SC/66b/BRG/06

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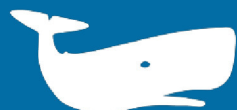

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**Total Mercury Concentrations in Epidermis and Advancements in Steroid Hormone Analyses in Gray Whale Blubber:**

**Significance for Western Gray Whale Biopsy Research**

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**ABSTRACT**

The IWC Scientific Committee has repeatedly stressed the critical need for studies on reproductive status, genetics, and feeding ecology of the western gray whale (*Eschrichtius robustus*) subpopulation, which is currently designated as critically endangered by the IUCN. Biopsy samples from western gray whales off Sakhalin Island have been collected in collaboration with Russian scientists since 2011. These samples provide precious material amenable to hormone, genetic and stable isotopes of C and N analyses, as previously reported by our team (Gendron *et al.* 2015, Bickham *et al.* 2015, Bickham *et al.* 2013). Maximizing the scientific information that can be mined from these samples is critical. Here, we report on mercury analyses in epidermis and enhanced hormone analyses optimized for gray whale blubber that further widen data mining. Due to the extremely small size of the biopsy material available from western gray whale samples, these analyses were conducted on tissue from fifteen stranded eastern gray whale individuals for processing and analytical chemistry optimization purposes. The methodologies described in this report can be performed in western gray whale biopsy samples obtained from live, free-ranging individuals with more certainty related to tissue mass and other characteristics of biopsy samples.

Total mercury concentrations ([THg]) were analyzed in three distinct layers of the epidermis to assess diet and food web ecology of the gray whale. Gray whale epidermis has three distinct morphological and functional layers: the protective outer layer (stratum corneum); the middle layer, (stratum spinosum); and the regenerative layer (stratum basale). The [THg] were at expected levels and low relative to data published in other whales. The [THg] was lower in the corneum layer whereas the depths designated as D1 and D2 (subsections of the spinosum) had similar [THg]; this may reflect uniformity of that stratum. As water content and [THg] varies by strata care should be taken in which layers are used for this and similar chemical analyses and subsequent interpretations.

We previously validated reproductive hormone profiling in gray whales (Gendron *et al.* 2015). Subsequently, the amount of blubber needed for the progesterone extraction and pregnancy determination was successfully reduced and optimized from 150 mg to 50 mg (wet weight) using samples from stranded eastern gray whales (Gendron *et al.* 2015). These optimizations and developments were performed using an enzyme-linked immunosorbent assay (ELISA) for blubber steroid hormone analysis. The ELISA methodology allows for the analysis of only one steroid hormone at a time and analyses of multiple hormones would thus likely require more sample mass than what is available from the current standard skin biopsy. In order to bypass this limitation we report, here, on the development of a liquid chromatography tandem-mass spectrometry (LC-MS/MS) methodology that allows multiple steroid hormones to be detected simultaneously and using a single 50 mg gray whale blubber sample. The method development was performed on stranded eastern gray whale samples and included: 1) optimization of steroid hormone extraction for LC-MS/MS analyses, 2) successful simultaneous detection of endogenous progesterone, testosterone, and hydrocortisone from a 50 mg ww blubber sample, and 3) validation of cholic acid as surrogate internal standard for steroid hormone quantitation.

Further optimization is currently underway and includes 1) further validating the LC-MS/MS methodology by comparing it to a paired ELISA analysis, 2) analyzing other cetacean blubber samples to validate a cetacean-wide protocol, and 3) incorporating additional steroid hormones to the current panel to produce a more complete reproductive and fitness/stress assessment.

# INTRODUCTION

Cetacean health research is a relatively new field and while it is rapidly expanding, many knowledge gaps still remain. Due to a plethora of logistical hurdles associated with working with these species, data on population dynamics, health information, and relative fitness is lacking for most free-ranging populations of cetaceans. However, this information is vital when developing scientific-based management and conservation plans for these populations. The IWC Scientific Committee (SC) has thus repeatedly stressed the critical need for studies on reproductive status, genetics, and feeding ecology of the western gray whale (*Eschrichtius robustus*) subpopulation, which is currently designated as critically endangered by the IUCN. Biopsy samples from western gray whales off Sakhalin Island have been collected in collaboration with Russian scientists since 2011. These samples provide precious material amenable to hormone, genetic and stable isotope analyses, as previously reported (Gendron *et al.* 2015, Bickham *et al.* 2015, Bickham *et al.* 2013). Maximizing the scientific information that can be obtained from these samples is critical. Here, we report on mercury analyses based on specific epidermal layers and enhanced hormone analyses optimized for gray whale blubber. Due to the relatively small mass of the biopsy material available from western gray whale samples obtained from Sakhalin, these method development analyses were conducted on tissue from fifteen stranded eastern gray whale individuals for optimization purposes. The methodologies described in this report have been optimized so that they can now be performed in western gray whale biopsy samples obtained from live, free-ranging individuals.

