Ringed Seal (*Phoca hispida*) Blubber Cortisol Concentration as an Indication of Chronic Stress

By

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Abstract

Ringed seals (*Phoca hispida*) in the Canadian Arctic are subject to a variety of environmental and anthropogenic stressors that stand to potentially compromise population health and survival. Typically, animals exposed to chronic stressors initiate a stress response resulting in cortisol production, which results in physiological and behavioural changes designed to maintain homeostasis under the influence of the stressor. Chronic stress can affect reproduction and survival, and effects on individuals are often manifested at the population level. In a variety of marine mammal species, cortisol concentration in blubber has been used as an indicator of chronic stress. Cortisol extraction techniques were developed for Ringed seal blubber and fur samples. Blubber cortisol was found to be a reliable indicator of the condition factor ratio of blubber depth to core diameter. Blubber cortisol concentration in individual Ringed seals and relationship with seal condition was shown to alter depending on season and age class. The findings of this study are an important first step in developing an understanding of how this ice obligate species has and may respond to environmental stressors and will assist with developing conservation strategies.

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Dedication

To my younger brothers and sister: Matthew, Collin, Isaac and Harmony- Siblings by birth, friends by choice. All of you are brilliant individuals who can accomplish anything you set out to.

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Chapter 1

1.1 Introduction

Natural systems are changing worldwide at an unprecedented rate (IPCC 2013). Nowhere are the challenges associated with these changes, more prevalent than at the poles. Anthropogenic influence has altered the arctic landscape primarily in the form of climate change. Dramatic ecosystem alteration has been observed in the form of increasing surface temperatures (Rayner et al. 2003), decreasing sea ice cover and extent (Gagnon and Gough 2005, Maslanik et al. 2007, Perovich 2011, Galley et al. 2012, Heide-Jorgensen et al. 2013), longer open water seasons (Maslanik et al. 2007), decreasing ocean salinity due to melting permafrost and sea ice (Rabe et al. 2011, Morison et al. 2012), and dramatically rising sea levels (Arendt et al. 2002, Morison et al. 2012). The marine habitat modification that results from these influences facilitates further ecosystem disturbance by creating opportunities for increased shipping traffic (Huntington 2009), oil and mineral exploration (Alter et al. 2010), and direct human interactions with arctic wildlife (Hovelsrud et al. 2008, Moore and Huntington 2008).

There is an undeniable link between the environment and survivability of arctic marine mammal inhabitants (Laidre et al. 2008, Laidre et al. 2015) but we are yet to understand the synergistic or cumulative effects of all potential stressors on them (Moore and Huntington 2008, Eraud et al. 2009). Many studies have demonstrated correlations between changing environmental variables and the indications of health for both populations and individuals (Burek et al. 2008, Evans et al. 2010). Whether or not an individual is 'healthy' can be determined by investigating any deviation from what is biologically normal or baseline (Burek et al. 2008) and whether they are able to adapt, if there is a change, without compromise to survival and fitness (Sterling 2012). These deviations include changes to condition, immunity, reproduction and physiology (Burek et al. 2008, Evans et al. 2010)

1.2 Determining Health

1.2.1 Condition

In biological studies the physical condition of an individual is one of the most common means of determining health status. It is a potentially non-lethal, minimally intrusive measure and many indices have already been established in the literature. Indices have been developed for various species using morphometric measurements (Anderson et al. 1972, Ryg et al. 1988, Bolger and Connolly 1989, Pitt et al. 2006) specific to the organism being studied. For arctic marine mammals measurements for length, girth, blubber thickness and mass are generally used to establish a value from which health of the individual can be inferred (Ryg et al. 1988, Castellini et al. 2009, George et al. 2015).

Due to cold temperatures in the Canadian Arctic a thick blubber layer with high lipid content is crucial for maintaining internal temperatures (Worthy and Edwards 1990) and so a thicker blubber layer can be an indicator of the animal's health (Peig and Green 2009). Blubber thickness has been determined via biopsy dart in bottlenose dolphins (*Tursiops truncatus*) (Van Dolah et al. 2015), ultrasound for stellar sea lions (*Eumetopis jubatus*) and harbour seals (*Phoca vitulina*) (Mellish et al. 2004) or post mortem measurements from community harvested animals (Ryg et al. 1990, Harwood et al. 2000, Harwood et al. 2014). Lipid content can also be an important measure of condition as has been demonstrated in Polar bears (*Ursus maritimus*) (McKinney et al. 2014).

1.2.2 Immunity

Development and maintenance of the immune system is energetically costly; as such, if an individual is forced to elicit an immune response, other physiological processes are hindered thus compromising their health (McKean et al. 2008, Eraud et al. 2009). Activation of the immune system is directly correlated with decreases in body mass and size resulting in higher

casualties due to predation in doves (*Streptopilia decaocto*) (Eraud et al. 2009) as well as Galapagos sea lions (*Zalophus wollebaeki*) (Brock et al. 2013). Brock et al. (2013) determined that there is a trade-off between a growing animal's investment in immunity or resistance to starvation and that in the absence of influences that initiated an immune response, sea lions will grow according to food availability. Immunological studies analyze blood chemistry and infer health status based on changes in antibody and haptoglobin concentrations, increases in white blood cell count and the presence of pathogens or parasites (Harvell et al. 2002, Krafft et al. 2006, Marcogliese and Pietrock 2011, Brock et al. 2013).

A compromised immune system will result in a depletion of an animal's ability to successfully overcome infection or disease (Agusa et al. 2011). Organochlorine and heavy metal exposure have been linked to immunosuppression in ice seals (Lavigne and Schmitz 1990, Kakuschke et al. 2005) and presence of these contaminants can also be related back to low condition and reproductive rates (Nyman et al. 2003). The presence of potentially health compromising pollutants can be assessed by testing an animal's blubber since the tissue is known to be a site of bioaccumulation (Agusa et al. 2011, Welfinger-Smith et al. 2011). Prolonged exposure to some contaminants can also result in hyperactivity of the Hypothalamic – Pituitary – Adrenal cortex axis (HPA axis) causing further complications to health and survival (Engelhardt 1982).

1.2.3 Reproduction

A marine mammals' reproductive potential can be used as an indication of individual and population health. On an individual level, decreased recruitment ability can be measured by determining ovulation rates (Chambellant et al. 2012) and are correlated to low condition (Lockyer 1987, Harwood et al. 2000) and contaminant exposure (Letcher et al. 2010, Dietz et al.

2015). The presence of organochlorine pollution has been shown to cause reproductive disturbances in ringed seals (*Phoca hispida*) (Nyman et al. 2003) as well as sterility (Harding and Harkonen 1999). A population's reproductive success can be determined by examining changes in the population size and range over time through aerial surveys (Frost et al. 2004). Negative impacts on population health can be due to environmental changes such as increased temperatures and ice availability (Tynan and DeMaster 1997, Stirling and Smith 2004, Ferguson et al. 2005, Kovacs et al. 2011) as well as stresses due to prey availability (Miller et al. 2011).

1.2.4 Stress

The physiological effects of chronic stress include negative impacts on all of the above listed indicators of health (Condition, Immunity and Reproduction) (Sapolsky et al. 2000, Romero 2004). The endocrine stress response often correlates to the health of an animal so determining changes in stress hormone concentrations within an animal's blood and tissue can be an effective tool for assessing the present and future well-being of individuals as well as populations (Sheriff et al. 2011).

1.3 The Stress Response

The endocrine stress response in organisms is elicited following exposure to a stressor (internal or external stimulus) that pushes physiological systems away from homeostasis. Hormones released in this context work to return the organism to a normal physiological state (Selye 1950, McEwen and Stellar 1993). An individual who experiences an external stimuli that causes their body to elicit a physiological response in order to maintain homeostasis, has experienced a stressor. In endocrine stress response studies, homeostasis can be interpreted as the baseline stress hormone levels prior to stressful stimulation.

There are two categories of stress hormones that are released upon exposure to a stressor; catecholamines and glucocorticoids. The catecholamines include the hormones epinephrine and norepinephrine which are responsible for the "fight or flight" or the acute response (Cannon 1915). The chronic stress response is characterized by the release of glucocorticoids (Selye 1956); corticosterone being the dominant hormone in birds, reptiles, amphibians and some rodents and cortisol the dominant for teleost fish and most mammals (Romero 2004).

A hormonal cascade is initiated upon detection of a stressor, which subsequently causes the release of norepinephrine, epinephrine and cortisol (or corticosterone) (Figure 1.1). Detection of a stressor by the brain sends a neuronal signal to stimulate the hypothalamus causing the release of corticotropin releasing hormone (CRH), which activates the pituitary gland. The pituitary gland releases adrenocorticotropic hormone (ACTH), which then stimulates the adrenal cortex, causing the release of cortisol into the blood stream. In mammals, cortisol is transported primarily by corticosteroid binding globulins (CBG) to target cells (Hiller-Sturmhofel and Bartke 1998, Romero 2007, Peckett et al. 2011, Lattin and Romero 2015). Once in the circulation, bound and free cortisol are responsible for inhibiting growth and reproduction, modifying the immune system response, modifying behaviour and increasing the blood's glucose concentration (Romero 2004). The system is regulated by a negative feedback mechanism (Hiller-Sturmhofel and Bartke 1998). Once the stressor is alleviated, blood glucocorticoid levels typically decrease back to baseline levels within 30-60 minutes (Romero 2007), the physiological effects, however, persist (Romero 2007). If the stressor does not cease, the physiological strain on the individual will cause wear on the body thus affecting their ability to overcome stressors in the future (McEwen and Stellar 1993).

The goal of regulation or a return to baseline hormone levels is not to maintain consistency of the internal milieu rather to be able to adjust the internal environment to best facilitate survival and reproduction. This is the premise of allostasis in which the organism constantly re-evaluates the needs and allocation for resources available and makes accommodations to survive during exposure to the initial stressor (Sterling and Eyer 1989).

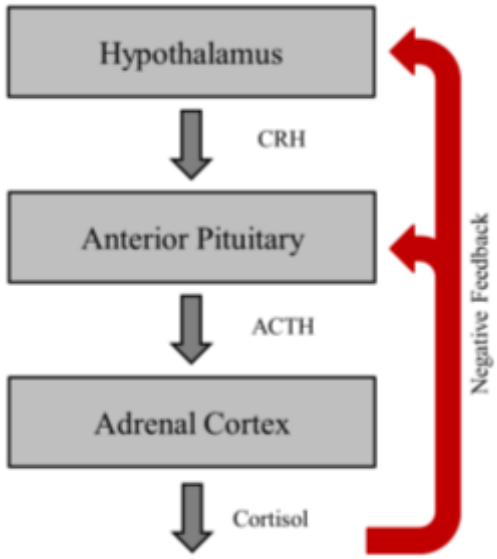


Figure 1.1- Representation of Stress Response (Hypothalamic-pituitary-adrenal axis) CRH (Corticotropin Releasing Hormone) and ACTH (Adrenocorticotrophic Hormone).

1.4 Study Species: Arctic Ringed seal (*Phoca hispida*)

Ringed seals serve both major ecological and socioeconomic roles in the Canadian Arctic. Their presence is ingrained in the Inuit's cultural identity (Borre 1991), economics through trade (Smith 1987, Pearce et al. 2010) and subsistence (Borre 1994, Harwood et al. 2000, Kuhnlein et al. 2004). They are considered a sentinel species due to their high trophic level, long life spans, and blubber stores, which bio-accumulate contaminants, metals and steroids (Bossart 2006, Moore 2008). As an ice-obligate species, ringed seal fitness is positively correlated to sea ice. They are, however, presently among the least sensitive to climate-induced

habitat change relative to other arctic mammal species due to their circumpolar distribution, flexible habitat requirements and large population (Laidre et al. 2008).

1.4.1 General Biology, Distribution and Life History

Ringed seals are the smallest of the true seals (Phocidae) (McLaren 1958) with pups measuring as little as 65cm (Smith 1987) and adults growing to be approximately 135cm with a life expectancy of approximately 45 years (McLaren 1958). They are well adapted for life on ice and in water due to their thick blubber layer, the absence of ears, expandable skin between each digit, hind limbs that have evolved for swimming, and retractable sex organs: characteristics that reduce drag and aid in streamlining for efficient swimming (Ridgway 1972, Riedman 1990).

They are generalist predators who will adapt to prey availability (Labansen et al. 2011) but primarily feed on ice associated prey such as gadids, euphausiids, amphipods and mysids (McLaren 1958, Lowry et al. 1980). Ringed seals will gather in areas of high zooplankton activity in order to feed but the older individuals will venture into deeper water where they can take advantage of higher energy prey such as larger polar cod and squid (Born et al. 2004, Young et al. 2010, Chambellant et al. 2013).

The ringed seal's primary predators are polar bear (*Ursus maritimus*) (McLaren 1958, Smith and Stirling 1975, Stirling 2002) and Arctic fox (*Alopex lagopus*) (Smith 1987). Recently, killer whales (*Orcinus orca*) have also become a potential predator in the eastern Canadian Arctic ocean because their range has expanded as a result of climate change (Higdon and Ferguson 2009).

Ringed seals are comprised of 5 subspecies whose overall distribution correlates directly with changing sea ice cover in the northern hemisphere and overlaps with some competing phocid species (McLaren 1958, Smith et al. 1991, Teilmann et al. 1999, Kelly et al. 2010a)

(Figure 1.2). Due to their dependence on sea ice and snow depth for both feeding and breeding, they are especially susceptible to environmental change. In the winter, ringed seals are aggregated towards areas of land-fast and pack ice that also have high quality snow cover (Reeves 1998).

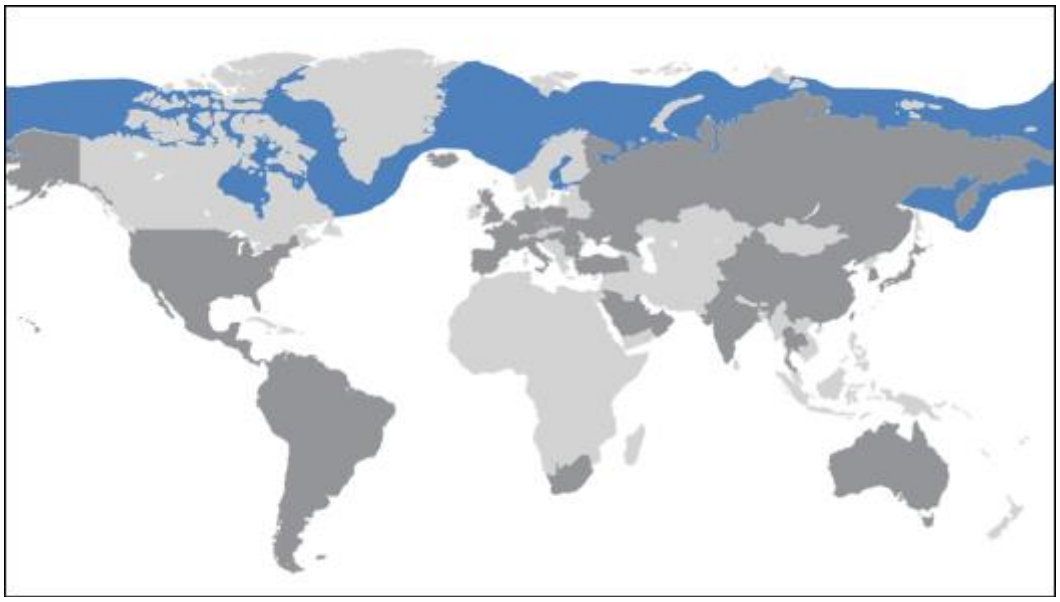


Figure 1.2- Estimated global distribution and range of the ringed seal indicated in blue

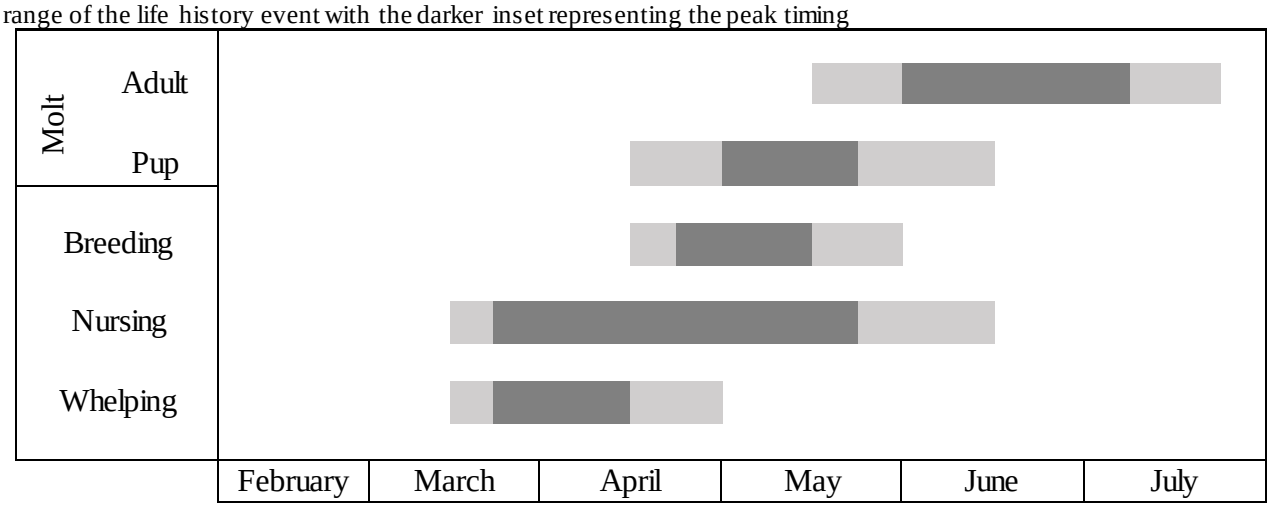
Female ringed seals reach sexual maturity at 5.61 years and will typically give birth to one pup per year (Smith 1987). The female undergoes a 3.5 month delayed implantation so they begin to ovulate while weaning the young from the previous year (McLaren 1958). Active gestation lasts 241 days and the pups are born from mid-March to mid-April each year (Smith 1987) but are suckled for 6 weeks (McLaren 1958) (Table 1.1).

Due partially to their small size, ringed seal pups require a shelter in order to maintain body heat and so sufficient snow depth is required for the creation of a birthing lair (McLaren 1958). Lairs are found where a minimum of 20cm to a maximum of 150cm of snow depth is present with access to a hole in the ice which allows for efficient escape/access to the water below (Smith and Stirling 1975, Kovacs et al. 2011). They serve as shelter from the elements as

well as predators. A lair with a seal in it can be 0-2°C despite the outside temperatures which can be lower than -25°C (Smith et al. 1991).

