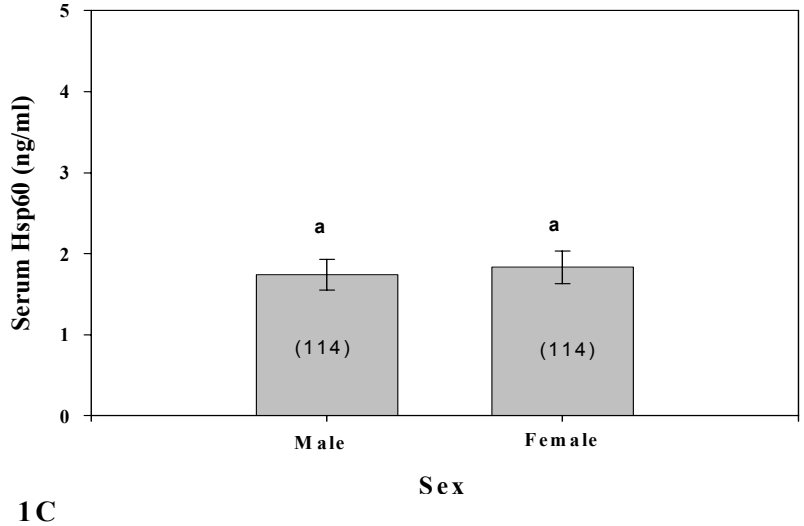
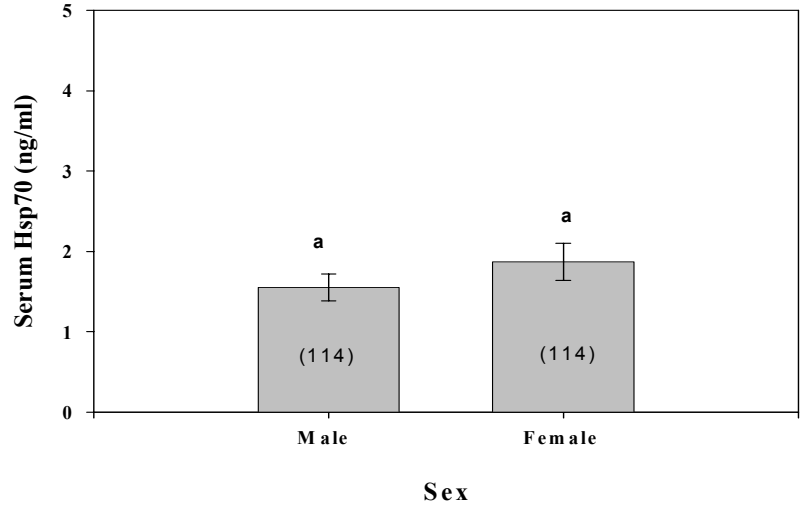
150
100
50
0
Cortisol (ng/ml)
a
a
(114)
(114)
Male
Female
Sex