Hormone analyses provide fundamental information for the assessment of the health and fitness status of cetacean populations. Marine mammal hormone analyses is a relatively new field of study being a little under 15 years old, but already crucial work has been done in this area. Progesterone analyses have been utilized to assess the reproductive status, more specifically pregnancy status, in the dugong, *Dugong dugon*, the common bottlenose dolphin, *Tursiops truncatus*, the short-beaked common dolphin, *Delphinus delphis*, the northern right-whale dolphin, *Lissodelphis borealis*, the Pacific white-sided dolphin, *Lagenorhynchus obliquidens*, and the minke whale, *Balaenoptera acutorostrata* (Burgess *et al.* 2012, Perez *et al.* 2011, Kellar *et al*. 2006, Mansour *et al*. 2002). Testosterone analyses have been utilized as a potential marker of male reproductive status in the short-beaked common dolphin, *D. delphis* (Kellar *et al.* 2009). Glucocorticosteroid analyses have been conducted in the harbor porpoise, *Phocoena phocoena*, the short-beaked common dolphin, *D. delphis*, and the common bottlenose dolphin, *T. truncatus* to assess their stress and relative fitness levels (Bechshoft *et al.* 2015, Kellar *et al.* 2015, Fair *et al.* 2014). We selected three hormones (progesterone, testosterone, and cortisol) to generate a reproductive and fitness assessment panel in gray whale.

Our analyses focus on the blubber matrix. While there are other matrices to measure steroid hormones in free-ranging whales such as from blow or feces, collection of these samples can prove difficult due to complex sampling conditions and the elusive nature of many cetaceans (Hogg *et al.* 2009, Thompson *et al*. 2014, Rolland *et al.* 2005, Hunt *et al.* 2006, Burgess *et al.* 2012). Skin and blubber biopsy collection provide a reliable access to samples amenable to multiple analyses in addition to hormone analyses including genetic analyses, stable isotopes of C and N analyses, and fatty acid analyses (Gendron *et al.* 2015, Bickham *et al.* 2015, Bickham *et al.* 2013, Herman *et al.* 2005). Hormone analyses in cetaceans has been previously conducted in the blubber matrix, but so far, almost exclusively from large blubber samples obtained from stranded and subsequently necropsied individuals. Methodologies compatible with the small blubber samples available from live, free-ranging individuals

are critically needed, especially if they can be conducted concurrently with genetic analyses, sex determination, stable isotope analyses, and fatty acid analyses.

The current published methodology employed to measure steroid hormones from blubber relies on an enzyme-linked immunosorbent assay (ELISA). There are benefits and limitations associated with this methodology. Some of the benefits associated with ELISAs are that 1) it is the current accepted methodology to perform this analysis in the blubber matrix and generates publishable data, and 2) it is a relatively inexpensive and fast assay. Unfortunately, the ELISA assay generates relatively highly variable results due to cross reactivity and lack of consistent reaction efficiency. To produce reliable quantitation data using an ELISA, large amount of sample (75-150 mg) must be extracted and analyzed in triplicate to compensate for the variability of the ELISA (Kellar *et al.* 2006). This is costly and sometimes unrealistic in terms of sample availability, especially in the context of blubber biopsy samples collected from free-ranging cetaceans. Another limitation of performing an ELISA assay on a blubber sample is the generation of data for a single hormone at a time.

Given the great need for health and fitness data in free-ranging cetacean populations and the limited size of the blubber samples obtained from these individuals, our goal with this work was to develop a methodology for detecting and quantitating steroid hormones from the blubber matrix that is more practical. Based on hormone analyses using analytical chemistry techniques in other species, we selected a methodology to address the need for specific, sensitive analyses compatible with small sample size. A more complete health and fitness assessment and hormone profile is produced by analyzing multiple steroid hormones (hydrocortisone, testosterone, and progesterone). For optimization purposes we had available to us blubber samples collected from necropsied eastern gray whales. Future directions of research involve including additional hormones in our analyses, including estradiol and other glucocorticosteroids, to produce a more complete assessment of the individuals.

Analyzing the total mercury accumulated in tissues can be used as another measure to assess the health for this species. This type of analysis is also an indicator of the population's environment and food chain health status as most marine mammals occupy the top of their respective food chains (Pauly *et al.* 1998). Work with total mercury analysis has been previously conducted in other cetacean species such as the harbor porpoise, *P. phocoena*, the white-beaked dolphin, *Lagenorhynchus albirostris*, the pantropical spotted dolphin, *Stenella attenuata*, the spinner dolphin, *Stenella longirostris*, the common bottlenose dolphins, *T. truncates,* and the Risso's dolphin, *Grampus giseus*, but not yet in the gray whale and not commonly in the epidermis (Siebert *et al.* 1999, Chen *et al.* 2002). This analysis is typically performed in necropsy tissue rather than in biopsy tissue from live individuals. Here, we report on total mercury analysis of these individuals using the epidermis from skin biopsy samples. For optimization purposes we had available to us samples collected from necropsied eastern gray whales. In this study gray whale epidermis was divided into three distinct morphological and functional layers: the protective outer layer (stratum corneum), the middle layer (stratum spinosum), and the regenerative layer (stratum basale). Total mercury concentrations (THg) were analyzed in these distinct layers of the epidermis to assess diet and food web ecology of the gray whale. Future directions of research involve performing this analysis on a biopsy sample collected from a live, free-ranging individual with known minimal mass for analyses and possible epidermal layer variability.

