ABSTRACT

Lifetime Profile of Testosterone in a Blue Whale Using its Waxy Earplug

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Matrices such as ice cores and tree rings have been analyzed for chemical profiles that yield telling data on environmental changes, climate change, and contaminants [1, 2]. Much like these layered matrices, earplugs of the baleen whale (e.g. *Balaenoptera musculus,* blue whales) are hypothesized to contain lipophilic chemical secretions of hormones, in addition to other anthropogenic chemicals. Whales secrete earwax into their ear canals like all other mammals; however, the earwax remains compacted within the ear meatus for the entirety of the whale's lifespan, resulting in the formation of a layered earplug. The layers of the earplug, called lamina, have been used to age whales, as each layer represents migratory and feeding patterns over time. It was hypothesized that lipophilic chemicals would be secreted along with the lipophilic cerumen matrix over time, yielding an earplug that contains an archive of a whale's life of chemical exposures and secretions. A method was successfully developed to analyze the chemicals within the earplug. This thesis focuses on the feasibility of extracting testosterone from a male blue whale earplug sample.

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LIFETIME PROFILE OF TESTOSTERONE IN A BLUE WHALE USING ITS WAXY EARPLUG

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By

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CHAPTER ONE

Introduction

Blue whale background

The blue whale, *Balaenoptera musculus*, is an elusive, endangered species whose numbers have declined to a fraction of post-whaling numbers. Estimates of today's population of blue whales are 6,000-8,000 [3]. The genus *Balaenoptera* consists of several species, including Fin (*Balaenoptera physalus*), Blue (*Balaenoptera musculus*), and Sei (*Balaenoptera borealis*) [3]. Whales are completely aquatic air breathing mammals, diving up to 14.7 minutes while foraging [2]. Whaling documents have estimated blue whales lifespan to range from 50 to 110 years, while their estimated age of sexual maturity ranges from 5-15 years. Blue whales typically swim at 6-8 km/hr, but can reach speeds up to 20-30 km/hr when threatened [3]. Blue whales are the largest mammals on earth, weighing an average of 80,000-130,000 kg, and measuring an average length of 25 meters in males and 26 meters in females [1, 2]. Blue whales are identified in field studies by their long, streamlined shape, with their head forming less than 25 percent of total length [3]. Blue whale's throats are grooved, and are colored marine blue grey or mustard if covered with diatoms [3].

Most of the research currently available on blue whales attempts to delineate feeding and migration patterns. Findings on feeding behaviors are based on observational encounters. Blue whales almost exclusively feed on krill, *Euphasia superba*, during seasons that last up to 120 days [4, 3]. Feeding in brightly lit surface waters where krill

congregate in shoals, blue whales allegedly find their prey by making ultrasonic bursts of sound to echolocate masses of krill [3]. The species of *Balaenoptera* are classified as swallowers, in that they gulp their food and expel water from the sides of their mouth. Blue whales, along with fin whales, are known as rorquals; this type of feeding is characterized by lunging at the prey, rapidly engulfing large quantities of schooling krill [4]. Blue whales, like other baleen whales, filter feed with baleen, a keratinized comb like structure that hangs from the roof on the whales' mouth. The size of blue whales' baleen bristles corresponds to their exclusive feeding on krill [3].

While little behavioral data is available other than feeding observations, blue whales have been tracked by various methods to map out their habitat and migration patterns. A general trend that studies find is that after feeding in polar waters, blue whales tend to migrate in groups of 3 or 4 to tropical waters, where they breed and fast for several months [3]. Blue whale's migration patterns have been extrapolated from whaling documents revealing a broad longitudinal range in warmer tropical waters during the winter and narrower clusters at northern latitudes during summer months [5]. Analysis of sound recordings produced by observed whales has suggested that different geographic groups, or stocks, have different calls that can be used to determine the overall distribution of the animal in addition to verifying the geographical boundaries of different stocks [6]. Noting blue whale observations over a period of years has also been attempted in an effort to map migration patterns, despite consistently low sample sizes [7]. Radio tagging and satellite location of blue also is used to map out possible migration routes, breeding, and calving locations; this technology found that blue whales travel in zigzagged patterns while foraging, travelling in a migratory pattern that supports previous

north- southward routes [8]. Radio tags have been also utilized to verify diving patterns; tagged blue whales in one study demonstrated a range of 95-96.5% of time spent submerged, had dives lasting from 3.7 to 7.2 min, and had an average dive depth of 103 m. [9].

