Determining Baseline Stress-Related
Hormone Values in Large Cetaceans

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LONG-TERM GOALS

The long-term goals of this project are four-fold: 1) determine stress-related hormone (cortisol) baseline values from reconstructed lifetime hormone and contaminant profiles in both historic (<1970s) and contemporary archived whale earplugs (>1970s), 2) determine the potential relationship between stress-related hormones and contaminants concentrations within an individual earplug, 3) compare and contrast stress-related hormones and contaminants levels between historical and contemporary earplug samples (among species), and 4) determine the potential relationship between stress-related hormones recovered in earplugs with hormones recovered from blubber. From these data, we will ***establish species specific baseline levels that can be used as a comparative tool in future cetacean stress research***. The results of this study will contribute to improving mitigation strategies through improved assessments of the potential impacts of anthropogenic activity.

OBJECTIVES

Historically, whale earplugs have been used to age large cetaceans. This rudimentary technique which consists of vertically slicing the earplug and counting the light and dark lamina is similar to tree ring-dating techniques. We are now able to combine historical uses (aging techniques) with a unique analytical approach to reconstruct chemical profiles (chronology) from an individual whale. ***These reconstructed chemical profiles provided a unique window into stress-related hormone (cortisol, aldosterone, T_3 and T_4) concentrations and variability during periods of stress, such as development and contaminant exposure.***

1.1 *Objectives*

1. Determine stress-related hormone baseline values from reconstructed lifetime hormone and contaminant profiles in both historic (<1970s) and contemporary archived whale earplugs (>1970s).

2. Determine the potential relationship between stress-related hormones and contaminants concentrations within an individual organism (earplug).

3. Compare and contrast stress-related hormones and contaminants levels between historical and contemporary earplug samples (among species).

4. Determine the potential relationship between stress-related hormones recovered in earplugs with hormones recovered from blubber.

APPROACH

Sectioning whale earplugs
Aliquots of each layer of the frozen whale earplug will be sectioned using a high-speed drill (Dremel 4000 High Performance Rotary Tool, Racine, WI). Earplugs will be sectioned longitudinally through a band saw at approximately 0.5–1 fpm. Serial sections will be labeled and stored at -80 °C. For lamina discrimination (aging), each section will be photographed using a high-resolution digital camera (12MP) and photographic software (Canon U.S.A$^®$).

Stress-related hormone radioimmunoassay technique
Cortisol, aldosterone, hormones thyroxine (T_4) and triiodothyronine (T_3) levels in each identified lamina will be determined utilizing Correlate-EIA kits (ENZO Assay Design). Aliquots of cerumen/lamina will be homogenized and transferred to a (2:1) chloroform:methanol solution where lipids will be extracted for 60 minutes at 160 °C in a Soxtec 2043 (Foss$^®$, Eden Prairie, MN) and subsequently pipetted into polypropylene tubes coated with antibodies against the hormones tested. One milliliter of radio-labeled hormone will be added and incubated at 37 °C for 45 min before decantation. The amount of antibody-bound labeled hormone will be assessed against standard calibration curves.

Contaminant extraction methodology
POPs will be extracted from cerumen homogenates using enhanced pressurized liquid extraction (ePLE), which combines pressurized liquid extraction (PLE) with adsorbent cleanup techniques into a single automated technique (Robinson et al. *in revision*; Figure 5). ePLE will be performed using an accelerated solvent extractor (ASE 350, Dionex, Salt Lake City, UT) with 66 mL ASE extraction cells. The final method will consist of homogenization of an aliquot of whale cerumen (~0.25 g) with sodium sulfate (baked at 500 °C for 12 hours and allowed to cool) utilizing a mortar and pestle. Cerumen homogenates will be placed on top of pre-cleaned sorbents (with an order of basic alumina oxide, silica gel and florisil from top to bottom) within the ASE cell. The sorbents will be pre-cleaned using 1:1 (v/v) dichloromethane (DCM):hexane (HEX) under the following ASE conditions: 100 °C, 1500 psi, and 50% rinse volume. Cerumen homogenates will be spiked with isotopically-labeled surrogate standards to correct for target analyte loss during sample preparation and will be allowed to come to equilibrium for 1 hr prior to extraction. Next, cerumen homogenates will be extracted with DCM:HEX (1:1) under the same ASE conditions as described above except with a 150% rinse volume. ASE extracts will be concentrated to ~0.3 mL using a Turbo Vap II from Caliper (Hopkinton, MA), then transferred to a gas chromatography (GC) vial and spiked with isotopically-labeled internal standards prior to analysis.

