Variation in blubber cortisol as a measure of stress in beluga whales of the Canadian Arctic

By

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Abstract

Exposure to stressors in free-living mammals can be measured via glucocorticoid hormones (i.e.cortisol) concentrations collected from a variety of tissues. Adipose tissue in mammals acts as a reservoir storing various hormones released from the vascular system over time. Using adipose from marine mammals (blubber) as a tissue for extracting cortisol, a known stress hormone in mammals, provides a means for measuring cortisol concentrations not associated with capture stress. Beluga whale (*Delphinapterus leucas*) range is limited to the Arctic where recent changes in climate are exaggerated and historic commercial hunting practices have reduced certain beluga whale populations. Our objectives were to 1) compare cortisol concentration differences among archived blubber samples with varying quality and with blubber depth, 2) compare blubber cortisol from beluga whales in a high-stress entrapment event to whales harvested during seasonal subsistence hunts, and 3) compare blubber cortisol among beluga whale populations in relation to conservation status, diet, sex, age and year sampled. Blubber samples that showed signs of deterioration (yellowed) had lower cortisol concentrations, indicating low quality samples should be excluded for most reliable and repeatable results. The deepest blubber, nearest the muscle, contained the highest concentration of cortisol compared to the middle and outer depths, suggesting the inner depth as a reliable and repeatable measure of comparative cortisol values. Blubber cortisol concentrations from entrapped whales were 7 times higher than concentrations from seasonal harvests, indicating blubber cortisol concentration is useful as a measure of high stress. Blubber cortisol was higher in the threatened Cumberland Sound population when compared to three other healthy populations, supporting blubber cortisol as a measure of population health.

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Thesis Format

The following thesis is in manuscript format. I wrote chapters 1, 2 and 3 as individual manuscripts each with their own abstract, introduction, methods, results, discussion, references and figures. The overall introduction and conclusion include a summary of the background and findings of my research beyond what is included in each chapter. I used first person plural in this thesis in preparation for publications with my co-authors. I also used log-transformed cortisol values in all figures to match the accompanying data analysis. All untransformed values are listed in the text for comparison and application to future studies.

Northern communities collected all blubber samples during their subsistence hunts. These samples were stored at Fisheries and Oceans Canada until I accessed them for the purpose of research for this thesis. I performed all hormone analysis, including extractions and radioimmunoassay. I prepared all samples for stable isotope analysis including sample selection, drying and homogenization and sent samples to the Chemical Tracers Lab at the University of Windsor (Windsor, ON). Beluga whale age obtained from a single tooth cementum or dentine growth layer group, sex and other biological data were obtained through various labs at Fisheries and Oceans Canada (Winnipeg, MB). I performed all data analysis and wrote all chapters with guidance of my committee.

Thesis Introduction

Recent changes in the Arctic include decreases in seasonal sea ice extent (2.7 ± 0.5% per decade) (Parkinson and Cavalieri 2002, Parkinson et al. 1999; Parkinson et al. 2008), thinning of sea ice (Rothrock et al. 1999), declining perennial ice (mutli-year ice) (Comiso 2002) and warming (Comiso 2003). As the ice melts, retreats, and thins, solar radiation is absorbed and further warming occurs (Parkinson et al. 2008). This warming process is projected to have cascading effects on arctic communities including plants, invertebrates and marine mammals (Post et al. 2009). Physical changes in the abiotic factors of the Arctic will likely affect marine organisms indirectly through changes in prey availability. From 1980-2002, Gaston et al. 2003, identified a shift in prey availability from arctic cod to capelin in northern Hudson Bay by assessing the diet of thick-billed murres. Climate change will also affect marine organisms by increasing access to historically seasonal predators or predators that previously resided in southern distributions. Higdon and Ferguson (2009) quantified the number of killer whale sightings in Hudson Bay and found an exponential increase over the past century. Lastly, marine organisms will likely be exposed to increased human activity and noise, which is known to affect whale feeding and behavior (Kilabuk 1998).

Beluga whales (*Delphinapterus leucas*) are medium sized odontocetes in the family monodontidae and are abundant throughout many areas of the Arctic. Beluga whales are long-lived (60-80 years). They breed in early spring (April – June), gestation is about 15 months and they give birth in July- August. They reproduce on average every 3 years nursing calves for about 2 years. Their range is limited to the Arctic and Subarctic, where climate change is most apparent (Post et al. 2009). They live in close association with sea ice and use sea ice for shelter and refuge from predators (Huntington 1998; Heide-Jørgensen et al. 2009). Most beluga whales migrate each year from estuaries and rivers to areas of moving ice flow edges during winter, while some

beluga whale populations have short or no migration. Regardless of migration distance, beluga whales are ice-associated species and spend much of their life surrounded by sea ice. They move in accordance with the fluctuations of sea ice. According to fossil records, beluga whales have persisted through ice-free periods in the past (O'Corry-Crowe et al. 2010); however, the current rate of sea-ice decrease in the Arctic is unparalleled. This rate of change leads to questions about whether beluga whales can endure ice-free periods and the collapse of ice-obligate prey bases. Although studies of beluga whale stomach contents have shown a fairly diverse array of prey items (McLeod et al. 2008), other studies using stable isotope and fatty acid analysis have shown beluga whales select for arctic cod (Loseto et al. 2009, Marcoux et al. 2012), a more energy-dense prey within the arctic ecosystem (Harter et al. 2013). The most adaptive response would be to switch to alternative prey sources but energetic demands of marine mammals are highly debated (Lavigne et al. 1985) and therefore a dietary shift may not be sustainable. Beluga whales are a long-lived K-strategist species that adapts slowly. Beluga whales reproduce every 2-3 years, with females reaching breeding maturity at ~8 years and males at ~13 years (Luque et al. 2007). If the Arctic continues to warm at the projected rate (Comiso 2002), some beluga whales alive today will witness the first ice-free summer in the Arctic.

Threats from an organism's environment trigger a stress response system intended to help an individual respond to a stressor and increase survival (Romero and Butler 2007). In vertebrates, the stress response activates the hypothalamus-pituitary-adrenal axis (HPA), which responds to the stressor by producing and/or releasing hormones (glucocorticoids, mineralcorticoids and catecholamines) from specific organs (Romero and Butler 2007). The response includes the release of corticotrophin-releasing hormone (CRH, released within a few seconds) from the hypothalamus, followed by a release of adrenocorticotropic hormone (ACTH, released within 10 seconds) from the

anterior pituitary, and finally the secretion of glucocorticoids (cortisol, cortisone, and corticosterone, released within minutes) from the adrenal cortex (Thompson and Geraci 1986, Sapolsky et al. 2000, Romero and Butler 2007). The stress response system is advantageous for temporary stress (acute), but when faced with prolonged stressors (chronic), the continued release of glucocorticoids has deleterious effects including muscle wasting, bone thinning, lowered immune system, neuronal damage, decreased reproduction and if left unchecked, death (Clark et al. 2006). Chronic stress can often result in profound effects on individual organisms, populations and ultimately ecosystems (Romero 2004).

By measuring hormones associated with the stress response system, ecologists can compare stress hormone levels between threatened and healthy populations and among areas with low and accelerated sea ice loss in order to identify areas of most concern. Cortisol is an established indicator of adrenal activity related to stress in marine mammals (Thompson and Geraci 1986; Romero and Butler 2007). If changes in cortisol are monitored over time, ecologists may be able to use beluga whale blubber cortisol as a measure of the impact of these changes to other areas of the biotic community.

Current measures of cortisol in tissues of marine mammals have been limited to blood, feces, saliva and hair (Amaral 2010). However, these measures require capture (blood, saliva and hair), are affected by capture stress (blood and saliva), are difficult to obtain (feces), or are impossible to obtain from whales (hair) (Amaral 2010). To date, no published research exists on blubber cortisol concentrations in marine mammals. Blubber cortisol concentrations can be obtained non-lethally with a remote biopsy dart and are unlikely to reflect stress associated with collection (Kellar et al. 2006).

The objective of this thesis is to introduce blubber as a tissue for cortisol extraction and to apply the use of blubber cortisol to examine the ecology of beluga

whales from several areas throughout the Canadian Arctic. Our hope is that ecologists will be able to apply this research on a greater scale to other marine mammals.

In the first chapter, we use a method from Kellar (2006) for progesterone hormone extraction from blubber to obtain cortisol concentrations, a known measure of stress in marine mammals (Thompson and Geraci 1986; Romero and Butler 2007), from archived beluga whale blubber collected from Inuit subsistence harvests (Appendix I). Next we examine cortisol degradation in archived blubber samples to identify degraded samples for exclusion or control in further studies because degraded samples could suggest false trends (Amaral 2010). Finally, we examine variation in cortisol concentrations due to blubber collection depth. Blubber composition varies with depth (Krahn et al. 2004; Struntz et al. 2004), suggesting cortisol may vary as well.

Chapter 2 applies the methods from chapter 1 to compare cortisol concentrations in whales from a high stress event (ice entrapment) to whales from a healthy group (seasonally harvested whales) to validate blubber cortisol as a reflector of long-term stress in beluga whales. No measure of high stress blubber cortisol currently exists and it is important to establish high and low levels of cortisol for comparison (Amaral 2010). We also test for differences between sexes in trapped whales and among ages in both trapped whales and healthy whales. Age and sex are important factors affecting cortisol levels across many species (Drafta et al. 1982; Sapolsky et al. 1984; Nicolson et al. 1997; Kudielka et al. 2004).

Chapter 3 applies the previous two chapters' findings to compare cortisol concentrations in healthy beluga whale populations with a threatened population to discover whether population status is reflected in blubber cortisol levels. We also tested for dietary effects using stable isotope analysis of skin on blubber cortisol concentration in all populations. Dietary shifts resulting in increased starvation risk will likely result in

the release of glucocorticoids (Romero and Butler 2007). As with chapter 2, we also tested for the effect of age and sex on cortisol concentration in all populations.

This thesis provides information on the use of blubber as a tissue for cortisol extraction and tests factors that may influence cortisol concentration. We examine the use of blubber cortisol in beluga whales because of their distribution throughout the Arctic, their history of commercial exploitation, and relatively high abundance. With the dramatic changes occurring in the Arctic, we expect the results of this thesis to help contribute to the understanding of marine ecosystems in response to this unparalleled change.

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Chapter 1: Influence of sample degradation in archived blubber samples and variation in blubber cortisol concentrations with blubber depth in beluga whales

Abstract

Various biological tissues have been used to assess cortisol concentrations in animals such that a short-term to long-term estimate of activation of the physiological stress response can be obtained. In recent years, blubber has been demonstrated to be a good candidate tissue from which to extract steroid hormones. However, the effects of sample storage and variation in blubber depth on glucocorticoid hormone concentrations are unknown. Our objective was to identify a method for extracting cortisol from blubber in beluga whales (*Delphinapterus leucas*) and to evaluate the concentration of hormones in relation to blubber depth and degradation from long-term storage. We extracted cortisol from blubber of beluga whales by modifying an existing progesterone-blubber extraction method. Visible degradation of samples increased with time spent in storage. Cortisol concentrations were lower in degraded samples, but time in storage did not affect cortisol after controlling for sample quality. Cortisol concentrations increased with blubber depth, with highest concentrations in blubber closest to the muscle. These results show that cortisol in blubber samples collected and archived prior to extraction may be degraded and should be used with caution. However, high quality samples without visible degradation after long-term storage can still yield useful measures of cortisol concentration. Additionally, sample depth should be considered and controlled for during sample collection. Our findings provide necessary information for developing accurate sampling protocols for extracting cortisol from blubber of marine mammals, including sampling by biopsy dart.

Introduction

An organism's ability to acclimate to various challenges in their environment drives individual survival and ultimately the adaptation of a species (Boonstra 2005). A

complex neuroendocrine system persistent in all vertebrates stimulates the release of a series of hormones ultimately mobilizing energy stores and triggering specific behaviors during periods when a stressor is perceived (Boonstra 2004). Exposure to short-term stressors and the subsequent release of associated hormones (e.g. glucocorticoids) are often beneficial for organisms (i.e. acute stress) (Möstl and Palme 2002). However, if the stressor persists and related hormones continue to be released (i.e. chronic stress) the stress response system breaks down tissues and organs hindering vital functions, ultimately causing illness, decreased reproduction and death (Boonstra et al. 1998, Boonstra 2005).

By measuring glucocorticoids in tissues that reflect long-term exposure to a stressor, it is possible to monitor changes in the stress experienced by individuals over a period of time (Davenport et al. 2006; Bortolotti et al. 2008; Saco et al. 2008; Okuliarova et al. 2010;). Stress hormones reflect the amount of disruption in an organism's homeostasis (Hennessy et al. 1979; Reeder and Kramer 2005) and more broadly, may be a measure of population health. They offer a valuable tool for measuring health in ecological systems where adverse change is evident.