The pups are born with a temporary fur layer called the lanugo which, when dry serves as a form of insulation. The birthing lair is necessary shelter in order to avoid the risk of hypothermia (Smith et al. 1991). A period of extensive blubber loss from fasting during the energy intensive months of June and early July coincides with the peak annual molt (McLaren 1958). When basking and molting season is over the seals need to increase their dietary intake in order to attain peak body condition (highest blubber content) in time for freeze up (Figure 1.3) (Young and Ferguson 2013). During the freeze up when there is a large ice extent, the ringed seal will stay close to the breeding sites until spring (Martinez-Bakker et al. 2013)

Table 1.1 – Life history summary of Arctic ringed seals modified from Kelly et al. (2010b). Bars indicate the typical range of the life history event with the darker inset representing the peak timing



1.4.2 Potential Stressors for the Ringed Seal

Internal stimuli are the anticipated physiological stressors that the ringed seal will encounter and is evolutionarily prepared for. These include life history events such as breeding, lactation or molting. Thus, cortisol levels will vary naturally by season in relation to these events

(Riviere et al. 1977, Ashwell et al. 1986, Routti et al. 2010) and potentially due to seasonal diet variations (Oki and Atkinson 2004).

External stimuli are the environmental stressors that the individual is forced to adapt to. In the Arctic most of these stressors are directly related to the habitat changes resulting from climate change. For ringed seals, these changes include but are not limited to changes in predator type such as killer whales (Higdon and Ferguson 2009, Higdon et al. 2013), the introduction and spread of new diseases to immunologically naïve populations such as morbillivirus (Duignan et al. 1997) and brucellosis (Forbes et al. 2000), range shifts of other species which result in competition for available prey such as harbour seals in the Hudson Bay (Bajzak et al. 2013) and the decline of sea ice extents or sufficient snow cover, which is necessary for the ringed seal's life cycle (Born et al. 2004, Stirling and Smith 2004, Ferguson et al. 2005, Moore and Huntington 2008). Due to their ice-obligate life history, ringed seals are highly susceptible to the impending changes to the arctic ecosystem and will only survive if they are able to overcome and adapt to changes that they are exposed to.

1.5 Objectives

There is a lack of understanding of what the long term, physiological effects of a changing arctic ecosystem will have on the health of the ringed seal. This thesis aimed to expand that knowledge base in order to facilitate more informed and accurate conservation and management decisions. By analyzing archived samples this thesis addressed the following hypotheses;

1. Blubber acts as an indicator of exposure to long term chronic stress in ringed seals (Chapter 2).
2. Cortisol levels in blubber can be used to determine the condition of ringed seals

(Chapter 3).

In chapter 2, I examine the relationship between the cortisol stored in archived ringed seal fur and blubber in order to determine whether blubber can be used as a proxy to determine chronic stress exposure. I also examine whether different analytical and extraction methods would result in comparable results in the concentration of cortisol measured in blubber. The analyses in chapter 3 examines whether the trends in changing ringed seal condition over time are mirrored by blubber cortisol concentration and determine, to what degree the two are related. Chapter 4 summarizes the implications and potential applications of the findings from this thesis.

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Chapter 2 Validation and Comparison of Extraction and Measurement of Cortisol from the Blubber of Arctic Ringed Seals (*Phoca hispida*)

2.1 Introduction

Logistical barriers such as international boundaries, monetary cost, the expansive range and dangers associated with the north make research in the Arctic more difficult than in other parts of the world (Polyakov et al. 2014). Therefore, the value of an interdisciplinary approach to conservation studies has become increasingly important as this ecosystem changes. The health of marine mammals reflects the health of the ecosystem (Burek et al. 2008) and the survival of an individual is determined by their ability to adapt and their capacity to cope with stresses such as environmental change (Koolhaas et al. 1997). Studying an organism's physiological response to potential stressors and the ultimate effect on the population as a whole is one aspect of an emerging discipline known as conservation physiology (Wikelski and Cooke 2006). Establishing a method to measure stress in an individual aids in the assessment of population health and the development of conservation strategies. Implementing conservation physiology principles to arctic research will aid in understanding the causes of species issues such as low recruitment and a decreased ability to thrive in an ever-changing environment (Sheriff et al. 2011).

Methods to analyze both acute and chronic stress responses in animals have been developed and applied to numerous ecological studies; however, there is a lack of integrated knowledge when it comes to arctic marine mammals specifically. In many vertebrate species, the baseline glucocorticoid (GC) levels vary naturally based on the season or time of day. When exposed to an external stimulus, a change in GC concentration can be detected in the blood within 3-5 minutes (Romero and Reed 2005). Once the stimuli ceases, GC levels typically return to their starting point within 30-60 minutes but the physiological effects can persist (Romero

2007). Plasma GC levels in response to a stressor have been examined in various aquatic (Norris et al. 1999, Tryland et al. 2006, Hogg and Rogers 2009), terrestrial (Christison and Johnson 1972), and avian species (Scheuerlein et al. 2001) but require the capture and restraint of the individual in order to acquire a sample. Saliva cortisol levels also increase rapidly and have been used as an indicator of stress in domestic dogs (*Canis lupus familiaris)* (Bennett and Hayssen 2010). The collection of saliva is considered significantly less intrusive than blood, therefore eliminating the concern of sample collection that will likely result in heightened plasma levels of GCs.

The analysis of GC levels in excrement (feces and urine) is another frequently used method to determine stress levels in animals (Creel et al. 2002, Pride 2005, Stephen and Ledger 2006, Gobush et al. 2014). Collection of these samples is the least intrusive method of stress analysis as the animal does not need to be handled or disturbed. Analysis of excrement hormone levels depend on opportunistic sample collection and as such can place limitations on the nature of questions under investigation (Gobush et al. 2014). The samples are not always collected immediately and are therefore susceptible to contamination and degradation which can compromise the resulting hormone concentration levels. In many wildlife species, especially those that are difficult or impossible to handle, excrement analysis is the only feasible method to assess the endocrine stress response (Hunt et al. 2006).

If a stressor persists, the hypothalamic-pituitary-adrenal axis (HPA axis) will continue to produce GC hormones thus maintaining a heightened concentration in the circulation and potentially resulting in a decreased efficacy of the stress response (Sterling and Eyer 1989). Glucocorticoids in circulation can be sequestered and stored in various tissues reflecting long term circulating concentrations. In avian species, the dominant GC produced during a stress

response is corticosterone. The cells in a growing feather are highly vascularized and accumulate compounds such as circulating hormones in the keratin structure as they grow (Bortolotti and Barlow 1988). A study of the corticosterone concentration levels along the extent of partridge (*Alectoris rufa*) feathers demonstrate that as a stressor is applied to the animal, the GC concentration stored at the point of growth increased (Bortolotti et al. 2008), a mechanism similar to that seen in mammalian fur.

One of the most common tissues used as a measure of chronic stress exposure in mammals is fur or hair since it is not influenced by factors such a circadian rhythms (Macbeth et al. 2010). Glucocorticoids enter the hair shaft at a volume that is proportional to the free moving hormone that is in the blood (Macbeth et al. 2010). The method of entrance into the hair follicle is via passive diffusion from the dermal papilla where it is taken up during the growth phase of the hair follicle. The hormone is incorporated into the hair as it grows and therefore represents a specific time (Bennett and Hayssen 2010, Ashley et al. 2011). As such, fur offers a historical record of the individual's circulating level of GC's as far back as the length of time that the fur was growing (Sheriff et al. 2011).

A novel approach to the analysis of chronic stress in marine mammals has been demonstrated in beluga (*Delphinapterus leucas*), the common dolphin (*Delphinus delphis*) and harbour seals (*Phoca vitulina*) by extracting stored cortisol from the animals blubber (Kellar et al. 2015, Trana et al. 2015, Kershaw and Hall 2016). Blubber is the superficial, lipid rich tissue that is loosely attached between the epidermis and muscle of aquatic mammals. It has evolved parallel between species therefore showing similarities in function and structure across organisms (Koopman et al. 2002). It's primary function is to actively control the passage of heat from the core of the animal to the environment (Parry 1949) and to act as a form of stored energy

that is metabolized as needed (Pond 1992). It is composed of three distinguishable layers that are potentially related to differences in fatty acid composition, stable isotopes present and metabolic activity resulting in lipid mobilization (Strandberg et al. 2008, Bagge et al. 2012). As such, the blubber layer has been used as a tissue to assess general condition (Ryg et al. 1988, Castellini et al. 2009) and dietary composition in marine mammals (Young et al. 2010, Watt and Ferguson 2015).

Blubber accumulates contaminants and hormones by passively diffusing from the capillaries found throughout the lipid (Deslypere et al. 1985, Mead 1986) acting as a sink for molecules that are unrelated to the primary functions of the tissue. As a result of this tendency to bio accumulate coupled with marine mammals low detoxification capacity (Bossart 2006, Brown et al. 2014), blubber has long been used in conservation studies to assess environmental pollution levels including Persistent Organic Pollutants (Savinov et al. 2011, Welfinger-Smith et al. 2011, Gaden et al. 2012) and heavy metal contamination (Kakuschke et al. 2005, Agusa et al. 2011a, Agusa et al. 2011b).

Growth and reproduction rates can be examined by measuring the progesterone levels stored throughout the profile of the tissue (Kellar et al. 2006, Kellar et al. 2009). Cortisol has a similar base structure to progesterone and is also a lipid soluble hormone that can freely diffuse through the blubber layer (Deslypere et al. 1985). Extraction of the hormone from the blubber requires the application of highly lipophilic solvents in order to separate the two. Two techniques of hormone extraction from blubber have been employed in the present study that varied in the strength of non-polar solvents used as well as the time required and complexity of the process.

Two different measurement techniques have been compared and include a classic competitive binding assay alongside multi-detection measurement using liquid chromatography

mass spectrometer/mass spectrometer (LC MS/MS). Radioimmunoassay (RIA) is commonly used in hormone analysis and involves competition for binding on an antibody between the hormone of interest and the same hormone labelled with a radioisotope. Once equilibrium is established in the assay, the percent of the total labelled antigen which is bound is inversely proportional to the concentration of unlabelled antigen which is naturally present in the sample. Liquid chromatography-mass spectrometry (LC MS) is a powerful analytical tool that is increasingly being used to measure small steroids and molecules (Monaghan et al. 2013). It is highly specific and able to measure multiple analytes at the same time. When deciding on an analytical technique, factors such as time, nature of the sample and accessibility of the required equipment must be considered.

Both extraction and analytical techniques offer different benefits dependent on the tissue or analyte being measured. Choosing the appropriate method may impact the calculated concentration of hormone present in the blubber tissue therefore care must be taken to choose a method that ensures the most consistent and reliable results.

Arctic research is costly and labour intensive but multiple agencies and programs have established relationships with Inuit hunters who are on the frontlines and have seen firsthand the changes to the arctic ecosystem. The Department of Fisheries and Oceans Canada, has been collecting and archiving various biological samples for over 3 decades. Within the extensive library of samples available are hundreds of ringed seal (*phoca hispida*) tissue samples that have been collected by Inuit hunters in many northern Canadian communities. Measuring changes in GC levels in wildlife and understanding those changes are important in assessing the well-being of a population and developing effective management and conservation strategies (Sheriff et al. 2011) in the Canadian Arctic. The aim of this study was to determine which extraction and

analytical technique is best suited for the archived samples of fur and blubber and whether the results acquired can be used to further facilitate the understanding of the changes seen and new challenges faced by the ringed seal.

2.2 Materials and Methods

2.2.1 Sample Collection

2.2.1.1 Ulukhaktok, Northwest Territories, Canada

A community hunt, led by the Hunters and Trappers Organization in Ulukhaktok (formerly known as Holman), Northwest Territories, takes place annually during the summer months along the shore of Prince Albert Sound off the Victoria Island coast (Figure 2.1). Seals are harvested non-selectively with no preference given to size, age or sex (Harwood et al. 2012). Blubber thickness was measured at the mid-point along the sternum and girth measurements were taken at both the axillary and hip regions. Samples of complete sculp cross sections obtained from the subsistence hunts that took place in June of 2005-2012 were used for this study. Upon collection, samples were wrapped in aluminum foil and kept in individually labelled whirl-pack bags. These samples were approximately 200g and included the fur, skin and 3 blubber layers up to the outer muscle layer. The samples were stored frozen in coolers at the hunting camp until weather and ice conditions permitted transport to community freezers where samples were then stored at approximately -20°C. Sex was determined by visual analysis in the field. Age was determined in the Ulukhaktok lab by extracting a canine tooth from the lower jaw and counting the dentinal lines in a cross section. Cementum lines were counted, if readable (Smith 1973, Harwood et al. 2012). Upon completion of the hunting season and age analysis, samples were then shipped frozen to the Freshwater Institute in Winnipeg, Manitoba where they were stored at -25°C in archive.

2.2.1.2 Arviat, Nunavut, Canada

Ringed seal blubber, epidermis, lower jaw bones and corresponding morphological measurements for each individual sampled, were collected by members of the Arviat Hunters and Trappers Association during the fall subsistence hunts in 2007-2012. The hunt takes place annually along the shores of this western Hudson Bay community (Figure 2.1). The harvest coincides with the winter freeze up and typically takes place from the end of October through the beginning of November. Seals were non-selectively harvested and the demographics of the individuals in the hunting grounds show equal distribution at this point in the season (Smith 1973). Tissue samples and blubber thickness measurements were consistently collected from the mid-ventral region of the animal and girth measurements were obtained from the axillary region. Blubber samples that ranged in mass from 100-500g were collected and stored in individually labelled plastic bags. Fur was collected by cutting off an approximately 3cm wide by 6cm long section of the mid-ventral edge of the sculp which was also stored in individually labelled plastic bags. Sex was determined by visual analysis in the field and verified genetically in the lab. Until the end of that seasons hunt, samples were stored in community freezers at -20^{o}C and then shipped frozen to the Freshwater Institute in Winnipeg, Manitoba where they were stored at -25^{o}C in archive. In order to determine the age of the individuals, canine teeth were extracted after softening the periodontal membrane via submersion and soaking of the lower jaws in a heated water bath for 2-4 hours. The teeth were then sent for age determination to Matson's Laboratory in Montana, Utah. Seal ages were determined by identifying and counting the growth layer groups (GLGs) within the cementum layers on mounted, longitudinally sliced, sections of teeth from each individual (Stewart et al. 1996).

Figure 2.1- Ringed seal samples were collected from the subsistence hunts in 1- Ulukhaktok (Holman), Northwest Territories and 2- Arviat, Nunavut.

2.2.2 Sample Preparation and Analysis

Archived blubber and fur samples were chosen at random and scored for quality of sample as either high or low (Figures 2.2). Archived fur samples were selected based on whether they had corresponding blubber samples of high quality. Blubber tissue samples that appeared yellowed, dried or rotting were assessed as low quality and were not used for this study. A pilot study showed that low quality samples had low or undetectable steroid concentrations, suggesting that sample quality influenced steroid measurement as previously described for Beluga whale blubber samples (Trana et al. (2015). Samples deemed as high quality appeared pink in colour and lacked any signs of degradation. Blubber samples were obtained using a

scalpel to cut an even, cross sectional piece of tissue (Figure 2.2). Subsamples were taken in 1 gram duplicates (n=28) and transferred to a 15mL plastic vial for the purposes of comparison to fur. For development of extraction and analysis methods, 1 gram quadruplicate samples (n=45) were obtained, transferred to 15mL plastic vials and separated into 4 treatment groups (Table 2.1). Samples were freeze dried for approximately 48 hours at -50 °C, sealed and stored at -25°C prior to analysis.

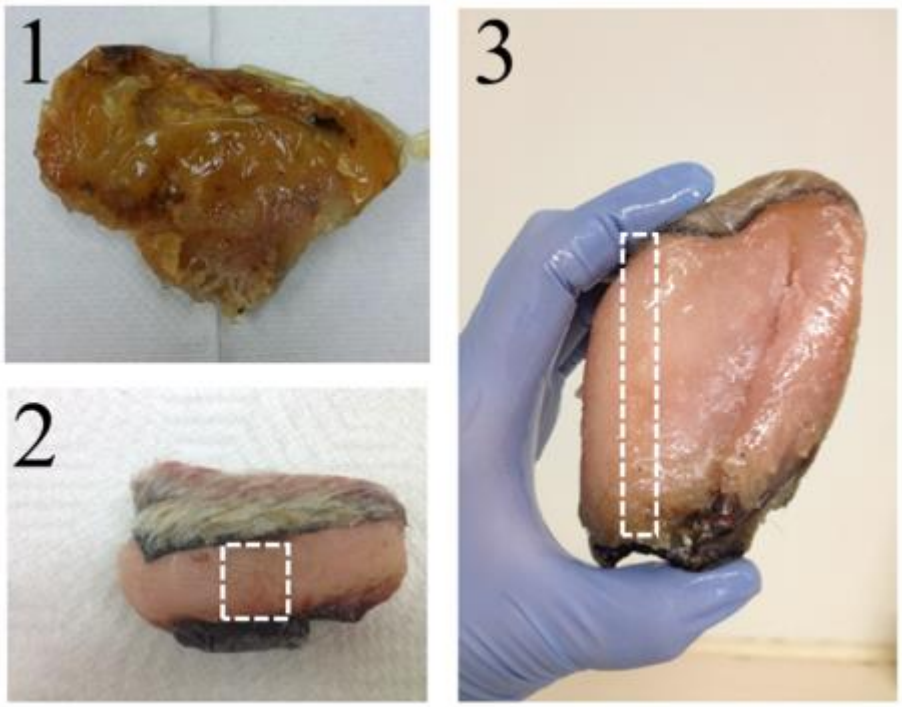

Figure 2.2- Tissue subsamples of varying qualities from field collection for - Ulukhaktok (Holman), Northwest Territories (2) and Arviat, Nunavut (1 and 3). Yellowed, dry or rotting samples were considered poor quality (1) whereas high quality samples appeared pink (2 and 3). Example of a blubber subsample that includes all 3 layers is outlined in white

Figure 2.3- Rinsed and dried fur samples of varying quality. Samples that contained residual lipid residue post rinsing were considered low quality (A) whereas high quality samples appeared dry and free of contamination (B, C).

Table 1.1- Summary of extraction and analytical methods. For each treatment n=45. LC/MS/MS = liquid chromatography/mass spectrometry/mass spectrometry; RIA = radioimmunoassay.