1B


5
4
3
2
1
0
Serum Hsp60 (ng/ml)
a
a
(114)
(114)
Male
Female
Sex

1C

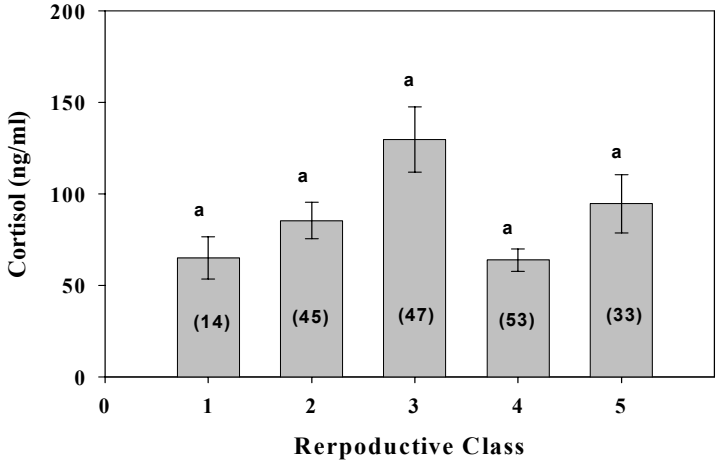

5
4
3
2
1
0
Serum Hsp70 (ng/ml)
a
a
(114)
(114)
Male
Female
Sex

Effect of Polar Bear Reproductive Class on Stress Indicators

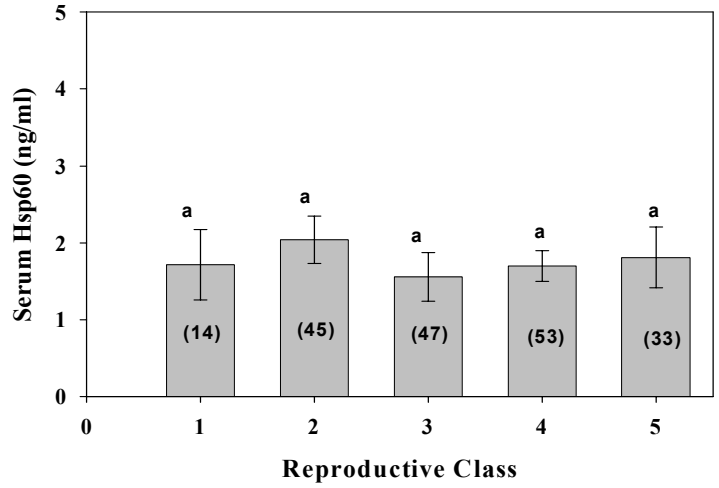
Polar bears were further separated into five different reproductive classes for analysis to determine if the indicators were influenced by age and reproductive status. The reproductive classes were solitary adult female, adult female with dependent offspring, juvenile/subadult female, adult male, and juvenile/subadult male. There was no statistically significant differences noted in serum cortisol (Fig. 2A) hsp60 (Fig. 2B) or hsp70 levels (Fig. 2C) in polar bears when reproductive classes were compared.

Figure 2. Effect of reproductive class on polar bear serum stress indicators. Polar bears of different reproductive class were compared for serum cortisol (A), hsp60 (B) and hsp70 (C) levels. The reproductive class include: 1-solitary adult female, 2-adult female with dependent offspring, 3-juvenile/subadult female, 4-adult male, 5-juvenile/subadult male. Values represent mean ± SEM. Sample size shown in parentheses; bars with the same letters are not statistically different (ANCOVA with age as a covariate (A) and a Sidak test; $P<0.05$).

2A


Cortisol (ng/ml)
200
150
100
50
0
a
a
a
a
a
(14)
(45)
(47)
(53)
(33)
0
1
2
3
4
5
Rerpoproductive Class

2B


Serum Hsp60 (ng/ml)
5
4
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2
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0
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a
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2
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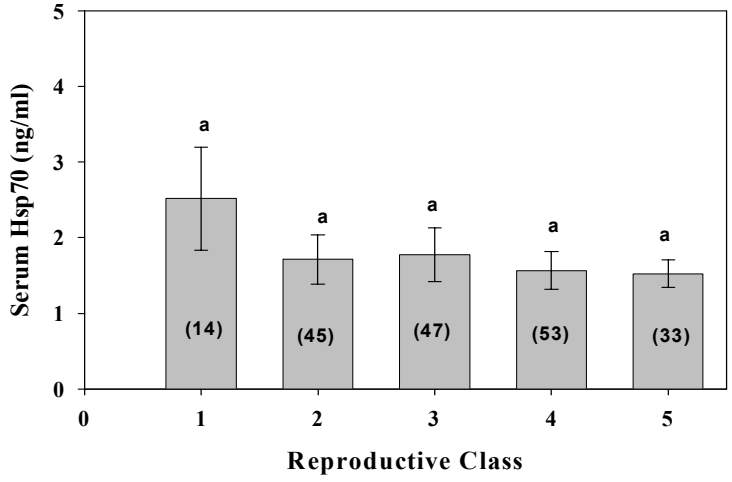
4
5
Reproductive Class

2C


Serum Hsp70 (ng/ml)
5
4
3
2
1
0
a
a
a
a
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(14)
(45)
(47)
(53)
(33)
0
1
2
3
4
5
Reproductive Class

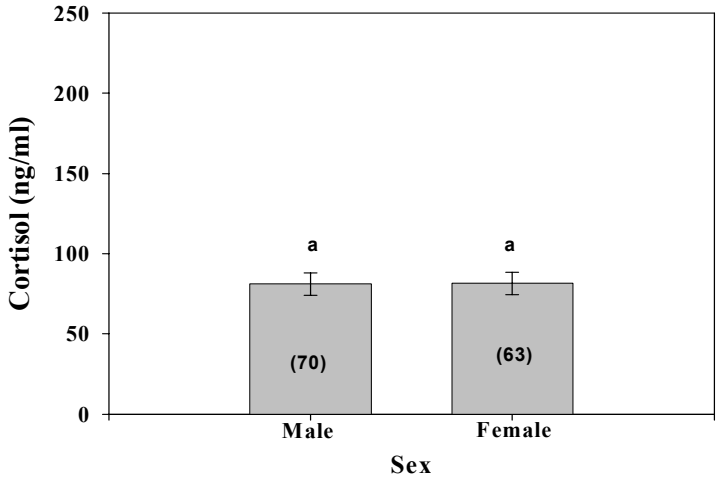
Appendix 4 Grizzly Bear Biological and Environmental Analysis