In the Arctic, recent decreases in seasonal sea ice extent (Parkinson and Cavalieri 2002) and increased warming (Comiso 2003) have begun to affect certain species and are expected to have cascading effects on surrounding communities (Post et al. 2009). Physical changes in the Arctic will likely affect ice-adapted whales, like belugas (*Delphinapterus leucas*), indirectly through changes in prey availability (Gaston et al. 2003), increased predator access (Higdon and Ferguson 2009) and increased human development (Kilabuk 1998).The range of beluga whales is limited to the Arctic and Subarctic, where climate change is most apparent. They live in close association with sea ice and use sea ice for shelter and refuge from predators (Huntington 1998). Beluga whales prefer fish such as arctic cod (Loseto et al. 2009; Marcoux et al. 2012),

an ice-associated species that has decreased in areas where sea ice has decreased (Haug et al. 2013; Hop and Gjøsæter 2013). The killer whale (*Orcinus orca*) is the main predator of beluga whales and sightings have increased in certain areas of the Arctic (Higdon and Ferguson 2009; Ferguson et al. 2011; Higdon et al. 2012).

Measuring concentrations of cortisol, a hormone associated with stress in mammals, in tissues of cetaceans has been limited to blood, feces, and saliva (Amaral 2010). However, collecting blood and saliva requires capture and often reflects capture stress, while the collection of feces, although non-invasive, is often difficult and samples can become contaminated by sea water during collection (Amaral 2010). Blubber may be collected non-lethally with the use of remote biopsy and is unlikely to reflect stress associated with collection (Kellar et al. 2006). Blubber, like hair, may reflect stress over a longer period of time and show whether prolonged exposure to these potential stressors are correlated with a long term increase in so-called stress hormones such as cortisol. However, despite reported measurement of other steroid hormones (progestins, androgens, and estrogens) from blubber of cetaceans (Amaral 2010) there are currently no published studies describing extraction and measurement of cortisol from blubber.

Archiving samples for future analyses is useful only when the effects of degradation are understood (Amaral 2010), as the use of degraded samples may suggest false trends in cortisol levels. In marine mammals, blubber composition varies with depth, with changes in lipid concentration with depth as well as structural differences in fatty acids, adipocytes and connective tissue (Krahn et al. 2004; Struntz et al. 2004). These differences have been attributed to the metabolism and turnover of adipose tissue in mammals (Struntz et al. 2004). For example, fatty acid samples from blubber closest to the muscle consist of long and short carbon chains, reflecting prey fatty acids and the area of metabolic activity, whereas shallow blubber depths are an accumulation of shorter chain carbon fatty acids that are not readily broken down and

function as a structural molecule (Koopman et al. 1996). Understanding exactly how cortisol concentrations vary with blubber depth will help interpret results and optimize future sampling protocols.

In this study we modified a method for extracting progesterone in blubber (Kellar et al. 2006) to accommodate cortisol in blubber of beluga whales. We then examined the influence of tissue degradation on cortisol levels in archived blubber samples and assessed changes in concentration of cortisol in blubber in relation to depth from skin.

Methods

Sample collection – All blubber samples were collected during Inuit subsistence hunts (for details see Harwood et al. 2002) and archived in -40˚C freezers at Fisheries and Oceans Canada, Winnipeg, Manitoba. We examined the effects of degradation on cortisol concentration using 929 samples (500 males, 256 females and 173 unknown sex; ages 1-64 yrs) collected throughout the Canadian Arctic (Eastern Beaufort Sea, Eastern High Arctic, Western Hudson Bay and Cumberland Sound) and we ranked quality of the blubber samples according to tissue color: 1) orange, brown or red (lowest quality), 2) yellow, 3) yellow edges, pink in center, and 4) pink (highest quality). The samples for our research were collected as a single 1 gram (g) sub-sample through all blubber depths from each individual.

We investigated the effect of blubber depth (distal to proximal) on cortisol concentration. We chose to investigate depth from skin using samples collected from the subsistence harvest of the Eastern Beaufort Sea beluga whale population by Inuit communities near Hendrickson Island, Northwest Territories (See Chapter 3; Figure 3.1, Lat: 69˚29', Long: -133˚35') to control for possible intra-population effects. We subsampled for blubber depth effects by removing nine 1g samples from each individual hunter collected sample, collected in a 3 x 3 grid pattern to examine both vertical and

horizontal variation in cortisol (i.e., based on depth or position; Figure 1.1). We removed outer edges of all blubber sub-sections to avoid potential contamination. We placed each 1g sample in a weighed and tared 15mL plastic vial and freeze-dried them for 72 hours to remove water.

Sample extraction – We modified the extraction method used for blubber progesterone in Kellar et al. (2006) to accommodate a larger sample mass, which was necessary to obtain detectable levels of cortisol. We added 2mL of anhydrous ethanol to each 1g sample. We vortexed the sample for 30 seconds, pressed the sample with a metal rod to separate connective/vascular tissue from lipid, removed the ethanol-lipid mixture and transferred it to a 15mL glass vial. We rinsed the remaining tissue with an additional 2mL of ethanol and added the rinse to the 15mL glass vial, leaving the connective/vascular tissue behind. We vortexed the lipid-ethanol mixture for 2 minutes, centrifuged the mixture (3,000rcf, room temperature, 10 min), and placed the sample in a nitrogen evaporator until all ethanol was removed and only lipid remained. We added 2mL of ethanol: acetone mix (4:1), vortexed the samples for 2 minutes and then centrifuged (3,000rcf, room temperature, 10 min) and evaporated as before. We added 1mL of ethyl ether to the remaining lipid layer, vortexed for 2 minutes, centrifuged (3,000rcf, room temperature, 15 min) and evaporated as before. We added 1mL of acetonitrile to the remaining lipid layer, vortexed for 5 minutes and added 2mL of hexane to the acetonitrile-lipid sample mixture. We vortexed the mixture for 2 minutes and then centrifuged (3,000rcf, room temperature, 20 min). We removed the hexane (top layer) with a glass pipette and added an additional 2mL of hexane to the acetonitrile-lipid mixture. We vortexed the mixture for 2 minutes, centrifuged (3,000rcf, room temperature, 20 min) and removed and discarded the hexane again. We collected the acetonitrile layer, placed it in a plastic 2mL micro-centrifuge tube and evaporated it to dryness using

a nitrogen evaporator. We placed the evaporated samples in a -40°C freezer until measurement.

Radioimmunoassay– We added 250µL of a RIA buffer composed of 10mL phosphate buffer (71.6g $Na_2HPO_4.2H_2O$, 15.3g $NaH_2PO_4.2H_2$ in 1liter milli-Q (ultrapure) water), 0.9g NaCl, 0.5g Bovine Serum Albumin and 90ml milli-Q water to each extracted sample residue and vortexed the sample for several minutes until it was dissolved into solution. We prepared each sample tube with 100µL of sample mixture and 100µL tritiated cortisol (5000 disintegrations per minute, dpm) (PerkinElmer, Waltham, Massachusetts). We mixed labeled cortisol and the sample briefly before adding 100µL of 1:3200 dilution of cortisol antibody (Fitzgerald Industries, Acton, Massachusetts, product code 20-CR50). 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. We incubated the mixture for 1 hour at room temperature and then at 4°C for a minimum of 12 hours. After incubation we added 100µL of a charcoal-dextran buffer solution (2.5g charcoal, 0.25g dextran in 50mL RIA buffer) to the mixture, vortexed the mixture for 15 seconds, incubated the charcoal-sample mixture for 15 minutes at room temperature before centrifuging samples at 4°C (2500rpm for 30 minutes). The supernatant was poured into scintillation vials and 4mL of scintillation fluid (Ultima Gold, PerkinElmer, Waltham, Massachusetts, USA) was added to the vial containing the sample. The vial was gently mixed and counted for radioactivity for 5 minutes in a scintillation counter (Tri-Carb® 3110TR, Perkin Elmer INC, Waltham, Massachusetts, USA). Each assay contained a standard concentration curve measured in triplicate of 10 concentrations ranging from 0.05 to 25ng/mL. We ran each sample in duplicate.

Validation - Extraction efficiency was measured by adding a known (250,000dmp) amount of radioactively labeled cortisol to the sample prior to extraction. Samples were

counted after extraction and remaining labeled cortisol was measured. The mean extraction efficiency was 77% ± 4%; we measured inter-assay variation by including a pooled sample in each of 22 assays and we measured the intra-assay variation by including 20 pooled samples within one assay. We took the mean of the pooled sample groups over the standard deviation and expressed the value as a percent to show sampling variability among samples. The inter-assay and intra-assay coefficients of variation were 14% (n = 22) and 6% (n = 20) respectively. We tested for parallelism and found the native cortisol and the standard cortisol to be parallel (Figure 1.2). We also examined sample color quench to control for any cloudiness in the samples by double diluting a sample pool spiked with 260,000dpm cortisol in triplicate for 10 dilutions and then measuring the difference between each dilution and a blank containing buffer only. The most concentrated samples were color quenched by less than 1% (Figure 1.3).

Data analysis – Statistical analyses were performed in JMP® 10 (SAS Institute Inc. 2012). Cortisol concentrations were log-transformed to improve normality. We investigated the effect of time in storage on sample quality using an ordinal logistic regression, and examined the effect of sample quality and time spent in storage on cortisol concentration using a general linear model, followed by Tukey Kramer's HSD post hoc test to compare quality levels. We used an ANOVA to compare cortisol concentrations among blubber depths and positions while accounting for individual, followed by Tukey Kramer's HSD post hoc test for comparisons of blubber depth.

Results

Sample quality decreased with increasing time in storage (Figure 1.4; ordinal logistic regression, df = 1, $X^2 = 363.65$, $p < 0.0001$) and affected cortisol concentrations (Figure 1.5; $F_{3,912} = 9.07$, $p < 0.0001$). Samples of highest quality (rank 4, no degradation) had higher cortisol concentrations (0.43 ± 0.02ng/g) than ranks with more degradation (rank 1: 0.31 ± 0.05ng/g, rank 2: 0.34 ± 0.03ng/g, rank 3: 0.30 ± 0.02ng/g)

(Tukey Kramer's HSD, $p < 0.0081$), which did not differ from each other (all $p > 0.91$). However, time in storage did not affect cortisol when the effect of sample quality was controlled for in the same model ($F_{4,912} = 0.19$, $p = 0.67$).

Cortisol concentrations differed among blubber depths (vertical sections) (Figure 1.6; $F_{28, 214} = 93.41$, $p < 0.0001$), with mean cortisol concentration higher in the inner depth (closest to the muscle, 0.49 ± 0.11ng/g) (Tukey-Kramer HSD, $p < 0.0001$) compared to the middle (0.33 ± 0.08ng/g) and outer depths (0.31 ± 0.06ng/g) (closest to skin) ($p \leq 0.015$). The range of cortisol concentrations was 0.036 – 4.3ng/g in the inner depth, 0.043 – 3.2ng/g in the middle section, and 0.049 – 2.0ng/g in the outer depth. We found no difference in cortisol concentration among positions (horizontal sections) (ANOVA, $F_{28, 214} = 1.71$, $p = 0.184$).

Discussion

Our results indicate that blubber samples archived over years prior to extraction should be assessed for sample degradation prior to testing for ecological patterns. Many metabolites degrade over time due to various processes (Elliott and Peakman 2008) and although freezing decreases degradation, cortisol extracted from frozen saliva samples still degraded after one year (Garde and Hansen 2005). Additionally, fecal samples contain bacteria that metabolize hormones within hours after defecation (Khan et al. 2002). Immediate extraction of cortisol followed by archival may preserve blubber cortisol more effectively over a longer storage time and would be worth testing.

Steroid hormones enter and accumulate in adipose tissue by diffusion (Mead 1963, Dolezel et al. 1991) and are metabolized slowly when compared to other tissues (Kellar et al. 2006). Little is known about incorporation and depletion rates of hormones with blubber depth, and rates likely vary among life stages and seasons. Effects of blubber depth on fatty acids, contaminants and adipocyte size are due in part to differences in metabolic activity with blubber depth (Struntz et al. 2004). Blubber

thickness in beluga whales varies seasonally with foraging habits, increasing from 8cm in spring to 30cm after summer (Huntington 2000). Progesterone concentrations in dolphins, do not change with blubber depth (Kellar et al. 2006), while blubber depth effects on contaminants vary among species (Krahn et al. 2004). Changes we found in blubber cortisol with depth suggest a complexity worth considering. To avoid depth effects in studies examining blubber cortisol, samples should be taken through the entire blubber column uniformly or consistently from the same depth. Based on our results, samples taken from the inner blubber depth would likely provide the most robust data, accommodating both samples containing low concentrations and the effect of blubber depth.