While extensive effort has been put forth into mapping blue whale's geographic presence, biopsies for physiological analysis are not available as they are in other mammals. The only report of blue whale structures is provided by an 1883 document by Tullberg that histologically details baleen formation in fetuses [10].

Previous hormonal studies

Due to their open ocean habitat, blue whales are rarely observed, and consequently are not routinely sampled in scientific studies. More baleen whale species have been studied by collecting blubber or fecal samples in order to elucidate everything from contaminants to hormone profiles. For example, lipophilic steroid hormones have been measured in minke whale (*Balaenoptera acutorostrata*) blubber samples to determine pregnancy status of female whales [11]. Fecal samples have also been used to elucidate endocrine profiles of right whales (*Eubalaena glacialis*), including glucocorticoid levels [12]. Hormones have also been detected in lung mucosa from whale blows in humpback whales (*Megaptera novaeangliae*) and North Atlantic right whales (*Eubalaena glacialis*) [13]. Serum testosterone levels and sperm concentrations have been studied in captive killers whales (*Orcinus orca*) to evaluate their reproductive seasons [14]. A major setback in these sampling methods is that the endocrine data

yielded provides only a snapshot of hormone secretions; there are no lifetime profiles available of any whale.

Testosterone in a blue whale

This thesis focuses on the concentrations of testosterone recovered in a male blue whale earplug. While large baleen whales may not be able to be serially sampled as is possible with terrestrial mammals, it is hoped that the hormone profiles yielded by earplug samples will provide insight into their reproductive cycles and age of sexual maturity.

Steroid hormones are a class of lipophilic compounds that ultimately stimulate transcription of specific genes, leading to a cellular response [15]. In general, hormones are secreted by endocrine glands into the bloodstream, by which they reach their target cells. The binding of the hormone to its target receptor triggers a second messenger response within the cell, which is then amplified [15]. The result of this cascade is the activation of specific transcription factors that will initiate translation of specific proteins, which in turn will initiate a cellular response [15]. Sex hormones are responsible for the changes that occurs throughout a mammal's life, including birth, puberty, aging, and death [15]. Sex hormones also play a role in maintaining homeostasis and changing behaviors, especially sexual behaviors [15].

Testosterone is a hormone that is an indicator of male sexual maturity in mammals, initiating changes in behavior and physiological sexual secondary characteristics [15]. A surge of testosterone occurs on onset of male sexual maturity across all mammalian species, where it plays a marked role in changes in behavior [15,

16]. Testosterone is essential for the development of sperm and male physical features such as large muscle mass and the growth of reproductive organs [15]. Testosterone is also linked to aggressive behaviors in some mammals [15]. In a typical mammal, the male fetus produces testosterone before birth, at which this hormone becomes inactive until puberty [15]. The increase in testosterone secretion leads to phenotypic changes in mammals, such as enlargement of gonads, in addition to stimulating aggression and mating behaviors [15].

Changes in mammalian hormone levels are usually determined by collecting blood at different physiological states [15]. There are several constraints in sampling when designing a hormone study on captive or free-ranging whales. Limitations include access to samples, blood collection protocols, and interpreting results. For example, fluctuations of hormone levels that occur within minutes or hours are critical to document in order to fully understand hormonal activity [15]. The size and rarity of encounters of blue whales makes this method of analysis unfeasible for studying lifetime hormone profiles.

