ABSTRACT

Bowhead Whale Earplug Can Be Used for Lifetime Chemical Analysis

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In nature, there are several proxies used in aging; there are coral and carbon signals, sediment and ice cores contaminants, dendrology in trees, and whale earplugs. For over a century, whale earplugs have been used as a proxy for age in baleen whales [14]. Whale earplugs contain wax that is accumulated and preserved over a lifetime and because feeding and fasting from migration patterns results in different colored waxy secretions, the age of the whales can be estimated. Thus far, earplugs have been recovered in some baleen whale species as well as the sperm whale (a toothed whale). It was previously thought that the bowhead whale (Balaena mysticetus) did not produce earplugs. However, this research reveals that the bowhead whale does produce earplugs and that these earplugs can be used as an aging matrix [15]. Our results come from a bowhead whale believed to be a female 50-year-old whale. Previous research determined that aging consists of counting light and dark lamina laid down biannually [15]. Using gross morphology analysis of this female bowhead plug it was discovered that lamina follow a typical pattern [20] A novel technique was developed to separate the bowhead lamina. This process revealed 65 distinct laminae, which were analyzed for cortisol and testosterone composition. The objective of this thesis was to determine a method to separate lamina in a bowhead whale earplug.

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BOWHEAD WHALE EARPLUG CAN BE USED FOR LIFETIME CHEMICAL ANALYSIS

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CHAPTER ONE

Introduction

Bowhead Whale Background

Bowhead whales, *Baleana mysticetus*, are native mainly to the Northern Hemisphere [1]. They are constantly among colder climates and spend both winter and spring surrounded by ice [1]. While there are major gaps in the exact location of various population stocks of bowhead whales, there appear to be five distinct populations [1]. These populations include three in the Eastern Arctic (the Hudson Bay Stock, the Davis Strait Stock, and the Spitsbergen Stock) as well as a population in the Western Arctic called the Bering-Chukchi-Beaufort Stock, and finally a stock in the Okhotsk Sea [1]. As of 2001 the BCB (Bering-Chukchi-Beaufort) stock was approximated through comparative photography and passive acoustics to have 10,545 individuals with a 3.4% rate of increase [2]. The BCB stock is the largest Bowhead whale subpopulation. Even with these rising numbers, bowhead whales are considered an endangered species [3].

Bowhead whale stocks are at risk from several environmental and anthropogenic factors. The species as a whole has also been exploited by the overhunting of whalers before commercial laws made in 1914, which has contributed to their reduced numbers and endangered status. Today, the bowhead whale also faces threats from its ocean environment through heavy toxin levels [3]. The high levels of toxic metals have been shown to store in the whales' systems and eventually degrade vital organs [3]. Most commonly there are high amounts of cadmium found in bowhead liver and kidneys [5].

Whales accumulate toxic metals through their diets, and there is a linear relationship between whale age and amount of toxin accumulated indicating these animals do not metabolize these toxins [3]. Bowheads can also face dangers of contaminant exposure through oil spills and entanglement in fishing gear [6]. Given the bowheads importance to the natives in Alaska and importance as an ecosystem sentinel and model, understanding the physiology of the bowhead is extremely important

Because of the bowhead's great size and aquatic habitat, its morphology is probably the least understood of all mammals [13]. What is known is that the bowhead whale can be up to 19 meters in length and can weigh upward of one hundred or more tons [5]. Females are slightly larger than males, however males have larger flippers than females [5]. Their head is about one-third of their body, and their fluke is another third [5]. These whales have blubber up to 35 cm in thickness to be used as an energy reserve, for thermoregulation and for buoyancy [5]. They have an extraordinarily large mouth, which happens to be the longest of all the baleen whales, which makes it easy for bowheads to feed on large quantities of small krill [5]. Bowheads also have an extremely well-muscled tongue, as well as a convex baleen rack, which suggests that the tongue directs prey into the post lingual process of the whale [6].

Bowheads have a very wide range of food sources including sixty kinds of invertebrates with emphasis on copepods and euphausiids [1]. However, they feed mainly on zooplankton, especially *Clanus hyperboreus* and *Clanus glacialis* [7]. These zooplanktons are found below 12 meters on the water column and on the shelf [7]. Bowheads continuously keep their mouths open while swimming and use their baleen plate with long thin bristles to take in large quantities and filter their select prey [9].

Bowhead whales feed mainly in the summers, and migrate seasonally. However, unlike other baleen whales, bowhead whales do not migrate out of the Arctic waters into a more temperate or tropical environment [8].

Since bowhead whales predominantly live among ice they must thermoregulate accordingly. Not only do these whales have a very thick layer of blubber to insulate themselves, but they incorporate regional heterothermy, meaning that different parts of their bodies can be different temperatures simultaneously [7]. This way when the whale is immersed in the icy waters it can conserve heat and energy. The bowhead whales' fluke also aids in thermoregulation by containing large arteries surrounded by veins which are used in counter current heat exchange. In other words, the fluke's heat can be sacrificed in order for the whale's core body to remain warm around 33.8 °C [5,7]. These large mammals manage ice coverage by breaking through sea ice up to twenty centimeters thick with their skull [5,8] or holding their breath for long periods until they find an ice-free region[8].

It is thought that bowhead whales can live well past one hundred years old, some as long lived as two hundred years old [5,8]. This makes the bowhead whale the longest living mammal on earth [9]. Bowhead whales reach sexual maturity at about twenty years old [10]. Females are 13-13.5 meters in length when they reach sexual maturity and males are 12-13 meters in length [11]. Their mating season is typically in March [9]. Gestation last about 13-14 months and females give birth about every 3-4 years [9,11,21]. Whales are mammals and hence employ lactation strategies post live birth [9].