Method #	**1**	**2**	**3**	**4**
Analysis	LC/MS/MS		RIA	
Extraction	Methanol	Acetonitrile	Methanol	Acetonitrile

2.2.2.1 Blubber Cortisol Extraction

Method 1; Methanol extracted, LC/MS/MS analyzed

Each sample was spiked with 200pg/μL of deuterium (d_4) labelled cortisol, immersed in 4mL of methanol, capped and placed in an ultrasonic water bath set (Fisher Scientific Ultrasonic Bath 9.5L) to 45khz for 60 minutes at 25 °C. The tubes were then removed from the bath and left at -10 °C for ~24 hours to allow for full saturation of the tissue by the solvent. A glass rod was then used to homogenize the lipid phase and the remaining tissue was compacted to the bottom of the sample vial. While at room temperature, the aqueous phase was then transferred to a new vial (Vial B) and the remaining connective tissue, as well as the rod, was double rinsed with 1mL of methanol which was then also transferred to the second vial labelled for that specific sample. Vial B was then centrifuged at room temperature for 1 min at 7000rpm (Thermo Scientific

Sorvall ST16) in order to separate the lipid layer from the methanol. Cortisol is soluble in methanol so should separate from the lipid and be present in that layer. The vial was then transferred to the -80 °C freezer for 5 minutes in order to solidify the lipid layer thus making it easier to draw the top methanol layer off. The top layer was removed with a glass pipette and transferred to a third vial (Vial C) specific to each sample. The second was double rinsed with 1 mL of methanol with each rinse being added to Vial C. Vial C contained only methanol, a theoretically known amount of d_4-labelled cortisol and an unknown amount of native cortisol extracted from the blubber tissue. Vial C was evaporated to dryness on a nitrogen evaporator at 40 °C for ~40minutes. Once dry, 200µL of methanol was added to the vial which was then vortexed to reconstitute the sample. The 200µL was then transferred to a 1.5mL microvial suitable for use on an LC/MS/MS (Liquid Chromatography coupled with mass spectrometry) instrument. The microvials containing sequestered hormone, labelled cortisol and 200µL of methanol were then run on the LC/MS/MS in 2µL injections with 16 minute intervals. The intensity of the d_4 labelled cortisol was used to calculate the concentration of native cortisol from each sample and was determined using instrument specific software (Analyst 12 Analytical Technologies and Applied Biosystems: Concord, Ontario).

Method 2; Acetonitrile extracted, LC/MS/MS analyzed

The technique for steroid extraction was modified from Kellar et al. (2006). Each sample vial containing 1g of freeze dried blubber was spiked with 200pg/µL of d_4 labelled cortisol and 2mL of ethyl alcohol was added. Sample vials were then capped and left to soak at 25 °C in an ultrasonic bath set to 45 kHz for 24 hours. A glass rod was then used to homogenize the lipid phase and the remaining tissue was compacted to the bottom of the sample vial. At room temperature the aqueous phase was then transferred to a new vial and the remaining connective

tissue was double rinsed with 1mL of ethyl alcohol which was then also transferred to the second vial. The ethyl alcohol from each sample was then evaporated off in a nitrogen evaporator at 40°C. Once dry, 1mL of a 4:1 Ethyl alcohol:acetone mixture was added to each of the vials which were then vortexed again and evaporated down to dryness on the nitrogen evaporator. This step was repeated with 1mL of Ethyl Ether. The sample vials were then removed from the nitrogen evaporator and 2mL of hexane and 1mL of acetonitrile was added to each sample and then vortexed for 5 minutes. While at room temperature, sample vials were then capped and centrifuged at 3000rpm for 20 minutes. Following centrifugation two distinct phases were visible within the vial. The phase of greater volume, which contained a mixture of hexane and lipid, was considered waste and removed from the vial while ensuring not to disturb the acetonitrile phase. To remove as much lipid from the sample as possible, this step was repeated a second time. The remaining acetonitrile was transferred by pipette, to a 2mL centrifuge tube and evaporated to complete dryness by nitrogen evaporation in a 40 °C water bath. The dried samples were reconstituted in 200µL of methanol which was then vortexed. The 200µL was then transferred to a 1.5mL microvial suitable for use on the LC MS/MS and analyzed as described above

Method 3; Methanol extracted, RIA analyzed

The extraction method here is identical to that detailed in method 1 with the exception that the sample was not initially spiked with d_4-labelled cortisol. Once the extracted sample was transferred to the microvial and evaporated to dryness, it was sealed and stored at -25 °C prior to analysis by radioimmunoassay (RIA).

Method 4; Acetonitrile extracted, RIA analyzed

The extraction method here is identical to that detailed in method 2 except that the sample was not initially spiked with d_4-labelled cortisol. Once the extracted sample was transferred to the microvial and evaporated to dryness, it was sealed and stored at -25 °C prior to analysis by radioimmunoassay (RIA).

2.2.2.2 Fur Cortisol Extraction

Fur was collected by shaving samples of the epidermis which that were obtained from the mid-ventral region of the animal. Care was taken to ensure that broken skin was not included in the sample prior to rinsing. The cleaning and extraction of fur samples was modified from Ashley et al. (2011). In order to eliminate any contamination such as blood, oils or dirt from the surface of the fur samples, each sample was submersed and vortexed in a mild detergent mixture (Sunlight™ detergent 1% solution in ~25 °C distilled water) for 30 seconds and then rinsed with distilled water at room temperature. The sample was then triple rinsed in methanol for 30 seconds to remove any remaining surface contamination. The fur was then transferred to labelled aluminum weigh boats and left to dry in a fume hood at room temperature for 24 hours. Once dry, the fur was cut with scissors into fine pieces, weighed into 50mg samples and transferred to a mini centrifuge tube. Samples that appeared to remain contaminated were deemed as low quality and were discarded (Figure 2.3). To each tube, 1mL of methanol was added and the samples were left to soak for 48 hours. The supernatant was then removed and transferred to a new vial. The remaining fur was double rinsed in 500µL of methanol which was added to the new tube. Samples were then evaporated down to complete dryness by nitrogen evaporation in a 40 °C water bath and then stored at -25 °C prior to RIA analysis.

2.2.2.3 RIA Analysis

Methods 3 and 4 were analyzed using radioimmunoassay (RIA) following similar published protocols Ryan et al. (2011). Extracted samples were reconstituted in 250µL of ice cold RIA buffer (10mL Phosphate buffer (71.6g $Na_2HPO_4.2H_2O$ and 15.3g $NaH_2PO_4.2H_2O$), 90mL Milli-Q water, 0.9g NaCl and 0.5g Bovine serum albumin; pH 7.4) 100µL aliquots of the re-suspended samples were then added to separate assay tubes. Standards with a known concentration of cortisol were established in triplicate. On ice 100µL of cortisol-specific antibody (Fitzgerald Industries, NY, USA catalogue number 20-CR50) (1:8000 dilution) and 100µL of 5000 $\pm$250 disintegrations per minute (DPM) tritium-labelled cortisol (GE Healthcare, NJ, USA) was added to all assay tubes. The tubes were briefly vortexed and allowed to incubate at room temperature for 1 hour followed by 12-16 hours at 4°C. Post incubation, the reaction was stopped by adding 100µL of dextran coated charcoal (50mL RIA buffer, 0.25g dextran, 2.5g charcoal) to each assay tube and allowing the tubes to sit on ice for 15 minutes. The tubes were then centrifuged for 30 minutes at 4°C and 2500rpm. The supernatant was decanted into 7mL scintillation vials and 4mL of scintillation fluid (Ultima Gold AB, Perkin Elmer, Waltham, MA, USA) was added to each vial. The tubes were placed on a scintillation counter (Tri-Carb 3110TR, Perkin Elmer) and counted for 5 minutes. According to the manufacturer cross-reactivity of the antibody used was; 100% for cortisol; 5.7% for 11-deoxycortisol; 3.3% for corticosterone; 36% for prednisolone; and < 0.7% for cortisone. Extraction efficiency was determined by adding a known volume of tritium labelled cortisol to a sample, proceeding with the cortisol extraction protocol detailed above, and then measuring the level of radioactivity still present in the sample post extraction. The fur mean extraction efficiency was determined to be 81 ± 3% (n=3) and acetonitrile extracted blubber 60 ± 4% (n=11). Inter-assay variation was

calculated as 11 ± 3% (n=4) and 20 ± 5% (n=23) for fur and blubber respectively and intra-assay variation was calculated as 3 ± 1% (n=4) and 6 ± 3% (n=5) for fur and blubber respectively.

2.2.2.4 LC/MS/MS Analysis

In order to correct for the recovery of individual isomers in each sample, a labelled recovery internal standard (LRIS) was added to each sample prior to extraction and analysis (200pg/❍L of deuterium (d_4) labelled cortisol, Wellington Laboratories, Guelph, ON). To correct for instrument performance, labelled standard was added to extracted samples (n=8) immediately prior to being run on the LC/MS/MS rather than before extraction. High performance liquid chromatography (HPLC) separations were achieved using the Agilent 1100 series HPLC (Agilent Technologies G1312A). This system contains a vacuum degasser, binary pump and an autosampler. A 100µL syringe was used to draw a volume of 2µL (draw and eject speed 200 µL/min) from each vial/sample run. The column used was a Grace Genesis C_{18} analytical column (50mx2.1mm i.d., 4µm particle size). A mobile phase of optima grade methanol and water at a flow rate of 300µL/min was used. The elution program started at an initial composition of 80:20 water/methanol and increased to 100% methanol in 6 minutes. This was held for 6 minutes and then returned to the starting ratio in 2 minutes. The column equilibrated for 8 minutes between each sample. After a maximum of 10 samples had been run, the column was flushed with 100% methanol for 16 minutes prior to the method continuing on more samples. A Sciex API 2000 triple quadrupole mass spectrometer (Applied Biosystems MDS model 017378-E) was used in ESI negative ion mode. Quantitation was achieved by monitoring the specific ion transitions using a predetermined set of parameters (Table 2.2). The response of the d_4-labelled cortisol under MS/MS using multiple reaction monitoring (MRM)

was used to calculate the amount of native cortisol present in samples. Equipment cleaning took place between every 30 samples (maximum).

Table 2.2- Additional parameters for MS/MS method

Parameter	**Abbreviation**	**Value (units)**
Curtain gas	CUR	55 (a.u.)
Sheath gas	GS1	55 (a.u.)
Turbo gas	GS2	40 (a.u.)
Ionspray voltage	IS	-4200 (V)
Turbo-gas temperature	TEM	500 (^{o}C)
Declustering potential	DP	-21 (V)
Focusing potential (Cortisol)	FP	-290 (V)
Focusing potential (d_4-Cortisol)	FP d_4	-340 (V)
Entrance potential	EP	-10 (V)
Quad 1 offset	IQ1	-1.2 (V)
Quad 3 offset	IQ3	-4.5 (V)
Collison gas	CAD	9 (a.u.)
Collision cell entrance potential	CEP	-20 (V)
Collison energy	CE	-12 (eV)
Collision cell exit potential	CXP	-18 (V)

2.2.3 Statistical Analysis

All Statistical analyses and graphing was conducted using JMP 12. Individuals were categorized into age classes which were based on biologically significant reproductive ages/stages (Table 2.3). The age class categorization ((Pups (0-1), Juveniles (1-5) and Adults (>5)) also accounted for general changes in behaviour, physiology and size (McLaren 1958). Values for cortisol present were adjusted based on the sample tissues respective extraction efficiency for all RIA analyses. A MANOVA was done in order to determine whether the variables of location, sex, year and age class, as well as interactions between the statistically significant variables, had a significant effect on the concentrations of cortisol extracted from fur and blubber samples. Distributions of sample cortisol concentration were assessed for normality using Anderson-Darling test and outliers were removed based on Grubbs test values.

A one way ANOVA was then applied in order to do a direct comparison between the cortisol measured in the two tissues.

Comparison of extraction techniques and subsequent measurement of cortisol from blubber was assessed using a standard regression analysis of the methanol and acetonitrile method and was conducted separately for the 2 extraction methods analyzed by LC/MS/MS and RIA.

Table 2.3- Demographics of the samples analyzed from the communities of Arviat, NU and Ulukhaktok, NT. Samples from different years have been combined by age class and sex.

Community	Pup (< 1 year old)		Juvenile (1-5 years old)		Adult (> 5 years old)	
	Male	Female	Male	Female	Male	Female
Arviat	1	1	2	3	4	3
Ulukhaktok	0	0	2	3	7	1

2.3 Results

Data sets for fur and blubber cortisol concentrations were normally distributed once log transformed. Of all the variables assessed there were no significant interactions and only age class was found to have a significant effect on cortisol concentration for blubber and fur (Table 2.4). Average fur cortisol concentration differed between adults (1.42 ± 0.16 SE ng/g), juveniles (0.92 ± 0.15 ng/g), and pups (0.81 ± 0.37 ng/g) as it did in average blubber; adults (0.10 ± 0.01 SE ng/g), juveniles (0.25 ± 0.03 ng/g), and pups (0.94 ± 0.06 ng/g).

Table 2.4- MANOVA results for cortisol concentration in blubber (model $R^2 = 0.62$) and fur (model $R^2 = 0.38$) in ringed seals collected from Arviat, NU and Ulukhaktok, NT from 2007-12 and testing for location, sex, year, and age class. Blubber cortisol was extracted using Method 4.

Blubber	***df***	**F**	**P**
Location	1	0.44	0.52
Sex	1	0.003	0.96
Year	4	0.27	0.89
Age Class	2	13.37	0.0006
Model	8, 16	3.89	0.008

Fur	***df***	**F**	**P**
Location	1	0.02	0.88
Sex	1	0.27	0.61
Year	4	0.53	0.72
Age Class	2	3.04	0.07
Model	8,16	1.41	0.26

Further analysis revealed a relationship between fur and blubber cortisol concentrations for the juvenile seals (R^2=0.64, F(1,9)=13.95, p=0.006) but not for the adults (R^2=0.09, F(1,12)=1.18, p=0.30). Average cortisol concentration was consistently higher for fur than blubber within all variables (Table 2.5). A small sample size (n=2) for the cortisol concentrations measured in blubber and fur of pups prevented further analysis from being conducted on this age class. When pups are removed from the data set, there are statistically significant differences

found between adult and juvenile mean fur cortisol concentration ($t(21)=-2.29$, $p=0.03$), *df* 1, $F=4.08$) and to a greater extent mean blubber ($t(21)=4.79$, $p<0.0001$). However, a positive trend in the relationship between fur and blubber cortisol concentrations can be seen for the two younger age classes (Figure 2.4).

Table 2.5- Average Cortisol concentrations with standard error by sample location and sex separated by age class i) Adult, ii) Juvenile and iii) Pup. Blubber cortisol was extracted using Method 4.

i.)

	Sex		**Location**	
	Male (n=11)	Female (n=2)	Arviat (n=7)	Ulukhaktok (n=6)
Blubber	0.10 ± 0.02	0.2 ± 0.03	0.12 ± 0.02	0.09 ± 0.02
Fur	1.39 ± 0.16	0.87 ± 0.18	1.48 ± 0.23	1.46 ± 0.25

ii)

	Sex		**Location**	
	Male (n=4)	Female (n=6)	Arviat (n=5)	Ulukhaktok (n=5)
Blubber	0.27 ± 0.06	0.11±0.05	0.24±0.04	0.25 ± 0.05
Fur	1.01 ± 0.2	1.85 ± 0.67	0.75 ± 0.12	1.11 ± 0.23

iii)

	Sex		**Location**
	Male (n=1)	Female (n=1)	Arviat (n=2)
Blubber	0.13	0.41	0.27 ± 0.15
Fur	0.92	1.19	1.06 ± 0.14

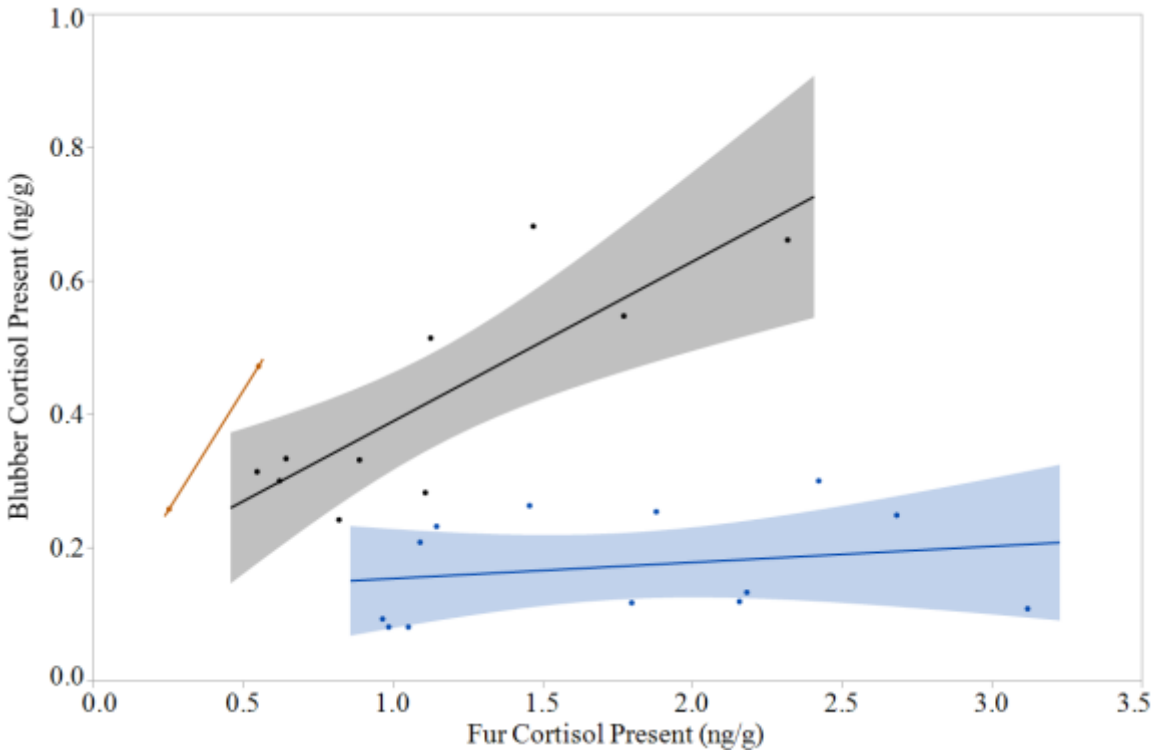

Figure 2.4-Regression analysis for correlating fur by blubber cortisol concentrations (ng/g) for Adult (Blue, $Y=0.129+0.0242^{x}$, $R^2=0.05$), Juvenile (Grey, $Y= 0.149+ 0.239^{x}$, $R^2= 0.69$) and Pup (Orange, $Y= 0.075+0.717^{x}$, $R^2=1$). Shaded areas represent the regression line 95% confidence fit.