The whale earplug

This study uses the whale earplug to analyze the lifetime profile of hormones secreted by a male blue whale. In order to provide some insight into blue whale's largely unknown lives, this study, Robinson et al. (in press), utilized an earplug sample which was shown to contain secreted lipophilic hormones and other lipophilic chemicals.

Several species of whales secrete ear wax (cerumen) into their ear canals throughout their lives, eventually forming a long, multilayered earplug [17]. The wax or

cerumen is composed of lipids, waxes, and keratin and is chronologically secreted over time and compacted into layers, called laminae [19]. Each light and dark lamina indicates migration season and feeding patterns, and the number of layers has been shown to be a reliable indicator of whale age [17, 18].

This study sought to use the earplug to determine testosterone levels found in each layer of the earwax in order to elucidate endocrine trends. It was hypothesized that testosterone would be secreted with the cerumen, producing lifetime hormone profiles for this animal. This hypothesis is based on other studies that demonstrate lipophilic chemical's affinity to lipophilic matrices for secretion, such as studies on the lipophilic chemicals found in whale blubber [20]. The concentrations of chemicals found within the earplug proved to be analyzable and quantifiable, demonstrating that cerumen is a viable matrix for further research. In addition, testosterone concentrations will be assessed versus other hormones (Trumble et al. in prep) in order to ascertain interactions during mammalian development. Investigations that use human earwax as a diagnostic tool rather than plasma or urine samples may enable doctors to easily diagnose diseases [21]. Cerumen has also been proposed to be a helpful matrix to evaluate exposure to hazardous substances in dogs [22]. The wealth of chemical profile information that can be gained from a single cerumen sample may be important in the ability to further study these rare mammals, in addition to utilizing a noninvasive sampling method for diagnostics and other mammal research.

CHAPTER TWO

Materials and Methods

Chemicals

Reagent grade or better chemicals were commercially purchased and stored according to the manufacturers recommendations. Testosterone enzyme immunoassay (ADI-900-065) kits were purchased from Enzo Life Sciences (Farmingdale, NY).

Earplug samples

The whale earplug sample was donated by the Santa Barbara Museum of Natural History (Santa Barbara, CA) under National Marine Fisheries Service permit No. 17157-00 issued to Stephen J. Trumble. The 25.4 cm long blue whale earplug had been harvested post-mortem in 2007 after a ship struck the whale along the coast of California. The earplug was preserved at -30 °C. Figure 1 provides a visual of the whale ear.

Sectioning the earplugs

Upon receipt of the earplug, the sample was labeled and stored at -80°C. To improve accessibility to the internal lamina, the earplug was longitudinally sectioned up to four times depending on its thickness using an ultra-fine-toothed band saw (Vectrax Vertical Variable Speed Band Saw, MSC Industrial Supply Co., Melville, NY) operating from 350–1200 rpm. The earplug was manually moved through the band saw at approximately 0.5–1 fpm. For lamina discrimination, each section was photographed

using a high-resolution digital camera (12MP) and photographic software (Canon U.S.A®). Each lamina layer was removed from the frozen longitudinal section under 20x magnification using a high-speed drill (Dremel 4000 High Performance Rotary Tool, Racine, WI). Aliquots of each layer were placed into vials with polytetrafluoroethylene caps and stored under nitrogen at -30 °C.

Testosterone radioimmunoassay technique

In preparation for testosterone assays, cerumen subsamples were homogenized to a finely ground powder and added to 3 ml of phosphate buffered saline (PBS; pH 7.0) and vortexed for 60s using a VWR VX-2500 Multi-Tube Vortex Mixer (VWR International, Radnor, PA). Five microliters of diethyl ether was added to the solution and vortexed for an additional 60s. The samples were capped under nitrogen and frozen for two hours. The resulting unfrozen portion of the extract was decanted into a 15ml centrifuge tube and dried under a gentle stream of nitrogen for 2 hours or until all remaining liquid was gone. Assay buffer was added to the final residue and the tubes were frozen at −20°C until analysis.

