

Cortisol levels in beluga whales (*Delphinapterus leucas*): Setting a benchmark for Marine Protected Area monitoring¹

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Abstract: Beluga whales (*Delphinapterus leucas*) are facing profound changes in their habitat, with impacts expected at the individual and population level. Detecting and monitoring exposure and response to environmental stressors is necessary for beluga conservation and management of human activities. Cortisol has proven as a useful tool to assess stress on wildlife. Cortisol was measured in three blubber layers and plasma in subsistence-hunted beluga whales from the summers of 2007 to 2010 using an HPLC/MS/MS. We assessed the effect of biological and biochemical factors. Cortisol ranged from undetectable to 17.8 ng/g in blubber and 2.5 to 61.2 ng/mL in plasma. Concentrations were highest in the inner blubber layer likely reflecting circulating levels. All tissues were significantly higher in 2008 for reasons that remain unclear. Cortisol levels were on par with resting levels in captive belugas. Best fit models for cortisol revealed age to be an important determinant along with length and blubber thickness. Lack of relationships with biochemical factors such as organic contaminants suggests current cortisol levels are not significantly influenced by present contaminant concentrations. Our findings support the use of middle and outer blubber tissues for an integrated measure of chronic stress that are less subject to the influence of acute stress.

Key words: hormones, marine mammals, physiology, Beaufort Sea.

Résumé : Les bélugas (*Delphinapterus leucas*) font face à de profonds changements au niveau de leur habitat, avec des impacts prévus sur le plan individuel et de la population. La détection et la surveillance de l'exposition et de la réponse aux agresseurs environnementaux sont nécessaires pour la conservation des bélugas et la gestion d'activités humaines. Le cortisol s'avère un outil efficace afin d'évaluer le stress sur la faune. Le cortisol a été mesuré dans trois couches de graisse et dans le plasma de bélugas provenant de chasse de subsistance au cours des étés de 2007 à 2010, et ce, en utilisant un « HPLC/MS/MS ». Nous

Received 6 June 2017. Accepted 3 November 2017.

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¹This article is part of a Special issue entitled "The beluga summit: knowledge sharing of the eastern Beaufort Sea beluga whale".

Lisa L. Loseto currently serves as Co-Editor; peer review and editorial decisions regarding this manuscript were handled by John Iacozza and Greg Henry.

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avons évalué l'effet de facteurs biologiques et biochimiques. Les concentrations de cortisol allaient d'indétectable à 17,8 ng/g dans la graisse et de 2,5 à 61,2 ng/mL dans le plasma. Les concentrations étaient les plus hautes dans la couche de graisse intérieure reflétant probablement des niveaux circulants. Tous les tissus étaient significativement plus élevés en 2008 pour des raisons qui demeurent incertaines. Les niveaux de cortisol étaient égaux à ceux à l'état de repos chez les bélugas captifs. Les modèles de meilleure adaptation pour le cortisol ont révélé que l'âge est un déterminant important ainsi que la longueur et l'épaisseur de graisse. Le manque de corrélations avec des facteurs biochimiques comme les contaminants organiques suggère que les niveaux de cortisol actuels ne sont pas significativement influencés par les concentrations de contaminants actuelles. Nos constatations soutiennent l'utilisation de tissus de couches de graisse moyennes et extérieures pour la mesure intégrée de stress chronique qui sont moins vulnérables aux effets de stress aigu. [Traduit par la Rédaction]

Mots-clés : hormones, mammifères marins, physiologie, mer de Beaufort.

Introduction

The Arctic has experienced warming at twice the global average (IPCC 2013) and as a consequence, Arctic marine ecosystems are being affected by associated changes in ocean productivity, species ecology, and human activity (AMAP 2011b). Top predators, such as marine mammals, represent some of the most vulnerable species to climate change impacts (Laidre et al. 2008; Laidre et al. 2015). In addition to climate change, marine mammals face additional risks from shipping and associated noise, commercial fishing, contaminants and resource exploration and extraction (AMAP 2011a, 2011b; Moore et al. 2012; Reeves et al. 2014). Growing concerns about the body condition in marine mammals, seabirds, and forage fish species in the Beaufort Sea underscore the need for new assessment tools and approaches to inform managers and stakeholders (Harwood et al. 2015; Laidre et al. 2015). Cortisol levels offer a tool or means to measure stress levels in marine mammals at both an individual and population level (Atkinson et al. 2015).

Beluga whales (*Delphinapterus leucas*) are hypothesized to be a moderately sensitive species to climate change impacts (Laidre et al. 2008). As such, belugas can serve as valuable indicator species because of their circumpolar distribution, trophic level and accessibility for samples from ongoing subsistence harvests, and circumpolar monitoring programs. In the well-studied Eastern Beaufort Sea (EBS) beluga population, researchers have documented a decline in growth rates over recent decades, raising concern of climate change-mediated impacts (Harwood et al. 2014). Although the population appears healthy and is estimated at approximately 40 000 individuals (Hill and DeMaster 1999), their large home range spanning the Bering Sea to the Beaufort Sea, are regions that have experienced pronounced changes linked to a warming climate (e.g., loss of sea ice (Stroeve et al. 2012), reduced land-fast ice extent (Yu et al. 2014), changes in primary productivity (Brown and Arrigo 2012)) as well as offshore oil and gas exploration and development (Reeves et al. 2014).