## METHODS

Collection and Storage: Eastern Gray Whale Samples

Tissues from stranded animals were obtained through collaboration with The Marine Mammal Center (TMMC). All samples were shipped on dry ice and stored in -80°C freezer until processing and analyses. Inventory and hormone analyses were performed at The Institute of Environmental and Human Health, Lubbock, TX. Due to the nature of the sample collections, subsamples with ample amounts (mass) of dermis and hypodermis were received from stranded animals. Thus, sample mass is not reported in the detailed inventory of eastern gray whale samples presented below in Table 1 as they were relatively robust.

Histology

We used archived, frozen gray whale epidermis (ENP) subsamples (n=16) that were collected from stranded animals. The tissues were fixed in 10% formalin, routinely processed, sectioned at approximately 5 µm, placed on glass slides, stained with Hematoxylin and Eosin (H&E), and viewed using light microscopy. Photomicrographs of the sections were taken using an Olympus dp71 camera (Olympus America, Inc, 3500 Corporate Parkway, Center Valley, PA 18034). Layers of the epidermis were measured using Image J image analysis software (http://imagej.nih.gov/ij/). We measured: stratum corneum, stratum spinosum, top layer spinosum, and the ratio of top of spinosum: whole spinosum. Each layer was measured 5 times and the average was used. Mercury concentrations were determined from the corneum at 1mm depth and from the stratum spinosum at 3 depths taken in 3 mm increments.

Total Mercury Concentration ([THg]) Analysis

Gray whale epidermis was described as three distinct morphological and functional layers: stratum corneum, stratum spinosum, and stratum basale (Reeb *et al*. 2007). Epidermis from 15 eastern gray whales (Table 1) was subsampled to investigate total mercury concentrations ([THg]) for these different epidermal layers (depths). Based on detailed structural analysis, the epidermis was divided into 4 subsamples – the outer corneum, and the spinosum was divided into 3 equal depths, Depth 1 was the outermost section, adjacent to the corneum, while Depth 3 was innermost.

Subsamples were freeze dried. Water content was calculated by subtraction of dry mass from wet mass. Dried samples were ground to a fine, homogenous powder with a cryomill. [THg] was measured, in triplicate using a direct mercury analyzer (DMA80 tri-cell) with a detection limit of 8 ppb (dry mass basis). Three whales (all calves) had [THg] below the detection limit and have not been included in statistical comparisons of epidermal layers. Thus, our reported data represent 12 whales. For one whale we had both dorsal and ventral samples and analyzed both.

Steroid Hormone Analyses

Steroid hormone extraction was performed as described previously in other cetacean blubber samples (Mansour *et al*. 2002, Kellar *et al*. 2006; Perez *et al*. 2011). Eastern gray whale blubber samples obtained from stranded animals were received from The Marine Mammal Center (F. Gulland and others) and were used for the validation and optimization of the LC-MS/MS methodology in the gray whale species. Due to the small size of biopsy samples, it was necessary to use the smallest amount of blubber possible, 50 mg.

Table 1: Eastern Gray Whale Sample Inventory

| Whale ID | TTU ID | Sex | Age | Tissue Freshness | Species | Collection Date | COD* | Collection Location |
|---|---|---|---|---|---|---|---|---|
| C-68 | ER-13-031 | F | Calf | Fresh | Gray Whale | 10/10/1997 | Maternal Separation | Point Arena, CA |
| C-115 | ER-13-095 | F | Adult | Fresh | Gray Whale | 5/1/2000 | Unknown | San Francisco |
| C-117 | ER-13-088 | M | Adult | Moderate | Gray Whale | 5/5/2000 | Unknown | Sausalito, CA |
| C-118 | ER-13-091 | M | Adult | Moderate | Gray Whale | 5/10/2000 | Unknown | Alameda, CA |
| C-119 | ER-13-094 | M | Adult | Fresh | Gray Whale | 5/11/2000 | Unknown | Richmond, CA |
| C-125 | ER-13-097 | M | Adult | Moderate | Gray Whale | 5/31/2000 | Unknown | Richmond, CA |
| C-126 | ER-13-093 | M | Juvenile | Fresh | Gray Whale | 5/31/2000 | Unknown | Richmond, CA |
| C-139 | ER-13-092 | M | Calf | Moderate | Gray Whale | 2/25/2001 | Maternal Separation | Morro Bay, CA |
| C-179 | ER-13-028 | M | Juvenile | Fresh | Gray Whale | 3/27/2004 | Unknown | San Francisco, CA |
| C-209 | ER-13-089 | F | Pup | Fresh | Harbor Porpoise | 6/30/2005 | Maternal Separation | Samoa, CA |
| C-232 | ER-13-030 | F | Calf | Moderate | Gray Whale | 5/24/2007 | Boat Strike | Richmond, CA |
| C-320 | ER-13-026 | F | Juvenile | Moderate | Gray Whale | 5/12/2010 | Unknown | San Francisco, CA |
| C-341 | ER-13-090 | M | Adult | Fresh | Gray Whale | 4/19/2011 | Boat Strike | San Francisco, CA |
| C-343 | ER-13-027 | F | Juvenile | Fresh | Gray Whale | 6/11/2011 | Boat Strike | Pescardero, CA |
| C-317 | ER-13-029 | M | Juvenile | Moderate | Gray Whale | 4/21/2010 | Unknown | Richmond, CA |