In humans, cortisol concentrations can differ between sexes, with age playing an important role in how differences occur (Kudielka and Kirschbaum 2005). It is possible that differences in cortisol between sexes and among ages exist. However, our sample set contained few females because of harvest biases toward males so we reserved sex and age differences in blubber cortisol of beluga whales for a more complete data set. In dolphins, Kellar et al. (2006) found homogeneity of progesterone concentration with sampling locations on the body. This study also showed no difference in hormone concentrations with blubber depth, suggesting a difference between whale species or a difference in progesterone and cortisol incorporation or metabolization within blubber. It is important to know if cortisol varies with sample location in order to assess differences due to other factors. Additionally, cortisol may vary seasonally and with reproductive status as within blood of some marine mammals (Gardner and Hall 1997) and is worth investigation in blubber cortisol in order to improve our understanding of blubber cortisol variability. Additional measures of blubber cortisol associated with exposure to high and low stress scenarios would be useful for providing concentration reference points.

Our results support the use of blubber for obtaining cortisol concentration and provide useful information on controlling for quality, degradation, and variation along sample depth in archived samples. After considering sample quality and depth, the measurement of blubber cortisol levels adds an additional method for collecting data indicating individual and population health in response to stressors in beluga whales and other marine mammals.

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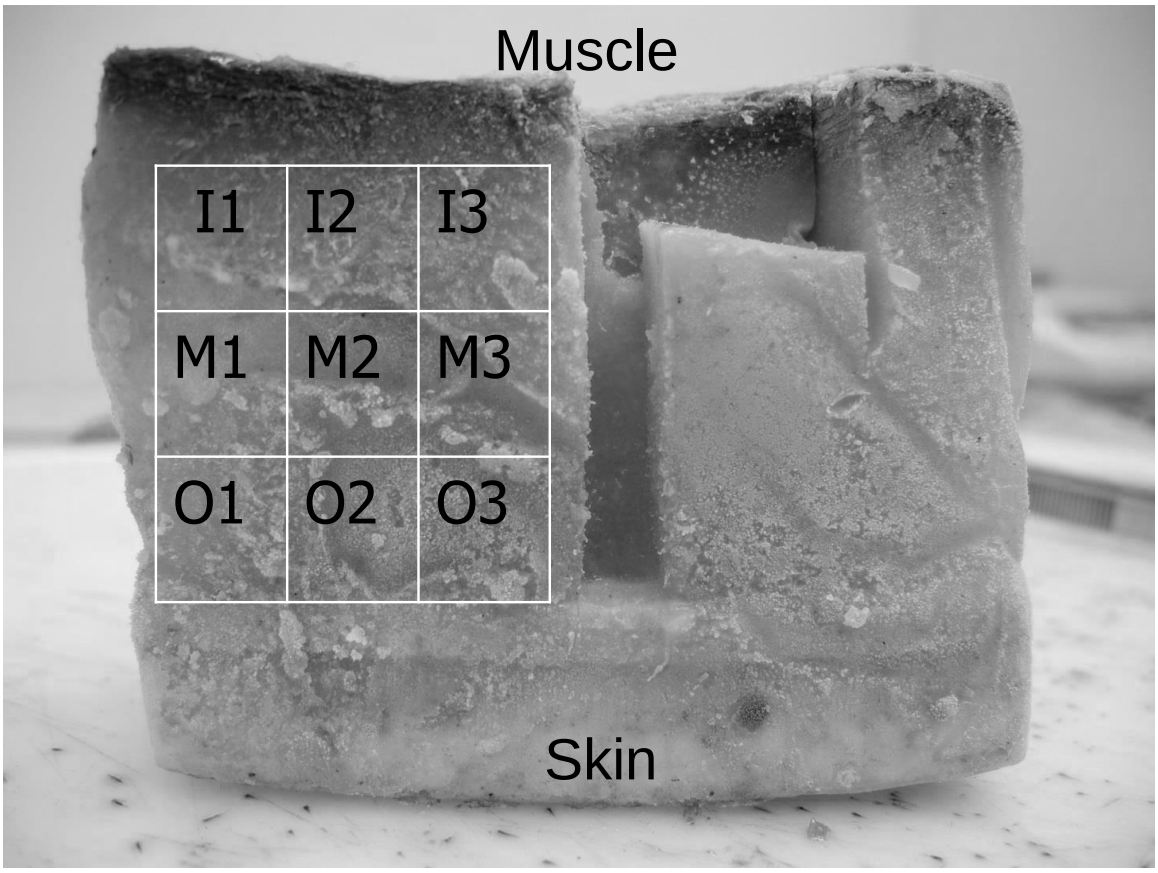

Figure 1.1. Beluga whale (*Delphinapterus leucas*) blubber depth sub-sampling method. Sample depth noted by letters (I = inner, M = middle and O = outer) and sample positions noted by numbers (1-3).

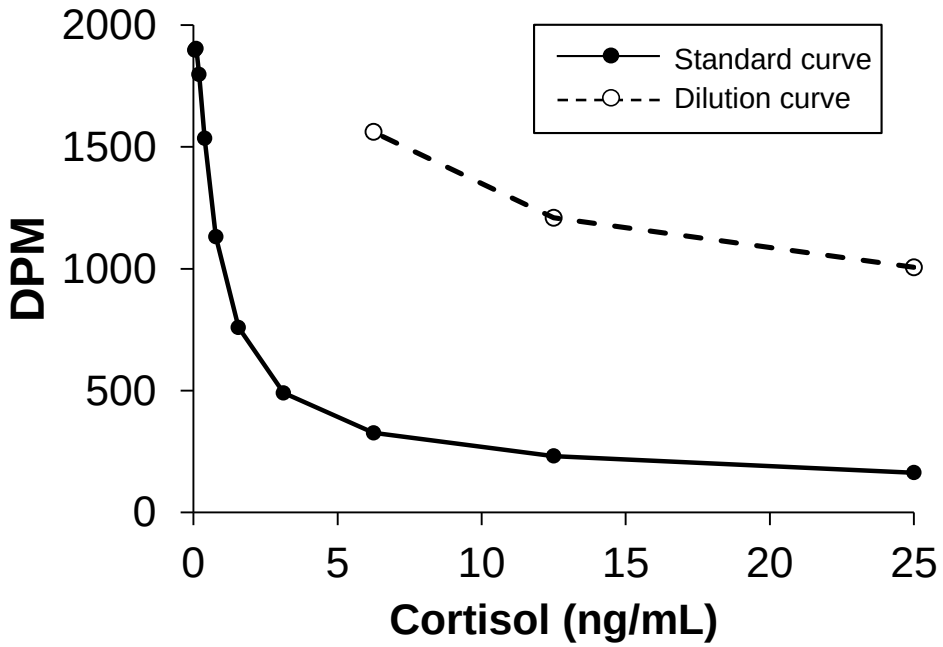

Figure 1.2. Disintegrations per minute (DPM) and cortisol (ng/g). Standard curve ranging from 0.05 to 25ng/mL compared to a three-point dilution curve of 0.36 to 1 ng/mL. The approximately parallel lines represent the lack of cross reactivity.

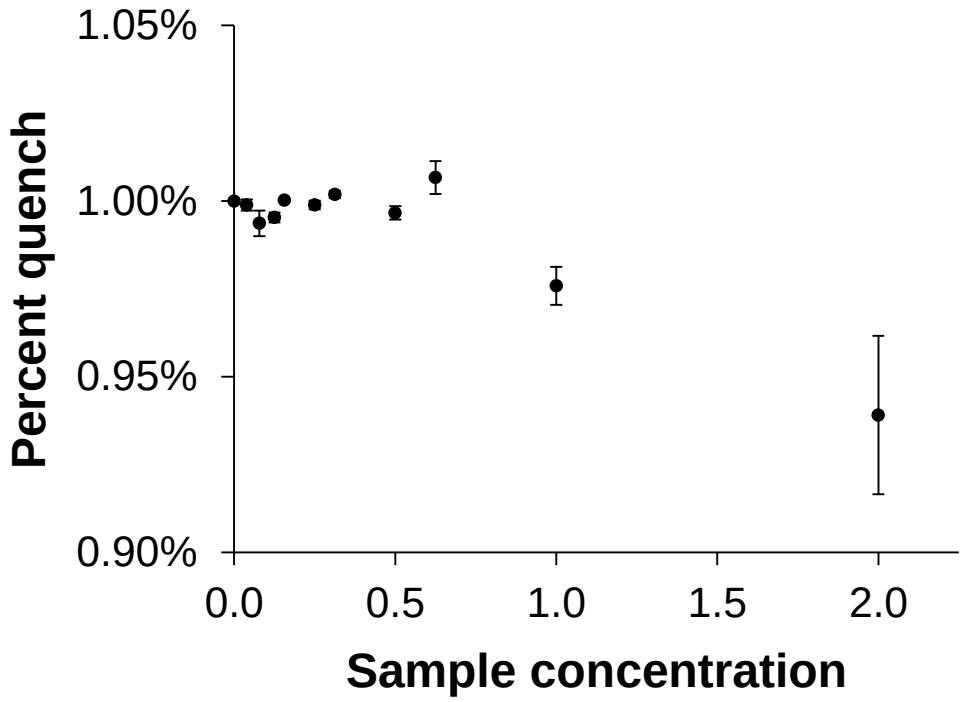

Figure 1.3. Percent quench and sample concentration (2.0 represents twice the amount of an average sample while the point at 0.0 represents a blank). Points represent the mean ± SE of each of the three trials.

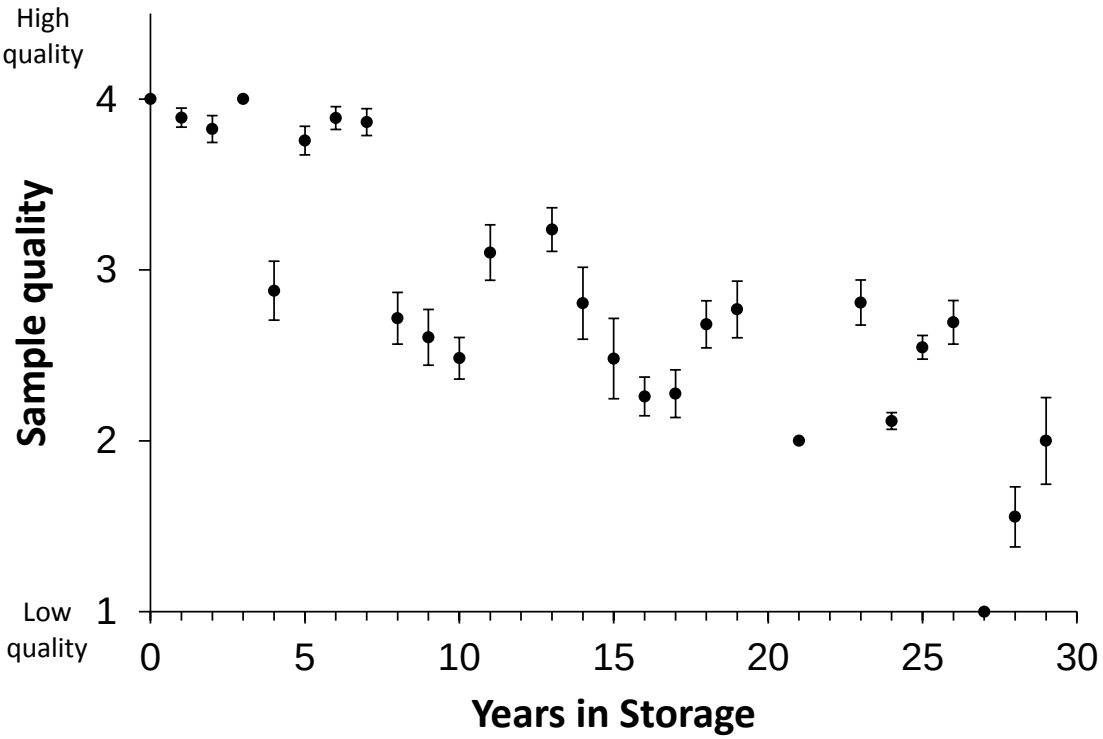

Figure 1.4. Quality of beluga whale (*Delphinapterus leucas*) blubber samples (mean ± SE, log-transformed ng/g; 1 lowest quality, 4 highest quality) decreased with time spent in storage.