Contaminant Extract Analyses
The analysis of pesticides, PCBs, and PBDEs will be performed using a 7890 gas chromatograph coupled to a 5975 mass spectrometer (Agilent Technologies, Santa Clara, CA) in electron capture negative ionization (ECNI) or electron impact (EI) with selective ion monitoring. One microliter of sample extract will be injected utilizing an Agilent 7683 Injector in a pulsed splitless mode (pulse at 20 psi until 0.74 minutes). The injection port will be set to 300° C. Chromatographic separation will be achieved using a DB-5 capillary column (J&W, 30 m x 0.25 mm i.d.; 0.25µm film thickness). All

analytes except for *p,p'*-DDT, *p,p'*-DDE, and *o,p'*-DDE will use an oven temperature program of 120 °C, held for 1 min, ramped to 275 °C at 4 °C min^{-1}, then ramped to 320 °C at 6 °C min^{-1}, and held for the final 5 minutes. The total run time will be 52.25 minutes. Helium (99.999%) will be used as the carrier gas, and methane (99.999%) will be the buffer gas. The ECNI ion source and quadrupole mass analyzer temperatures will both be set to 150 °C.*p,p'*-DDT, *p,p'*-DDE, and *o,p'*-DDE will be analyzed using the same instrumentation and parameters as described above except in EI mode with an oven temperature program of 120 °C for 1 minute, and then ramped at 4 °C min^{-1} to 250 °C for a total run time of 33.5 minutes and a source temperature of 230 °C. The quadrupole mass analyzer temperature will be 150 °C.

Quality Assurance and Quality Control (QA/QC)
Target analytes will be identified based on retention times (±0.05 min) as well as a quantitative to qualitative ion response ratio (±20%). Ion responses ratios will be based on continuous calibration verification standards analyzed prior to sample analysis. All target analytes will be identified using a single quantitative ion and two qualitative ions, except for *p,p'*- DDT, which has one quantitative ion and one qualitative ion. Following these QA/QC guidelines, a representative chromatogram showing the first identification of trans-chlordane and trans-nonachlor in cerumen is provide in Figure 6. Target analyte concentrations will be determined using a calibration curve with at least seven points ranging several orders of magnitude. Target analyte calibration curves will plot the response dependent concentration factor of the target analyte (concentration of target analyte divided by the concentration of its surrogate standard) versus the concentration dependent response factor of the surrogate standard (response of the target analyte divided by the response of its surrogate standard). Target analyte calibration curves will be linear and forced through the origin, and have coefficients of determination (r^2) of at least 0.99. Surrogate recoveries will be quantified using internals standards spike prior to analysis.

Technical Approach
Objective 2:
Regression techniques (linear, curve fitting) will be used to assess the relationships among age (based on age/lamina counts), hormone, and contaminant concentrations (lipid mass basis) among species of large whales sampled. While estimating concentrations based on lipid mass will account for foraging changes, we will also eliminate the effect of body size in this comparative analysis. A majority of the earplugs from museums holdings have associated lengths at time of sampling. We will back calculate age at length regression using published length-mass regressions ($\log_e M_{mn} = a+b \log_e L_{max}$) and estimate growth curves and, hence, eliminate the effect of body size in our hormone and contaminant analyses. For example, published blue whale regressions (male, $r^2 = 0.97$, n = 6; a = -7.347, b = 2.329) were used to determine mass at age (lamina counts).

Technical Approach
Objective 3:
Baseline hormone concentration will be determined within specific earplugs. To determine significant changes in hormone levels from baseline, a Wilcoxon signed-rank tests will be performed. Based on previous results, we anticipate a positive skew of the hormone distributions, therefore values will be log transformed. Correlations between age, hormone and contaminant concentrations will be calculated using Pearson correlation coefficients. These measurements will include all hormones (aldosterone, T_3, T_4, and cortisol) as well as contaminants.

Technical Approach
Objective 4:
Stress-related hormone (cortisol, aldosterone, T_3 and T_4) levels in the earplug and corresponding blubber samples will be determined by radioimmunoassay techniques (see above). Correction factors will be determined for each hormone for each sample set analyzed. Mean values and therefore mean correction factors will be assigned to each species.

WORK COMPLETED

This award was announced in June 2014; thus far progress has been made in securing archived samples from the Smithsonian Museum as well as the London Museum of Natural History. Our team has also been in contact with marine mammal stranding networks along both U.S. coasts as well as in Spain to be notified in the event of a baleen whale stranding. Our team has added one graduate and two undergraduate students as well as one post-doctoral student to this project for the intial year. We are in the early phases of this study (ordering consummables) but have already analyzed one blue whale earplug for cortisol and persistent organic comtaminants as well as begun aging one bowhead earplug collected in 2013 (>1970s) from Barrow Alaska which will be compared to an archived bowheaed earplug sample collected in 1964 (Los Angeles County Museum of Natural History; <1970s).