Effect of Grizzly Bear Sex on Stress Indicators

Grizzly bears were separated by sex to determine if any difference in serum based stress indicators existed between male and female bears. There was no significant sex effect on serum cortisol (Fig. 3A), hsp60 (Fig. 3B) or hsp70 levels (Fig. 3C) in grizzly bears. These analyses were carried out using BCI and body mass as covariates for serum hsp60 and hsp70, respectively.

Figure 3. Effect of grizzly bear sex on stress indicators. Serum from male and female grizzly bears were compared for cortisol (A), hsp60 (B) and hsp70 (C) levels. Values represent mean ± SEM. Sample size shown in parentheses; bars with the same letters are not statistically different (ANCOVA; capture method as a fixed factor, no covariates; $P<0.05$).

3A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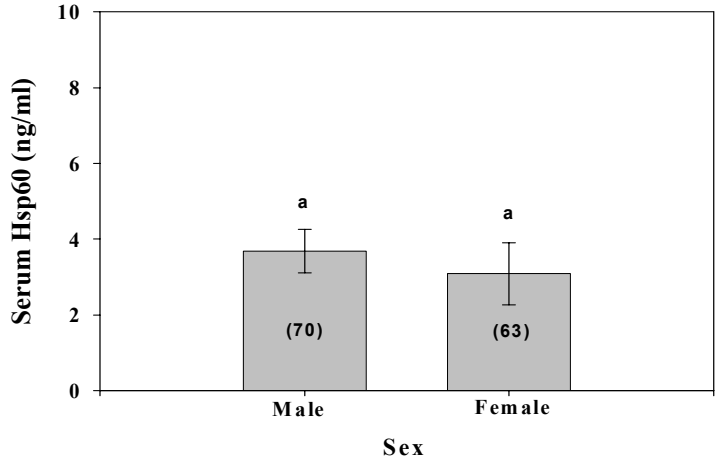
150
100
50
0
Cortisol (ng/ml)
a
a
(70)
(63)
Male
Female
Sex

3B


10
8
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Serum Hsp60 (ng/ml)
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(70)
(63)
Male
Female
Sex

3C


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0
Serum Hsp70 (ng/ml)
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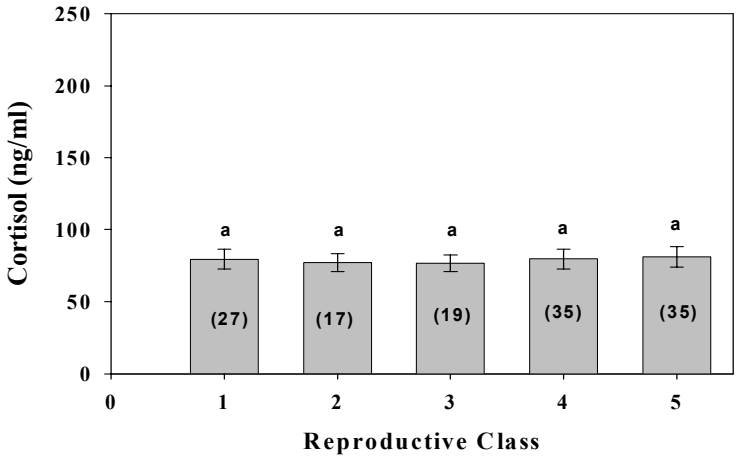
(70)
(63)
Male
Female
Sex