*COD = cause of death

To optimize the LC-MS/MS steroid hormone extraction, it was determined to be best to filter the samples through a regenerated cellulose filter to avoid causing damage to the liquid chromatography column and emitter due to the complex and lipid-rich nature of the blubber matrix. An experiment was conducted to ensure that there was minimal steroid hormone loss due to sample filtration. Four blubber sections (no epidermis) were analyzed from each of four eastern gray whale samples: two 50 mg sections and two 50 mg sections spiked with 300 ng/g of progesterone (P4) to serve as a positive control and to calculate the extraction efficiency for the non-spiked paired sample (16 total samples). This progesterone level is within the range reported in pregnant cetaceans (Kellar *et al*. 2006). A blank and a blank spiked at 300 ng/g were analyzed as quality control samples and for the purpose of determining extraction efficiency. The 50 mg sections were placed into separate homogenization tubes and homogenized on a FastPrep® 24 benchtop homogenizer (MP Biomedicals, Solon,OH). The homogenate was washed using ethanol and run through a series of steroid extractions using ethanol, acetone, diethyl ether, acetonitrile, and hexane. After the addition of each solvent, samples were mixed by vortex, centrifuged, and evaporated with dry nitrogen gas using a Biotage TurboVap® LV (Biotage, Charlotte, NC). Before the final evaporation step, one 50 mg extract and one spiked 50 mg extract from each individual whale was filtered by syringe using a cellulose filter. After the final extraction P4 levels were quantified via an ELISA assay kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's protocol. Samples and standards were placed into 96 well plates treated with goat anti-mouse IgG. Progesterone alkaline phosphatase conjugate and progesterone monoclonal antibody were added to all wells, excluding blanks and specified control wells. Plates were incubated at room temperature on a plate shaker for 2 hours then washed in triplicate with tris buffered saline. P-nitrophenyl phosphate in buffer was added to each well and incubated for 45 min at room temperature without shaking. A stop solution of trisodium phosphate in water was added to each well and the plate was read on a Synergy 4 plate reader (BioTek, Winooski, VT) with an absorbance of 405 nm with a correction of 570 nm. After calculating amounts of progesterone following the instructions provided within the ELISA kit (Enzo Life Sciences, Farmingdale, NY), extraction efficiencies were calculated by subtracting the amount of progesterone in a sample from the amount of progesterone in the same-weight spiked sample and dividing by spike amount (300 ng/g). Progesterone concentrations were corrected according to the corresponding extraction efficiency.

We successfully determined that filtration does not affect steroid hormone concentrations with our ELISA results since there was no significant difference in progesterone concentrations of samples that were filtered when compared to their non-filtered paired sample (see Results). Samples for LC-MS/MS analysis were filtered by syringe and cellulose filter, evaporated, centrifuged for 15 minutes at 3750 g, and dissolved in 50 µL of a 60:40 solution of LC-MS grade water:acetonitrile with 0.1% formic acid. Samples were stored in the -80$^0$C freezer until LC-MS steroid hormone analysis. Samples were spiked with a surrogate internal standard of 5 nM cholic acid for the purposes of quantitation. Samples were diluted 1:10 and 1 ul was injected onto a C18 reversed-phase column. A flow rate of 300 nL/min was used in the chromatography with nanospray ionization into the Thermo LTQ XL linear ion trap mass spectrometer. The scanning conditions of the instrument were optimized to detect the afore-mentioned hormones of interest. By performing tandem-mass spectrometry, we scanned for the parent mass and the most prominent fragment mass to accurately identify our target hormones at the reproducible retention times.

Standard curves for hydrocortisone, testosterone, and progesterone quantitation were generated using cholic acid as a surrogate internal standard with the following concentrations: 0.05 nM (15.72 fg/µL), 0.1 nM (31.45 fg/µL), 0.5 nM (157.23 fg/µL), 1 nM (314.47 fg/µL), 5 nM (1.572 pg/µL), and 10 nM (3.14 pg/µL). Cholic acid concentration was held constant at 5 nM and concentrations were reported as peak area ratios. The use of surrogate internal standards can be very useful (e.g. Kunze, et.al, 2015), and cholic acid was chosen due to its steroidal structure and lack of measurable accumulation in the blubber samples tested.