On the day of the assay, cerumen aliquots were thawed and vortexed in 3 times for 30 seconds each. Testosterone levels were measured using a commercially-available enzyme immunoassay kit (ADI-900-065; Enzo Life Sciences, Farmingdale, NY) with a standard curve range between 5.67 $pg \cdot ml^{-1}$-2000 $pg \cdot ml^{-1}$. The reported interassay coefficient of variation (COV) ranged from 9.3%-14.6%. To control the measurement error contributed by all extraction and quantification steps, each sample was extracted and measured at least two times and reported as the average nanograms of testosterone

per subsample mass (ng g^{-1}). Extraction efficiency was determined for each group by spiking selected subsamples with dilutions of testosterone. The extraction efficiency was calculated as the amount of quantified testosterone in the spiked samples subtracted by the quantified amount in the non-spiked samples, the total of which was divided by the amount of testosterone added prior to extraction. Cerumen spiked with testosterone had an average percent recovery of 100.9 % (range 82.5%-104.8%).

CHAPTER THREE

Results

Triplicate recoveries

Hormone extraction techniques for testosterone in cerumen was tested to determine extraction efficiencies. Cerumen spiked with testosterone had an average percent recovery of 100.9 % (range 82.5% to 104.8%).

Hormone concentration results

The age of this blue whale was estimated to be 12 years using the counting of light/dark lamina method used by Roe, 1968. Testosterone was extracted from each of the 24 lamina and thus produced, for the first time, lifetime hormone values for a single male blue whale. Testosterone concentrations reveal age at sexual maturity to be approximately 9 years where testosterone concentrations increased 9000 fold over baseline values.

Baseline testosterone levels (1.0 ng g^{-1}; 0.05 ng ml^{-1}) were measured in the terminal (24) lamina. Testosterone levels increased from birth to approximately three years of age (6.5 ± 1.9 ng g^{-1}, 0.16 ± 0.02 ng ml^{-1};123 ± 27 % above baseline) where levels declined (2.3 ± 1.0 ng g^{-1}, 0.05 ± 0.02 ng ml^{-1}; 41 ± 24 % above baseline) until age 114 months (9.5 years), whereupon levels increased over baseline to a maximum of 370 ng g^{1} (6.6 ng ml^{-1}).

CHAPTER FOUR

Discussion and Conclusions

Discussion

The analysis of a single whale earplug yielded a lifetime profile of seasonal testosterone concentrations sequestered throughout the animal's lifetime. Testosterone concentrations sequestered in the cerumen ranged from approximately 1 ng g^{-1} to 370 ng g^{-1} (0.1-23.0 nmol/L) during the lifespan of this blue whale (Figure 2a). While this impressive range has not been recorded from individual baleen whales, Kjeld et al. calculated serum testosterone ranges from male fin whales (N = 278 whales) of 0.1-40.2 nmol/L with no age of sexual maturity calculated [23]. Kellar et al. sampled blubber from male short-beaked common dolphins (*Delphinus delphis*) for one year, recovering testosterone levels of mature males (14.3 ± 3.0 ng/g) that were significantly higher than those of pubertal (2.5 ± 0.5 ng/g, P =0.006) and immature animals (2.2 ± 0.3 ng/g, $P <$ 0.0001) [24].

The increase of androgens during postnatal development is a key factor defining puberty in male mammals [25]. The significant increase in testosterone observed during this study at approximately 120 ± 6 months (9.5 ± 0.5 years) provides strong evidence of age at sexual maturity and refines previous estimates of 5-15 years [26]. In an analysis of the sexual maturation and reproductive seasonality observed in male killer whales (*Orcinus orca*), Robeck and Monfort (2006) reported mean testosterone concentrations peaked during sexual maturity (6.0 ± 3.3 ng ml^{-1}) at approximately age 11 years [14].