Effect of Grizzly Bear Reproductive Class on Stress Indicators

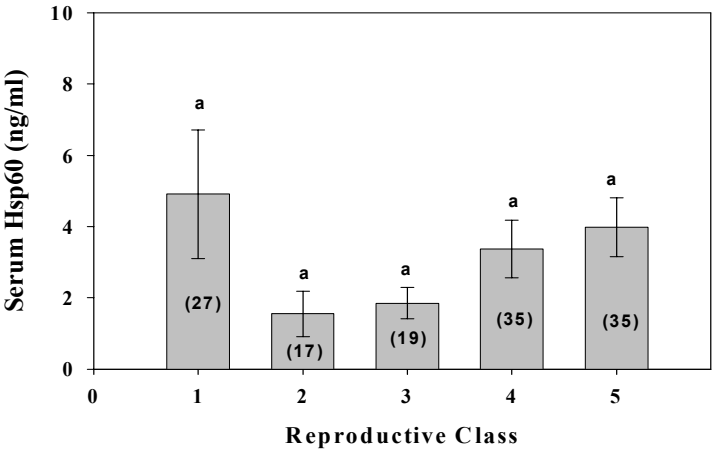
Grizzly bears were organized into different reproductive classes during field sampling, including solitary adult female, adult female with dependent offspring, juvenile/subadult female, adult male and juvenile/subadult male. This categorization was achieved through field observations and known life history data from previous captures. There was no significant differences in serum cortisol (Fig. 4A), hsp60 (Fig. 4B) or hsp70 (Fig. 4C) levels between bears of different reproductive classes.

Figure 4. Effect of grizzly bear reproductive class on stress indicators**.** Grizzly bears of different reproductive classes were analysed for serum cortisol (A), hsp60 (B), and hsp70 (C) levels. Values represent mean ± SEM. Reproductive classes: 1-solitary adult female, 2-adult female with dependent offspring, 3-juvenile/subadult female, 4-adult male, 5-juvenile/subadult male. Sample size shown in parentheses; bars with the same letters are not statistically different (ANCOVA; capture method as a fixed factor, no covariates; $P<0.05$).

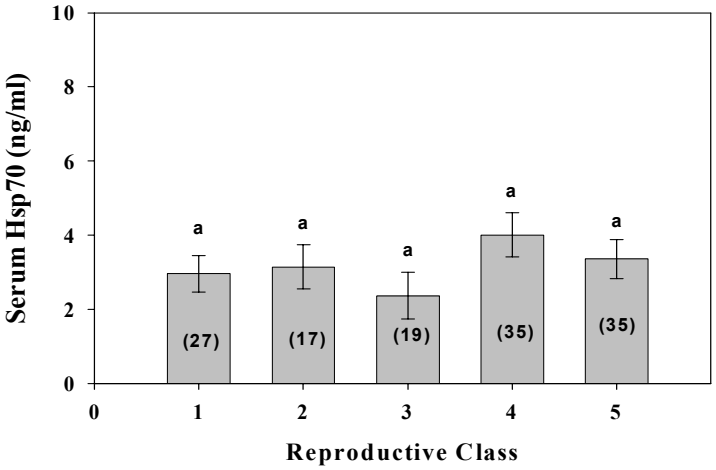
4A


Cortisol (ng/ml)
250
200
150
100
50
0
a
a
a
a
a
(27)
(17)
(19)
(35)
(35)
0
1
2
3
4
5
Reproductive Class

4B


Serum Hsp60 (ng/ml)
10
8
6
4
2
0
a
a
a
a
a
(27)
(17)
(19)
(35)
(35)
0
1
2
3
4
5
Reproductive Class

4C

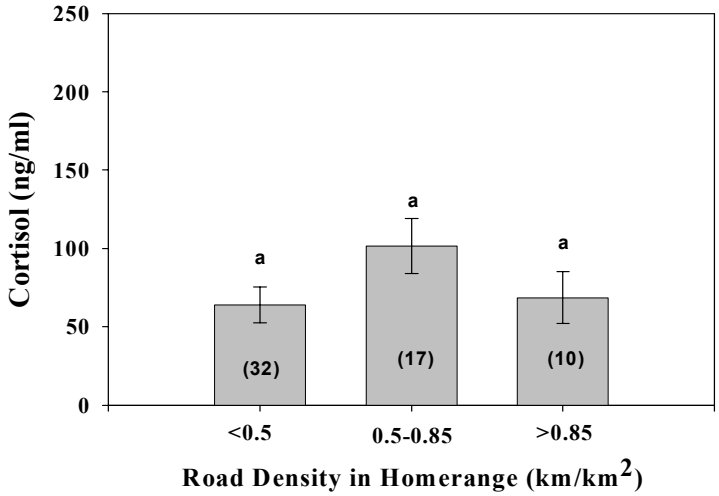

Serum Hsp70 (ng/ml)
10
8
6
4
2
0
a
a
a
a
a
(27)
(17)
(19)
(35)
(35)
0
1
2
3
4
5
Reproductive Class