## RESULTS

Total Mercury Analysis

Table 2: Total Hg concentrations (wet and dry basis) and water content (% $H_2O$) in epidermis of stranded eastern gray whales by histologically defined layers described in the Methods section as mean, standard deviation (S.D.), and range.

| | ***Corneum*** | ***Depth1*** | ***Depth2*** | ***Depth3*** |
|---|---|---|---|---|
| ***$THg_{wet}$ (ppb)*** | | | | |
| **Mean ± S.D.** | 19.95 ± 6.32 | 17.17 ± 4.55 | 15.26 ± 4.55 | 13.24 ± 4.40 |
| **Range** | 13.34 - 34.18 | 13.25 - 26.01 | 10.42 - 25.22 | 8.30 - 24.19 |
| ***$THg_{dry}$ (ppb)*** | | | | |
| **Mean ± S.D.** | 52.49 ± 9.80 | 59.16 ± 13.84 | 56.88 ± 16.07 | 47.23 ± 16.49 |
| **Range** | 34.22 - 65.97 | 43.08 - 93.74 | 37.31 - 98.92 | 29.58 - 90.44 |
| ***% $H_2O$*** | | | | |
| **Mean ± S.D.** | 0.609 ± 0.077 | 0.710 ± 0.029 | 0.732 ± 0.024 | 0.719 ± 0.028 |
| **Range** | 48.2 – 73.0 | 66.7 – 75.5 | 68.7 – 77.4 | 66.2 – 76.3 |

$THg_{wet}$ ranged from 10 – 34 ppb, while $THg_{dry}$ ranged from 30 – 99ppb. Water content was extremely variable (48.2 – 77.4%), which can affect differences in $THg_{wet}$ among layers.

Comparisons among layers were made using repeated measures ANOVA on ranks, followed by a Tukey test (Fig. 1). There are significant differences in all variables ($THg_{dry}$, $THg_{wet}$ and % $H_2O$). $THg_{wet}$ decreased from the outermost layer (corneum) to the innermost layer (D3), however, the higher concentration of $THg_{wet}$ in the corneum is likely related to the relatively lower %H2O in that layer. The pattern of variability of $THg_{dry}$ is more complex, with a tendency for the concentration to be lowest in the innermost (D3) and outermost (corneum), and higher in the intermediate layers.