Beluga whales continue to be an important part of a traditional subsistence harvest by the Inupiat in Alaska and the Inuvialuit in Northwest Territories, Canada (McGhee 1988; Huntington et al. 1999; Harwood et al. 2002). As part of the Canadian beluga management plan, a harvest monitoring program has been in place for over 30 years (Harwood et al. 2002; FJMC 2013). To conserve the long-term health of the beluga population, the Tarium Nirvutait Marine Protected Area (TN MPA) was instated in 2010 in the Mackenzie Estuary, where they form a summering aggregation (DFO 2013). As such, there is a legal obligation to use appropriate indicators to assess performance of the MPA and insure a thriving health population (Gazette 2010). More recently, in 2017, a second MPA was designated in the Western Canadian Arctic (Anguniaqvia niqiqyuam).

Stress hormones, such as cortisol, have been suggested as indicators for the early detection of changes to beluga health (Loseto et al. 2010). Cortisol is a glucocorticoid hormone that has various functions, including regulation of energy metabolism, maintenance of growth and development, and responses to stress influencing the physiology and endocrinology of the reproductive system (Moberg 1991; Dobson and Smith 2000). Cortisol has been used as an indicator of stress response and overall population health for a wide range of mammals (Sheriff et al. 2011; Atkinson et al. 2015). It has been quantified in many matrices such as blood (serum/plasma), urine, feces and hair, as well as in blubber and the blow from cetaceans (St. Aubin et al. 2001; Schmitt et al. 2010; Macbeth et al. 2012; Palme et al. 2013; Thompson et al. 2014; Kellar et al. 2015; Trana et al. 2015). Cortisol has also provided insight into stress associated with contaminants, such as persistent organic pollutants (POPs) that include PCBs and PBDEs (Verboven et al. 2010; Bechshoft et al. 2012b). EBS beluga have been monitored for POPs to provide baseline levels, assess foodweb biomagnification, and evaluate impacts to health (Braune et al. 2005; Tomy et al. 2009; Desforges et al. 2013; Noël et al. 2014). Vitamins A (retinol) and E (tocopherol), like cortisol, have served to reveal toxicological effects associated with POP exposure (Routti et al. 2005; Mos et al. 2007). Recently vitamins A and E were identified as useful biomarkers of contaminant-mediated effects in the EBS beluga whales (Desforges et al. 2013).

While cortisol may be a potentially useful indicator of stress or condition, interpreting levels requires an understanding of the natural variability within a given species as well as the possible confounding influences of endogenous and exogenous compounds. For instance, cortisol levels can reflect diurnal and seasonal cycles as well as size, sex, and age of individuals (Rosen and Kumagai 2008; Myers et al. 2010; Kellar et al. 2015). Endogenous compounds such as vitamins A and E have also been shown to interact with glucocorticoid homeostasis and functioning; both vitamins appear to diminish glucocorticoid stress responses in organisms and thus antagonize the hypothalamic–pituitary–adrenal (HPA) axis. Understanding the effects of confounding variables and developing a benchmark for beluga cortisol levels are essential for the interpretation of results and the assessment over-time and across studies (Atkinson et al. 2015).

We set out to determine and characterize benchmark cortisol levels and associated determinants for the EBS beluga using harvested whales. The biological factors including age, sex, length, blubber thickness, and variation among the tissues of blubber and plasma were considered along with year of collection. Given the observed relationships between organic contaminants (PCBs, PBDEs) and vitamins A and E, we also assess for biochemical relationships between cortisol organic contaminants and vitamins A and E. Findings from this study will provide recommendation for the use of cortisol as an indicator for long-term monitoring of stress in beluga whales in a marine protected area. Tissues used were collected over four consecutive summers that were analyzed for vitamins and organic contaminants that were previously published (Desforges et al. 2013) (Supplementary Table S1²) and measure cortisol using a simple liquid extraction method followed by high-performance liquid chromatography tandem mass spectrometer (HPLC/MS/MS).

Methods

Study design

Beluga tissues were collected from harvested whales at Hendrickson Island, near the community of Tuktoyaktuk, within the Tarium Niryutait Marine Protected Area in the

²Supplementary Table S1 is available with the article through the journal Web site at <http://nrcresearchpress.com/doi/suppl/10.1139/as-2017-0020>.

Northwest Territories, Canada (Fig. 1). For consistency and analyses for trends among biochemical the blubber and plasma samples analyzed were the same as those in Desforges et al. (2013) (Supplementary Table S1²). A total of 66 whales were sampled over four consecutive summers from 2007 to 2010. Over 80% of the whales were adult males, due to hunter biases of typically selecting for larger sized males and whales without calves (Supplementary Table S1²). Age estimates ranged from 15 to 60 years with a mean of 31 ± 1.4 (age estimates based on one growth layer group (Stewart et al. 2006)). Beluga length ranged from 351 to 513 cm with a mean of 410 ± 4.4 cm. Blubber and plasma samples were taken from each whale within hours of the harvest. Blood was collected directly from the jugular vein into heparinized plasma separation tubes (Becton-Dickson, USA). Blood was centrifuged on site, and plasma was collected and kept frozen at -80 °C. As per the standardized skin/blubber sample collection, full-depth blubber samples were taken slightly dorsal to the pectoral flipper. This location was selected for several reasons, including comparability to biopsy sampling, accessibility when sampling whales on shore and finally this location is distant from potential influences of structural interferences of the dorsal ridge for complementary analyses (e.g., fatty acids). The blubber/skin sample was wrapped in solvent-rinsed tinfoil, frozen at -20 °C on site, stored in portable freezers and shipped to Fisheries and Oceans Canada (Sidney, BC) where they were stored and protected from light at -80 °C within two weeks of collection. Blubber samples can be kept for several years, but degradation of the blubber sample and hormone levels can occur (Trana et al. 2015). All blubber samples extracted in this study, were visually pink and free of discolouration, with no notable degradation occurring.