Effect of Road Development in Grizzly Bear Homerange on Stress Indicators

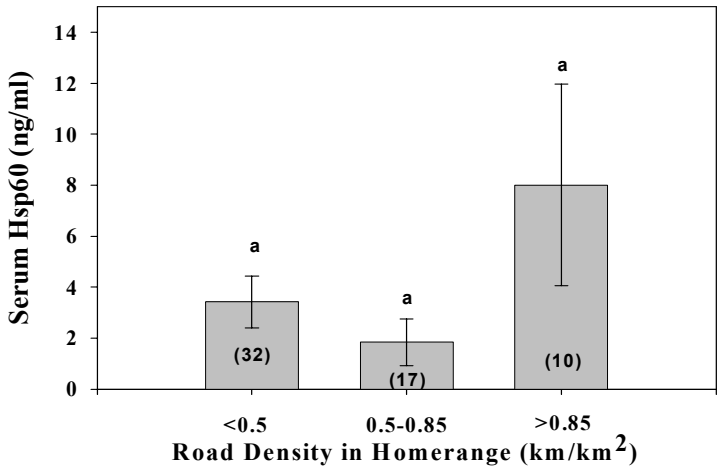
Road density is determined in the bears 95% kernel home range for that particular year. There was no observed statistical significance in serum cortisol (Fig. 5A), hsp60 (Fig. 5B) or hsp70 (Fig. 5C) levels in grizzly bears between different road densities in the bears homerange.

Figure 5. Effect of Road Development in Grizzly Bear Homerange on Stress Indicators. Grizzly bear serum was analyzed on the basis of road development in their homerange for differences in cortisol (A), hsp60 (B), and hsp70 (C). Values represent means ± SEM Sample size shown in parentheses; bars with the same letters are not statistically different (ANCOVA; capture method as a fixed factor, no covariates; $P<0.05$).

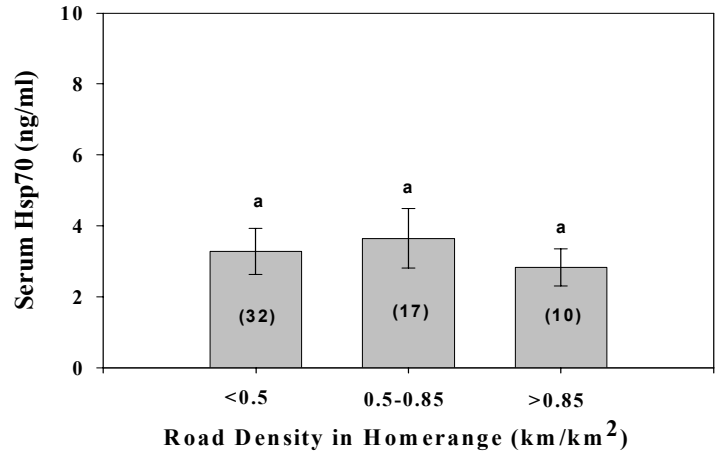
5A


250
200
150
100
50
0
Cortisol (ng/ml)
a
a
a
(32)
(17)
(10)
<0.5
0.5-0.85
>0.85
Road Density in Homerange (km/km2)

5B


14
12
10
8
6
4
2
0
Serum Hsp60 (ng/ml)
a
a
a
(32)
(17)
(10)
<0.5
0.5-0.85
>0.85
Road Density in Homerange (km/km2)

5C


10
8
6
4
2
0
Serum Hsp70 (ng/ml)
a
a
a
(32)
(17)
(10)
<0.5
0.5-0.85
>0.85
Road Density in Homerange (km/km2)

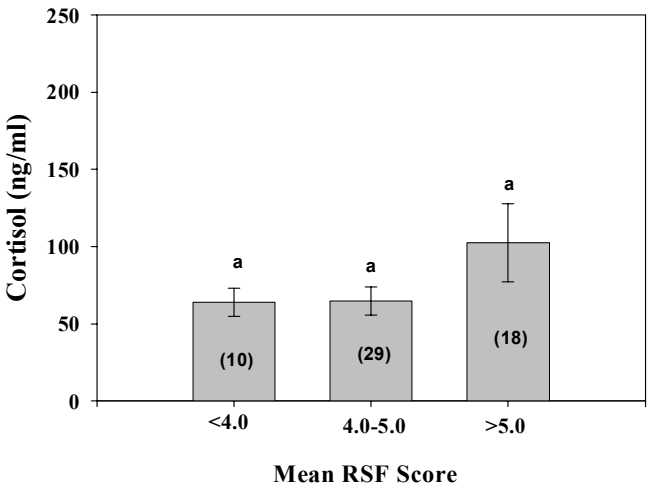
Effect of Resource Selection Function on Grizzly Bear Stress Indicators

Mean resource selection function (RSF) score is a measure of the habitat attributes available to the bear in its 95% kernel home range for that particular year. The home range was broken down into pixels (30 m x 30 m) and each pixel was associated with an RSF score (Boyce et al. 2002).