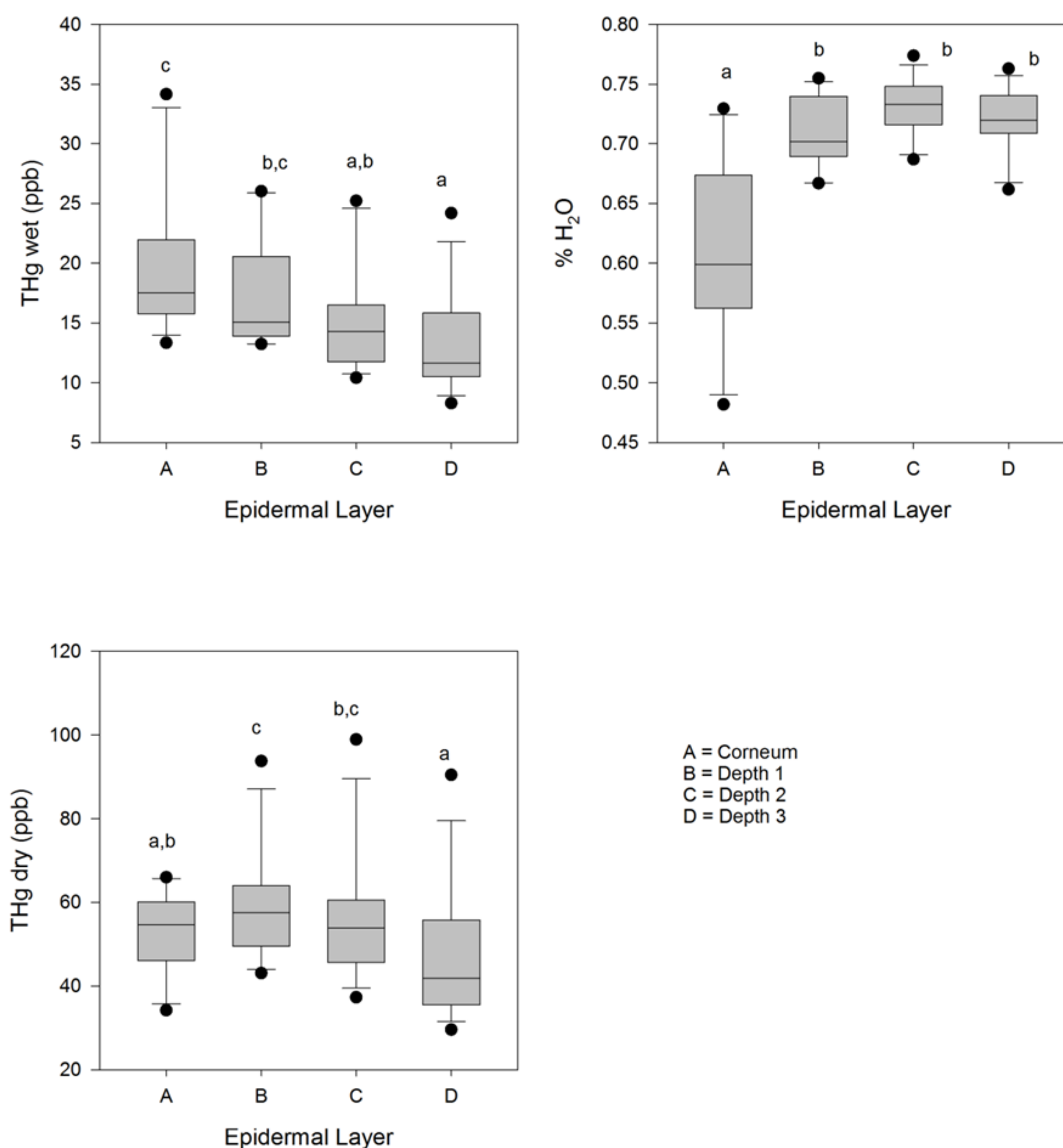


Figure 1: Comparisons among layers (A, B, C and D as described in legend) were made using repeated measures ANOVA on ranks, followed by a Tukey test (Fig. JMC). There are some significant differences in all variables ($THg_{dry}$, $THg_{wet}$ and %$H_2O$); letters a, b and c are used to designate which means are statistically similar (share same letter) or are statistically different (do not share a letter). $THg_{wet}$ decreased from the outermost layer (corneum) to the innermost layer (D3), however, the higher concentration of $THg_{wet}$ in the corneum is likely related to the relatively lower %$H_2O$ in that layer. The pattern of variability of $THg_{dry}$ is more complex, with a tendency for the concentration to be lowest in the innermost (D3) and outermost (corneum), and higher in the intermediate layers.

Concentrations of THg ([THg]) were at expected levels and low relative to other whales, especially odontocetes (Table 3).

Table 3: Total Mercury Concentrations ([THg]) reported in tissues of marine mammals

| Species | Tissue Type | [THg] µg/g (dry weight) | Reference |
|---|---|---|---|
| *Delphinapterus leucas* | Muscle | 1.33 ± 0.66 | Wagemann *et al.* 1998 |
| *Delphinapterus leucas* | Liver | 27.06 ± 24.67 | Wagemann *et al*. 1998 |
| *Delphinapterus leucas* | Skin | 0.78 ± 0.41 | Wagemann *et al*. 1998 |
| *Delphinapterus leucas* | Blubber | 0.103 ± 0.005 | Wagemann *et al*. 1998 |
| *Monodon monoceros* | Muscle | 1.03 ± 0.37 | Wagemann *et al*. 1998 |
| *Monodon monoceros* | Liver | 10.77 ± 8.02 | Wagemann *et al*. 1998 |
| *Monodon monoceros* | Skin | 0.59 ± 0.04 | Wagemann *et al*. 1998 |
| *Monodon monoceros* | Blubber | 0.04 ± 0.03 | Wagemann *et al*. 1998 |
| *Pusa hispida* | Muscle | 0.41 ± 0.37 | Wagemann *et al*. 1998 |
| *Pusa hispida* | Liver | 28.64 ± 29.31 | Wagemann *et al*. 1998 |
| *Lagenorhynchus albirostris* | Muscle | 5.86 ± 4.27 | Siebert *et al.* 1999 |
| *Lagenorhynchus albirostris* | Liver | 77.77 ± 123.79 | Siebert *et al.* 1999 |
| *Lagenorhynchus albirostris* | Kidney | 7.6 ± 8.19 | Siebert *et al.* 1999 |
| *Phocoena phocoena* | Muscle | 12.7 ± 53.3 | Siebert *et al.* 1999 |
| *Phocoena phocoena* | Liver | 38.8 ± 76.5 | Siebert *et al.* 1999 |
| *Phocoena phocoena* | Kidney | 9.6 ± 21.2 | Siebert *et al.* 1999 |
| *Odontoceti and Delphinidae* | Liver | 370 ± 525 | Endo *et al.* 2002 |
| *Odontoceti and Delphinidae* | Kidney | 40.5 ± 48.5 | Endo *et al.* 2002 |
| *Odontoceti and Delphinidae* | Lung | 42.8 ± 43.8 | Endo *et al.* 2002 |

Steroid Hormone Analyses

Table 4: Results of sample filtration study

| Sample | Sex | Age | Sample Weight (g) | Progesterone ng/g | Extraction Efficiency |
|---|---|---|---|---|---|
| C139 | M | Calf | 0.05 | 1.7510 | 79.00% |
| C139 Filtered | M | Calf | 0.05 | 1.5657 | 71.99% |
| C68 | F | Calf | 0.05 | 2.5429 | 73.20% |
| C68 Filtered | F | Calf | 0.05 | 3.0487 | 64.72% |
| C118 | M | Adult | 0.05 | 2.0392 | 79.43% |
| C118 Filtered | M | Adult | 0.05 | 1.3562 | 70.29% |
| C320 | F | Juvenile | 0.05 | 4.1694 | 68.80% |
| C320 Filtered | F | Juvenile | 0.05 | 1.2632 | 60.74% |

Filtration of samples using a regenerated cellulose filter as a processing step before injection onto a liquid chromatography column and subsequent mass spectrometry, did not have a significant effect on the steroid hormone (progesterone) concentrations detectable in the

blubber matrix as measured by ELISA assay. Any variation in progesterone concentration is relatively minor and can be attributed to the variation associated with the ELISA assay due to cross reactivity variables and reaction efficiency of the antibody with the target steroid hormone. Extraction efficiency was within the range previously reported for the steroid hormone extraction used (Kellar et al. 2006, 2009).