There have been studies involving seasonal hormone oscillations in captive cetaceans but because of logistical and monetary constraints fewer data regarding androgen levels in baleen whales have been collected. The relationship between serum testosterone concentrations and maturity state has been previously documented for several toothed cetacean species including *Phocoenoides dalli*, *Delphinapterus leucas*, *Tursiops truncatus* as well as the fin whale [27, 28, 29, 30]. Other species have also used testosterone concentrations to evaluate maturity states and reproductive seasonality. Keller et al. measured blubber testosterone in male short-beaked common dolphins (*Delphinus delphis*) during one year to estimate reproductive seasonality [24]. Captive Hawaiian monk seals (*Monachus schauinslandi)* have been bimonthly sampled for androgens by serum and saliva samples in a one year period, revealing a seasonal pattern of androgen secretions [31]. Testicular enlargement, a symptom of testosterone secretion, has also been used to determine age of maturity of male ringed seals (*Phoca hispida*) [32]. In each of these studies, testosterone found to be a key indicator of reproductive maturation and seasons.

Another chief finding by Trumble et al (in prep) yielded from this sample is that testosterone levels peaked just prior to the maximum to increases in the stress hormone cortisol levels (Figure 2b). As blue whale reproductive behavior is not well documented, no conclusions can be drawn pertaining to this occurrence.

Endocrine profiles of mammals have only previously been provided by methods using matrices such as blubber, feces, and blood serum samples. These methods only provide the hormonal activity of the mammal for the single moment when the sample was taken. In contrast, a single earplug sample yields a seasonal hormone profile as a function

of age in this whale. The hormone profiles provided by the earplug reveal a whale's developmental changes and possible responses to environmental cues. In addition to pinpointing pubertal age of male blue whales, the finding of a simultaneous peak of testosterone and cortisol represents a novel finding across all mammalian species, as other mammals have not been found to provide a matrix that yields a lifetime profile of hormone secretions. Analysis of chemical profiles can also yield data on anthropogenic chemicals that the whale is exposed to over its entire lifetime. The use of this new matrix for chemical analysis will likely provide a wealth of information on the previously enigmatic blue whale. This new method using earplug as a matrix provides multitudes of other research opportunities, such as testing female blue whales, other earplug-forming whale species, and providing insight into the presence of anthropogenic chemicals.

Conclusions

A method of successfully analyzing the hormone chemical profile of a single whale blue whale earplug sample was determined. This finding confirms that hormones and other lipophilic chemicals accumulate in cerumen, just as in blubber. The cerumen layers proved to yield measurable concentrations of hormones which in turn can be analyzed. These findings show that earplugs are a valuable matrix for the study of this rare mammal. In addition to providing endocrine life profiles of blue whales and other earplug-secreting whales, measurements of anthropogenic contaminants can be made to visualize the persistence of concentrations of chemicals over time for environmental and whale health studies. The finding of a simultaneous peak of testosterone and cortisol in

the male blue whale sample needs to be further investigated for applications to behavioral studies and mammalian endocrinology.