The relationship between cortisol concentration measurements obtained for acetonitrile and methanol based extraction techniques with analysis by LC/MS/MS was found to be significant ($R^2=0.37$, $F(1,23)=12.67$, $p=0.002$) (Figure 2.5), but when using the data obtained from measurement with the RIA, the relationship between the two extraction techniques was not significant ($R^2=0.006$, $F(1,23)=0.123$, $p=0.728$) (Figure 2.6).

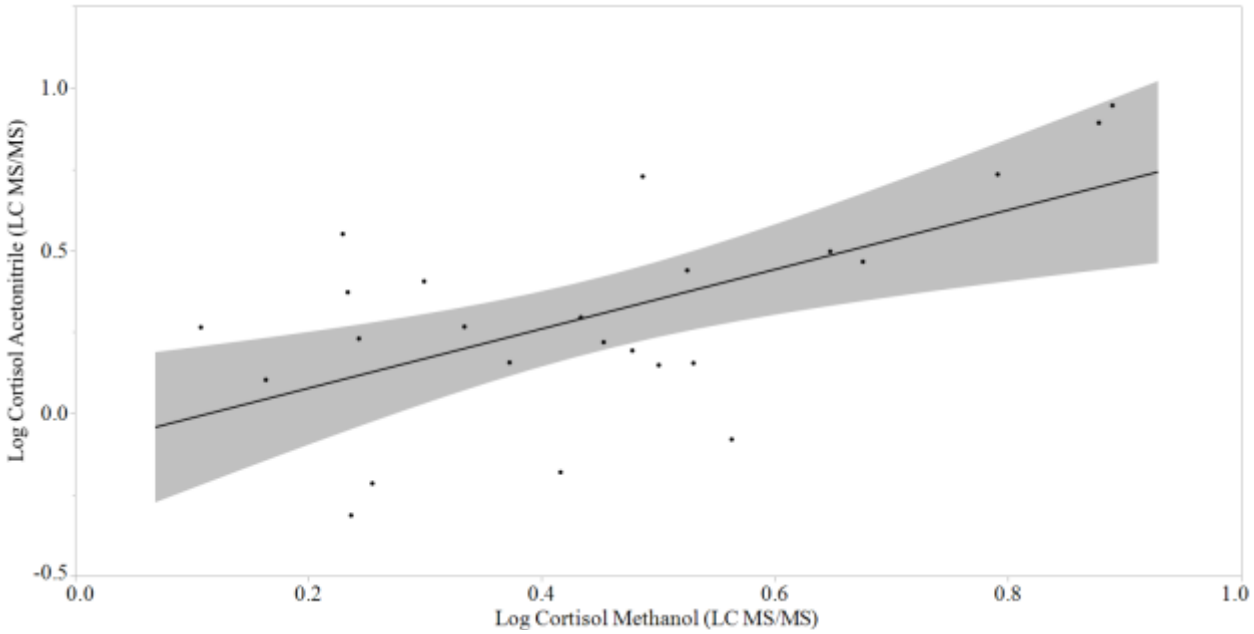

Figure 2.5- Log cortisol concentrations determined using Acetonitrile and Methanol extraction methods analyzed via LC MS/MS ($Y=-0.1054+0.912^{x}$, $R^2= 0.365$). Shaded areas represent the regression line 95% confidence fit.

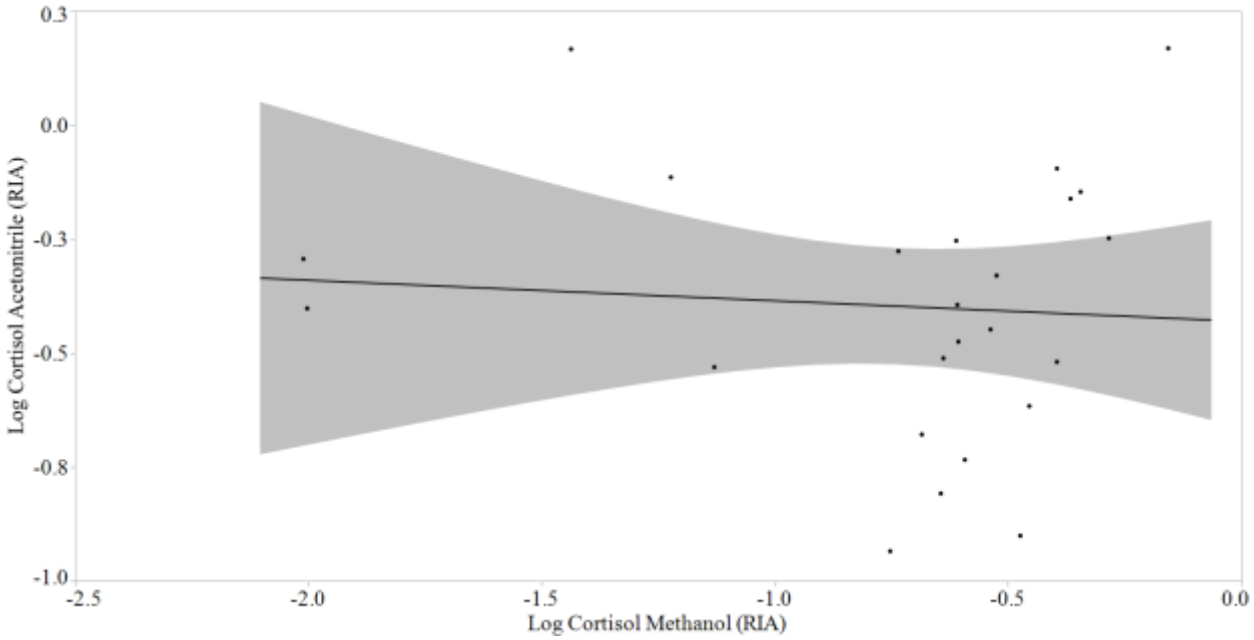

Figure 2.6- Log cortisol concentrations determined using Acetonitrile and Methanol extraction methods analyzed via RIA ($Y=-0.4301-0.0451^{x}$, $R^2=0.006$). Shaded areas represent the regression line 95% confidence fit.

An assessment of LC/MS/MS ion suppression was made by extracting blubber (n=8) with methanol and acetonitrile based techniques and then intentionally fortifying the extracts

with a known amount of d_4-Cortisol. The results from this study, suggest that d_4-Cortisol is subjected to greater matrix suppression (mean: 37.7% ± 3.1%) when acetonitrile was used as the extracting solvent (Figure 2.7).

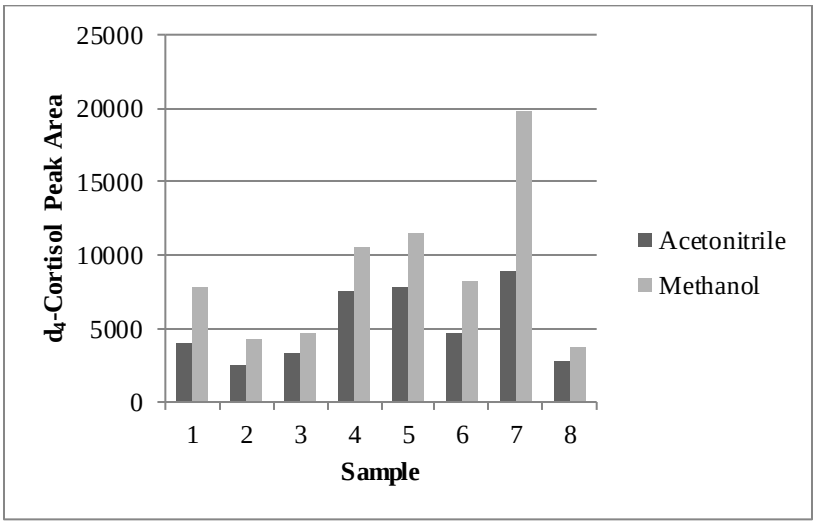

Figure 2.7- Suppression analysis from LC/MS/MS for the two extraction methods, Acetonitrile and Methanol (n=8).

The mean cortisol concentration for both acetonitrile and methanol based extractions was higher when using LC MS/MS (2.64 ± 0.42, 3.18 ± 0.37 respectively) analysis versus RIA (0.50 ± 0.07, 0.27 ± 0.03 respectively) (Figure 2.8). Extraction methods under both analytical techniques show a significant relationship (LC/MS/MS- $r(22)=0.81$, $p=0.05$ and RIA- $r(22)=0.24$, $p=0.004$) as well as between analytical techniques (Acetonitrile $r(22)=0.86$, $p=0.0001$ and Methanol $r(22)=0.338$, $p=0.0001$).

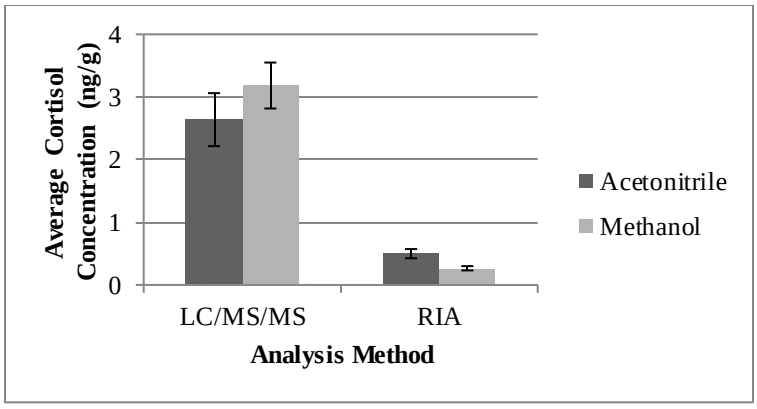

Figure 2.8- The mean concentration with standard error of cortisol measured from blubber using Acetonitrile or Methanol extraction techniques from the same individuals and analyzed via LC/MS/MS or RIA (n=24 for each point).

2.4 Discussion

2.4.1 Matrices for assessing Chronic stress

The samples analyzed for the fur and blubber cortisol concentrations were from different communities which represent different life history phases that the individuals were experiencing upon capture due to the difference in the time of year that the samples were collected. This needs to be considered when assessing a potential relationship between our measurements. The cortisol concentrations found in both matrices are significantly affected by age class. Individuals from within the pup age class were only from Arviat, NU whereas the adult and juvenile samples are pooled from both communities. Although the rate of sequestration of cortisol into fur is understood to mirror the circulating hormone level at the point of growth, the exact mechanism by which cortisol concentrations are established in blubber is unknown.

2.4.1.1 Fur

Ringed seals undergo a full molt annually which overlaps with other energy intensive life history events such as breeding and whelping (McLaren 1958, Ling 1970). The full cycle lasts approximately 12 weeks for adults (Smith 1987) during which fully grown seals spend a large period of time basking (Born et al. 2004, Kelly et al. 2010a). Basking on sea ice reduces the

thermal stress of entering and exiting the cold water as well as facilitating fur growth by keeping epidermal temperatures at a level that facilitates pelage regeneration (Smith et al. 1991, Kelly et al. 2010b). Regeneration coincides with the molt of the previous season's coat and is facilitated by high levels of circulating thyroid hormone (John et al. 1987). The most rapid regrowth occurs near the end of the molt cycle when thyroid hormone levels rapidly increase (Ashwell et al. 1986, Shero et al. 2015) and is slowed by the activation of the HPA axis which triggers the production and release of cortisol into the blood (Riviere et al. 1977). Following this phase, circulating thyroid and cortisol levels decrease causing a reduction in metabolic rate and subsequently the reduced mobilization rate of energy reserves (Routti et al. 2010, Shero et al. 2015).

The adult and juvenile seals' fur is not the major source of insulation (Ling 1970) but does provide a thin air barrier of separation between the external environment and the body (Liwanag et al. 2012). In contrast, neonate pelage, known as the lanugo, is essential for maintaining internal core temperatures (St Aubin and Geraci 1986). Lanugo growth takes place in utero and is fully shed 4-6 weeks after birth (Smith 1987). At the point when the pups were sampled in Arviat, they would have fully completed the shedding of their lanugo and would have a fur coat that began to grow approximately 5.5 months prior. The cortisol measured in the pup fur would not be reflective of the time period after they were feeding on their mother's milk and spending a large amount of time in the subnivean lairs. During this time glucocorticoids are transferred via lactation to the pup, affecting the baseline circulating hormone levels (Sheriff et al. 2011). This study showed an average pup blubber cortisol level that is lower than that in the older age classes but this calculation is based on only two Arviat samples and therefore may not accurately reflect the population average.

The juvenile and adult coats from Arviat began growth one month later than the pups (approximately 4.5 months old). The Ulukhaktok, NT juvenile and adult seals were sampled near the end of the molt cycle at the point when the fur collected was almost fully grown and new. The hormone that was extracted from the fur is a reflection of the circulating cortisol concentrations at the point of growth (Bennett and Hayssen 2010) therefore despite the large time difference between completions of growth and sampling, if the individuals from the 2 separate locations have similar baseline circulating levels, the yield of our analysis should be comparable. Indeed there was no significant difference in fur cortisol concentration between the locations which is why the adult and juvenile samples were pooled despite community of origin (Table 2.4 and 2.5).

2.4.1.2 Blubber

There are multiple factors that could affect blubber cortisol levels such as circulating hormone levels and subsequent sequestration, rate of metabolism and rate of retention within the tissue. Emerging research suggests that despite these unknowns, the concentration of stress hormone found within blubber can be an indication of exposure to chronically stressful circumstances (Kellar et al. 2015, Trana et al. 2015, Kershaw and Hall 2016). Analysis of blubber cortisol concentrations minimizes the effects of sampling on stress hormone levels obtained due to the lag time expected with the uptake of cortisol into the blubber layer (Kellar et al. 2015). There are further benefits to the expanding knowledge of the relationship between the stress response and blubber tissue because, as with this study, it allows for the use of archived samples to establish a better understanding of the potential changes to the physiological response of ringed seals to chronic stressors over decades.

Relationships between cortisol concentrations in blubber and age class were difficult to assess due to the small sample size. When pups were excluded from analysis, there was a distinct difference between stored cortisol levels in juveniles and adults. If stored cortisol reflects circulating levels, this finding would indicate that there may be differences between HPA activation in these two age classes. Rate of lipid metabolism is affected by circulating cortisol levels which influences blubber thickness, an integral component of condition (Pond 1992).The finding in chapter 3 suggest that there is no difference between the condition of adults and juveniles despite which community the samples originated from. Similar condition suggests that circulating levels are comparable between these age classes and that although rate of metabolism is a function of cortisol circulation, the difference in cortisol extracted from the blubber is due to one of the unknown mechanisms of hormone sequestration and retention.

As previously reported, variations in cortisol levels in the blubber may be an indication of seasonal variation in circulating glucocorticoid levels (Kershaw and Hall 2016). Community hunts and sample collection times associated with high stress life history events tend to produce blubber tissue with elevated cortisol concentrations. Although the relationship between cortisol concentrations found in fur is generally not predictive of the actual levels found in blubber, this trend in seasonal variation is similar therefore blubber likely can be used as an indicator of chronic stress exposure for juveniles.

2.4.2 Determining Cortisol Concentration

2.4.2.1 Extraction from Blubber

The quantification of hormone levels in biological samples such as blubber, require extraction in order to isolate the analyte in question and purify the sample to ensure the least amount of interference with the assay. Blubber is primarily composed of lipid which is soluble in

nonpolar solvents therefore organic solvents such as hexane, ether, chloroform, methanol and acetonitrile are commonly used for hormone extraction. Effective lipid separation from the analyte in question is essential for the high sensitivity analytical tools used to determine the overall concentration. Failure to effectively 'clean' a sample could result in compromised results and potentially damage analytical equipment. An ideal solvent would completely separate the lipid from the sample while being safe to handle, inexpensive and readily available.

In this study, the two extraction methods used both employed the use of common solvents for hormone isolation. Using methanol as the extraction solvent resulted in consistently higher yields than the acetonitrile based method when analyzing on the LC MS/MS (Figure 2.7). The samples used to compare the two methods were the same and the polarity index values for acetonitrile and methanol are comparable (6.2 and 6.6 respectively), however, the later method had far fewer steps which could account for this variation in yield. The extensive process required for the acetonitrile based extraction included the use of multiple other solvents and required more sample transfer steps than the methanol extraction. Although the end solvents qualities are similar, the capacity for sample loss was greater with the acetonitrile method.

The benefits to using the methanol extraction technique with blubber are numerous. It is inexpensive, safe and readily stocked in chemistry laboratories relative to the solvents required for the acetonitrile based extraction technique. The ultimate determining factor for selecting an appropriate extraction method should be accuracy. Although, there were no certified reference materials available, this study compared overall trends of cortisol and as such, was not entirely dependent on method accuracy or absolute cortisol amounts. Despite the potential for a loss of sample with the steps employed by the acetonitrile method, the higher correlation between the

acetonitrile extracted samples despite the analytical technique employed suggests that it should be the preferred method of cortisol extraction from blubber.

2.4.2.2 Analysis of Cortisol Extract

LC MS/MS and RIA are common analytical techniques used to determine the concentration of various analytes including glucocorticoids. Our results show that, despite the extraction method used, the concentration of cortisol measured in blubber extract samples is consistently higher when analyzed on the LC MS/MS versus RIA. Many direct comparison studies between these two analytical techniques are based on human plasma or serum samples but no one method consistently shows higher yields (Janse et al. 2011, Xu et al. 2014, Tran et al. 2015). Despite larger concentration values there is consistently high correlation between the two techniques suggesting that in most studies, either method is suitable. Variation in the volume of return could be dependent on the analyte in question, antibody used and tissue that the sample is extracted from and is not necessarily a reflection of the overall efficiency of the technique.

Liquid Chromatography coupled with Mass Spectrometry

LC MS/MS couples the physical separation of the analytes in question from all other components of a sample by forcing a liquid mobile phase of the sample through a column designed to separate molecules based on molecular size. The purpose of the mass spectrometry is to measure the mass-to-charge ratio of the separated compounds in order to ultimately determine the composition or amount of that compound within the sample.

This analytical technique is highly sensitive and specific which may result in an increased quality of measurements obtained (Monaghan et al. 2013). It is a beneficial technique when samples available are limited because small volumes are required for analysis therefore the

source sample can be re-run multiple times without the need to prepare more extracted samples (Hogg 2009) additionally, the same sample can be used to assess multiple analytes at a time. Unfortunately, set up and maintenance can be prohibitively expensive.

The acetonitrile extraction method showed greater suppression than the methanol meaning that the detection response for cortisol was lower with acetonitrile, however, the high correlation between the two extraction methods imply that either will give comparable results with this analytical technique. However, based on both analytical techniques acetonitrile extraction provides more consistent results.