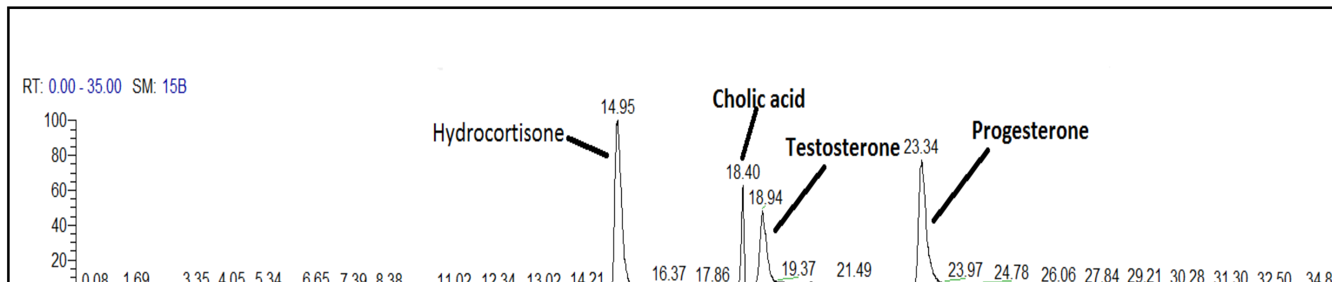


Figure 2: Endogenous blubber hormone and cholic acid spike detection via LC-MS/ MS from a 50 mg ww sample.

The three hormones selected were detected using the mass spectrometer. Hydrocortisone has a retention time of 14.95 minutes; testosterone has a retention time of 18.94 minutes, and progesterone has a retention time of 23.24 minutes. Neither the endogenous hormones nor the blubber matrix affected the peak area of the cholic acid, which has a retention time of 18.40 minutes. All three steroid standard curves exhibited linearity over the range of 0.05 - 10 nM (15.72 fg/ul – 3.14 pg/ul). The standard curve for progesterone is presented in Figure 3. Hydocortisone and testosterone had $R^2$ values of 0.9979 and 0.9915, respectively.

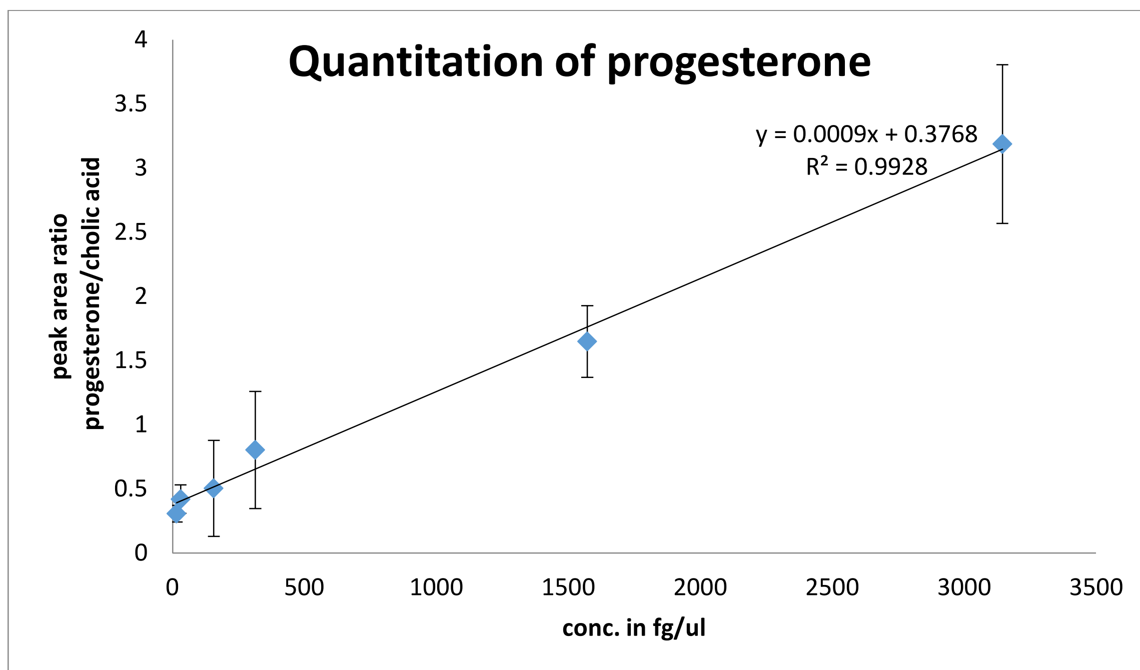


Figure 3: Standard curve for progesterone generated using cholic acid as a surrogateinternal standard.