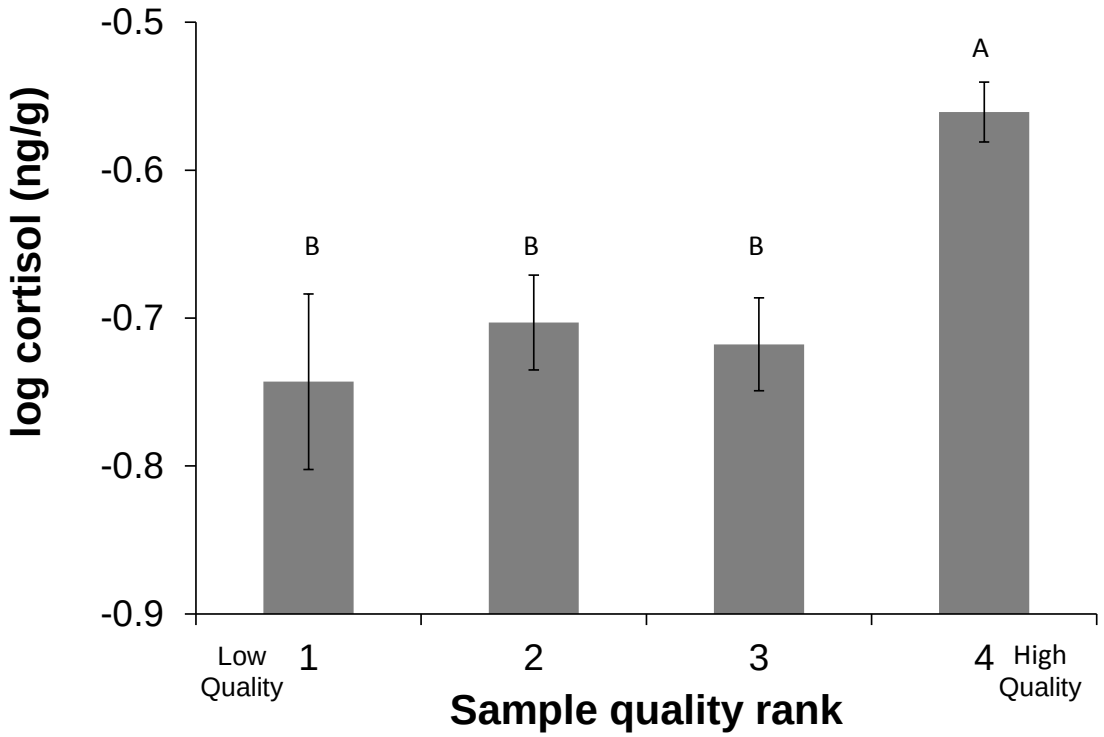

Figure 1.5. Effect of sample quality (1 lowest, 4 highest) on cortisol concentration (mean ± SE, log-transformed ng/g) in beluga whale (*Delphinapterus leucas*) blubber. Ranks with the same letter did not differ (n = 917).

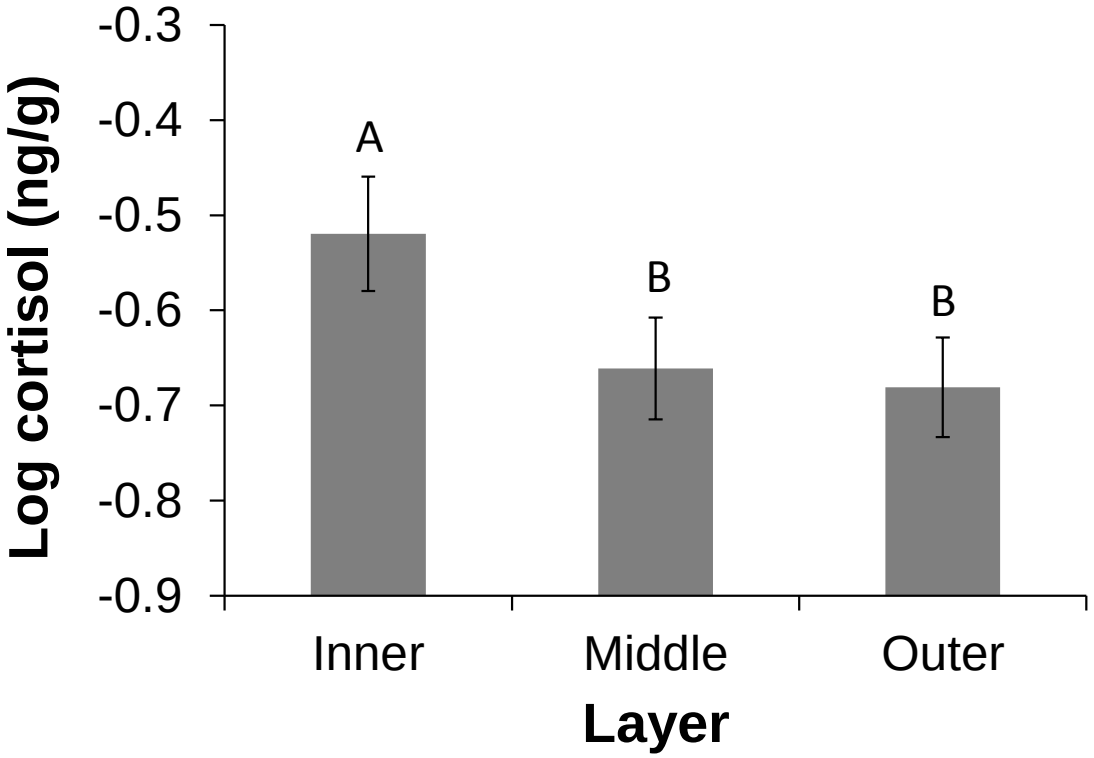

Figure 1.6. Cortisol concentrations (mean ± SE of log-transformed ng/g) in beluga whale (*Delphinapterus leucas*) blubber were higher in the depth closest to muscle (inner) compared to the shallow depth closest to skin (outer). Depths with the same letter did not differ (n = 917).

Chapter 2: Increased blubber cortisol in ice-entrapped beluga whales

Abstract

Entrapments of whales in sea ice occur occasionally in the Arctic and often last several weeks, resulting in emaciation or death of whales. These highly stressful events provide a unique opportunity for investigating the dynamics of stress physiology in otherwise healthy marine mammals, allowing us to examine their physiological response to a prolonged stressor (chronic stress). By measuring cortisol in blubber we expect a reflection of chronic stress rather than acute stress because adipose tissue is less subject to rapid changes in blood cortisol levels, reflecting stressors experienced over a longer period of time, and should not be affected by stress associated with sampling. We measured blubber cortisol of 36 whales entrapped November 2006 in Husky Lakes basin and 26 whales from the same population (Eastern Beaufort Sea) during regular seasonal harvests in July of 2006 and 2007. Cortisol concentrations were seven times higher in blubber from entrapped whales (1.76 ± 0.32 ng/g) compared to whales from regular seasonal harvests (0.26 ± 0.042 ng/g) and appeared to increase with whale age. Our results provide a measure of blubber cortisol from a “high stress” event, and show cortisol as a useful measure of stress in beluga whales.

Introduction

In the Arctic, seasonal fluctuations in sea ice create a dynamic landscape where conditions change daily, especially during spring and fall when freeze-up and melt occur. During the fall freeze-up, areas of open water close gradually before freezing over entirely and whales in these areas can become trapped, unable to access open water (Weaver and Richard 1989). Entrapments are more likely in specific areas of the Arctic, such as inlets, sounds, lakes and straits (Harwood 2007). These areas are thought to attract whales because of abundant fish and other prey (Weaver and Richard 1989). Entrapments occur periodically and are considered a natural event causing emaciation

or death of arctic whales, particularly monodontids (beluga and narwhal) (Weaver and Richard 1989). Beluga whales, whose distribution is limited to the Arctic and Subarctic, are particularly susceptible to entrapment events because they live in close association with sea ice, using it for shelter and refuge from predators (Huntington 1998).

During stressful events (e.g. entrapments), a complex neuroendocrine system persistent in all vertebrates stimulates the release of a series of hormones, causing an increase in glucocorticoid (GC) hormones such as cortisol, the primary GC in marine mammals (Oki and Atkinson 2004). The release of glucocorticoid hormones following exposure to short-term stressors is often beneficial for organisms (i.e. acute stress) (Möstl and Palme 2002) and serves as a physiological mechanism for mobilizing energy stores and triggering behaviors that aid in escape or defense (Boonstra 2004). However, if the stressor persists and related hormones continue to release (i.e. chronic stress) the stress response system breaks down tissues and organs, hindering vital functions and ultimately causing illness, decreased reproduction and death (Boonstra et al. 1998; Boonstra 2005).

By measuring stress hormones in tissues that reflect long-term exposure to a stressor, it is possible to monitor changes in the stress experienced by individuals over a desired time (Davenport et al. 2006; Bortolotti et al. 2008; Saco et al. 2008; Okuliarova et al. 2010). In pigs, plasma progesterone enters adipose tissue within 16-50 hours (Hillbrand and Elsaesser 1983) and other steroid hormones in vertebrates, like cortisol, may enter adipose tissue at a similar rate, where they accumulate over a period of time (Mead et al. 1963). Collecting blood and saliva requires capture and often reflects capture stress, while the collection of feces, although non-invasive, is often difficult and samples can become contaminated by seawater during collection (Amaral 2010). Blubber can be collected non-lethally with the use of remote biopsy and is unlikely to reflect stressors associated with collection (Kellar et al. 2006). Blubber, like hair

(Davenport et al. 2006; Manenschijn et al. 2011), may reflect stress over a longer period and indicate whether prolonged exposure to these potential stressors are correlated with a long-term increase in cortisol.

Entrapments generally occur over an extended period so we can use samples collected from entrapped whales to show a measure of "high stress". Currently there is no published measurement of cortisol from blubber during a highly stressful event. Identifying a measure of "high stress" also provides support for the use of blubber as a measure of chronic stress that we can use for comparison when examining the health of the beluga whale population across their range.

We examined differences in blubber cortisol between beluga whales trapped in the ice during freeze-up and beluga whales harvested during annual subsistence hunts. We also examined differences in cortisol from trapped whales among ages and between sexes.

Methods

Study Area – Husky Lakes, in northern portion of Northwest Territories, Canada, are the inner most inlets extending off Liverpool Bay into the Beaufort Sea (Figure 2.1). Some beluga whales from the Eastern Beaufort Sea population enter Liverpool Bay in late July or early August and in some years whales go farther inland, entering Husky Lakes (Higdon and Ferguson 2012). Beluga whales from this population typically begin migrating west to the Bering Sea in mid-August and early September, but occasionally whales remain in various basins of Husky Lakes and become trapped when their only exit to the Arctic Ocean freezes over (Higdon and Ferguson 2012). In 2006 freeze up in Husky Lakes began in September, and 250 whales were counted at the surface during an aerial survey on September 6th (Fisheries Joint Management Council 2008a). On November 14th, following 7 additional whale count surveys that documented the deteriorating condition of these whales, management actions were taken to remove

whales; the remaining 37 entrapped whales were removed by the local community and a survey the following summer found 8 additional whale carcasses thought to be related to this event (Fisheries Joint Management Council 2008a).

Sample collection – Blubber from entrapment samples were collected from the 2006-entrapment event in Husky Lakes basin in November and other samples were collected during the seasonal subsistence harvest from the same population (Eastern Beaufort Sea) in July of 2006 and 2007; all samples were frozen and archived at Fisheries and Oceans Canada, Winnipeg, Manitoba in -40°C freezers. A tooth was extracted from each whale for aging based on one growth layer group of cementum/dentine deposited annually (Luque et al. 2007). We took 1g sub-samples through all blubber layers to avoid effects of blubber depth and selected samples without any visible discoloration, which reflects sample degradation (see Chapter 1). We removed outer edges of all blubber sub-sections to avoid potential contamination.

We extracted cortisol from blubber samples using a modified version of the Kellar et al (2006) method for extracting blubber progesterone (Chapter 1). Cortisol concentrations were measured by radioimmunoassay; method validation steps included assessing inter-assay variation, intra-assay variation, parallelism, extraction efficiency and sample quench (Chapter 1).

Data analysis – Statistical analyses were performed in JMP® 10 (SAS Institute Inc. 2012). Cortisol concentrations were log-transformed to improve normality. We tested for differences in cortisol concentrations between the July 2006 and July 2007 harvest using a t-test. Because our July harvest samples did not contain any known females, we tested for sex differences in entrapped whale cortisol concentrations using a t-test. We then examined the effect of source (entrapment vs subsistence harvest) and age on cortisol concentrations in both entrapment and seasonal harvests using a general linear model.

Results

We measured cortisol concentrations in 29 entrapped whales (26 males and 3 females), 8 whales harvested in July 2006 (all unknown sex) and 18 whales harvested in July 2007 (12 males and 6 of unknown sex). Samples from the 2006 and 2007 subsistence harvests did not differ in cortisol concentration ($t = -0.51$, $df = 24$, $p = 0.613$), so these samples were pooled for comparison with entrapped whales. Likewise, cortisol concentrations did not differ between males and females from the entrapment ($t = 0.39$, $df = 27$, $p = 0.697$). We found no interaction between whale age and source (entrapment event or seasonal harvest; $F_{1, 37} = 1.68$, $p = 0.203$), so re-ran the model with no interaction term. Cortisol concentrations in samples from the entrapment event (1.76 ± 0.32ng/g) were seven times higher than in samples from the subsistence harvests (0.249 ± 0.042ng/g) (Figure 2.2; $F_{1, 38} = 19.85$, $p < 0.0001$), and marginally increased with age of whales (Figure 2.3; $F_{1, 38} = 4.02$, $p = 0.052$).