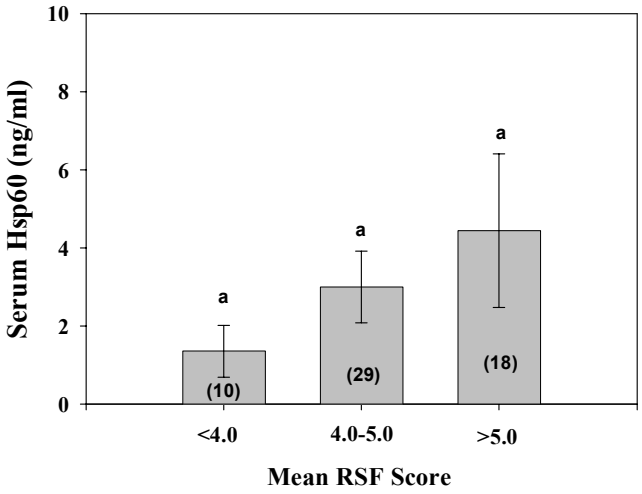
There were no significant differences in serum cortisol (Fig. 6A), hsp60 (Fig. 6B) or hsp70 (Fig. 6C) levels when grizzly bears were compared on the basis of homerange mean RSF scores.

Figure 6. Effect of resource selection function on grizzly bear stress indicators. The mean RSF score of the homerange for a particular bear was used to sort the sampled bears. Grizzly bear serum was analyzed for serum levels of cortisol (A), hsp60 (B) and hsp70 (C) levels. Values represent means ± SEM. Sample size shown in parentheses; bars with the same letters are not statistically different (ANCOVA; capture method as a fixed factor, no covariates; $P<0.05$).

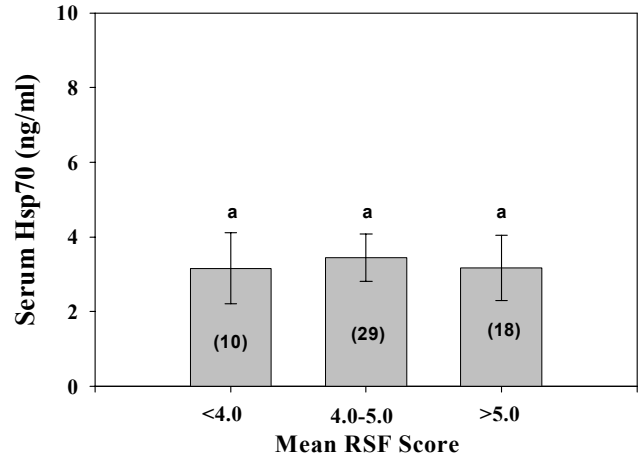
6A


250
200
150
100
50
0
Cortisol (ng/ml)
a
a
a
(10)
(29)
(18)
<4.0
4.0-5.0
>5.0
Mean RSF Score

6B


10
8
6
4
2
0
Serum Hsp60 (ng/ml)
a
a
a
(10)
(29)
(18)
<4.0
4.0-5.0
>5.0
Mean RSF Score

6C

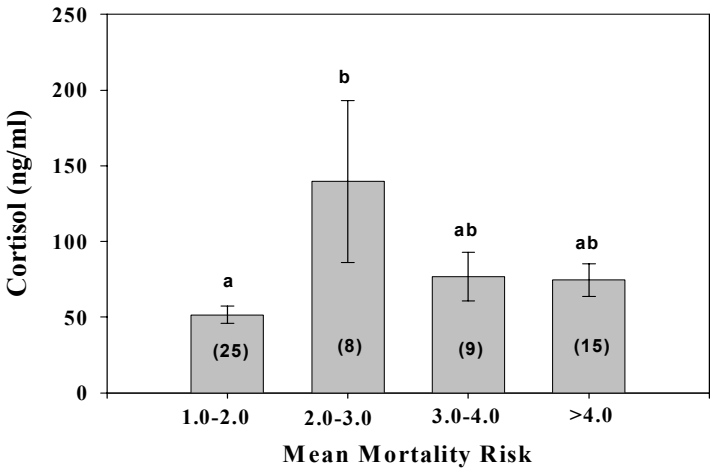

10
8
6
4
2
0
Serum Hsp70 (ng/ml)
a
a
a
(10)
(29)
(18)
<4.0
4.0-5.0
>5.0
Mean RSF Score