## CONCLUSIONS

Total Mercury Concentration ([THg]) Analyses

Concentrations of THg ([THg]) were at expected levels and low relative to other whales, especially odontocetes. Of the epidermal strata tested, [THg] was lower in the corneum layer. This may be due to loss of mercury through frequent shedding of the outer layer of the epidermis given that grey whales shed their stratum corneum while feeding in the benthic layer. Similar shedding has been demonstrated in beluga whales. Unlike the corneum, D1 and D2 had similar [THg]. This likely was because they are similar subsections of the spinosum, and may reflect chemical and/or morphological uniformity of that stratum. A publication on biopsy use optimization featuring the present work on microanatomy and mercury analyses as well as previous work presented to the IWC on stable isotopes will be submitted soon.

Steroid Hormone Analyses

Due to the lipid-rich nature of the blubber matrix, an extensive amount of wash steps are taken to ensure matrix interferences are minimized in the steroid hormone determination. Initially analytical chemistry methods, specifically liquid chromatography-tandem mass spectrometry, displayed matrix interferences as observed in the total ion chromatogram. Mainly, blubber matrix components interfered with the steroid hormone separations and caused blockages in the emitter. Because of this, a filtration step of the samples was added to prevent damage to the liquid chromatography column, transfer tube and mass spectrometer. Before implementing this change in the steroid hormone extraction from the blubber matrix, the effects of filtration were tested by quantitating progesterone from the blubber matrix of four individuals using and ELISA assay. No significant detectable loss of progesterone was observed in the filtered samples when compared to the non-filtered samples. From this, it was determined that the filtration of the sample was appropriate and necessary for steroid hormone analysis using LC-MS/MS.

Hydrocortisone, testosterone, and progesterone were detected in the blubber matrix, as seen in the mass spectrum obtained from the extract of a 50 mg ww blubber sample (Figure 2). The cholic acid internal standard did not interfere with this detection and has proved reliable in the quantitation of steroid hormones from this matrix. Although the use of an individual stable-isotope labeled internal standard to quantitate each steroid hormone would increase the precision of quantitation with this methodology, the use of cholic acid as a structurally similar surrogate, is less expensive and does not require the use of a separate standard for each steroid, and demonstrates the utility of nanospray mass spectrometry in the analysis of these steroids from a highly sample-limited, complex matrix. The detection of these hormones from one 50 mg ww blubber sample is significant because the quantity of data generated from this minimal amount of sample was not possible with the ELISA methodology. Steroid hormone detection from the blubber matrix using LC-MS/MS has broad implications with regards to biopsy samples collected from live, free-ranging cetaceans where the blubber sample is very mass-limited. This is especially applicable and significant to the western gray whale population and the biopsy samples collected from these individuals. Population dynamics, health information, and relative

fitness information are essential in generating a scientifically-based conservation plan for this population, and this hormone analysis of these limited biopsy samples aids in providing such information without consuming the sample in its entirety.

Validation of this method will be submitted for publication soon. Further optimization is currently underway and includes 1) comparing the quantitation of steroid hormones from LC-MS/MS analyses to the current accepted methodology of steroid hormone quantitation from the blubber matrix (the ELISA), 2) optimizing the conditions of negative ion mode on the mass spectrometer to analyze hormones such as estradiol which ionize predominantly in the negative form, 3) adding more hormones to the current panel of hormones to generate a more complete assessment of the individuals, and 4) analyzing the steroid hormone profiles of other species of marine mammals using LC-MS/MS techniques. Other goals associated with this study are 1) measuring progesterone in adult gray whale females of known reproductive status, and 2) further reducing minimum sample size required for assessing the reproductive status of an individual. Once progesterone levels in pregnant or recently pregnant females are measured, we will investigate the suitability of further reducing the amount of blubber needed for pregnancy determination from 50 mg to 30 mg ww. We hypothesize that this smaller amount of material may be suitable for pregnancy determination but not absolute progesterone quantitation.

**Permits and Acknowledgments**.— We thank Frances Gulland (The Marine Mammal Center, Sausalito, CA) for providing eastern gray whale tissues. We thank Teri Rowles (National Marine Fisheries Service, Silver spring, MD) for providing the required permits for this research (NMFS Office of Protected Resources' Marine Mammal Health and Stranding Response Program (MMHSRP) permit 932-1905-MA-009526). ). Dr. Marianne Lian assisted with chemical analyses at the UAF. We thank Koen Broker (Shell Global Solutions International) and Jennifer Dupont (ExxonMobil Upstream Research Company) for their efforts to conceive and obtain initial funding for this project. We thank Mike Swindoll, Mike Scott, Lucie N'Guessan and Azivy Aziz for technical information, encouragement, organizational help, and invaluable assistance implementing this program. This study was funded by Exxon Neftegas Limited (ENL) and Sakhalin Energy Investment Company (SEIC). The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding parties.

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