Sample preparation and extraction

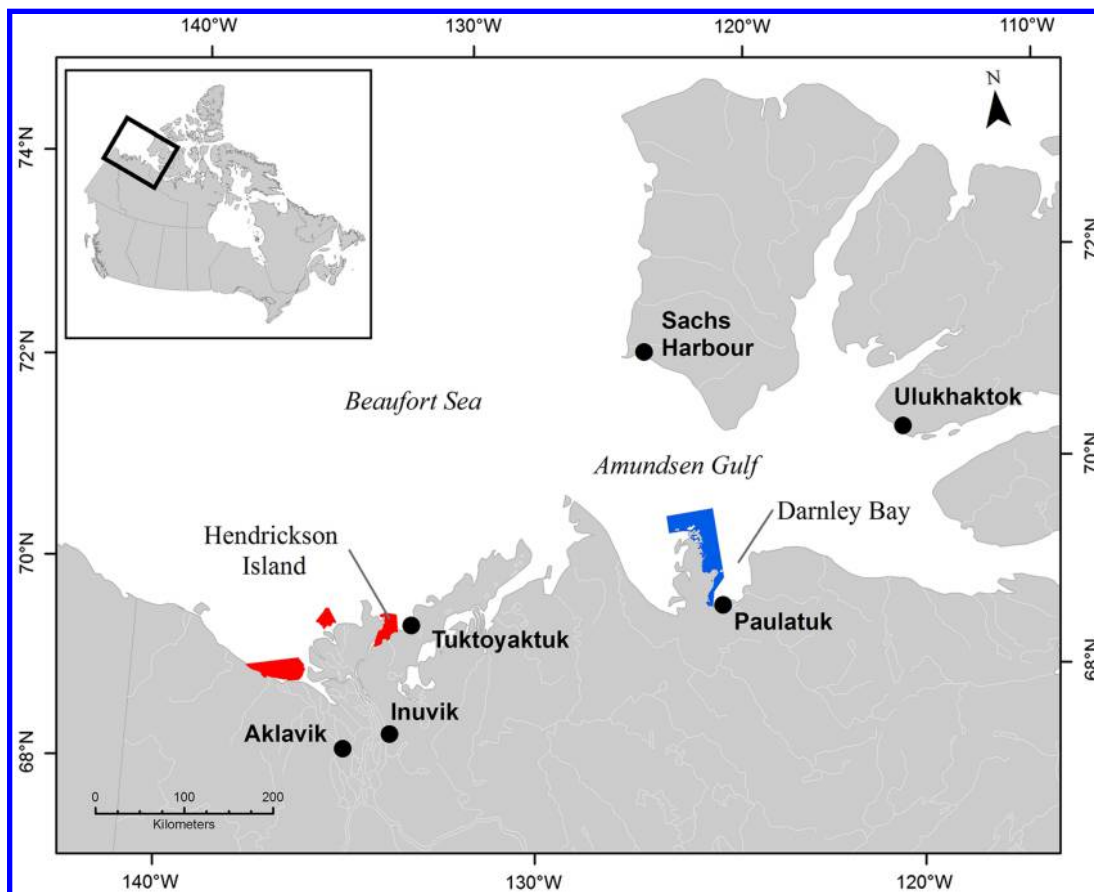
Cortisol in plasma and blubber was extracted by a liquid extraction. Plasma was thawed and vortexed to ensure it is homogenous. We spiked 400 μ L of plasma with 10 μ L of 500 ng/mL d₄-cortisol as an internal standard, then added 3 mL of 9:1 hexane:ethyl acetate, vortexed (1 min) and followed by centrifuging (4000g for 5 min). The samples were then frozen at -80 °C for 7 min. The supernatant was transferred to a clean test tube and these steps were repeated with 3 mL of 3:2 hexane:ethyl acetate to maximize extraction efficiency of cortisol. Samples were then reduced in volume using nitrogen, and then brought to a final volume of 100 μ L using methanol that was vortexed prior to analysis.

For blubber extractions, stratification was analyzed; therefore, a 1.5 g piece of blubber was cut into its three layers, inner (closest to the muscle), middle, and outer (closest to the skin). Each section of the blubber was weighed and put into a 15 mL plastic vial and were freeze-dried for 48 h (FreeZone 6 liter Console Freezer Dry System, Labconco®, Kansas City, MO, USA). Three millilitres of methanol was added to the blubber, spiked with 10 μ L of 1.5 ng/ μ L of d₄-cortisol, and blubber was pressed with a glass rod until the interstitial tissue was pelleted at the bottom of the vial. The sample was sonicated in hot water (40 °C) for 50 min, vortexed (1 min) and centrifuged (4000g for 5 min). Supernatant was transferred to a new vial, and these steps were repeated by adding 3 mL of methanol to the precipitate, vortexed and centrifuged to maximize extraction efficiency. All the supernatant were combined, then the sample was reduced in volume with nitrogen and brought to a final volume of 200 μ L using MeOH.