Discussion

The elevated blubber cortisol concentrations from whales that had been trapped in the ice for over 2 months support the use of blubber cortisol as an indicator of long-term stress. Although our control samples were collected at a different time of year than the entrapped samples (July vs November respectively), the 7-fold difference in cortisol was much higher than the seasonal differences in plasma cortisol concentration found in harbor seals (Gardiner and Hall 1997), suggesting seasonality alone did not account for these differences. Seasonal differences in cortisol concentrations in mammals occur in most species tested but seasonal peaks vary among species and therefore each species must be considered and tested individually (Romero 2002, Romero et al. 2008). Seasonal differences are associated with changes in the energetic demands of the environment and changes in reproductive activity (Gardner and Hall 1997). In birds, reptiles and amphibians, glucocorticoid levels were highest during the breeding season,

and although these trends were not as clear for mammals, fewer studies exist on mammalian species with regard to seasonal and reproductive cycles (Romero 2002). If beluga whales experience seasonal fluctuations with increases during the breeding season, then the effect of entrapment on cortisol concentrations would be even greater than suggested by our results. Diurnal patterns of glucocorticoids in vertebrates are related to insulin production and are associated with fed and fasting states (Dallman et al. 1993). Although blood cortisol in harbor seals can also vary during certain hours of the day in winter (Oki and Atkinson 2004), it is unlikely that blubber from beluga whales would reflect diurnal variation in cortisol because adipose tissue is less subject to rapid changes in cortisol release compared to blood.

Our results suggested a relationship between cortisol and beluga whale age. Cortisol can change with age because of changes in metabolism, reproductive activity and senescence, and can be significantly elevated in older individuals when faced with a stressor because feedback mechanisms do not function as well with age (Sapolsky et al. 1984). Although the interaction between age and source (harvest, entrapment) was not significant, the data appear to suggest that while entrapment cortisol concentrations increased with age, the relationship was less evident in whales from the subsistence harvest (Figure 2.3). Age effects can be exaggerated in some species during stressful events due to a breakdown in the hippocampus in older individuals (Sapolsky et al. 1984), but a larger sample size may be needed to determine if this age effect occurs in beluga only during extremely stressful events like entrapments. In addition, the reproductive status of females may affect cortisol concentrations in some species, but our sample included only three known females and differences were not detected between sexes.

For the 2006 Husky Lake entrapment event we know freeze-up began in September and whales harvested between November 15th and 23rd appeared to be in

poor condition (Fisheries Joint Management Council 2008b; Kocho-Shellenberg 2010). However, it is difficult to identify when whales physiologically perceived the event as a stressor, instigating the initial release of cortisol. Monitoring whale behavior (ability to take normal breaths) in addition to collecting blubber biopsy samples throughout an entrapment event would provide an estimate for duration of stress. This information would be valuable as a tool for estimating the effect of stress on individuals that escape entrapments, and would also provide a measure of time from initial release of cortisol to when it is reflected in the blubber.

No published data of cortisol incorporation in blubber exist due in part to the difficult nature of monitoring these measures in a laboratory setting. Entrapments may provide a situation where cortisol incorporation into adipose tissue could be observed during a high stress event. Our cortisol measurements from this event provide a reference for measuring increased stress in other marine mammal populations and suggest this method could be used to measure and compare stress among healthy and threatened marine mammal populations.

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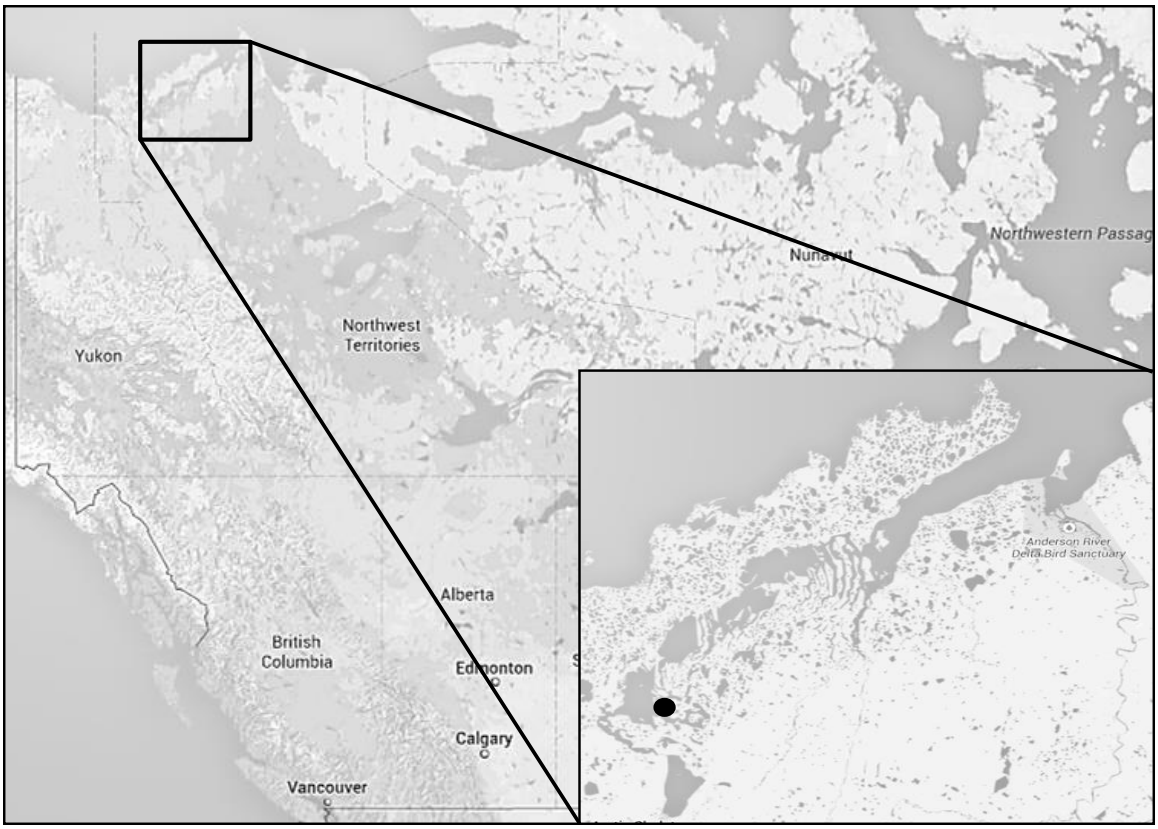

Figure 2.1. Location of the 2006 Beluga whale (*Delphinapterus leucas*) entrapment, Eastern Beaufort Sea, Husky Lakes, Canada.

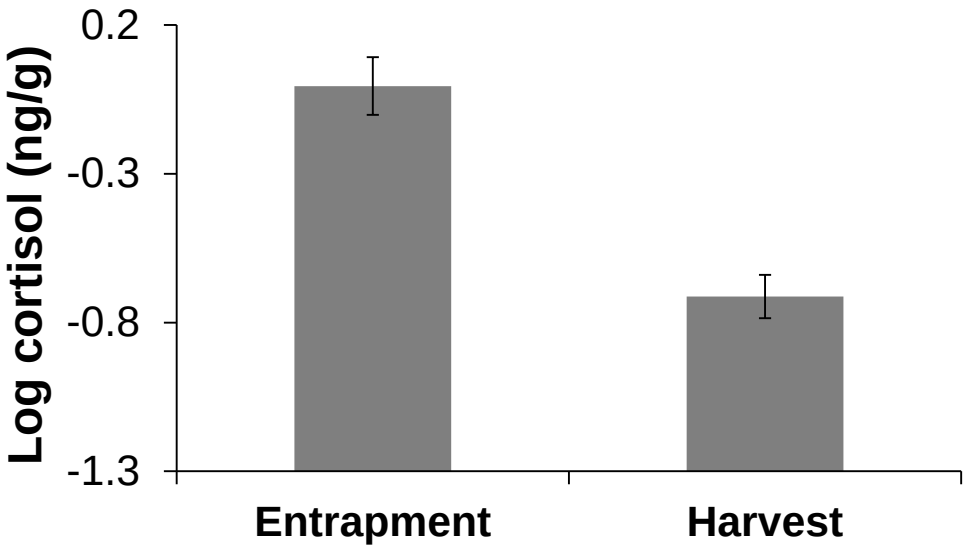

Figure 2.2. Cortisol concentration (mean ± SE of log-transformed ng/g) in blubber of beluga whales (*Delphinapterus leucas*) from the Husky Lake entrapment in November 2006 and the Beaufort Sea subsistence harvests in July 2006 and 2007.

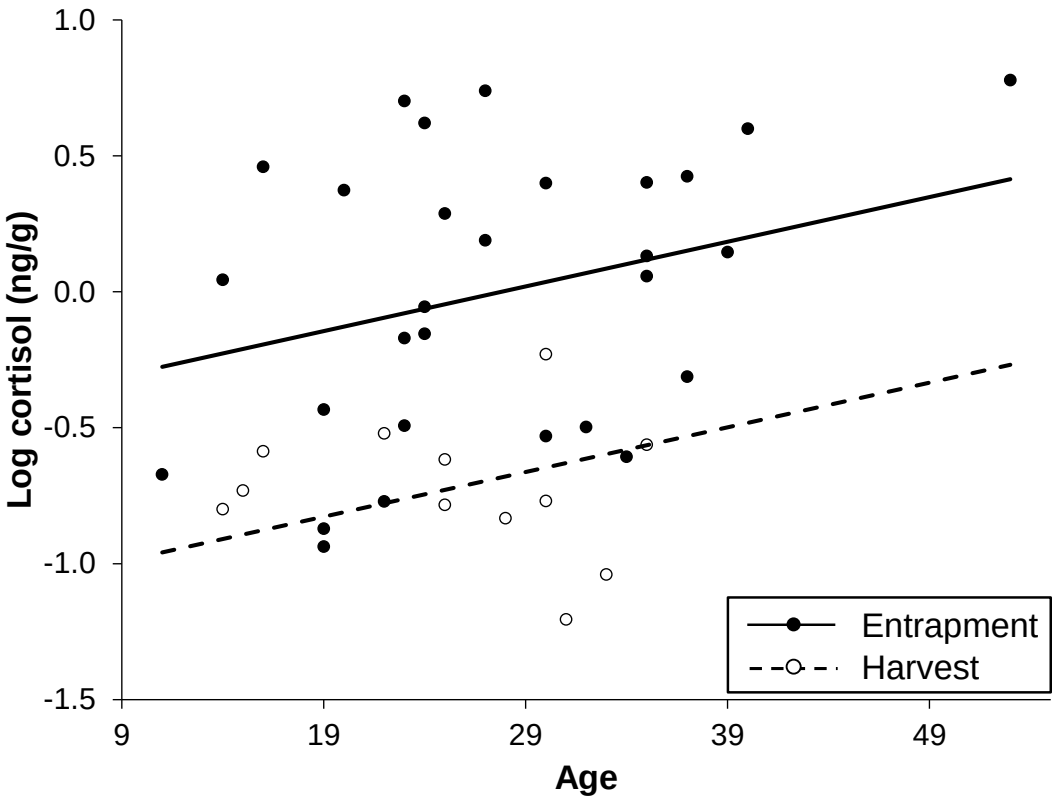

Figure 2.3. Cortisol concentrations (log-transformed ng/g) plotted against age in years obtained from a single tooth cementum or dentine growth layer group in beluga whales (*Delphinapterus leucas*) from the Husky Lake entrapment in November 2006 and the Beaufort Sea subsistence harvests in July 2006 and 2007.

Chapter 3: Higher blubber cortisol in threatened beluga whales of the Canadian Arctic

Abstract

Recent changes in the Arctic associated with increasing temperatures include declines in seasonal ice and changes in ice formation or structure, which could strongly affect odontocetes that live in close association with sea ice like beluga whales (*Delphinapterus leucas*). Beluga whales migrate each fall/winter with the ice edge; they use the ice for refuge from predators and consume several prey species that depend on ice for survival. If sea ice continues to decline, beluga whale populations may experience changes in prey availability and increased predator access. Cortisol, a hormone related to stress, may reflect changes in physiology before population size decreases, but no large-scale studies have examined cortisol in free-ranging whale populations. We measured blubber cortisol, which is less likely to reflect stress caused by sample collection than blood, in samples collected and archived from 1981-2010 from four beluga whale populations in the Canadian Arctic that differ in conservation status (i.e. healthy, special concern or threatened) and other population characteristics (migration distance, historical commercial harvest rate and predation pressure). Higher cortisol concentrations occurred in the population under highest concern (0.61 ± 0.07 ng/g) compared to three populations with lower conservation risk status (0.32 ± 0.08, 0.36 ± 0.27, 0.44 ± 0.04 ng/g). Blubber cortisol concentrations were unaffected by sex, age, or diet, but the relative importance of year sampled in our model selection suggests changes over time may be occurring in some populations. The increased cortisol concentration of the threatened population supports blubber cortisol as an indicator of population status. We suggest cortisol measurements from blubber be added to methods for the assessment of population health, particularly when samples can be collected via biopsy dart.