FIGURES

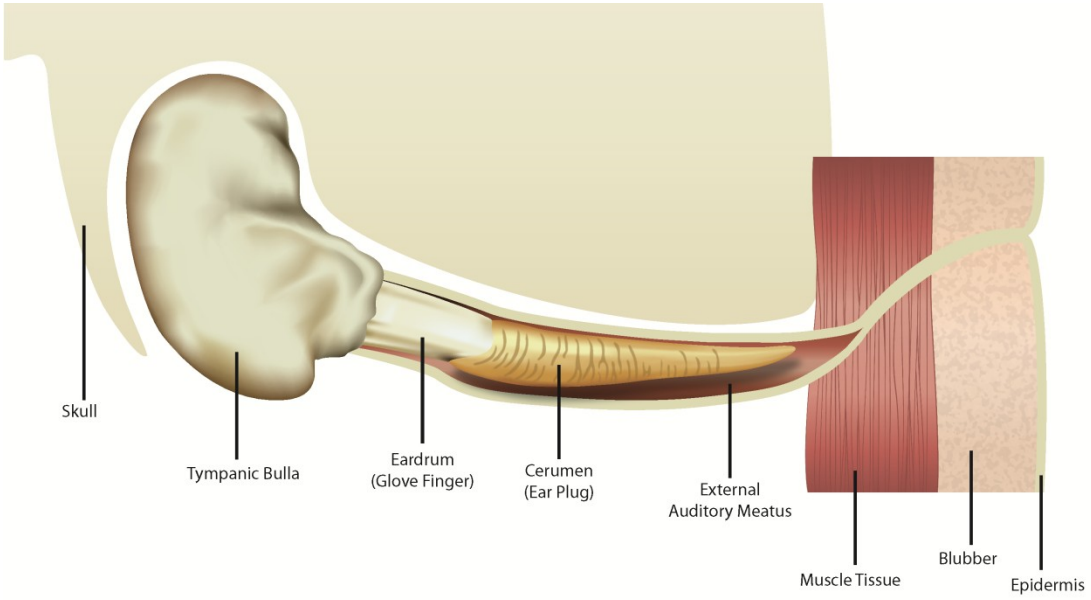

Figure 1 Schematic diagram of a whale internal ear canal [33, 34]

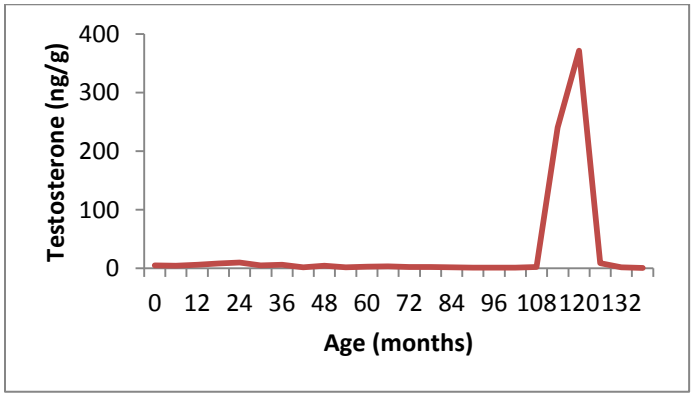

Figure 2a Testosterone concentrations (ng/g) vs estimated aged based on lamina position.

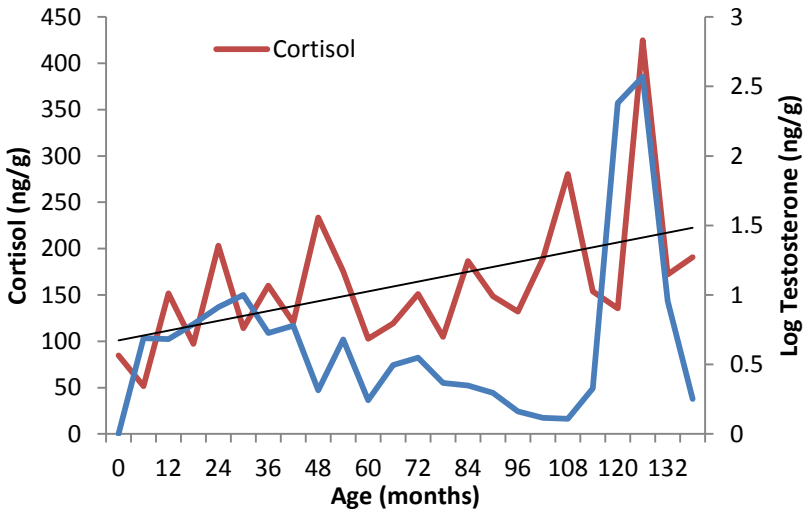

Figure 2b Concentration of cortisol (ng/g) and Testosterone (ng/g) vs estimated age based on lamina position. Cortisol is red, Testosterone is blue.

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