RESULTS

Blue whale
One blue whale earplug has been analyzed for contaminants and the stress hormone cortisol (N=1; 25 cm). This earplug was extracted from a shipstrike animal in 2007 (>1970s) and housed at the Santa Barbara Museum of Natural History until shipped to Baylor. Aging analysis produced an estimation of 144 months (± 6 months; dark and light lamina = 1 year) using high resolution digitial imaging. A baseline cortisol was established at approximately 12 months of age. While cortisol levels increased over the lifespan of this animal (Figure 1), two distinct increases, possibly corresponding with development (birth to juvenile and juvenile to sexual maturation), were detected using percent change over baseline cortisol concentrations. This inspection of pecent change from baseline shows initial developmental stress hormone patterns in the blue whale.
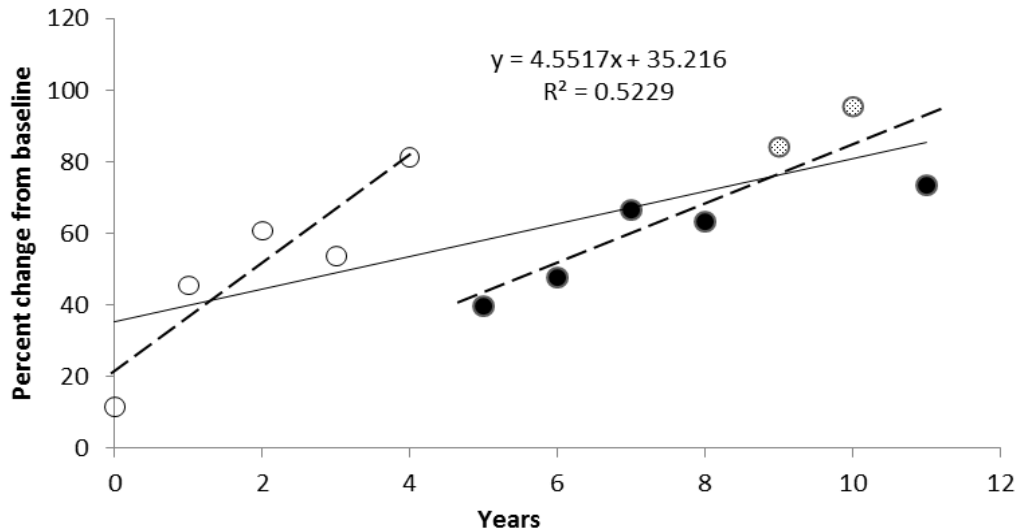
$$y = 4.5517x + 35.216$$
$$R^2 = 0.5229$$

Figure 1. Percent increase over baseline cortisol levels extracted from the lamina of a blue whale earplug collected in 2007 (>1980s). This represents a lifespan of a single whale.
Solid line represents overall regression between variables whereas open circles (dotted line) and dark circles (dotted line) indicate potential developmental periods.
The dotted circles represent age at sexual maturity.

Bowhead Whale
One earplug from a bowhead whale was acquired from Barrow Alaska collected from subsistence harvests in the summer of 2013 (B1-2013; 53 cm, 2000g). Aging of the earplug by counting lamina has begun. Distinct lamina, based on lipid content, has been verified in the lab (light layer = 52% lipid; dark layer = 24% lipid; Figure 2).

Figure 2. Cross section of medial portion of bowhead whale earplug showing light and dark lamina.

Plans
Over the next year our team will be collecting and analyzing archived and freshly extracted whale earplugs from sources including museums and stranding networks. We are in constant contact with various networks in the event that an animal beaches/strands along the coast of the U.S.

IMPACT/APPLICATIONS

The Office of Naval Research's Marine Mammal Physiology Program (Code 32) seeks to develop an understanding of the natural variation of stress markers; better understand and characterize the relationships among hormones or other biomarkers in different matrices; define and compare the quantitative and temporal relationships of hormones across the different matrices; and evaluate/ characterize the relationship between the physiological stress response in marine mammals and acoustic exposure and 'biologically significant' disturbances. This study is aimed at determining baseline stress-hormone concentrations as well as the influence of various anthropogenic influences (contaminants) on the stress response of large whales (mysticetes). Current methods associated with analyzing the effects of anthropogenic activities on the marine ecosystem are labor, time, and cost intensive while offering only a recent snapshot of an event. As a result, there are no feasible approaches to assess baseline stress-related hormones in marine mammals. Without such data, there is no context with which to interpret the biological significance or anthropogenic impact on individuals and populations. The proposed study will report baseline stress-related hormone levels from the whale earplugs archived in museums and offer a correction factor with those hormone levels taken from blubber. From these data, we will ***establish species specific baseline levels that can be used as a comparative tool in future cetacean stress research***. The results of this study will contribute to improving mitigation strategies through improved assessments of the potential impacts of anthropogenic activity.

Because we have access to samples dating back to the 1950s, lifetime profiles of these analytes for several species of large whales will provide, for the first time ever, a clear picture of the impact of anthropogenic disturbances (e.g. noise disturbance). We believe that this will assist the Navy in explaining the impact, or lack thereof, in marine mammals and stress associated with noise (low frequency). Our study will also improve our understanding of the natural physiological responses associated with development in marine mammals.

RELATED PROJECTS

There are no related products