Radioimmunoassay

RIA uses antibodies to measure the concentration of antigens in a sample. It is a sensitive *in vitro* analytical technique where a known quantity of radiolabelled antigen in question and antibody for that antigen are added to a sample. The unlabelled antigen present in the sample competes with the radioactive antigen for antibody binding sites. The higher the concentration of unknown antigen results in a lower amount of bound radioactive antigen and displacement of the labelled antigen is proportional to the amount of unknown. Studies that utilize RIA to assess hormone levels are common in the literature and have been used in a range of matrices including serum, hair, fecal material, feathers, saliva and blubber (Norris et al. 1999, Pride 2005, Ryan et al. 2012, Trana et al. 2015) therefore comparison of methods and values across studies are more straightforward with this method as opposed to the less published LC MS/MS technique.

Immunoassay interference can occur when there is a substance present within the sample that alters the measureable concentration of the analyte by interfering with the antibodies binding activity (Tate and Ward 2004). Detection of estrogen from baboon (*Papio cynocephalus*) fecal samples was higher when analyzed by RIA whereas testosterone returns were higher when

analyzed by LC MS/MS (Gesquiere et al. 2014). The low relative cortisol return with the RIA analysis in the present study could be due to the antibodies potential to cross react with other steroids that are present within the sample. Lower yields may also be the result of analyte independent interference such as matrix effects on the assay where foreign components present within the extract could compromise the ability to accurately obtain concentrations. Blubber is a novel matrix for hormone analysis and its high lipid content makes it particularly challenging to ensure a pure sample prior to analysis. The lower yields detected when methanol was used as the extraction solvent may indicate that methanol does not leave the sample as free of lipid as the acetonitrile and that RIA analysis is particularly sensitive to samples that are not clean. Disadvantages to this method include the dangers associated with the handling and preparation of the radioactive antigen and the inconvenience of required special training, certification and designated space required to prepare samples for analysis.

2.5 Conclusion

The unknown mechanisms responsible for the stored hormone that we collected from blubber samples make it difficult to directly compare to the concentrations collected from fur. Fur cortisol measurements reflect the circulating concentration at the point of growth whereas it is more likely that blubber cortisol stores reflect concentration stored in relation to the circulating level and rate of metabolism. Some studies suggest a relationship between fatty acid structure and the rate of metabolism (Spitzer et al. 1966, Connor et al. 1996, Soppela and Nieminen 2002, Mustonen et al. 2007). Shorter chain fatty acids are metabolized more efficiently potentially causing the longer chain fatty acids to accumulate (Mustonen et al. 2007). Different age classes and populations can feed at different trophic levels resulting in different fatty acid signatures

therefore the rate of metabolism and potentially rate of cortisol release could be dependent on nutrition. Both matrices can be used as an indicator of chronic stress but blubber hormone levels are likely more specifically related to nutritional stress.

Both techniques of extraction and analysis have benefits and drawbacks therefore selection of the appropriate methods is ultimately dependent on accessibility and the nature of the study question. Methanol is less costly and labour intensive but the consistency between acetonitrile extracted samples and the two different analytical techniques suggest that the latter may be the more suitable option. The two analytical techniques both represent reliable methods of analysis for studies where the comparative levels of hormones are of the greater interest than the absolute values.

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Chapter 3 Chronic stress and Condition in Arctic Ringed Seals (*Phoca hispida*)

3.1 Introduction

Due to anthropogenic pressures the Arctic ecosystem is in a rapid state of modification relative to historical data. The Arctic is an environment that on the surface, seems barren and simplistic but it is a complex and sensitive system where even minor shifts and alterations can cause extreme impacts to its inhabitants (IPCC 2013). Sea ice is one of the defining physical features of the arctic and it is rapidly changing in composition and extent (Galley et al. 2012). An integral component of the ecosystem; the ice, is essential to the survival of marine and human inhabitants that depend on it. It functions to provide habitat and a means to determine migration paths for marine mammals (Kovacs et al. 2011, Bajzak et al. 2013, Heide-Jorgensen et al. 2013). The direct dependence on the ice varies by species but in studies of the impact and extent of climate change on the marine arctic ecosystem as a whole it is useful to determine the impact that the changes have on the health and well-being of those species most dependent on it.

The Ringed seal (*Phoca hispida*), is an ice obligate species in the Canadian arctic, whose fitness is directly correlated to sea ice (Laidre et al. 2008). The timing of key life history events such as breeding and molting, has evolved to align with normal annual environmental changes (Moore and Huntington 2008). Their life history reliance on sea ice means that they will be among the first to show the impact of climate change (Ferguson et al. 2005). Ice dependence, along with their high trophic level, long life span and the bioaccumulation of elements in tissues, make them an optimal study species for various types of investigation (Bossart 2006, Moore 2008). Additionally, ringed seals maintain significant cultural and economic importance to many northern communities (Borre 1991, Duhaime et al. 2002, Kuhnlein et al. 2004, Brunborg et al. 2006, Kuhnlein et al. 2008).

Paradoxically, the wide circumpolar distribution, large population and generalist prey tendencies, may make the ringed seal the least sensitive to climate induced habitat change relative to other arctic marine mammals, however, this does not mean that they are free of risk (Laidre et al. 2008, Moore 2008, Moore and Huntington 2008, Labansen et al. 2011). Assessment classifications for the ringed seal have changed over time due to the exponential acceleration in habitat modification witnessed in the arctic (Sundqvist et al. 2012). According to the Species at Risk Act, ringed seals are categorized as 'Not at Risk' (COSEWIC 1989) but a more recent status assessment listed them as 'Threatened' (Kelly et al. 2010), which illustrates the importance of constant review and a development of a greater understanding of how population health may be impacted.

Traditional methods to determine the health of ice seals at the individual level typically include morphometric measurements (e.g. length, mass, girth, blubber depth (Mammals 1967)) to establish a rating specific to the index being used. Ryg et al. (1990) established a direct relationship between condition, as determined by such an index, and the overall mass blubber percent. As such, condition of ice seals has been determined by measuring the overall blubber content, via the mass of the sculp or by calculating a condition index value. For ringed seals in the Beaufort region, condition indices have shown a significant trend in decreasing annual mean condition over the past two decades which correlates to changes in sea ice (Harwood et al. 2012a). Alternative methods for determining the condition of various species include the analysis of body chemistry in relation to these indices. Blood haptoglobin concentration of ringed seals from Svalbard, Norway has been used to assess general health due to its direct relationship to condition (Krafft et al. 2006). Conductive techniques that examine the composition and deposition of adipose tissue are being used on both terrestrial and aquatic species (Wirsing et al.

2002, Pitt et al. 2006). Alternatively, gravimetric analysis of the adipose tissue in sea birds and polar bears has been shown to accurately determine condition (Jacobs et al. 2012, McKinney et al. 2014). The synthesis, mobilization and storage of hormones can also be used as an indication of health in seals. Condition and reproductive success has been determined by quantifying changes in thyroid and cortisol hormone levels in the blood of Weddell seals, Harbour seals and dolphins (Renouf and Noseworthy 1991, Kellar et al. 2015, Shero et al. 2015)

Ringed Seal Stress

Ringed seals experience both internal and external stressors that result in the initiation of their endocrine stress response. The response includes the activation of the Hypothalamic-Pituitary Axis and subsequent release of the glucocorticoid, cortisol, into the circulation (Selye 1956, Sterling and Eyer 1981). Cortisol is always present at a baseline level in the circulatory system but in the presence of a stressor, becomes elevated. Normal life history events are related to seasonal, annual or circadian cycles that will result in a variation of this baseline level independent of external stressors (Riviere et al. 1977, Ashwell et al. 1986, Oki and Atkinson 2004, Routti et al. 2010b). These cortisol concentration fluctuations alone should be within a range that a healthy seal can manage and survive, however, when external stressors are applied, the individual is forced to reallocate energy to overcome the stressor (Sterling and Eyer 1981); energy that otherwise may be essential to maintain the health of the individual or reproductive success (Burek et al. 2008).

The effects of external stressors on ringed seals in the Canadian arctic resulting in poor body condition have been observed by Inuit hunters and are consistent with findings of multiple biological studies. As with many other arctic marine mammals, the major climate change related

challenges that ringed seals experience are related to; ecosystem and habitat changes and their impact on stress and subsequently health. The extent to which these stressors are present varies depending on the region and may be linked to climate change as it has been shown that the response of the arctic ecosystem to climate change varies depending on latitude and the water bodies that the seals inhabit (Galley et al. 2012).

Ecosystem and habitat changes have negative effects on the health, survival and reproduction of arctic ringed seals in a number of ways. Sea ice extent is decreasing throughout the ecosystem but southern regions have also recorded changes to volume and nature of precipitation which directly affects the salinity, ice and snow thickness that is crucial for the life history of the ringed seals and survival of pups (Stirling and Smith 2004, Ferguson et al. 2005). Rising temperatures have resulted in the introduction of new or an increased number of predators to seal habitat (Higdon et al. 2013). Climate change presents the risk of the introduction of pathogens into immunologically naïve ringed seal populations which can result in epizootics (Lynch et al. 2011) such as the morbillivirus and brucellosis outbreaks in 1988 and 1996 respectively (Heide-Jorgensen et al. 1992, Duignan et al. 1997, Forbes et al. 2000). Duignan et al. (1997) found that the highest prevalence of exposure to morbillivirus is when ringed seals whose ranges overlap with harp seals (*Pagophilus groenlandicus*). This is of concern because as temperatures change in the arctic, the range of competing species will likely increase in overlap with ringed seals (Moore and Huntington 2008, Bajzak et al. 2013). Higher temperatures and longer seasons will increase ringed seal susceptibility to new infectious agents if they are introduced (Bradley et al. 2005). In more recent years, hunters have reported seeing seals that appeared lethargic and presented with lesions similar to those observed with morbillivirus (symptoms described in Kennedy (1998)). Exposure to contaminants such as those that have

been shown to compromise the immune system (Kendall et al. 1992) and result in decreased reproduction (Harding and Harkonen 1999) in seals can also be considered as a major risk to their health.

In order to quantify the cumulative effects of human based environmental changes on the health of ringed seals, further study into direct biological changes are needed. We are generally familiar with the impact of life history events in fluctuations of circulating glucocorticoid concentrations but as far as baseline serum cortisol levels, there is great variation between individuals and species (St Aubin and Geraci 1986, Tryland et al. 2006). Cortisol levels consistently vary by season and in relation to the annual molt in harbour seals (*Phoca vitulina*) (Riviere et al. 1977) but remain constant during the fasting period in harp seals (*Pagophilus groenlandicus*) (Nordoy et al. 1993); both life history events are also experienced by ringed seals. Further complicating the interpretation of measured serum cortisol levels is the variation in the rate of HPA axis activity and how quickly the concentration of hormone found in the bloodstream can change (Romero and Reed 2005, Otovic and Hutchinson 2015). Measuring cortisol in a tissue that is less susceptible to sudden shifts in hormone concentration could allow us to obtain measurements that are more reflective of long-term HPA activity and feeding stress (Chapter 2). This study aims to determine whether there is a correlation between condition and the stress hormone stored in the blubber layer of ringed seals.

3.2 Materials and Methods

3.2.1 Sample Collection

3.2.1.1 Ulukhaktok, Northwest Territories, Canada

A community hunt, led by the Hunters and Trappers Organization in Ulukhaktok (formerly known as Holman), Northwest Territories, takes place annually during the summer months along the shore of Prince Albert Sound off the Victoria Island coast (Figure 3.1). Seals

are non-selectively harvested with no preference given to size, age or sex (Harwood et al. 2012b). Blubber thickness was measured at the mid-point along the sternum and girth measurements were taken at both the axillary and hip regions. Samples of complete blubber cross sections obtained from the subsistence hunts that took place in June of 2005-2012 were used for this study. Upon collection, samples were wrapped in aluminum foil and kept in individually labelled whirl-pack bags. These samples were approximately 200g and included the fur, skin and 3 blubber layers up to the outer muscle layer (Figure 3.2). The samples were stored frozen in coolers at the hunting camp until weather and ice conditions permitted transport to community freezers where samples were then stored at approximately -20°C. Sex was determined by visual analysis in the field. Age was determined in the Ulukhaktok, NT lab by extracting a canine tooth from the lower jaw and counting the dentinal lines in a cross section. Cementum lines were counted, if readable (Smith 1973, Harwood et al. 2012b). Upon completion of the hunting season and age analysis, samples were then shipped frozen to the Freshwater Institute in Winnipeg, Manitoba where they were stored at -25°C in archive.

3.2.1.2 Arviat, Nunavut, Canada

Ringed seal blubber, epidermis, lower jaw bones and corresponding morphological measurements for each individual sampled, were collected by members of the Arviat Hunters and Trappers Association during the fall subsistence hunts in 2007-2012. The hunt takes place annually along the shores of this western Hudson Bay community (Figure 3.1). The harvest coincides with the winter freeze up and typically takes place from the end of October through the beginning of November. Seals were non-selectively harvested and the demographics of the individuals in the hunting grounds show equal distribution at this point in the season (Smith 1973). Tissue samples and blubber thickness measurements were consistently collected from the

mid-ventral region of the animal and girth measurements were obtained from the axillary region. Blubber samples that ranged in mass from 100-500g were collected and stored in individually labelled plastic bags. Sex was determined by visual analysis in the field and verified genetically in the lab. Until the end of that season's hunt, samples were stored in community freezers at -20°C and then shipped frozen to the Freshwater Institute in Winnipeg, Manitoba where they were stored at -25°C in archive. In order to determine the age of the individuals, canine teeth were extracted after softening the periodontal membrane via submersion and soaking of the lower jaws in a heated water bath for 2-4 hours. The teeth were then sent for age determination to Matson's Laboratory in Montana, Utah. Seal ages were determined by identifying and counting the growth layer groups (GLGs) within the cementum layers on mounted, longitudinally sliced, sections of teeth from each individual (Stewart et al. 1996).

Figure 3.1- Ringed seal samples were collected from the subsistence hunts in 1- Ulukhaktok (Holman), Northwest Territories and 2- Arviat, Nunavut.

3.2.2 Sample Preparation and Analysis

Archived blubber samples were arbitrarily chosen and scored for quality of sample as either high or low (Figure 3.2). Blubber tissue samples that appeared yellowed dried or rotting were assessed as low quality and were not used in further analyses. A pilot study showed that low quality samples had low or undetectable steroid concentrations, suggesting that sample quality influenced steroid measurement as previously described for Beluga whale blubber samples (Trana et al. 2015). Samples deemed as high quality appeared pink in colour and lacked any signs of degradation. Blubber subsamples weighing 1 gram (n=816) were taken using a scalpel to cut an even, cross sectional piece of tissue (Figure 3.2) and then transferred to a 15mL plastic vial. Approximately 40% of all samples were subsampled in duplicate to assess consistency in preparation and analytical techniques. Samples were freeze dried for approximately 48 hours at -50 °C, sealed and stored at -25°C prior to analysis.

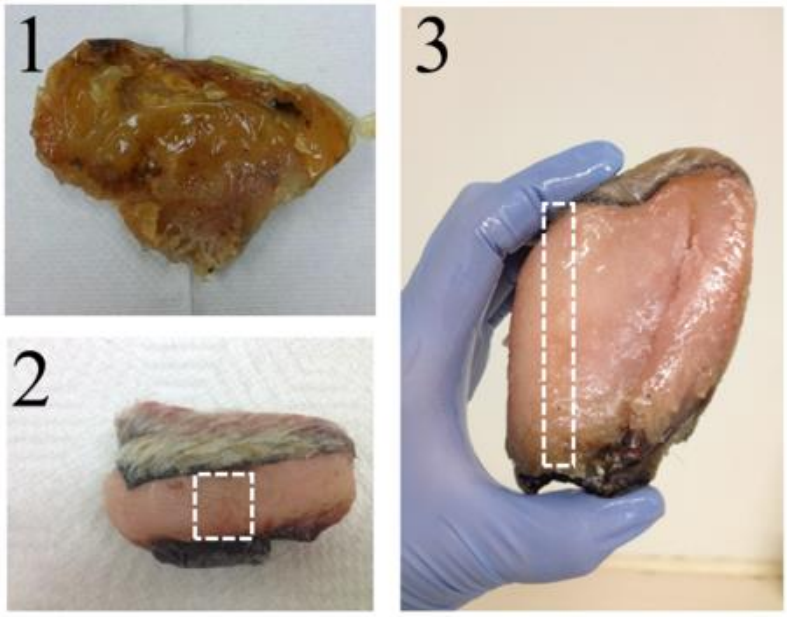

Figure 3.2- Tissue subsamples of varying qualities from field collection for - Ulukhaktok (Holman), Northwest Territories (2) and Arviat, Nunavut (1 and 3). Yellowed, dry or rotting samples were considered poor quality (1) whereas high quality samples appeared pink (2 and 3). Example of a blubber subsample that includes all 3 layers is outlined in white.

3.2.2.1 Cortisol Extraction

The technique for hormone extraction was modified from Kellar et al. (2006). Each sample vial containing 1g of freeze dried blubber had 2mL of ethyl alcohol added. Sample vials were then capped and left to soak at 25 °C in an ultrasonic bath (Fisher Scientific Ultrasonic Bath 9.5L) set to 45 kHz for 24 hours. A glass rod was then used to homogenize the lipid phase and the remaining tissue was compacted to the bottom of the sample vial. At room temperature the aqueous phase was then transferred to a new vial and the remaining connective tissue was double rinsed with 1mL of ethyl alcohol which was then also transferred to the second vial. The ethyl alcohol from each sample was then evaporated off in a nitrogen evaporator at 40°C. Once dry, 1mL of a 4:1 Ethyl alcohol:acetone mixture was added to each of the vials, which were then vortexed again and evaporated down to dryness on the nitrogen evaporator. This step was repeated with 1mL of Ethyl Ether. The sample vials were then removed from the nitrogen evaporator and 2mL of hexane and 1mL of acetonitrile was added to each sample and then vortexed for 5 minutes. While at room temperature, sample vials were then capped and centrifuged (Thermo Scientific Sorvall ST16) at 3000rpm for 20 minutes. Following centrifugation two distinct phases were visible within the vial. The phase of greater volume, which contained a mixture of hexane and lipid, was considered waste and removed from the vial while ensuring not to disturb the acetonitrile phase. To remove as much lipid from the sample as possible, this step was repeated a second time. The remaining acetonitrile was transferred by pipette, to a 2mL centrifuge tube and evaporated to complete dryness by nitrogen evaporation in a 40 °C water bath. The tube was then sealed and stored at -25 °C prior to analysis by radioimmunoassay (RIA).