Effect of Mortality Risk on Grizzly Bear Stress Indicators

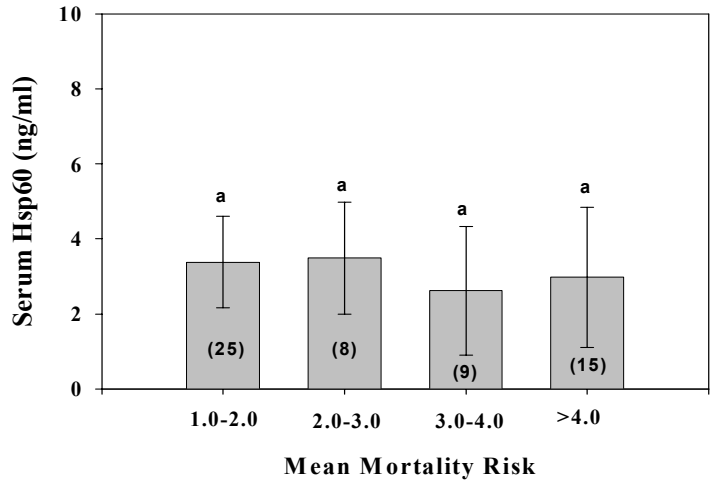
Mean mortality risk is an index determined by a number of factors in the habitat and is outlined by Nielsen et al. (2004). Briefly, it takes into account aspects of the bear's home range and the likelihood of its mortality based on previously studied attributes of documented grizzly bear mortalities. Grizzly bears in a homerange with a mortality risk of 2.0-3.0 had a significantly higher ($p=0.012$) level cortisol compared to the other groups (Fig. 7A). Serum hsp60 levels (Fig. 7B) and serum hsp70 levels (Fig. 7C) showed no significant differences based on mean mortality risk.

Figure 7. Effect of mortality risk on grizzly bear stress indicators. Serum level of cortisol (A), hsp60 (B) and hsp70 (C) were analyzed on the basis of the mean mortality risk of each grizzly bears homerange. Values represent means ± SEM. Columns represent means ± SEM. Sample size shown in parentheses; bars with the same letters are not statistically different (ANCOVA; capture method as a fixed factor, no covariates; $P<0.05$).

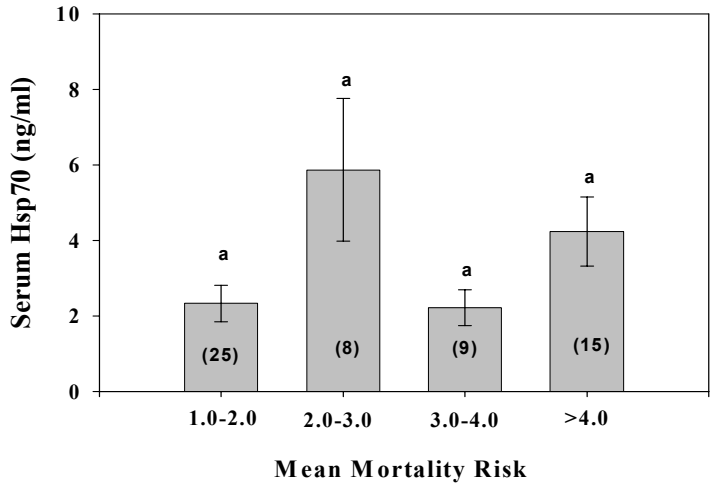
7A


250
200
150
100
50
0
Cortisol (ng/ml)
a
b
ab
ab
(25)
(8)
(9)
(15)
1.0-2.0
2.0-3.0
3.0-4.0
>4.0
Mean Mortality Risk

7B


10
8
6
4
2
0
Serum Hsp60 (ng/ml)
a
a
a
a
(25)
(8)
(9)
(15)
1.0-2.0
2.0-3.0
3.0-4.0
>4.0
Mean Mortality Risk

7C

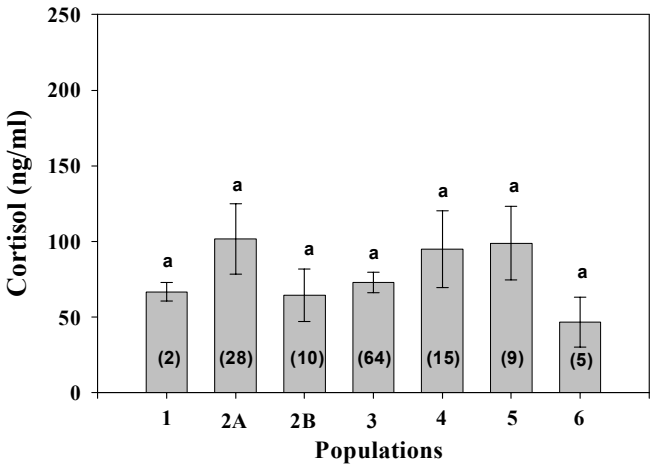

10
8
6
4
2
0
Serum Hsp70 (ng/ml)
a
a
a
a
(25)
(8)
(9)
(15)
1.0-2.0
2.0-3.0
3.0-4.0
>4.0
Mean Mortality Risk