LC/MS/MS conditions and sample analysis

Native and mass-labeled cortisol were analyzed by high-performance liquid chromatography tandem mass spectrometer (LC/MS/MS) using an average relative response factor (ARRF) model for quantification. Calibration was performed using ARRF with d₄-cortisol

Fig. 1. Beluga tissue samples collected from subsistence beluga hunts where sampling occurs at Hendrickson Island, a hunting area used by Inuvialuit of Tuktoyaktuk, Northwest Territories Canada. Red and blue areas refer to the two Marine Protected Areas (MPA), Tarium Niryutait (Red) and Anguniaqvia niqiqyuam (Blue). Map source for layers for MPAs: Fisheries and Oceans Canada.



as the internal standard. A single concentration calibration was used, whereby 10 ng/mL of standard was used at the beginning, end, and after each sample set to determine the RRF.

$$\text{RRF} = \frac{(\text{Area Cortisol}/\text{Area Cortisol} - \text{d4})}{(\text{Conc Cortisol}/\text{Conc Cortisol} - \text{d4})}$$

The average of all values was used for the ARRF. Quantitation was then determined by solving for the concentration of cortisol (by rearrange the ARRF equation) and then the concentration in the sample was calculated by multiplying the calculated concentration in the extract by the final extract volume and dividing by the original mass of the sample.

A Genesis C₁₈ analytical column (length 10 cm; inner diameter 2.1 mm; particle size 4 μm; Jones Chromatography, Chromatographic Specialities, Brockville, ON, Canada) was used with a gradient mobile phase of methanol:water (start 20:80 to 100:0) at a flow rate of 300 μL/min over 25 min. MS source conditions are as follows, scan type at MRM, polarity at negative, CUR at 40, CAD at 10, IS at -5500, TEM at 500, GS1 and GS2 at 60, ihe at ON and the electrospray negative ionization model was used. Detection of native and

mass-labeled cortisol was achieved using multiple reaction monitoring and by monitoring the transition m/z 361.1 [M-H] \rightarrow 282.1 [M-CH₃O], m/z [M-H] 365.0 \rightarrow 335.0 [M-C₅H₄O], respectively.

QA/QC procedural blanks consisting of MeOH were analyzed every 15 samples. The native hormones were not detected in our blanks, so blank correction was not necessary. Injections of methanol (3 μ L) were used as instrument injection blanks for HPLC/MS/MS, and were run with every six samples.

The method detection limit (MDL) was determined by spiking a methanol blank with a low level of native cortisol and then processed through the entire method. A spiked blank can be used for MDL and accuracy/precision determinations in the absence of a negative control sample (Magnusson and Ornamark 2014). The MDL was calculated to be 0.21 ng/g using a 5:1 signal to noise ratio. As described above, for quantification of cortisol, all samples were spiked with an internal standard prior to extraction (i.e., plasma: 400 μ L of plasma spiked with 10 μ L of 0.5 ng/ μ L for a total of 5 ng in plasma d₄-cortisol; blubber: 3 mL of methanol was added to the blubber, spiked with 10 μ L of 1.5 ng/ μ L of d₄-cortisol for a total of 15 ng in blubber) to create a ratio from which a known amount of analyte was determined and assessed against our calibration model. Samples measured as non-detects were included in statistical analyses by replacing ND with half of the MDL concentration. Duplicate samples were analyzed with every six samples to verify the repeatability of the analytical methods. Duplicate cortisol values were within 19% of each other. Average recovery for plasma and blubber was 79% ($n = 35$) and 30% ($n = 217$), respectively. The recoveries in blubber were lower than desired; however, the detection frequency was 96.2% and the standard deviation in d₄-recoveries was <20%, well below the Horowitz RSD for precision of 30% for concentrations at the 1 ng/g range thus supporting that the method has good method precision. It is important to note that for blubber tissue there is no available matched matrix standard reference material for cortisol, this is a limitation to the method that requires future consideration. However, for plasma we were able to use NIST SRM 971 to assess method accuracy. The certified value in SRM 971 is 250.1 ± 5.8 nmol/L and with our method, we determined a value of 202.7 ± 17.0 nmol/L. Our measured SRM concentrations were 81% of the certified value, and using the Horowitz factor at 250 nmol/L, we fall within the 25% RSD range limit, as such based on our QC data objectives we consider our method to be fit for purpose.

Contaminant and vitamin analysis

Detailed description of PCB, PBDE, vitamin A, and vitamin E analysis of the samples in this study is described in Desforges et al. (2013). The final contaminant data included 169 PCB congeners and 30 PBDE congeners, which excluded nona and deca PBDEs because of analytical difficulties; data reported herein refer to the lipid weight corrected sum of all the congeners for each contaminant group (Supplementary Table S1²). Vitamin A levels reported herein refer to total retinoids in blubber and liver (retinol, dehydroretinol, and retinyl esters) and retinol in plasma. Vitamin E levels include total tocopherols (α -, γ -, and δ -tocopherol).