3.2.2.2 RIA Analysis

Samples were analyzed using radioimmunoassay (RIA) following similar published protocols (Ryan et al. (2011). Extracted samples were reconstituted in 250µL of ice cold RIA buffer (10mL Phosphate buffer (71.6g $Na_2HPO_4.2H_2O$ and 15.3g $NaH_2PO_4.2H_2O$), 90mL Milli-Q water, 0.9g NaCl and 0.5g Bovine serum albumin; pH 7.4) 100µL aliquots of the re-suspended samples were then added to separate assay tubes. Standards with a known concentration of cortisol were made in triplicate. On ice 100µL of cortisol-specific antibody (Fitzgerald Industries, NY, USA catalogue number 20-CR50) (1:8000 dilution) and 100µL of 5000 $\pm$ 250 disintegrations per minute (DPM) tritium-labelled cortisol (GE Healthcare, NJ, USA) was added to all assay tubes. The tubes were briefly vortexed and allowed to incubate at room temperature for 1 hour followed by 12-16 hours at 4°C. Post incubation, the reaction was stopped by adding 100µL of dextran coated charcoal (50mL RIA buffer, 0.25g dextran, 2.5g charcoal) to each assay tube and allowing the tubes to sit on ice for 15 minutes. The tubes were then centrifuged for 30 minutes at 4°C and 2500rpm. The supernatant was decanted into 7mL scintillation vials and 4mL of scintillation fluid (Ultima Gold AB, Perkin Elmer, Waltham, MA, USA) was added to each vial. The tubes were placed on a scintillation counter (Tri-Carb 3110TR, Perkin Elmer) and counted for 5min. According to the manufacturer cross-reactivity of the antibody used is; 100% for cortisol; 5.7% for 11-deoxycortisol; 3.3% for corticosterone; 36% for prednisolone; and < 0.7% for cortisone. Extraction efficiency was determined by adding a known volume of tritium labelled cortisol to a sample, proceeding with the cortisol extraction protocol detailed above, and then measuring the level of radioactivity still present in the sample post extraction. The blubber mean extraction efficiency was determined to be 60 ± 4% (n=11). Inter-assay variation was calculated as 20 ± 5% (n=23) and intra-assay variation was calculated as 6 ± 3% (n=5).

3.2.3 Individual Condition Analysis

Condition was determined using 4 indices previously used for analysis of ringed seals and other marine mammals. These are: A) lipid percent, B) length mass based index, C) core to blubber ratio and D) percent blubber.

A.) A recently used measure of condition in polar bears is lipid percent (McKinney et al. 2014) but this measure has yet to be applied to ringed seals. A higher lipid percent value implies a better condition. To determine lipid percent, the mass of the remaining solid tissue from the extraction phase described above was weighed and a value for percent lipid calculated as:

$$\text{Lipid percent} = (\text{Sample mass prior to extraction} - \text{remaining solid mass}) \times 100$$

B.) The most commonly used condition index is one that includes morphological measurements for length, weight and blubber thickness as described by Ryg et al. (1990). This index was calculated as:

$$\text{Condition} = \sqrt{(\text{Length}/\text{Weight})} \times \text{Blubber thickness}$$

C.) For analysis of the relationship between condition and stress level, the protocol outlined in Castellini et al. (2009) was used, where a core to blubber ratio was calculated using the morphometric data acquired by the hunters while in the field. Using this index increased the sample size for the study and also decreased the potential for error from sample collection in the field. This ratio was calculated as;

$$\text{Total body diameter} = (\text{Axillary girth})/\pi$$

$$\text{Core diameter} = \text{Total body diameter} - 2(\text{Axillary blubber depth})$$

$$\text{The ratio of blubber depth to core diameter} = 2(\text{Axillary blubber depth})/\text{Core diameter}$$

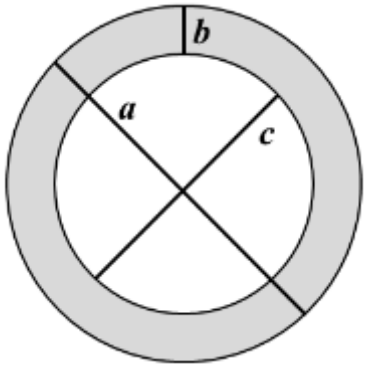

Figure 3.3-Axillary cross sectional representation of ringed seal where ***a***= Total body diameter, ***b***=Blubber depth and ***c***=Core diameter. Altered from (Castellini et al. 2009)

D.) Percent blubber was calculated using the sculp and overall weight of the individual upon capture. After a total body weight was determined in the field, the weight of the blubber layer, skin and fur alone (sculp) was recorded (Young and Ferguson 2013). The percent blubber was then calculated as:

$$\text{Percent blubber} = \frac{\text{Sculp weight}}{\text{Total body weight}} \times 100$$

3.2.4 Statistical Analysis

All Statistical analyses and graphing was conducted using JMP 12. Values for cortisol present were adjusted based on the sample tissues' respective extraction efficiency. Distributions of sample cortisol concentration were then assessed for normality using Anderson-Darling test and upon log transformation, the data met the necessary assumptions. Outliers were determined using a Grubbs test and were eliminated from further statistical analysis in order to maintain normal distributions. Individuals were categorized into age classes that were based on biologically significant reproductive ages/stages (Table 3.1). The age class categorization also

accounted for general changes in behaviour, physiology and size: pups (<1), juveniles (1-5) and adults (>5) (McLaren 1958).

Table 3.1- Demographics of the samples analyzed from the communities of Arviat, NU and Ulukhaktok, NT. Samples from different years have been combined by age class and sex.

Community	Pup (< 1 year old)		Juvenile (1-5 years old)		Adult (> 5 years old)	
	Male	Female	Male	Female	Male	Female
Arviat	53	62	65	74	111	134
Ulukhaktok	-	-	6	9	107	54

General Linear Model (GLM) and Multivariate Analysis of Variance (MANOVA) tests were performed in order to determine if there was a significant difference for both cortisol concentrations and condition value with the calculated variables sex, age class and community as well as any interactions between statistically significant variables. Least squares, linear regression analyses were used to examine the relationships between dependent variables. Potential trends over time were assessed using a Mann-Kendall test.

3.3 Results

The effect of variables age class, year and location differed among condition indices. Sex did not have a significant effect on condition for any of the indices. Condition A and D were significantly affected by the variables year and age class whereas both Condition B and C were affected by only location and year (Table 3.2). For the ringed seals in this study, blubber composition (A) is approximately 91$\pm$2% lipid and 5$\pm$1% water, which could vary due to year and age. Blubber Percent (Condition D) varied over the years examined but a difference between age classes was also found. Average blubber percent was the lowest in Adult (47.2+0.7%, n=152) and increased by age class Juvenile (48.8+0.9%, n=95) and Pup (50.0+0.9%, n=91). Condition index A was found to be significantly correlated with both condition index B (p=

<0.0001, *df* 528, R^2=0.0575) and C (p= <0.0001, *df* 604, R^2=0.0548). A high correlation between the two morphometrics based index values (Condition indexes B and C) (p= <0.0001, *df* 590, R^2=0.83) was determined (Figure 3.4).

Table 3.2- ANOVA results for Condition A (model R^2 = 0.14, n=632), Condition B (model R^2 = 0.43, n=554), Condition C (model R^2 = 0.18, n=638), and Condition D (model R^2 = 0.15, n=338), ringed seals for location (Arviat, NU and Ulukhaktok, NT), sex, year of sample collection (2003-2012) and age class (Adult, Juvenile, Pup).

Condition A	***df***	**F**	**P**
Location	1	2.24	0.14
Sex	1	0.002	0.97
Year	9	9.97	<0.0001
Age Class	2	3.03	0.045
Model	13, 618	7.61	<0.0001

Condition B	***df***	**F**	**P**
Location	1	258.21	<0.0001
Sex	1	0.48	0.49
Year	8	8.49	<0.0001
Age Class	2	0.91	0.40
Model	12,541	33.71	<0.0001

Condition C	***df***	**F**	**P**
Location	1	43.46	<0.0001
Sex	1	0.07	0.80
Year	9	6.02	<0.0001
Age Class	2	2.96	0.05
Model	13,541	33.706	<0.0001

Condition D	***df***	**F**	**P**
Sex	1	2.33	0.13
Year	7	6.32	<0.0001
Age Class	2	6.01	0.003
Model	10,327	5.58	<0.0001

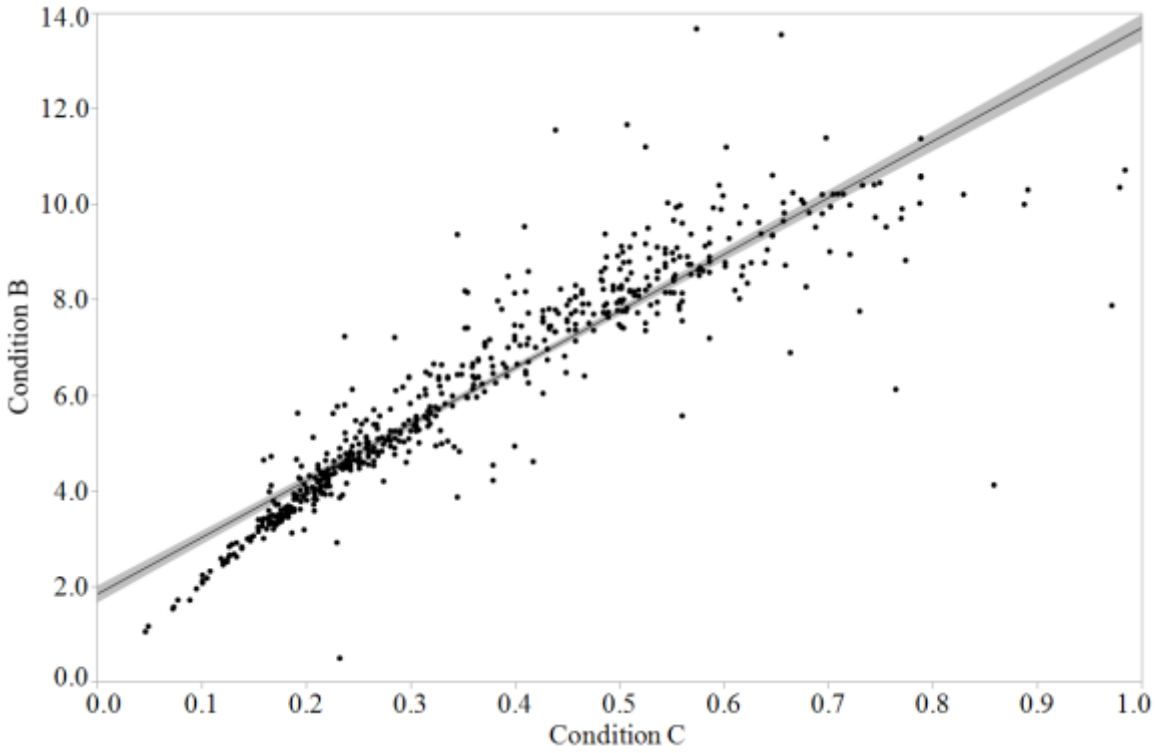

Figure 3.4- Regression analysis ($Y=1.83+11.85^{X}$) of the condition indices B and C (n=591). The shaded area represents the regression 95% confidence fit

Due to it's reduced variability and the larger sample size available compared to indices A and B, we chose to use Condition C for all further analysis. The combined average condition value for ringed seals from Arviat, NU from 2003-2012 (0.436 ± 0.007, n=506) is over twice that of ringed seals from Ulukhaktok, NT (0.206 ± 0.004, n=150). In the Arviat ringed seal population, the highest condition was from 2007 (0.568 ± 0.030, n=27) and the lowest, 2010 (0.365 ± 0.023, n=47) (Figure 3.5). The highest condition from Ulukhaktok's ringed seal population was recorded in 2011 (0.227 ± 0.013, n=19) and the lowest in 2010 (0.167± 0.009, n=19) (Figure 3.6). Analyzing locations separately indicated a significant effect of year on condition in Arviat NU ($F(7,506)=1.50$, $p=0.17$) but not in Ulukhaktok NT ($F(9,150)=9.58$, $p=0.0001$). The changes in condition seen over this period of time are not linear and are not significantly related to preceding

or subsequent years for either community as determined by a Mann-Kendall test for Arviat (p=0.72) and Ulukhaktuk (p=1).

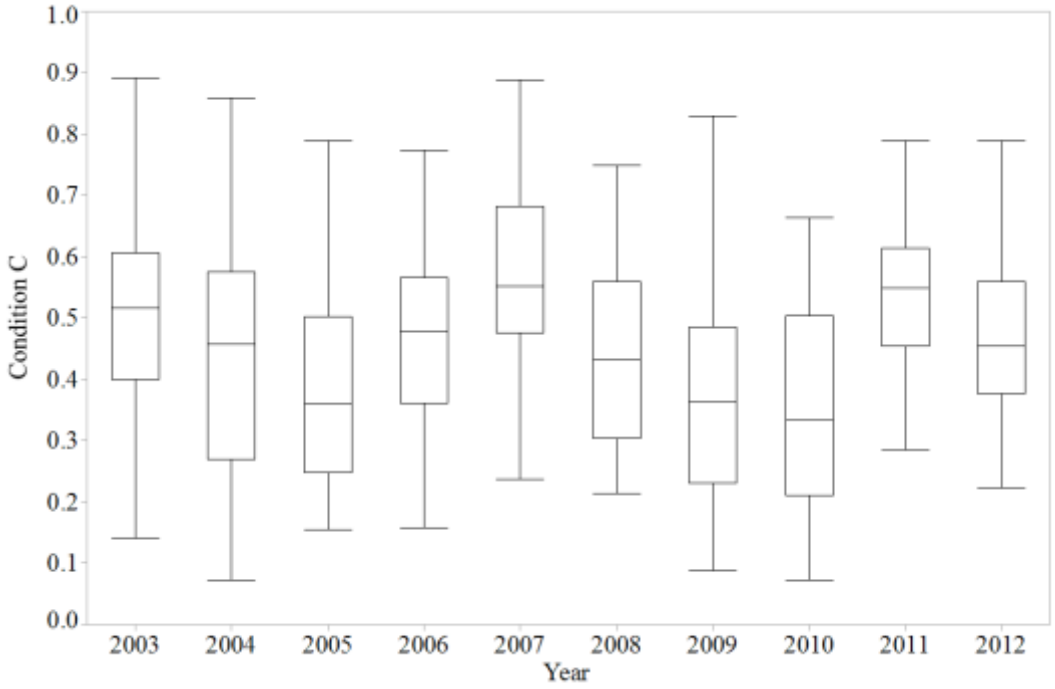

Figure 3.5- Condition, as determinined by Index C, over time (2003-2012) for the ringed seal populations (age classes pooled) in the community of Arviat, NU (n=506) (Fall sampling-Post peak feeding). Each box plot represents the median, interquartile range and 95% CI for the corresponding year.

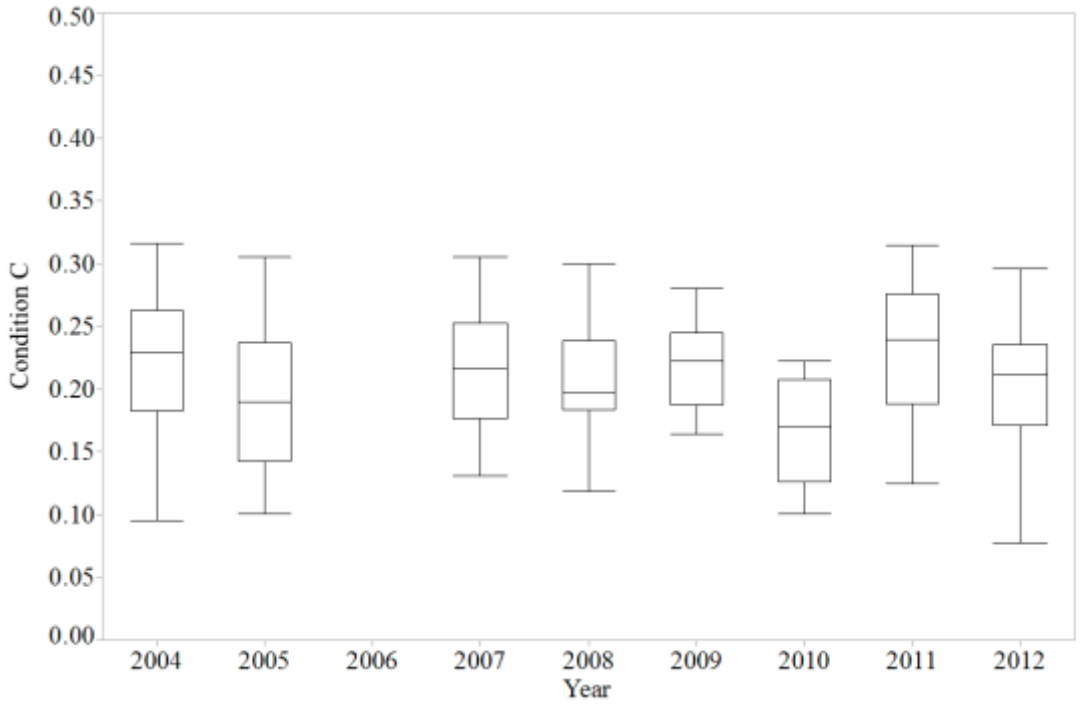

Figure 3.6- Condition, as determinined by Index C, over time (2004-2012) for the ringed seal populations (age classes pooled) in the community of Ulukhaktok, NT (n=150) (Summer sampling- post molt). Each box plot represents the median, interquartile range and 95% CI for the corresponding year.

Of the variables assessed, location and year were found to have a significant effect on both cortisol concentration and condition in ringed seals from both locations (Table 3.3). The interaction between year and location was also significant. In Arviat, age class was found to have a significant effect on blubber cortisol concentration, with greater estimates in adults (Least Squares Means -0.6299 ± 0.0282 SE), juveniles (-0.6490 ± 0.0459), and pups (-0.4868 ± 0.0549) but not on condition. The mean values of blubber cortisol concentration were adult (0.26 ± 0.05 SE), juvenile (0.19 ± 0.03 SE) and pup (0.32 ± 0.06 SE). In ringed seals sampled from Arviat, age class and year significantly influenced blubber cortisol levels, ($F(2,450)=3.68$, $p=0.03$ and $F(9,450)=2.64$, $p=0.006$ respectively). Comparing age classes, pups tended to have higher blubber cortisol levels than the other age classes in the same year (Figure 3.7). The changes in cortisol seen over this period of time are variable for Ulukhaktok but there was a significant increasing monotonic trend for all adults in Arviat ($p=0.04$) as determined by a Mann-Kendall test.