Introduction

Climate change is leading to changes in the arctic ecosystem. Recent decreases in seasonal sea ice extent (Parkinson and Cavalieri 2002) and increased warming (Comiso 2003) have begun to affect marine mammals (Stirling and Smith 2004; Høye et al. 2007) and will likely have cascading effects throughout the ecosystem (Post et al. 2009). Physical changes in the Arctic will likely affect beluga whales (*Delphinapterus leucas*) through changes in prey availability (Gaston et al. 2012), increased predator access (Higdon and Ferguson 2009) and increased human activity (Kilabuk 1998). Beluga whales are the most common odontocetes in the Arctic and are relatively accessible to researchers, making them an ideal study species for monitoring the impact of climate change on the biotic community. Their range is limited to the Arctic and Subarctic, where climate change is most apparent (Post et al. 2009). They live in close association with sea ice and use the sea ice for shelter and refuge from predators (Huntington 1998). Open waterways provide access to new areas of the Arctic for killer whales (*Orcinus orca*), a predator of beluga whales. Killer whale sightings have increased in certain areas of the Arctic (Higdon and Ferguson 2009), coinciding with declines in sea ice. Most beluga whales migrate each year from estuaries and rivers in summer to ice flow edges areas during winter. Estuaries appear be important for feeding (Seamen et al. 1982), molting skin and rearing young (Stewart and Lockhart 2005) and estuary systems including beluga whales and their prey are sensitive to change (Smith and Barber 2007; Ingram et al. 1996). Stomach contents indicate that beluga whales feed on a diverse array of prey items (McLeod et al. 2008). However, stable isotope and fatty acid analyses suggest beluga whales commonly select fish such as arctic cod (Loseto et al. 2009, Marcoux et al. 2012), an ice-associated species that has decreased in abundance where sea ice has decreased (Gaston et al. 2003). Decreases in sea ice will result in increased human marine traffic and inevitably development in the Arctic.

Local communities in areas of increased human marine traffic have observed decreases in beluga whale foraging and play behavior, and increases in alarm calling, an indication of stress (Kilabuk 1998).

Species conservation depends on informative assessments of population health (Wilson et al. 1996). Measures used to determine population health in marine mammals typically include surveys and population counts (Garner et al. 1999). For some species, counts can be fairly accurate (Wilson et al. 1996), but for marine mammals counts are associated with a substantial degree of error (Royle et al. 2007). Whale surveys count individuals present at the water surface and then correct for the number of individuals estimated to be below the water surface. For many marine mammals, time spent below the water surface can vary between individuals and among populations, seasons and weather (Garner et al. 1999) resulting in population estimation bias. Many of the measures of individual and population health require capture of beluga whales, which is difficult because of their size and logistical challenges of their habitat. However, biopsy dart does not require capture and allows collection of blubber samples that may be used for physiological assessment of health.

Cortisol can indicate changes in individual health by reflecting changes in cortisol concentration over time or between populations (Hennessy et al. 1979; Reeder and Kramer 2005). These measures of stress can be useful in a laboratory or captive setting, but they become more difficult to ascertain in free-living individuals because sample collection can cause stress and complicate the interpretation of hormone measurements (Amaral 2010). However, hormones measured in adipose tissue of pigs are unlikely to reflect stress associated with sample collection because of the longer time required for hormones to enter adipose tissue (Hillbrand and Elsaesser 1983). Measuring cortisol in free-living individuals from populations differing in status can provide a framework for understanding high or low stress among populations.

Age and sex can also affect stress physiology. As some organisms age, their stress response system wears down and feedback mechanisms that regulate hormone release do not work as well (Sapolsky et al. 1984). This age effect is reflected in the time it takes for cortisol to return to normal levels, which is longer in older individuals (Sapolsky et al. 1984). However, in normal, unstressed, humans cortisol levels are lower in older individuals (Drafta et al. 1982). Finally, human males and females exhibit differences in cortisol levels, depending on their age with older individuals having low responses and males showing the greatest age differences (Nicolson et al. 1997; Kudielka et al. 2004).

We measured blubber cortisol concentrations in beluga whales to examine several potential influences on cortisol levels. Our objectives were to compare blubber cortisol concentrations among four populations with different conservation status and to determine whether sex, age, or diet affected beluga whale cortisol concentrations, and whether these values have increased over the past three decades.

Methods

Study areas/populations – We measured cortisol concentrations of beluga whale blubber samples from four populations in the Canadian Arctic (Figure 3.1; Eastern Beaufort Sea, Eastern High Arctic, Western Hudson Bay and Cumberland Sound). These populations differ in conservation status (i.e. threatened, special concern or healthy) as well as other population characteristics (population size, summer and winter ranges and migration distance; Table 3.1).

Summer range of the Eastern Beaufort Sea beluga whale population is located off the north coast of the Yukon and Northwest Territories. Beluga whales in this area spend the summer in the Mackenzie River delta and farther west into Amundsen Gulf and Viscount Melville Sound (Richard et al. 2001). During fall, beluga whales migrate to the Chukchi and Bering Seas, where they overwinter (Department of Fisheries and

Oceans 2000). This population is considered healthy (Department of Fisheries and Oceans 2000), but may be negatively impacted in the future by increases in contaminant levels in prey, oil/gas exploration, and human development (Angliss and Allen 2008). Current contaminant levels within this population differ among segregated social groups due to differences in their foraging behaviors (Loseto et al. 2008). Beluga whales in the Eastern Beaufort Sea eat a variety of fish and invertebrates, but arctic cod is the most important prey source during summer (Loseto et al. 2009). There are few predators of beluga whales in the Beaufort Sea, with only 2-3 sightings of killer whales in these waters each decade (Higdon et al. 2013). Although polar bears may prey on beluga whales from this population during ice entrapment events and while on spring migration (Lowry et al. 1987a; Lowry et al. 1987b; Hobbs et al. 2008), predation is an unlikely stressor.

The Eastern High Arctic includes the northwest and northern waters surrounding Baffin Island, the area surrounding Devon Island, Somerset Island and Cornwallis Island, Nunavut and extends throughout the northern portion of Baffin Bay and the western coast of Greenland. The Eastern High Arctic population migrates from the Eastern Canadian Archipelago through Lancaster Sound and resides in the northern waters of Baffin Bay during winter (Richard et al. 2001). Eastern High Arctic beluga whale status is under special concern (Committee on the Status of Endangered Wildlife in Canada 2004). Surveys conducted off the coast of Greenland found that beluga whale numbers declined between 1981 and 1999 (Committee on the Status of Endangered Wildlife in Canada 2004). The population is composed of approximately 21,000 individuals and no record of original population size exists (Committee on the Status of Endangered Wildlife in Canada 2004). Exploitation of whales entering and residing off the coastal waters of Greenland is a concern (Smith and Martin 1994), and the distribution of beluga whales in this area has changed in response to increased human traffic (Kilabuk 1998).

Western Hudson Bay includes the western portion of Hudson Bay off the coast of Nunavut and Manitoba. Most beluga whales in Hudson Bay migrate to the Hudson Strait during winter until sea ice retreats (Richard et al. 2001) and separate during summer into distinct Western and Eastern populations. In Western Hudson Bay, the largest group of beluga whales spends their summer in the Nelson River, with smaller groups spending the summer in several other estuaries (Richard 1991; Richard 2005). The Hudson Bay beluga whales experienced severe population declines as a result of historical commercial fishing, and while the Western Hudson Bay population has subsequently increased, the Eastern Hudson Bay population remains low (Committee on the Status of Endangered Wildlife in Canada 2004). The Hudson Bay ecosystem is switching from ice-associated fish, such as arctic cod (*Boreogadus saida*), to north Atlantic fish, such as capelin (*Mallotus villosus*) and sand lance (*Ammodytes* sp.) (Gaston et al. 2003). Whereas most beluga whales generally rely on arctic cod, which are more energy-rich than other prey alternatives in the Arctic (Harter et al. 2013), beluga whales in Hudson Bay seem to rely heavily on capelin (Kelley et al. 2010).

Cumberland Sound extends east from Baffin Bay into Baffin Island, Nunavut. Beluga whales in this area have a short migration compared to the Hudson Bay and Beaufort Sea populations. During summer and fall, these whales reside in the western portion, where they are found deep in the estuaries of the Clearwater Fjord (Diemer et al. 2011; Department of Fisheries and Oceans 2002). In winter, the whales migrate east toward the mouth of the sound (Department of Fisheries and Oceans 2002). Cumberland Sound beluga whales were designated as threatened in 1990 by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) because the population had declined (Richard et al. 1990). Whether this population is recovering is unclear (Department of Fisheries and Oceans 2002). Beluga whales of Cumberland Sound forage primarily on arctic cod, turbot, and some invertebrates (Kilabuk 1998).

They have the highest contamination levels of any Canadian Arctic population, but the levels are much lower than in the St. Lawrence population (Department of Fisheries and Oceans 2002). Inuit hunters have reported increases in killer whale sightings in recent years (Higdon and Ferguson 2006; Diemer et al. 2011). The historically high presence of killer whales (Reeves and Mitchell 1988) and recent increases in killer whale sightings suggested predation risk could be an important stressor for this population.

Sample collection – Samples were collected from all four populations during seasonal harvests in May – Sept from 1981 – 2010. Skin and blubber samples from 458 harvested individuals were archived in freezers at Fisheries and Oceans Canada, Winnipeg, Manitoba. A tooth was extracted from each whale for aging based on one growth layer group of cementum/dentine deposited annually (Luque et al. 2007). We used highest quality blubber samples and sampled through all blubber depths based on criteria from our previous examination of degradation and blubber depth effects on cortisol (see Chapter 1), and removed outer edges of all blubber sub-sections to avoid potential contamination. We extracted cortisol from blubber using a modified version of a previously described method for extracting progesterone from blubber (Kellar et al. 2006). Cortisol concentrations were measured by radioimmunoassay and validated using inter-assay variation, intra-assay variation, parallelism, extraction efficiency, and sample quench (see Chapter 1).

Stable Isotope Analysis – Stable isotope ratios provide an estimate of an individual's diet because the isotopic signatures of a consumer's tissues reflect those of its food. We measured stable isotopes ratios of nitrogen and carbon in skin attached to each blubber sample; a thin slice approximately 0.5 grams was removed from the interior skin layer, rinsed with distilled water, and chopped finely. We freeze-dried the chopped sample for 36 hours to remove water, then transferred the dried skin to a vial and homogenized the skin with a spatula until it was a coarse powder. We shipped the vials to the University of

Windsor Chemical Tracers Lab for further homogenization using a mortar and pestle and lipid extraction using a 2:1 mixture of chloroform:methanol (McMeans et al. 2009). ~0.5 mg of the prepared sample was wrapped in a tin capsule and the nitrogen and carbon stable isotope ratios were measured on a continuous flow isotope ratio mass spectrometer (Delta V Advantage, Thermo Electron). Values are expressed using delta (δ) notation: $\delta X = 1000 \times [(R_{sample} \times R_{standard}^{-1}) - 1]$, where X = ^{15}N or ^{13}C and R is the ratio of the heavy to light isotope (^{15}N:^{14}N or ^{13}C:^{12}C). We adjusted carbon stable isotope ratios for oceanic Suess effect (addition of anthropogenic CO_2 depleted in ^{13}C; Quay et al. 2003).

Data analysis – Statistical analyses were performed in JMP® 10 (SAS Institute Inc. 2012). All cortisol data were log-transformed to improve normality. We examined a set of general linear models containing variables likely to influence cortisol concentration, including year collected, sex, age, and dietary information ($\delta^{15}N$ and $\delta^{13}C$), including different combinations of these variables and various interaction terms. We ranked these models based on Akaike's Information Criteria (AIC; Akaike 1974) to determine the most likely models for explaining variation in cortisol concentrations. We compared cortisol concentrations among populations using Tukey Kramer's HSD. Results are expressed as mean ± SE unless otherwise indicated.

Results

Cortisol concentrations varied among populations (Figure 3.2; $F_{3, 401} = 5.18$, $p = 0.0016$). Concentrations were higher in whales from the Cumberland Sound population (0.614 ± 0.08ng/g) than other populations (Eastern Beaufort Sea 0.363 ± 0.03ng/g; Eastern High Arctic 0.325 ± 0.08ng/g; Western Hudson Bay 0.442 ± 0.04ng/g) (Tukey-Kramer HSD, $p \leq 0.012$), which were similar to each other (all $p > 0.57$). The model containing population as the only predictor variable provided the most reliable and repeatable explanation for variation in cortisol concentrations, based on AIC values,

although the models including year sampled and the interaction between year and population had substantial support as well (Table 3.2). However, neither year ($F_{1, 401}$ = 0.033, p = 0.85) nor the interaction between year and population ($F_{3, 401}$ = 2.06, p = 0.10) were significant effects in the second best model, although the whole model was highly significant ($F_{7, 401}$ = 3.85, p = 0.0005). Models including sex, age and diet ($\delta^{15}N$, $\delta^{13}C$; Figure 3.3) had little effect on cortisol concentrations.