Statistical analyses

The effects of sex were assessed using a *t*-test for each tissue (i.e., inner, middle, outer blubbers, and plasma). To test for the effect of year and the layer of blubber on cortisol levels, a two-way ANOVA was used to enable the assessment of both factors independently and together for interaction effects, this was followed by pairwise analyses. Means are reported for various parameters with their associated standard errors. Pearson's correlation

was used to assess relationships between blubber layers and plasma. Systat 12 was used to run these univariate analyses.

To understand relationships between cortisol and biological factors and cortisol and biochemical factors, two stepwise multiple regression models were used for each tissue (i.e., inner, middle, outer blubber, and plasma). The biological model included the potential confounding biological factors of age, length, blubber thickness along with year. The biochemical model included both the endogenous compounds of vitamins A and E and the exogenous compounds PCBs and PBDEs along with year. For each criterion variable (inner, middle, outer blubber, and blood plasma cortisol levels), dependent variables (year, age, length, blubber thickness, inner vitamin E, middle vitamin E, outer vitamin E, inner vitamin A, middle vitamin A, outer vitamin A, PCB, PBDE) were tested using a stepwise regression model (R core team 2015). Due to the effect of sex in dependant variables (and disproportion of females among years), we tested for males only. The general equation (eq. (1)) for all regression models followed

$$(1) \text{ Co} = a_1V_1 + a_2V_2 + a_3V_3 + \dots + b$$

where Co is the cortisol level or predicted variable, V_1 to V_5 are exploratory variables, a_1 to a_5 are coefficients estimated by the model for each corresponding exploratory variable, and b is the slope estimated by the linear regression. Stepwise model selection was used to find significant relationships and best models were selected based on both p -values, adjusted R^2 , AIC (Akaike information criterion) values, and the AICs (measured as $\text{AIC}_1 - \text{AIC}_2$, where AIC_1 is the model being tested, and AIC_2 is the AIC value for the best fitting model).

Results

Cortisol measurements

Blubber cortisol levels ranged from undetectable to 17.8 ng/g, whereas plasma ranged from 2.5 to 61.2 ng/mL. There were no significant differences between sexes for all three blubber layers and plasma ($p > 0.2$). There were few females in this study ($n = 10$) relative to males ($n = 53$) and their absence in 2007 and 2010 made it challenging to assess the influence of sex as a factor. Although it may not be appropriate to compare blubber and plasma matrices for cortisol concentrations, converting ng/g and ng/mL to ppb is a straight conversion and demonstrates that the plasma concentrations (averaging 18.7 ppb) were 10× higher than blubber concentrations (averaging 1.1 ppb).

Because the sampling year may have influenced cortisol levels, we assessed for differences among blubber layers as well as years in a two-way ANOVA to check for interactions. Although blubber cortisol levels were found to significantly differ among years ($p < 0.0001$), there was no interaction between the year of sample collection and the layer of blubber being assessed ($p = 0.5$). The sampling year 2008 was significantly higher than the three other sampling years (Fig. 2). Cortisol levels differed among blubber layers ($p = 0.004$), with the inner layer being significantly higher (mean for males and females combined $1.7 \text{ ng/g} \pm 0.32$) and the middle and outer layers not significantly differing from one another ($p = 0.98$) (Fig. 2). Plasma cortisol levels did not differ among years ($p = 0.9$).

All blubber layers and plasma were significantly correlated with the exception of the correlation between middle blubber and plasma ($p = 0.17$; Table 1). Correlations were strongest among the three blubber layers. Plasma has the strongest correlation with inner blubber ($r = 0.62$; Table 1).

Cortisol relationships with biological and biochemical variables

To assess if biological factors such as age, length, and blubber thickness; and biochemical factors such as vitamins A, E, and Σ_{139} PCB, Σ_{30} PBDE, explained cortisol levels,

Fig. 2. Mean cortisol (ng/g) concentrations in beluga whale blubber tissue layers collected from 2007 to 2010 at Hendrickson Island, NT.

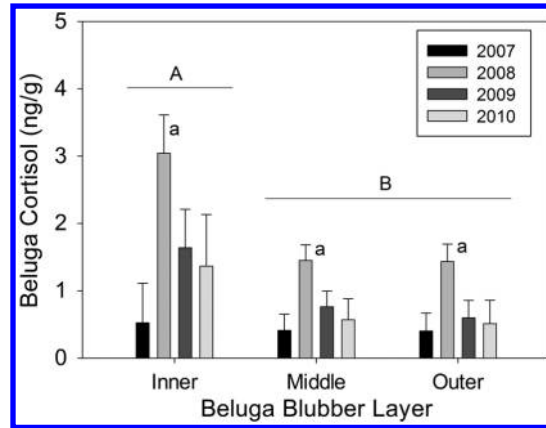


Table 1. Pearson’s correlation matrix for plasma and blubber layers sampled in beluga whales.

	Inner blubber ^a	Middle blubber ^a	Outer blubber	Plasma
Inner blubber	1.00			
Middle blubber	0.80	1.00		
Outer blubber	0.79	0.97	1.00	
Plasma	0.62	0.38 ^c	0.45 ^b	1.00

^aAll correlations in column are statistically significant ($p < 0.001$).

^b $p = 0.04$.