Table 3.3- ANOVA results for log cortisol concentration in blubber (model $R^2 = 0.286$) and log condition C (model $R^2 = 0.441$) in ringed seals (n= 588) controlling for location (Arviat, NU and Ulukhaktok, NT), sex, year of sample collection (2003-2012) and age class (Adult, Juvenile, Pup)

Cortisol (Log)	***df***	**F**	**P**
Location	1	12.654	0.0004
Sex	1	3.185	0.075
Year	9	3.369	0.0005
Age Class	2	3.672	0.026
Location*Year	1	68.44	<0.0001
Model	12, 575	19.232	<0.0001
Condition (Log)	***df***	**F**	**P**
Location	1	195.142	<0.0001
Sex	1	2.780	0.092

Year	9	5.081	<0.0001
Age Class	2	0.099	0.185
Location*Year	1	0.53	0.011
Model	12, 575	37.748	<0.001

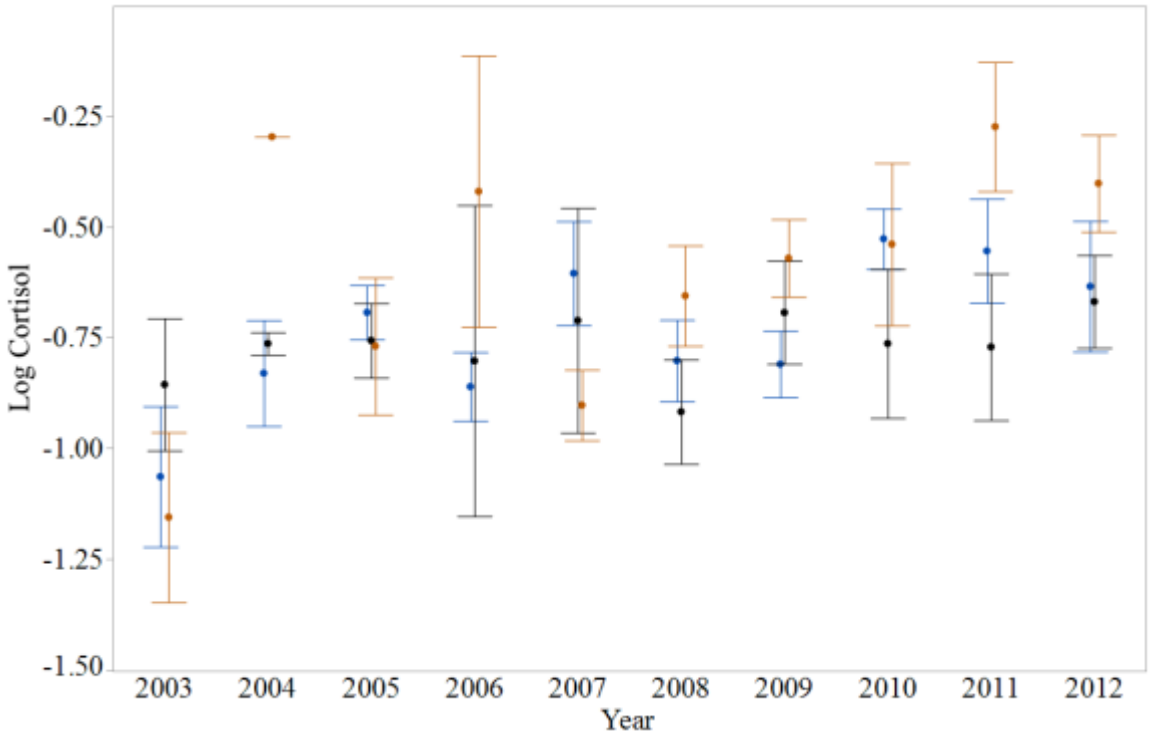

Figure 3.7- Average log blubber cortisol levels in the different age classes (Adult-blue, Juvenile-black, Pup-orange) of ringed seals from Arviat, NU (2003-2012). Data are expressed as a mean +/- SE.

Ulukhaktok ringed seal blubber cortisol concentrations were significantly affected by year ($F(7,157)=2.03$, $p=0.054$) but not by age class therefore the data for each year was pooled. Blubber cortisol levels showed a significant decrease in 2009 but in recent years has risen closer to previous levels (Figure 3.8).

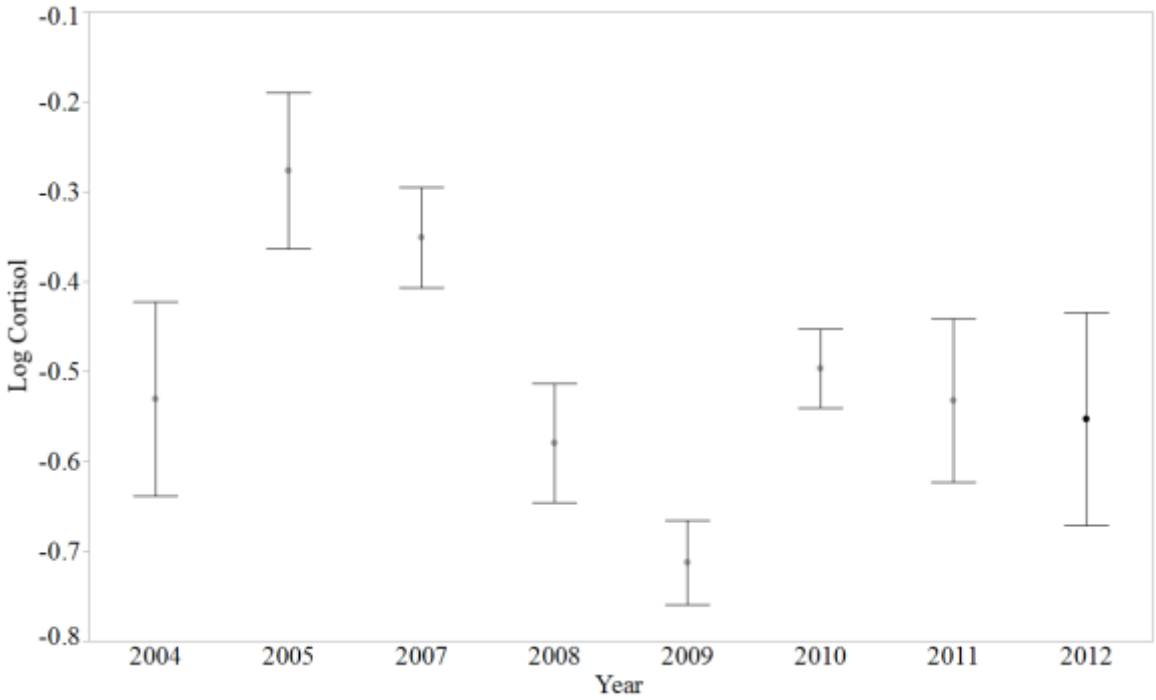

Figure 3.8- Average log blubber cortisol levels in all age classes of ringed seals sampled at Ulukhaktok NT between 2004 and 2012. Data are expressed as a mean +/- SE

In seals sampled in Arviat, NU, there were slightly different trends in the relationship between blubber cortisol concentration and condition in the different ringed seal age classes. Both Adult and juvenile seals show no significant correlation between condition and cortisol measurements (R^2=0.012, F(1,228), p=0.092 and R^2=0.02, F(1,127), p=0.114 respectively). Pups, however, do have a significant negative relationship between blubber cortisol concentration and condition (R^2=0.035, F(1,107), p=0.05) (Figure 3.9).

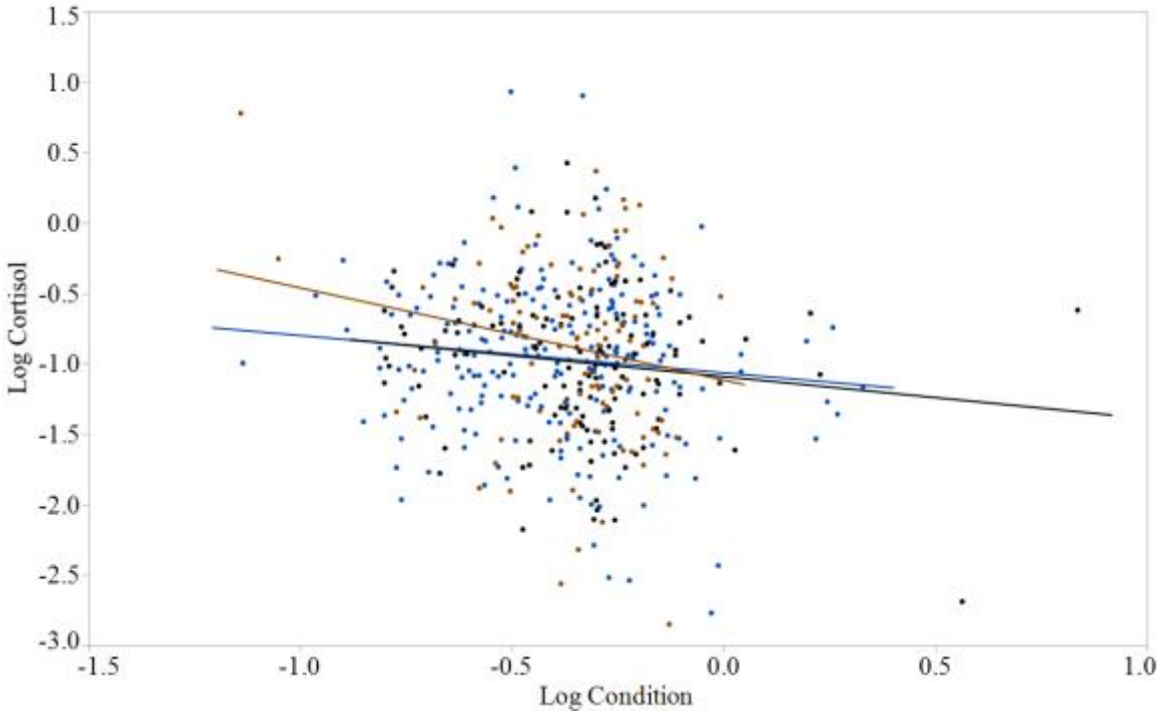

Figure 3.9- Regression analysis of log blubber cortisol concentration and log condition by age class (Adult-Blue, Juvenile- Black and Pup-Orange ($Y=-1.16-0.655^{x}$)) for ringed seals from Arviat, NU (2003-2012).

When year and location are considered, and only the age classes adult and juvenile are pooled, condition is determined to have significant effect on cortisol concentration in both the ringed seal population sampled from Ulukhaktok ($R^2=0.25$, $F(1,156)$, $p=<0.001$) and to a lesser extent, Arviat ($R^2=0.01$, $F(1,354)$, $p=0.05$) (Figure 3.10).

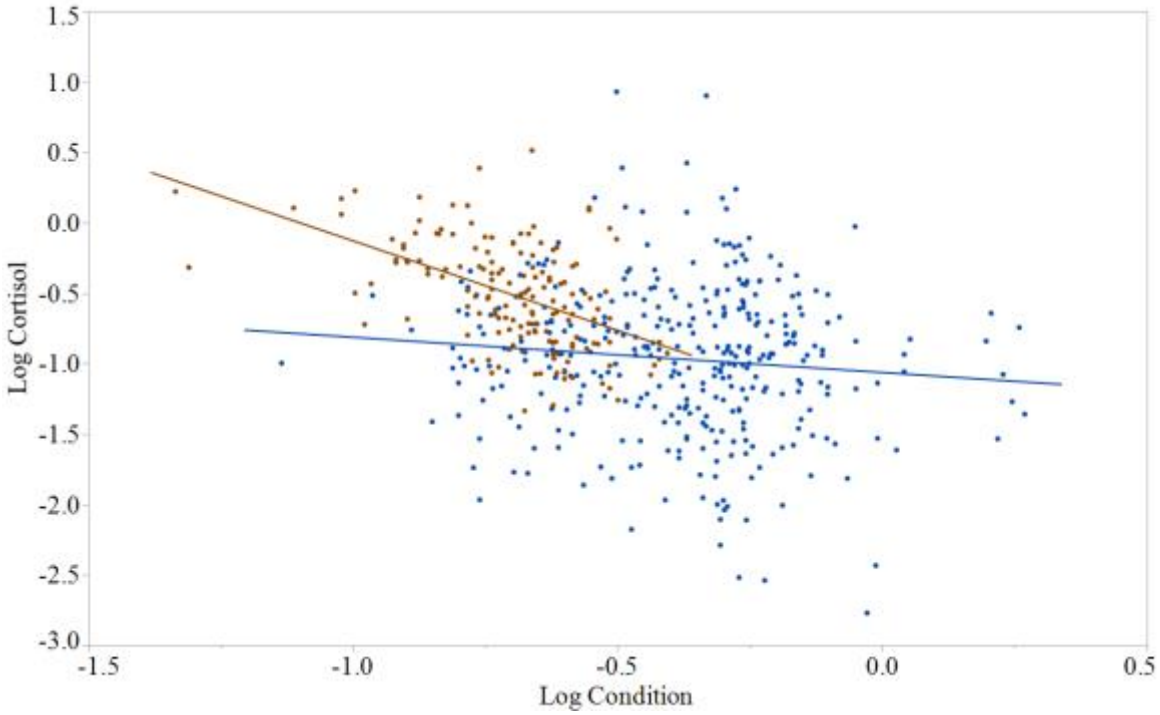

Figure 3.10- Regression analysis of log blubber cortisol concentration and log condition by locations, Arviat (Blue) ($Y=-1.062+0.2501^X$) and Ulukhaktok (Orange) ($Y=-1.4+1.272^X$) for adult and juvenile ringed seal samples, 2003-2012.

3.4 Discussion

3.4.1 Condition

Physical condition is often considered an indication of health for various terrestrial, avian and aquatic species across the animal kingdom. Traditional indices for phocid condition use blubber layer depth as well as measurements for mass and length. Blubber is the energy capital that has been accumulated through prey consumption by the individual and in the absence of a sufficient layer for the season, deficiencies in the animal's well-being can be assumed (Peig and Green 2009). Condition is dependent on the controlled uptake and release of lipid via the metabolism of this energy store (Pond 1992).

3.4.1.1 Indices

The lipid content within a full profile blubber sample (Condition A) will vary based on the composition of the whole blubber layer transect. The blubber profile is stratified into three visibly distinct sections that vary in function and composition (Strandberg et al. 2008, Bagge et al. 2012). The middle section will result in the highest variation in thickness because it contracts and expands based on lipid stored and energy metabolized (Young 1976, Strandberg et al. 2011). The inner and outer most sections have a higher vascular and connective tissue structure respectively, and are generally a consistent thickness despite overall profile depth (Strandberg et al. 2008). The composition of a blubber profile is similar along the length of the animal (Winter and Nunn 1950, Thiemann et al. 2006) but most morphometric based condition indices use the blubber depth measurement at the deepest point along the torso. A correlation between lipid content and morphometric based condition indices has been recently demonstrated in polar bear and sea bird tissue samples (Thiemann et al. 2006, Jacobs et al. 2012, McKinney et al. 2014) similar to ringed seals in the present study.

Blubber is composed of adipose cells which contain varying amounts of lipid, water, connective and vascular tissue (Mead 1986). The number of fat cells that an individual has is constant and based on nutrition (Young 1976), however, the lipid content of those cells is what varies in relation to blubber layer thickness, which changes seasonally. This layer's primary role is insulation and energy reserve which is dependent on thickness and water percent (Scholander et al. 1950, Young 1976). Water is inversely related to thermal conductivity therefore the percent of water found in blubber directly impacts the efficiency of internal heat retention and transfer (Bagge et al. 2012). Individuals with a higher percent of lipid are better suited to maintaining the animal's core temperature despite environmental temperature or increased heat releasing metabolic activity (Parry 1949, Worthy and Edwards 1990, Bagge et al. 2012).

Lipid content can be determined non-lethally through biopsy samples from which the overall mass of the blubber rather than the depth is the integral measurement. An even less invasive method to determine lipid percent is through a method called bioelectrical impedance in which a mild electrical current is passed through the tissue and total body water and lean mass can be determined based on the ease at which the current travels (Wirsing et al. 2002). It has proven to be most accurate in species of a high lipid composition thus may be a suitable method for determining wild marine mammal condition. Using methods to determine body composition rather than morphometric based condition indices may be beneficial as far as accuracy because cutting blubber tissue distorts the blubber depth measurement (Ryg et al. 1988) thus potentially altering the final measurement. Blubber is under condensed pressure prior to cutting therefore any measurement of depth taken from a dissected animal will have an unknown level of inaccuracy.

Measurements for Condition B and C are based on a conical model premise that all ice seals have a basic shape that is similar to two cones with bases aligned (Ryg et al. 1988, Castellini et al. 2009). Heat loss is a function of the ratio of wall thickness to radius of the cylindrical form. The joining point of the two cones marks the approximate highest girth and the deepest blubber depth (Gales and Burton 1987, McDonald et al. 2008). There presumably is a limit to how thick a ringed seals fat layer can accumulate to before there is no longer an energetic benefit. An increase in the radius of the body will decrease streamlining efficiency by increasing water resistance thus resulting in higher energy requirements to hunt or migrate (Ryg et al. 1988). The full length and mass of the individual, as dictated by the Committee of Marine Mammals (1967), are also critical components of most morphometric condition indices. This sets

limitations to which individual archived specimens can be used in a study as some of the measurements required are not always available.

Blubber is distributed over the body such that the ratio of blubber to core thickness is generally consistent (Ryg et al. 1988) therefore having access to just two measurements (girth with a corresponding depth measurement) enables the determination of condition based on an index such as Condition C (Castellini et al. 2009). The core of an individual does contain a moderate amount of lipid and in cases of extreme weight loss, thermal stability can still be achieved provided that this core weight decreases at a similar rate to the blubber layer (Ryg et al. 1988, Ryg et al. 1990, Castellini et al. 2009). If the individual is of a compromised condition, the total body diameter (*a*) will decrease as a result of the blubber depth (*b*) decreasing at a rate greater than the core diameter (*c*) (Figure 3.3). Condition indices that consider a ratio of core to blubber depth take into account that the core itself will change with age, reproductive status and condition so it is not surprising that neither of the variables for sex or age class were significant factors when determining condition by this index (Rosen and Renouf 1997). There are different consequences to health and wellbeing of an individual that has a 50cm core and 10cm thick blubber layer than a seal with a 55cm core and a 7.5cm blubber layer. For example, both seals have a 70cm diameter but the later has a smaller core to blubber ratio (0.40 and 0.27 respectively). If we determined condition using the Condition B index we would require an accurate mass measurement to account for difference between these two since the girth measurement is the same. There were more archived samples available that a core to blubber depth ratio could be determined compared to a morphometric condition index value and since there was a high correlation between the two morphometric based indices (Figure 3.4), Condition C was used for further analysis.