Population Based Differences in Grizzly Bear Stress Indicators

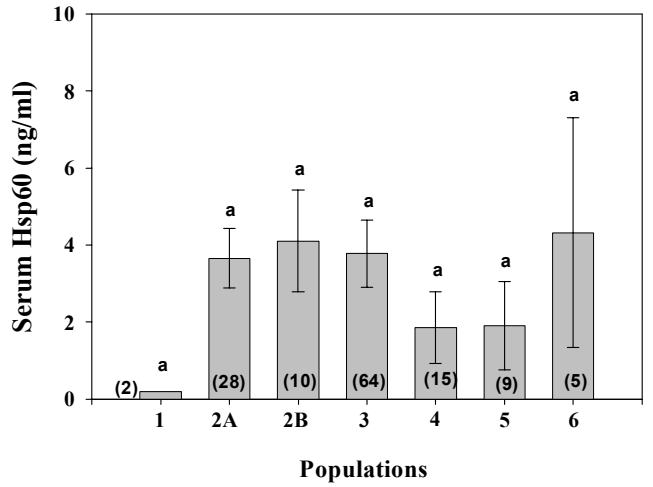
Populations were determined by DNA recapture data and GPS/radiotelemetry collar data collected on home ranges. The distribution of the different populations is shown in Fig. 4. For several of the designated populations, the sample sizes were small leading to large variance in the measured variables. Serum cortisol (Fig. 8A) and hsp60 (Fig. 8B) levels were not significantly different between populations. Serum hsp70 (Fig. 8C) levels showed wide variability across populations and the only significant difference observed was between population 2A (North of Hinton, AB) and 5 (south of Banff National Park)($p=0.045$).

Figure 8. Population based differences in grizzly bear stress indicators. Serum levels of cortisol (A), hsp60 (B) and hsp70 (C) were analyzed to determine population based differences in grizzly bears. Values represent means ± SEM. Population 1 is the northernmost population; population 2A includes areas is north of Hinton, AB; population 2B is east of Hinton, AB; population 3 lies within Jasper National Park; population is north of Banff National Park; population 5 borders Banff National Park to the south; population 6 is the southernmost population living south of Calgary, AB. Sample size shown in parentheses; bars with the same letters are not statistically different (ANCOVA; capture method as a fixed factor, no covariates; $P<0.05$).

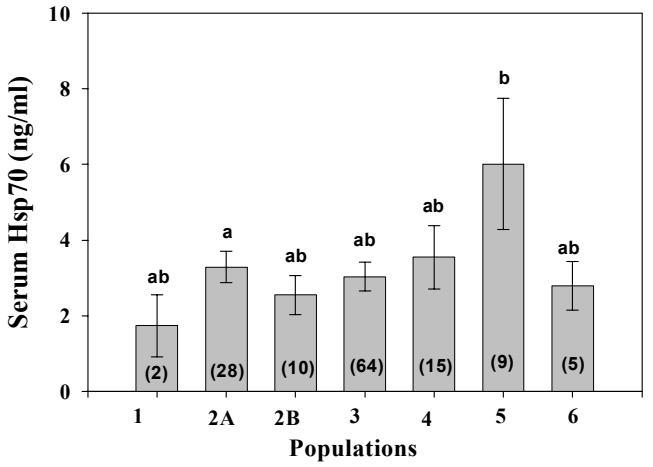
8A


Cortisol (ng/ml)
250
200
150
100
50
0
a
a
a
a
a
a
(2)
(28)
(10)
(64)
(15)
(9)
(5)
1
2A
2B
3
4
5
6
Populations

8B


Serum Hsp60 (ng/ml)
10
8
6
4
2
0
a
a
a
a
a
a
(2)
(28)
(10)
(64)
(15)
(9)
(5)
1
2A
2B
3
4
5
6
Populations

8C


Serum Hsp70 (ng/ml)
10
8
6
4
2
0
ab
a
ab
ab
ab
b
ab
(2)
(28)
(10)
(64)
(15)
(9)
(5)
1
2A
2B
3
4
5
6
Populations