^c $p = 0.17$.

we ran stepwise multiple linear regression models to evaluate best fit for each tissue. Overall the biological models had better fits with cortisol than the biochemical models (Table 2). Best fit models for inner and middle blubber revealed age to be a predominate variable, with continued good fits with blubber thickness, length, and year of collection. Cortisol increased with age, whereas trends with blubber thickness and length were negative. The best fit models for the outer blubber and plasma were not significant and had low model fits (Table 2). The biochemical models had low to no significance and poor fits with the only significant model measured between plasma cortisol and vitamin E (Table 2).

Discussion

Cortisol levels and variability

Cortisol exhibited differences among beluga tissues, with levels in plasma being 10 times higher than those in blubber. The high levels in plasma are likely responsible for elevated inner blubber cortisol levels, as it acquires hormones through passive diffusion from blood (Deslypere et al. 1985). Lower cortisol levels in middle and outer blubber that had weaker correlations with plasma cortisol, suggest that those blubber layers are not well penetrated by circulating blood and may better reflect a longer term integrative signal that is not as readily modulated by acute stress relative to inner blubber. The same observation was made for vitamin A in these whales in our previous study (Desforges et al. 2013), highlighting a

Table 2. Best fit models for biological factors and biochemical factors defined by regression models as determined by AIC and *p*-values.

Tissue	Variables	<i>p</i> -value	Adjusted R ²	AIC
Biological factor models				
Inner	Age (+)	0.001	0.17	95.22
	Age (+) + blubber thickness (-)	0.002762	0.1817	(+0.28)
	Age (+) + length (-)	0.004143	0.168	(+1.14)
	Age (+) + year (+)	0.005283	0.1597	(+1.65)
Middle	Age (+)	0.017	0.09087	-5.735
	Age (+) + blubber thickness (-)	0.04098	0.08642	(+1.20)
	Age (+) + Length (-)	0.05461	0.07566	(+1.81)
	Age (+) + year (-)	0.0596	0.07235	(+1.99)
Outer*	Length (+)	0.5253	-0.01329	-45.939
Plasma*	Blubber thickness (+)	0.6075	0.1165	133.51
Biochemical factor models				
Inner*	PCB (-)	0.4735	-0.00947	105.74
Middle*	Vitamin A (+)	0.08544	0.03917	-2.8587
Outer*	PBDE (-)	0.2612	0.0286	-46.847
Plasma	Vitamin E (-)	0.04493	0.1272	129.34

Note: + indicates a positive relation; - indicates a negative relation; * indicates there were no significant models for a tissue type and the best fitting model is presented instead. Statistically significant models ($p < 0.05$) are shown in bold.

common physiological mechanism linking these important hormones to different tissue compartments.

Differences were noted between sexes only for the innermost blubber layer; however, the small sample size for females precludes a complete assessment of the influence of sex on cortisol. Results in the literature regarding sex differences in cortisol are mixed in marine and terrestrial mammals, with some documenting differences among sexes in polar bears (Oskam et al. 2004; Macbeth et al. 2012) whereas others found no differences in polar bears, grizzly bears, and harbour porpoises (Eskesen et al. 2009; Macbeth et al. 2010; Bechshoft et al. 2013). The higher cortisol levels in the metabolically active inner blubber layer of females compared to males may be a reflection of higher stress conditions or energetic demands in reproductive females (Macbeth et al. 2012 and references therein). The timing of the beluga hunt (typically July) corresponds with the calving season, and with a 14 month gestation period, females are either in an early phase of gestation or have just entered post-partum phases, as such hunters typically avoid hunting female belugas (Harwood et al. 2002).

Highest cortisol levels measured in 2008 were consistent in all tissues and were not explained by differences in size, age, or blubber thickness. High levels in all tissues demonstrate that the middle and out layers were responsive to changes at a minimum of an annual period. Potential factors that may have resulted in the year 2008 (or in the months prior to the samples taken) having higher levels may include changes in prey availability/quantity, changes in predation pressures, increased anthropogenic-related stressors or other external environmental factors. The large home range of the EBS beluga and the absence of focused threat/stressor-response studies precludes our ability effectively evaluate potential stressors. At a regional scale, the fall sea ice minimums for the western Arctic hit significant lows for 2007 and 2008 at 32% and 28% of normal concentration (<http://www.ec.gc.ca/glaces-ice>). Such regional scale variables have cascading impacts on food webs and may explain recently observed declines in condition (Harwood et al. 2015) and are hypothesized to have altered the prey base and mercury exposure to these beluga whales (Loseto et al. 2015). Regional scale environmental influences have been observed in

polar bear fur cortisol levels, whereby inter-annual fluctuations in climate and ice cover (via the North Atlantic Oscillation index) strongly correlated (positively) with cortisol in East Greenland bears (Bechshoft et al. 2013). A continued time series of cortisol is required to assess climate change effects and other environmental factors on beluga cortisol levels.