Discussion

The higher blubber cortisol we found in the population that is most threatened indicates greater physiological stress compared to other healthy populations. Although the endangered Cumberland Sound population has recovered from historical commercial hunting and is considered stable, with a current harvest during community subsistence hunts, population numbers are still low compared to pre-commercial harvests (Richard et al. 1990; Department of Fisheries and Oceans 2002; Department of Fisheries and Oceans 2005). In 1980, the Cumberland Sound population was approximately 500 individuals (Department of Fisheries and Oceans 2005), but increased three-fold over the time of our sample collection. This population may be experiencing a dietary shift (Marcoux et al. 2012), and although we found no relationship between stable nitrogen or carbon isotope ratios and cortisol concentrations, evidence suggests prey shifts may affect age classes differently because of differences in foraging habits (Marcoux et al. 2012). All beluga whales from our study were adults and were likely harvested with a bias for larger males. Therefore, our sample may not capture the individuals experiencing the greatest dietary stress. However, the impact of a dietary shift negatively affecting specific age classes (breeding adults or juveniles) could affect the growth of the population.

If the changes in the Arctic over the past 30 years have adversely affected beluga whales, a corresponding increase in cortisol concentrations over time would be

expected. Our model selection results found substantial support for models including year sampled as explaining variation in cortisol concentration, suggesting a possible change in stressors over time for specific populations (Figure 3.4).

With declining sea ice, killer whales are expanding their range into more northern waters including Hudson Bay, where killer whale sightings have increased exponentially within the last decade (Higdon and Ferguson 2009). The northward movement of killer whales increases opportunity for predation on ice-adapted species like beluga whales (Ferguson et al. 2010). Increases in predation risk are possible triggers of the stress response system (Cowan and Curry 2000) and may lead to chronic stress in beluga whales.

Migration distance differs among the four populations (Table 3.1). When faced with local climactic changes, it may be advantageous to have a longer migration, which may decrease sensitivity to local changes in climate, diet, human development, traffic and contaminants. Beluga whale populations with larger migration distances also mix during winter, providing an opportunity for social networking and possibly interbreeding (O'Corry-Crowe et al. 2002). However, the actions of beluga whales during winter are not well understood, and genetic evidence suggests that populations do not interbreed (O'Corry-Crowe et al. 2002). Three of the most sedentary populations (Cumberland Sound and St. Lawrence in Canada and the Cook Inlet population in the United States) are also under the highest threat of extinction, supporting a possible advantage for migration. In some populations, some individuals may migrate while others do not, such as in Eastern Hudson Bay, James Bay and other isolated populations that use polynyas (area of open water surrounded by sea ice) for feeding and breathing (Postma personal communication 2010). Comparing stress levels of migratory and sedentary individuals within the same population would provide a measure of the population effects of migration for whales with similar resources. Alternatively, these sedentary populations

also have small population numbers. Perhaps smaller populations are less likely to migrate.

Current methods for assessing health of beluga whale populations are limited to aerial population estimates to assess declines (Garner et al. 1999). These surveys are associated with some error due to individuals not present at the water surface (Royle et al. 2007). We suggest measures of cortisol be used when assessing population health, specifically when blubber biopsies are part of monitoring protocols. If these measures are taken over time or compared to historical cortisol concentrations, it may be possible to identify populations at risk (undergoing changes of stress) before the population declines. Additional methods for monitoring population health include acoustic survey, individual physical measurements (girth, length, weight, birth weight and lipid index), and population characteristics (size estimates and age structure). Our results provide support for using blubber cortisol as a measure indicating population health for beluga whales and possibly other marine mammals.

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Table 3.1. Characteristics of the four beluga whale (*Delphinapterus leucas*) study populations, including population size estimates, conservation status, summer and winter areas, and approximate migration distances (measured in Arc Geographic Information System, Environmental Systems Research Institute online mapping package).

Population	Population size estimate	Conservation status[3]	Summering areas	Wintering areas	Approximate distance
Eastern Beaufort Sea	~32,000[1]	Not at Risk	Eastern Beaufort Sea	Southern Chukchi Sea	~2000km
Eastern High Arctic	~21,000[2]	Special Concern	North Somerset Island	East Baffin Bay	~1000km
Western Hudson Bay	~57,000[3]	Special Concern	Arviat	Hudson Strait	~1300km
Cumberland Sound	~2,000[4]	Threatened	Inner Cumberland Sound	Mouth of Cumberland Sound	~300km

[1]Harwood and Smith 2002, [2]Committee on the Status of Wildlife in Canada 2004, [3]Richard 2005, [4]Department of Fisheries and Oceans 2005

Table 3.2. Comparison of selected models for explaining variation in cortisol concentration in blubber from beluga whales (*Delphinapterus leucas*). Output for each model includes the coefficient of determination (R^2), error sum of squares (SSE), number of observations (N), number of parameters in the model (K), Akaike's Information Criterion corrected for small sample size (AIC_c), difference in AIC_c between each model and the best model (ΔAIC_c), and the relative information content of each model (weight). The relative importance of each variable is listed below. Variables included in the models are population (Eastern Beaufort Sea, Eastern High Arctic, Western Hudson Bay, and Cumberland Sound), year of sample collection (1981 – 2010), stable nitrogen and carbon isotope ratios obtained from skin ($\delta^{15}N$, $\delta^{13}C$), sex, and age obtained from a single tooth cementum or dentine growth layer group.

Model variables	R^2	SSE	N	K	AICc	ΔAICc	weight
Population	0.047	65.155	409	5	-741.2	0.0	0.476
Population, year, population*year	0.063	64.034	409	9	-740.0	1.2	0.260
Population, year	0.049	65.021	409	6	-739.9	1.2	0.258
Year	0.016	67.254	409	3	-732.3	8.9	0.006
Population, $\delta^{15}N$	0.056	61.002	387	6	-702.8	38.4	0.000
Population, δ15N, $\delta^{13}C$	0.044	60.994	387	7	-700.7	40.4	0.000
Population, $\delta^{15}N$, population* $\delta^{15}N$	0.066	60.382	387	9	-700.5	40.7	0.000
Population, $\delta^{15}N$, $\delta^{13}C$, year	0.058	60.889	387	8	-699.3	41.8	0.000
Population, $\delta^{13}C$	0.046	61.689	387	6	-698.4	42.7	0.000
Population, $\delta^{13}C$, population* $\delta^{13}C$	0.048	61.564	387	9	-693.0	48.2	0.000
$\delta^{15}N$	0.017	63.550	387	3	-693.1	48.1	0.000
$\delta^{13}C$	0.001	64.566	387	3	-686.9	54.2	0.000
Population, sex	0.120	45.133	310	6	-585.1	156.1	0.000
Population, sex, population*sex	0.137	45.133	310	9	-578.8	162.4	0.000
Population, year, sex	0.120	46.032	310	7	-576.9	164.3	0.000
Population, age	0.063	43.160	299	6	-566.4	174.7	0.000
Sex	0.064	48.978	310	3	-565.9	175.2	0.000
Population, age, population*age	0.067	42.979	299	9	-561.4	179.8	0.000
Age	0.002	45.963	299	3	-553.8	187.3	0.000
Population, $\delta^{15}N$, $\delta^{13}C$, sex	0.135	42.124	292	8	-548.8	192.3	0.000
Population, , $\delta^{15}N$, $\delta^{13}C$, age	0.065	40.758	281	8	-526.0	215.2	0.000
Population, sex, age, year	0.123	36.150	268	8	-520.3	220.8	0.000
Population, sex, age, year, $\delta^{13}C$	0.130	33.801	253	9	-490.5	250.6	0.000
Population, sex, age, year, $\delta^{15}N$, $\delta^{13}C$	0.132	33.721	253	10	-489.0	252.2	0.000

Variable	Relative importance
Population	0.994
Year	0.524
$\delta^{15}N$	0.000
$\delta^{13}C$	0.000
Sex	0.000
Age	0.000

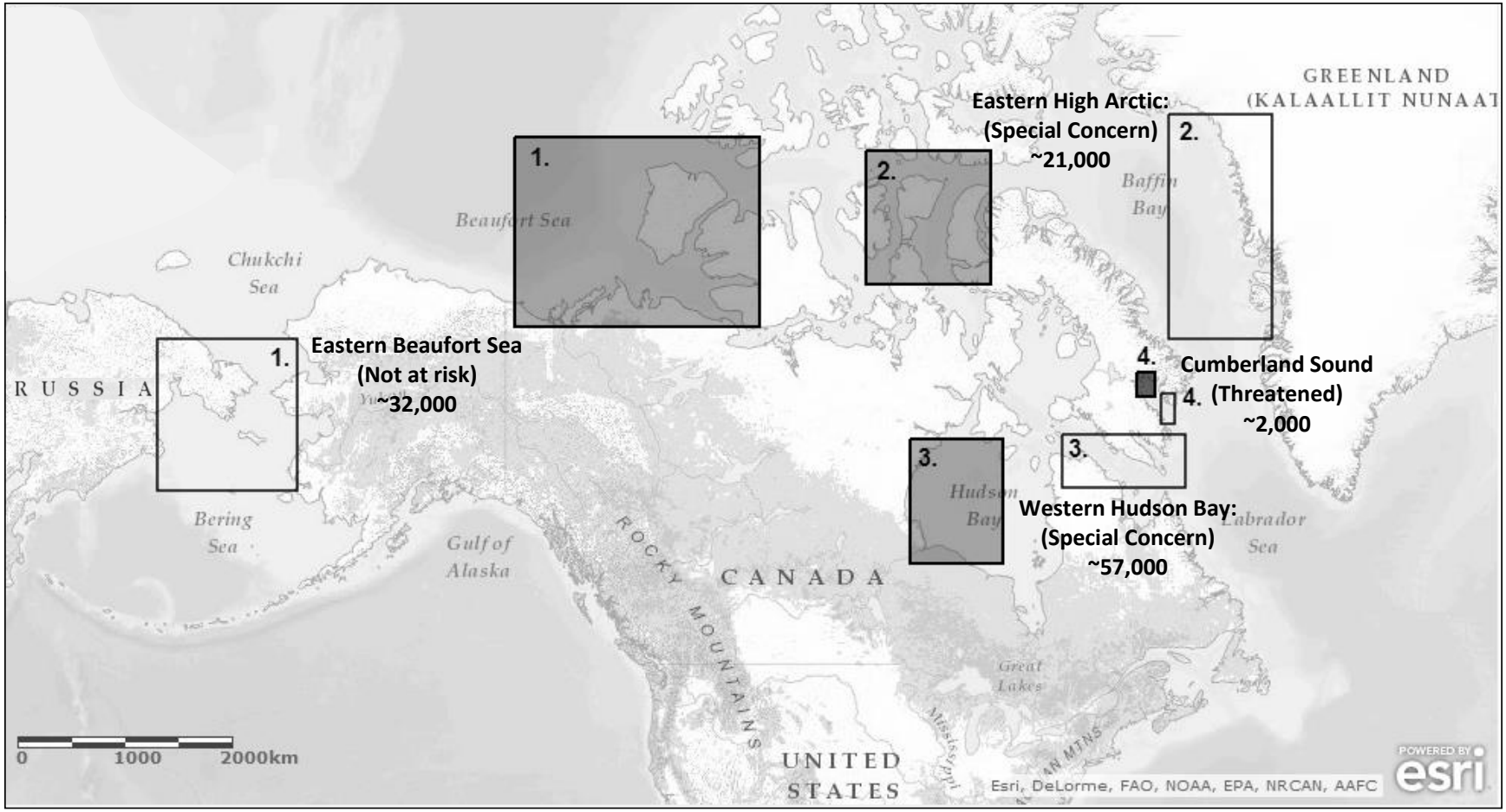

Figure 3.1. Beluga whale (*Delphinapterus Leucas*) study populations. Boxes represent summer (grey) and winter (black outline) areas for each population. 1. Eastern Beaufort Sea, 2. Eastern High Arctic, 3. Western Hudson Bay, 4.Cumberland Sound.