Sculp weight is a rudimentary method to determine overall composition of the individual in terms of the total percent blubber (Condition D). The sculp consists of the skin with the attached blubber separated from the core flippers and head. An accurate measurement of both the overall mass and sculp weight are sometimes difficult to obtain in the field, therefore many of the archived samples that we had access to do not have this measurement. Ryg et al. (1990) found a significant correlation between blubber content as determined from the sculp and a morphometric based condition index which approximated the blubber percent (Condition B).

3.4.1.2 Influences on Condition

Age Class

Our results found that unlike Condition B and C, Condition A and D were significantly influenced by age class. The composition of blubber tissue in younger seals contain relatively less connective tissue than adults (Jangaard and Ke 1968), which would account for age dependence in the variation in lipid percent. Stirling (2002) reported that following the six week weaning period, ringed seal pups are over 50% blubber, which is consistent with our finding for ringed seals from Arviat, NU. Over the period of time leading up to when these samples were collected, pups were entirely dependent on their mother's milk for nutrition. Lactation is extremely energy intensive and results in a food source that has high energy potential. This low sugar, high fat content milk is necessary for a pup's rapid growth, shedding of the lanugo and production of a new coat (Eisert et al. 2013). Some studies have found that adult ringed seals tend to consume higher proportions of fish, such as arctic cod, than younger individuals (Lowry et al. 1980, Labansen et al. 2007, Young et al. 2010). The differences in blubber and lipid percent found could be due to foraging habits such as age specific trophic feeding which would result in different energy intake and fatty acid signatures between the classes. Additionally the

adult individuals have the added energy expenditure required to rut, breed and whelp their young which will influence the rate of metabolism of their blubber layer.

Location

In the Hudson Bay community of Arviat, NU, seals were sampled during what should be the peak of their physical condition (Young and Ferguson 2013): after the height of their feeding and prior to the winter fast (Table 1.3). Their condition was significantly better than those from Ulukhaktok, NT for all ten years in this study. Arviat is located 1500km south east of Ulukhaktok in a region of the arctic that is subject to environmental changes and anthropogenic impacts that differ in nature, rate and degree.

Sea ice distribution and freeze up time is rapidly changing across the northern hemisphere and some models project an entirely sea ice free summer in the arctic as soon as 2030 (Wang and Overland 2012). The rate of this change varies based on latitude with the more southern regions, such as the Hudson Bay, are presently experiencing longer ice free periods than the higher latitudes (Galley et al. 2012). Due to the projected sea ice season, thickness and extent decrease, breeding may shift to taking place further north where sea ice is still present (Meier et al. 2004, Perovich 2011), growth rates may decrease (Sundqvist et al. 2012) and prey consumption may shift to lower trophic levels (Carroll 2013). Sea ice conditions have been directly attributed to significant negative impacts on growth, survival as well as condition in the Beaufort Sea ringed seal populations (Harwood et al. 2000).

Increased surface temperatures, a major reason for the shifts in sea ice demographics, have also been directly related to changes in phocid blubber thickness (Mellish et al. 2013). These warming trends over time are also responsible for changes to snow depth and precipitation rates in the Hudson Bay resulting in decreased pup survival (Ferguson et al. 2005). In the

absence of a suitable snow cover, pups become susceptible to freezing and predation as the lairs that they depend on for warmth and protection can easily be compromised.

As the sea ice and surface temperatures change, the range of potential predators and competition for prey will shift potentially compromising the survival and health of ringed seals. There has been no apparent increase in Killer whale (*Orcinus orca*) sightings in the Beaufort sea, however, the reduction in ice has led way to an exponential increase in sightings in the Hudson Bay, a water body where they have not historically been seen (Higdon and Ferguson 2009, Higdon et al. 2013).

3.4.2 Cortisol

The variables that impact the condition of ringed seals are among the same factors that cause changes to the production of cortisol. Cortisol levels will deviate from baseline circulating levels for a myriad of reasons that can be due to natural life history events, the internal stressors (Riviere et al. 1977) or external stressors such as those discussed.

Harbour (*Phoca vitulina*) and spotted seals (*Phoca largha*) have a high plasma cortisol concentration leading up to the molt which takes approximately 120-170 days. The peak of cortisol increase is followed by 2/3 of the fur growth, while thyroid hormone levels are heightened (Ashwell et al. 1986). This same trend is seen in Australian fur seals (*Arctocephalus pusillus*) (Atkinson et al. 2011). The ringed seal molt is about half of the length (McLaren 1958, Kelly et al. 2010) but if cortisol adjusts in the same manner in ringed seals then the highest circulating concentration should be in June for adults and May for pups with baseline returning 1 month after. Based on these assumptions of similar hormonal function in relation to natural life history events, in particular molting, the seals that were harvested in Ulukhaktok would have been sampled 1-2 months after their circulating cortisol levels were at their presumed highest and

the Arviat seals at their presumed lowest. The rate of uptake is one of the many unknowns about the relationship between blubber and hormones but in other mammals, similarly structured hormones such as progesterone are sequestered into adipose tissue and able to be detected at about 5-10 days after an acute stressor is applied (Hillbrand and Elsaesser 1983). If this rate of uptake is similar with cortisol and blubber, the tissue samples collected should be very close to a reflection of the trends in HPA axis activity as a result of internal stimuli.

Blubber accumulates steroid hormones by passively diffusing from the capillaries throughout the lipid (Deslypere et al. 1985, Mead 1986) and circulating free hormone is what is thought to be sequestered (Romero 2002). The amount of free cortisol available for uptake is regulated by transport binding globulin availability (Corticosteroid binding globulin (CBG for cortisol) (Lattin and Romero 2015). Baseline blubber cortisol calculations could vary based on the number of cortisol receptors available which changes seasonally and due to life history events (Desantis et al. 2013, Lattin and Romero 2015). The majority of mammal and vertebrate species have CBG levels sufficient enough to bind 90% of free GCs but the regulation of this availability can also be impacted by stressors (Desantis et al. 2013).

Just as there are many reasons that we might see an activation of the stress response, there are many degrees of threat perception and response by the individual. An individual may also have an existing condition that could affect their ability to effectively overcome a stressor (McEwen and Stellar 1993, McEwen 1998). Response to an acute external stressor is variable and based on genetics, developmental influences and experiences therefore adults may initiate a stress response of a lesser magnitude than naïve younger individuals (Weiner 1992, McEwen and Stellar 1993)

3.4.2.1 Relationship to blubber cortisol concentration

Age Class

Age class was found to affect cortisol levels in seals from Arviat, NU. Neonate pups may undergo a period of hypo-responsiveness to stressors as an evolutionary means to protect their developing bodies from the harm that a hyperactive HPA axis could cause (Romero 2004). Glucocorticoids (GC) can be transferred through the milk of a lactating mother to her pup therefore the cortisol measured in pups may not only be a product of initiation of the stress response but a combination of the HPA activation and cortisol transferred during feeding (Sheriff et al. 2011). A study that examined the relationship between lactating domesticated rats (*Rattus norvegicus*) high milk GC levels and it's progenies ability to better adapt to stressors in their adult life found that individuals that consumed higher concentrations of the hormone had lower stress induced GC release and displayed reduced fearfulness to acute external stimuli (Catalani et al. 2000).The pups that we examined, however, would be past these phases of their development and a more likely cause of their heightened cortisol levels would be the introduction of new stressors that they have not yet been able to familiarize or habituate to.

Location

The Ulukhaktok, NT seals were sampled during the whelp and molt period, both highly stressful life history events that have high energy requirements (McLaren 1958) and would be highly likely to cause natural shifts in the baseline cortisol levels of ringed seals. Arviat seal samples were taken after the molt and during the period of open-water feeding and fattening prior to the beginning of the fasting season. Although seasonal timing of sample collection is likely the most influential factor affecting the variation in condition and cortisol levels observed other, differences between the regions and thus seal populations cannot be discounted.

High quality sea ice conditions, which include the presence of land fast ice, facilitate the species ability to overcome population stressors such as increased hunting mortality and decreased fecundity (Meier et al. 2004, Laidre et al. 2008). As the sea ice and surface temperatures change, the range of potential predators and competition for prey will shift possibly compromising the survival and health of ringed seals. The open water and warming temperatures have also led to the presence of an increased number of harbour (*Phoca vitulina*) and harp seals (*Pagophilus groenlandicus*) in the Hudson Bay. Although harbour seals generally feed at a higher trophic level than ringed seals (Young et al. 2010), their overlapping distribution could have negative implications for the immunologically naïve ringed seal populations. Harp seals have been attributed to the epizootics that have caused massive die offs of other seal species in Europe and Eastern Canada (Heide-Jorgensen et al. 1992). Exposure to diseases can have negative impacts on body condition by compromising the developing immune system in pups and forcing the reallocation of energy reserves from normal growth and development (Brock et al. 2013).

As the sea ice retreats, making it easier to expand resource exploration, a new host of concerns for the health of the ringed seal has arisen. The development of offshore oil and gas exploration can result in the alteration of habitat and produces noise pollution which can impact marine mammal behaviour (Alter et al. 2010). Offshore drilling results in increased shipping traffic and the risk of pollution from oil spills (Huntington 2009). When removed from the source of contamination, ringed seals have a high hydrocarbon clearance rate however initial exposure can result in a cortisol increase of up to 400% above the baseline blood circulating levels (Engelhardt 1982). Prolonged exposure will result in hyperactivity of the HPA axis and subsequently many negative health impacts including muscle wasting, growth and immune

function suppression and inhibition of reproduction (Sapolsky et al. 2000). The complexity and variability of the arctic ecosystem makes it is more susceptible to spills and more difficult to clean up than other regions (Huntington 2009). In the presence of an environmental disaster such as an oil spill or leak, the negative ramifications for the ringed seal would be far reaching. Industrial development, including the exploitation of oil, is a very real threat in the Beaufort Sea specifically. Although all 8 arctic border nations have pledged to advance with development in a sustainable manner (Pietri et al. 2008), ongoing sovereignty disputes lead to an uncertain future for development breadth and potential ramifications to the ecosystem.

Contaminants

Environmental pollutants have the potential to impact the physiology of ringed seals thus compromising their health, reproductive capacity and survival. Environmental contaminants, such as persistent organic pollutants (POPs) and heavy metals, bioaccumulate in the large lipid reserves found within the blubber layer (Brown et al. 2014). As this layer is metabolized, some contaminants remain thus enriching their concentration within the blubber (Agusa et al. 2011).

Exposure to certain POPs can alter the expression of the mRNA in genes responsible for the encoding of nuclear receptor proteins such as peroxisome proliferator activated receptors (PPARs) (Routti et al. 2010a, Castelli et al. 2014). These receptors are critical in the regulation of metabolism in high concentration and lipid storage is low (Desvergne et al. 2006). Recent studies into lipid mobilization have also shown a link between the rates at which these contaminants are released and the level of lipophilicity of the fatty acids (FA) present within the blubber layer (Louis et al. 2016). Contaminants and FA with higher lipophilicity were more prevalently retained within the layer (Louis et al. 2016) and within seals vary based on diet (Gaden et al. 2012) as feeding at higher volumes and in higher trophic levels increase exposure

(Fisk et al. 2001) and the length of the FA chain respectively (Jangaard and Ke 1968, Aubail et al. 2010, Aubail et al. 2011).

Even if animals are able to acclimate to chronic pollutants, exposure may impact the magnitude of a stress response when exposed to a stressor. Immunotoxic pollutants can lead to hypersensitivity and autoimmunity in seals (Kakuschke et al. 2005). Norris found that brown trout (*Salmo trutta*), with the same starting baseline GC levels, responded differently to the application of an acute stressor depending on whether or not they were initially from a contaminated site (Norris et al. 1999). Chronic exposure to heavy metals resulted in decreased ability to effectively respond to a stressor even though their starting GC levels were the same as control fish. Immunological issues in harbour porpoises (*Phocoena phocoena*) have been tied to high mercury levels resulting in increased susceptibility to infectious diseases (Bennett et al. 2001).

3.4.3 The Relationship between Condition and Cortisol

There is a logical and proven connection between cortisol and condition as defined in this study. The main function of adipose tissue is the uptake, storage and controlled release of lipids in all mammals (Pond 1992). Cortisol is a catabolic glucocorticoid meaning that it is responsible for the production of energy (Sterling and Eyer 1981) which is accomplished via gluconeogenesis, a process by which glucose is produced from metabolism of the lipid stores (Hiller-Sturmhofel and Bartke 1998, Peckett et al. 2011, Shero et al. 2015). During periods of fasting, cortisol is secreted in order to mobilize the lipids that are stored thus depleting the volume of the blubber layer. The consequences of chronic cortisol secretion could include muscle wasting, growth and immune system function suppression as well as reduced reproductive success (Sapolsky et al. 2000).

3.5 Conclusion

Ringed seal populations are likely to decline over time due to environmental stressors (ACIA 2004, Kelly et al. 2010b). These external stressors are present across the arctic and have already been shown to impact ringed seal condition (Harwood et al. 2000). Baseline cortisol concentrations will change over time in relation to chronic stressors and the individual population's ability to acclimate to the changes (Baker et al. 2013). The major changes to the ecosystem are due to man-made variables resulting in primarily, climate change which is responsible for the shifts related to sea ice condition, prey availability, exposure to contaminants.

There are issues with the interpretation of HPA axis activation and circulating cortisol levels related to sample collection, circadian rhythm and bound versus unbound cortisol (Otovic and Hutchinson 2015). What complicates our interpretation of cortisol stores in blubber is a lack of understanding of the mechanics of glucocorticoid sequestration, release and storage. It is difficult to conclude whether the changes in condition and cortisol concentrations are due to the physical location that the samples were collected from or what point in the life history cycle the seals were. Nonetheless, given the trends that we observed in concentration between the two communities, we can ascertain that our measurements are likely reflective of the circulating hormone levels in the blood during stressful life history events.

Circulating cortisol levels are naturally highest during the breeding and molt season and return to baseline in the fall (Ashwell et al. 1986, Myers et al. 2010) whereas condition is lowest during the summer and highest in the late fall (Smith and Stirling 1975, Young and Ferguson 2013). During the fasting period (June and early July) (McLaren 1958) when the ringed seals have poor condition status, cortisol is secreted at an increased rate (Riviere et al. 1977) therefore we can conclude that cortisol is inherently related to condition. During peak cortisol release,

there is a relationship between condition and cortisol, however, during times related to low cortisol, there is no apparent relationship. The variation demonstrated in the measurements from the period of time when cortisol levels should be at baseline is a testament to the variation between individuals and their stress response. The exception to predictability of condition in relation to cortisol concentration is in pups. The range of cortisol concentrations may be attributed in part to individual health and interpretation of the environmental stressors that are causing the shift in population averages over time. The cortisol stress response often correlates with the health of the animal and is used to assess stresses on the population (Romero 2004). Measuring stored cortisol over time is an effective method to demonstrate the impact of external stressors on the population as a whole.

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Chapter 4

4.1 Conclusion

The importance of expanding our knowledge of ringed seal physiological changes cannot be overstated. Ringed seals will be among the first arctic marine mammals to experience the impact of climate change due to their life history dependence on sea ice (Moore and Huntington 2008, Martinez-Bakker et al. 2013), however, their adaptability to prey shifts coupled with their wide range and distribution have led to the perception that the species is at relatively less risk (Laidre 2008). Whether or not their physical adaptability to environmental change translates to efficient physiological adaptation is unknown. Whether they are able to maintain allostasis in the presence of these expanding stressors will dictate the extent that these negative impacts have on ringed seal reproductive and survival potential. This thesis aimed to expand that knowledge base in order to facilitate more informed and accurate conservation and management decisions all while minimizing the requirements for expanded sampling practices.

It has been established that chronic stress can be studied by examining concentrations of cortisol in fur and this study aimed to determine whether blubber could be used as a proxy for this assessment. We determined that blubber can be used to assess changes in chronic stress level, however, it cannot be confirmed whether the measurements that we obtained are a reflection of an increase in baseline circulating hormone levels at the time of sampling. The relationship between fur and blubber cortisol levels in juvenile seals suggests that although we cannot definitively conclude that cortisol concentrations in blubber mirror the circulating concentration, there is likely a relationship between the two.

When choosing an extraction and analytical technique, factors that need to be taken into account should include accessibility to equipment and the matrix that the hormone is being collected from. In order to avoid inaccurate results, extraction methods should ensure that a

sample is free of biological material that may interfere with measurement of the desired analyte as well as avoid analyte loss. Likewise, analytical techniques should be chosen based on consistency of and ability to overcome interference factors that may compromise results. For the analysis of cortisol from blubber the acetonitrile based extraction method provided the most consistency between analytical methods while minimizing the suppression of the returns from the LC MS/MS. RIA analysis resulted in greater variation between the concentrations measured in duplicate samples extracted under different measures, however, the methanol extracted sample had about half of the return of the acetonitrile.

The condition and chronic stress levels of ringed seals are changing over time, however, I was unable to pinpoint the causality of the observed changes in condition and stress. The condition of seals from Ulukhaktok was significantly lower than Arviat but these findings were likely a reflection of the effect of season rather than location on blubber thickness. There is a relationship between the stored cortisol levels and condition in seals that have recently completed a molt and in pups, however, there is no such relationship prior to the winter fast in adult and juveniles. Interpretation of these findings is difficult without understanding how cortisol is sequestered, stored and released from blubber tissue. Future studies should include developing a better understanding of these mechanisms.

Limiting factors to arctic marine mammal research include cost, labour intensity, jurisdictional issues (Pietri et al. 2008, Berkman and Young 2009) and safety (Ford et al. 2006). Maintaining, expanding and fostering the working relationships between researchers and Inuit communities is necessary, not only to reduce the impact of these factors by continuing to have access to community hunted samples but also to better understand the changes to the environment and arctic animals in real time. The impacts of climate change on the Inuit people's

quality of life are disproportionate to other Canadians (Morse 2010). As such, northern communities have a vested interest in and appreciation for sustainable wildlife populations and the development of research collaborations (Gearheard and Shirley 2007, Brook et al. 2009). The psychological and social well-being of the Inuit community has been tied to the practices of hunting and food sharing, which translates to the ability for a community to be self-sufficient and individuals to be capable to provide for their own (Borre 1994). Their use of the land is not only for subsistence but is also linked to their culture and identity. Cooperation with the Inuit communities is an integral component of the interdisciplinary approach to arctic research and respect for the connection between the land and the people is imperative. Respect is shown by not only communicating our findings with the communities but also using what they have already provided us with to learn as much as possible about what is happening to the land and animals that their livelihoods depend on. The benefits to developing novel methods to use the thousands of archived samples already made available to the Canadian government by the Inuit people are ecologically, financially and socially abundant.

The findings of this study are an important first step in developing an understanding of how this ice obligate species has and may respond to the increasing magnitude of environmental stressors and is critical to the development of conservation strategies for this species.

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