Cortisol comparisons: wild and captive

Comparing cortisol levels measured in this study with previous beluga cortisol studies is challenged by different methodologies, tissues, and conditions of study (wild vs. captive). To assist, we have made a table for comparison of our findings to other studies (Table 3). Baseline or benchmark plasma cortisol levels were determined from three captive beluga whales that were trained to voluntarily approach the investigator, and averaged 18 ng/mL (Schmitt et al. 2010) (Table 3). During a stressful event, the cortisol plasma levels increased, and ranged from 38 ± 34 to 79 ± 15 ng/mL and returned to baseline levels 12 h after the stressful event (Schmitt et al. 2010). Plasma cortisol levels from our study were on par with the resting baseline levels of the captive belugas and two times lower than the stressful event. We recognize that comparisons with captive studies are not equal since the living conditions may induce a chronic stress response (i.e., not a true baseline). We expected our beluga plasma cortisol to be high in response being chased during the hunt as was observed in previous studies of belugas and other cetaceans (Thomson and Geraci 1986; St. Aubin and Geraci 1989).

Plasma cortisol levels in live captured, wild belugas (Beaufort Sea, Hudson Bay, High Arctic) were double the measurements in our study, but lower than the induced stressful event in the captive belugas (St. Aubin and Geraci 1989; St. Aubin et al. 2001) (Table 3). The chase, capture, and restraint of a marine mammal can increase plasma cortisol levels (St. Aubin and Geraci 1989); however, we expected levels to be similar to our study given these belugas had also experienced a chase. St. Aubin et al. (2001) noted that 32 of the 115 beluga whales sampled were collected from a hunt (rather than live sampled), yet the authors did not report on any differences observed between hunted and live captured, nor was there comment on differences among the three populations sampled. It is unclear why such differences are observed among the studies; however, our means fall into St. Aubin et al. (2001) reported standard deviation. The authors noted a lack of size and age effects on cortisol levels (St. Aubin et al. 2001). Additional studies are needed to enable robust comparisons between live sampled to hunted sampled beluga cortisol levels. A factor for consideration when comparing studies is the date of study, as some studies were carried out over 20 years ago and methodologies and the associated sensitivities of instruments may have influenced cortisol measurements, in addition to sample preservation abilities.

Only one other study measured cortisol in inner, middle, and outer blubber for beluga (Trana et al. 2015). The study used samples from the same population evaluated here; however, samples were stored at warmer freezer temperatures (i.e., -40 °C vs. -80 °C in our study); additionally, a different extraction and detection method was used (radioimmunoassay kits for analysis). Trana et al. (2015) measured cortisol at three times lower for inner blubber and two times lower for middle and outer blubber (Table 3). This highlights a potential interference that requires further investigation of variables such as sample storage temperatures, extraction, and (or) analytical methods.

Cortisol associations with biological and biochemical factors

Determining the drivers and relationships between cortisol and biological and biochemical factors proved to be less significant than anticipated. Best fit models for biological factors highlighted age followed by age and other biological metrics (blubber thickness, length) to be important determinants of cortisol. Given the best fit biological models for plasma and

Table 3. Mean cortisol levels for comparison to other wild and captive studies. To allow for comparisons all concentrations are shown in ng/mL (plasma) and ng/g (blubber).

	Our study ^a	Trana et al. ^b	Schmitt et al. ^c	St. Aubin et al. ^d	St. Aubin and Geraci ^e
Location	Beaufort Sea	Beaufort Sea	Captive	Captive	Beaufort Sea Hudson Bay High Arctic
N	Dead sampled 62	Dead sampled 27	Live sampled, baseline 3	Out of water examination 3	Live sampled 115
Plasma (ng/mL)	Mean ± SD 18.68 ± 12.12	Mean ± SD	Mean ± SD 18.00 ± 7.10	Range 38–79	Mean ± SD 32.17 ± 16.43
Blubber (ng/g)	Inner 1.70 ± 2.55	0.49 ± 0.11			
	Middle 0.83 ± 1.1	0.33 ± 0.08			
	Outer 0.77 ± 1.16	0.31 ± 0.06			

^aMean includes both sexes and data across years 2007–2010.

^bMean includes both sexes and data across 2009–2010.

^cBeluga were originally caught in the Hudson Bay Churchill River region and were held captive and trained for 19 years at the time of this study.

^dStudy spans 15 years from 1983 to 1997.

^eBeluga were sampled in 1985 and 1987.

outer blubber were not significant we believe biological factors may not play a determining role in cortisol. This fits well with plasma cortisol because we know these levels reflect heightened stress induced from a chase and hunt. The lack of a significant biological relationship with outer blubber cortisol levels is interesting as it suggests this tissue is free of biological confounding factors, yielding it an ideal tissue for sampling and monitoring.

Cortisol was identified as a good indicator of condition in stellar sea lions as levels increased during periods of energy restriction and body mass loss (du Dot et al. 2009). While our models did not assess condition, the negative relationships with blubber thickness and length may lend support to this observation, whereby thinner, smaller whales may be in slightly poorer condition. The positive relationship with age may bolster this hypothesis because age, length, and blubber thickness are typically positively related. Findings suggest that age has an underlying influence on benchmark cortisol levels. In polar bears, sex, size, and life-stage interactions were important factors in defining hair cortisol levels, reflecting the various influences of reproductive stress and energetic demands of growth, fasting, and migration (Macbeth et al. 2012). These findings lend support to age being an important confounding variable influencing cortisol levels. With regards to condition, for our study, we did not observe any individuals in poor condition, a missing factor that may shed light on a condition–cortisol relationship.