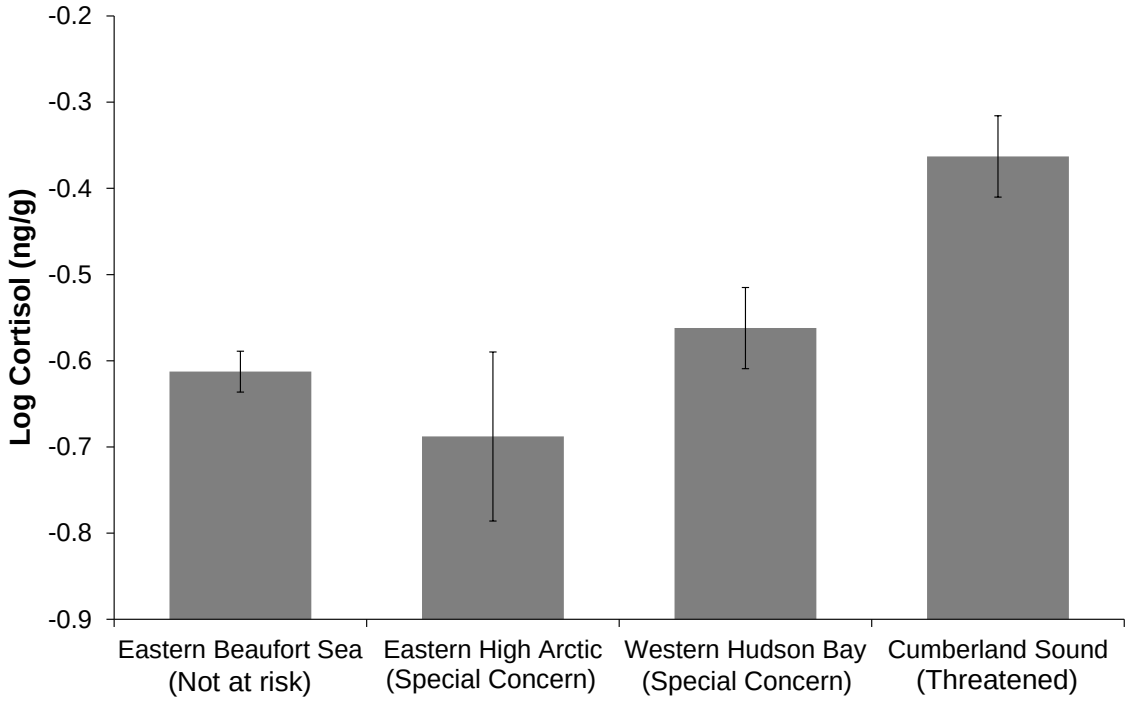

Figure 3.2. Beluga whale (*Delphinapterus leucas*) blubber cortisol concentration (mean ± SE log transformed ng/g) for each population from archived samples collected from 1981-2010.

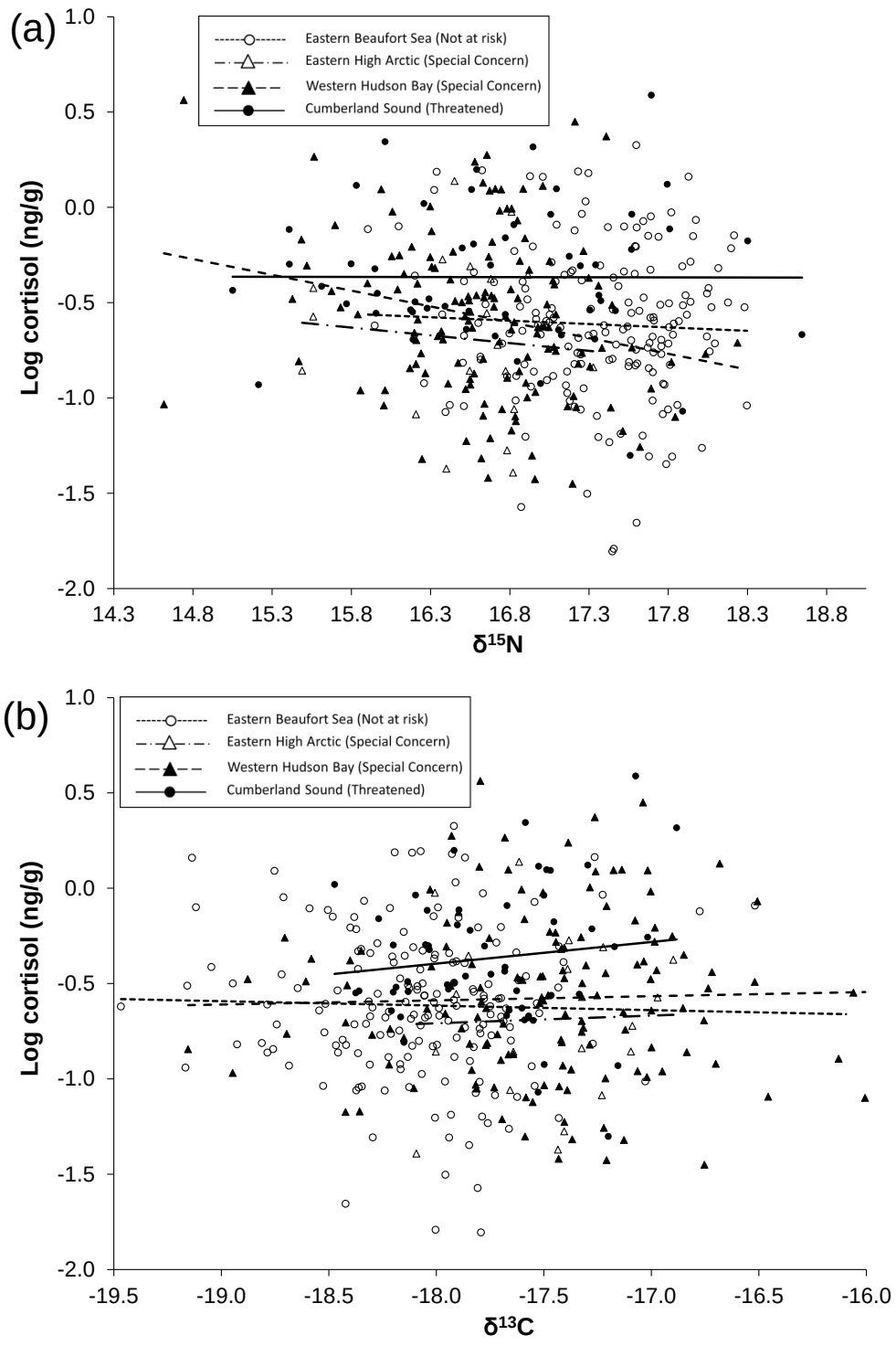

Figure 3.3. Log cortisol (ng/g) and stable nitrogen **(a)** and carbon **(b)** isotope ratios ($\delta^{15}N$, $\delta^{13}C$) from beluga whale (*Delphinapterus leucas*) blubber and skin respectively. Trend lines for the four whale populations indicate no relationship between cortisol concentrations and stable isotope ratios representing diet.

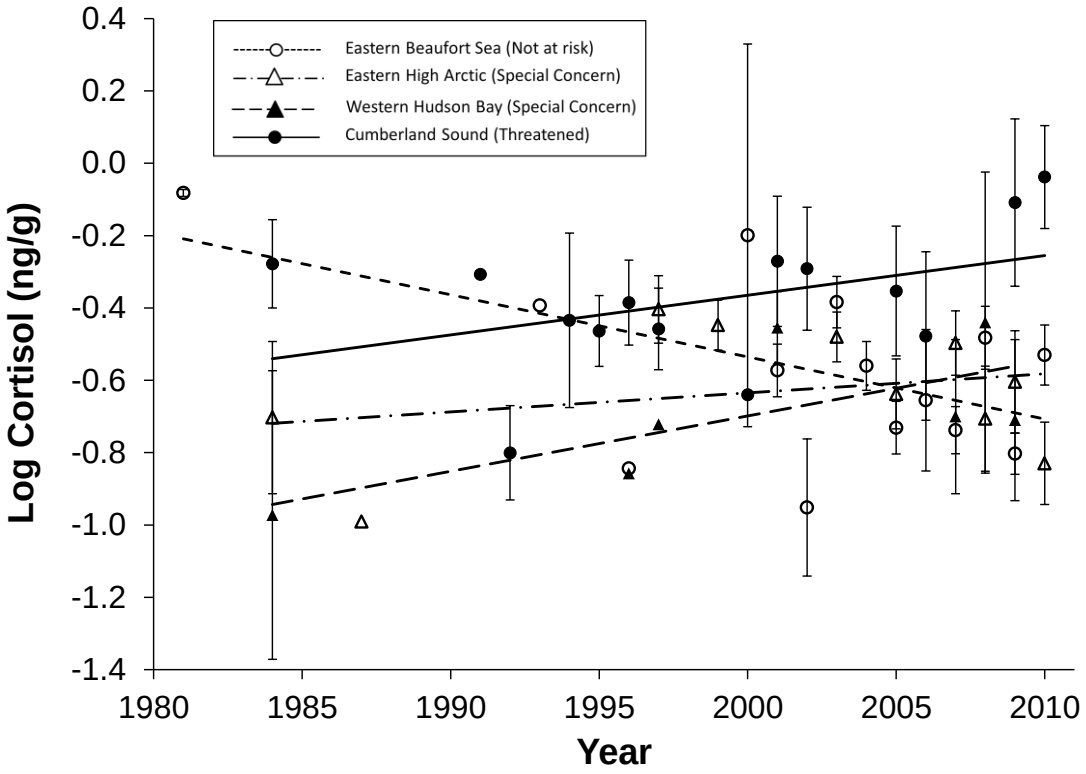

Figure 3.4. Blubber cortisol concentrations (mean ± SE log transformed ng/g) over time for each beluga whale (*Delphinapterus leucas*) population (Eastern Beaufort Sea, Eastern High Arctic, Western Hudson Bay and Cumberland Sound).

Thesis Conclusion

Recently, many studies have focused on the effects of warming temperatures on the Arctic and its inhabitants. If warming trends continue, the Arctic will likely become ice-free during the summer. Many species in the Arctic depend on sea ice for survival. Longer ice-free periods should cause a shift in the Arctic ecosystem to a less ice-associated regime. We expect ice-obligate species (e.g. polar bears, walrus) to become greatly reduced, ice associated species (e.g. beluga whale, narwhal, ring seal) to face major adaptive pressure, and ice-restricted, seasonal species (e.g. killer whale, humpback whale) to become year-round inhabitants of the Arctic (Moore and Huntington 2008). During this shift, many ice-associated species will lose habitat (Sterling and Smith 2004) and experience increased predation risk (Higdon and Ferguson 2009), increased competition, and a change in prey availability (Gaston et al. 2003).

Our research focused on a single ice-associated species, the beluga whale, using a stress-related glucocorticoid hormone, cortisol, to examine differences in stress among populations with differing conservation status. Additionally we wanted to look at effects of time, sex, age, and diet on stress in beluga whales. Before we could make these comparisons, we needed to find a method to measure cortisol in tissues that did not reflect capture stress, which was important because the samples came from harvested animals. We also wanted to use a tissue that would support the use of non-lethal and less-invasive techniques. The use of blubber for extracting cortisol is a novel technique, but blubber progesterone and testosterone extraction in whales is well-established (Mansour et al. 2002; Kellar et al. 2006; Kellar et al. 2009). Our results support the use of these same methods for extracting blubber cortisol. While developing the method for extracting of cortisol, we tested for degradation of cortisol with archived blubber tissue. Samples deemed of poor quality showed degradation in cortisol concentrations. However, the time samples spent in storage had less to do with reduced

cortisol than sample quality. We also tested for variation with blubber depth. Characteristics of blubber change with blubber depth in marine mammals and vary among species (Krahn et al. 2004; Struntz et al. 2004). Our results showed higher cortisol concentrations for depths closest to the muscle, likely due to metabolic activity and vascularization of this area (Struntz et al. 2004).

To test for blubber as an indicator of chronic stress, we measured cortisol in blubber from beluga whales harvested from an entrapment event, which can cause severe emaciation and death in whales (Weaver and Richard 1989). We found higher cortisol concentrations in the entrapped whales than whales harvested from the same population during subsistence hunts indicating that blubber cortisol can indicate chronic stress in beluga whales. Additionally, this measure provides a high cortisol concentration estimate, which can be used for comparison in future studies.

Historic threats, such as commercial hunting, of beluga whale populations in the Arctic caused major declines in many populations. Some populations have recovered and others have not (Harwood and Smith 2002; Innes et al. 2002; Department of Fisheries and Oceans 2005; Angliss and Allen 2008). Several agencies track beluga whale population numbers and recovery rates. Of the four populations we examined, the Cumberland Sound population is under greatest threat of disappearance according to the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) and had higher cortisol levels than any other population (Eastern Beaufort Sea, Eastern High Arctic and Western Hudson Bay). Differences in cortisol concentration among these populations were not related to diet as indicated by stable isotope analysis. We found some marginal support for differences in cortisol concentrations over time in certain populations, suggesting changes in stressors over time for some populations and not for others. Sex and age had no effect on cortisol levels for any beluga whale population,

suggesting that stressors are equal between males and females and among age classes.

Our investigation of factors influencing blubber cortisol in beluga whales supports the use of blubber cortisol in future studies of stress and shows how to apply blubber cortisol to examine population ecology. Poor quality samples should be excluded or controlled for and sample protocols should stress the importance of preserving samples well prior to extraction. Blubber samples from the inner depth will have the greatest concentration and variation. We recommend that future studies use blubber cortisol, along with other measures, to test for differences among groups and individuals experiencing varying stressors.

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