Results from the biochemical model had even fewer significant relationships between cortisol and the endogenous and exogenous compounds that were not consistent among tissues. The overall weak model fits may lend support to the lack of relationship between cortisol and circulating vitamin levels, as well as the lack for potential effects of PCB and PBDE on cortisol levels. Plasma was the only tissue to have a significant relationship, as measured with vitamin E. Note that the vitamin E was measured in inner blubber, not circulating with plasma. Because plasma cortisol likely reflects acute stress from the chase it is unclear how the relationship with blubber vitamin E is manifested. Few studies have evaluated the relationship between cortisol and vitamins, though some evidence suggests an antagonistic effect of vitamin A and E on glucocorticoids and the HPA axis

(Anstead 1998; Montero et al. 2001). Our most significant fit biochemical model was the negative relationship with vitamin E followed by a positive relationship with vitamin A. Controlled experiments in sturgeon (*Huso huso*) and dairy cows exposed to stressful events have found no association between vitamin E administration and increased cortisol levels (Mudron et al. 1994; Mudron et al. 1996; Falahatkar et al. 2012). However, pre-treatment with vitamin E in pigs reduced peak cortisol levels after stress challenge (Webel et al. 1998). Similarly, vitamin A (retinol and retinoic acid) has been found to antagonize the HPA-axis and cortisol production, and vice versa (Enwonwu and Phillips 2004; Marissal-Arvy et al. 2013), suggesting a possible mechanistic link between these vitamins and cortisol. This antagonism may be the result of interactions of active vitamin A compounds on glucocorticoid receptors and expression of dehydrogenase enzymes important for glucocorticoid activation (Anstead 1998; Marissal-Arvy et al. 2013). Further exploration of the opposing relationships between vitamins A/E and cortisol are needed to define direct interactions from cross correlation with common predictors.

Previous analyses identified biological factors as well as PCBs and PBDEs as important determinants of vitamin concentrations in these beluga whales (Desforges et al. 2013). It is important to note that the PCB and PBDE levels measured here are 7- and 12-fold lower than those measured in the St. Lawrence estuary beluga population, a population heavily burdened with contaminant loads (Hobbs et al. 2003; Raach et al. 2011). Levels of PCBs, PBDEs are correlated in beluga whales ($r = 0.63$) such that effects from the individual compounds are difficult to identify. Nonetheless, the different physicochemical properties of PCBs and PBDEs may cause differences in toxicokinetics and toxicodynamics, and to capture these, we included both contaminant groups in our analyses. We observed no significant relationships between contaminants and cortisol among the different tissues in this study. Lack of significant relationships may indicate the concentrations of POPs are such that they are not impacting or influencing cortisol levels. Studies on polar bear cortisol responses to POPs demonstrated a variation in relationships possibly owing to different tissue matrices (Oskam et al. 2004; Bechshoft et al. 2012a, 2012b). For example, cortisol and PCBs measured in polar bear hair demonstrated no relationship with organochlorines (OCs) (Bechshoft et al. 2012a), despite the negative trends observed between plasma cortisol and OC levels (Oskam et al. 2004). Cortisol relationships with biochemical factors were weak and suggest a lack of relationships with the endogenous and exogenous compounds or may point to our sample set not including individuals with high contaminant concentrations or in poor condition to build extremes into the dataset for a trend to be set.

Monitoring application for management

Our study lends support for the use of the middle and outer blubber layers to reflect resting, chronic, or integrative cortisol levels that are less susceptible to acute stress. This is in accordance with previous findings where plasma cortisol measurements were not ideal for monitoring the general state of beluga health due to reactivity to acute stress (St. Aubin et al. 2001). Middle and outer blubber tissues are also ideal tissue when considering storage degradation factors of heat, light, and oxygen exposure that the inner blubber and sample edges are readily exposed to (Trana et al. 2015); the use of the outer layer would allow for live biopsy collections. Lastly, these layers are known to reflect long-term storage of other compounds, such as fatty acids, vitamins, and contaminants, and have been suggested as an ideal tissue to reflect resting or chronic health (Kellar et al. 2015). The design of a monitoring program must consider factors that influence cortisol levels, such as individual biometrics, method of sample collection, the selection of tissue, sample storage prior to analysis, the extraction procedure and instrumentation used that will also preclude data comparison with other studies.

Establishing a benchmark, whether it be for cortisol or other physiological targets that respond to a stressor enables management to act when changes are observed. It is important that management not only have benchmarks for cortisol in beluga but also have reference points or targets to understand how much stress the population exposed to. Thus, cortisol provides an indicator tool that can be used for conservation management in the Tarium Nirvutait Marine Protected Area (Loseto et al. 2010). If there is an increase in disturbance from human activities (e.g., barges, vessels, seismic, and other commercial and industrial activities), we may be able to monitor physiological responses with changes in cortisol levels, and use references from other populations and captive studies, to be able to intervene if necessary.

Acknowledgements

This project was supported by multiple funding agencies and partners including Fisheries and Oceans Canada, the Northern Contaminants Program, the Fisheries Joint Management Committee, the Northern Students Training Program, and the Cumulative Impacts Monitoring Program. We thank F. and N. Pokiak for their years of dedication to the monitoring program, collecting samples in a consistent and concise manner for over 10 years at Hendrickson Island. We thank J. DeLaronde, A. MacHutchon, G. Boila, and B. Steward for laboratory support. We are grateful for the partnerships and support of Tuktoyaktuk Hunters and Trappers Committees and the beluga hunters who allowed us access to their whales for beluga tissue collections.

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