The Whale Earplug

In 1910 the first whale earplug was described by Lillie, who described the plug as a "solid plug of wax-like substance of fairly definite size and shape" [14]. Lillie also discovered the glove finger (tympanic membrane) of the whale and took the measurement of the earplug, finding it to be 5 inches in length [14]. Then in 1955, Purves discovered that there was a distinctive shape to the earplug, and that it was made up of an outer covering and a core. He further found that the outer covering was derived from the external auditory meatus lining and the core was a secretion of the glove finger and was made of light and dark laminae [14]. In 1959, Ichihara took the idea of light and dark laminae one step further and considered that the glove finger laid distinctive layers in correlation to their fat content (he found more fat content in the light layer) [14]. Ichihara stated that the dark lamina is formed by cells from the glove finger that increase in size because of the cells forming below itself which will eventually become fusiform and keratinized, and the light lamina is formed from cells that become rounded and destroyed by the break down of fats [14]. Much later Ichihara also found that the fin whale is born with a pair of prenatal lamina, a dark lamina and then a light [14].

More recently, Trumble et al. (2013) found that the whale earplug can be analyzed for hormones and contaminants, which when taken along with the layers can give a full lifetime chemical profile of the whale [15]. Their results indicated that the blue whale deposits one dark and one light lamina per year. Thus the age can be estimated by counting the pairs of light and dark lamina. Trumble's lab also tested for analytes such as cortisol, testosterone, organic contaminants and mercury [15]. This work reported the blue whale doubled its cortisol levels over baseline over the lifetime of the whale and sexual maturity of the whale was reached at10 years of age, which was more precise than previous estimates based on blubber biopsies [15].

Even though subsistence hunting has been ongoing for over 200 years, it was believed that the bowhead whale earplug did not lay earplugs with distinct lamina. However, we found that bowhead whale earplugs can be aged using lamina counts. The structure of the bowhead whale earplug is quite different from earplugs that have been researched previously. It has distinct layers, but the layers are neither in uniform lengths nor in a strictly linear fashion, and the plug has a relatively high water content. Therefore, the plug itself was more gelatinous and had to be frozen and dried prior to processing. Cortisol, a stress hormone, was assayed for each lamina and determined to have varying amounts of cortisol in each layer. Cortisol is an indicator of stress in mammals of a response to stressors in chemical, environmental, physical, and social factors [15]. Glucocorticoids are released into the bloodstream during the stress response; the more stress, the more glucocorticoid released. Each year the mammal should have varying levels of cortisol in their system for the various stressors they encounter, such as injury, pregnancy, etc. Cortisol levels along with the age count provided by the layers of lamina allow lifetime chemical profiles to be extrapolated.

Previous Aging Matrices in Whales

In young baleen whales, the baleen plate can be used to age a whale that is three years old or younger [12]. The seasonality can be determined by a growth difference of the cortical layer on the baleen plate [12]. Each two different cortical layers are equal to one year; these changes come from differences in season of feeding and non-feeding seasons of breeding [12]. The plate begins to develop when the whale is seven months old and continues to be viable as an aging technique until it is three years old, because after three years the plate begins to be worn down and the fetal and suckling periods are worn away [12].

Another way in which to age a large whale is through its accumulation of *corpora* in the ovary [12]. In this technique, all ovulation scars are counted, providing an age proxy, if given an estimation of sexual maturity in the species sampled [12]. In other words, in order for this method to be used the whale's average age at puberty and average annual increment of *corpora lutea* must be known [12]. Given the variation and the necessary information needed to calculate the age of a whale through corpora lutea accumulation, this method is not the most highly favored.

Feces from the Balaena have also been shown to be an indicator of age and reproductive state [13]. The whale's feces float, and as such can be collected and tested for androgen, progestin, and estrogen composition [13]. Fecal glucocorticoids were found to be low in immature and non-reproducing mammals, but high in severely injured or entangled whales [13]. Fecal glucocorticoids are also elevated during periods of gestation [13]. Glucocorticoid content is highest in pregnant females and second highest in adult males [13]. When fecal corticoid information is analyzed over several individuals in a population, using the androgen/estrogen ratio, the age and sex of an animal can be estimated.

Whale teeth are also a good indicator of whale age, as well as general health of the whale, reproductive health, and environmental factors [18]. Baleen whales do not have teeth, but for those whales that do, like the sperm whale, aging through teeth composition can be valuable. Depending on which whale is being aged, certain teeth must be chosen, for instance the sperm whale teeth to be tested are either the first mandibular or the un-erupted tooth from the upper jaw [18]. In order to evaluate a tooth, it must first be sliced in half and then polished. The tooth is then decalcified and cleaned further [18]. The samples are then thinly sliced and stained [18]. The tooth can then be used to determine the age of the whale by counting growth layers. A growth layers constitutes a cyclic repetition of incremental growth, with some sort of change between layers, such as dark vs. light, ridged vs. grooved etc. Age can then be estimated from the count of different layers [18].

Interestingly, "eye globes" or the whale's eye lens, can also be used to age baleen whales [19]. The eye lens nucleus contains amino acids that can be evaluated through aspartic acid racemization [19]. The amino acids in the eye lens can be in either D or L enantiomers. Whales can only produce the L enantiomer, thus the ratio of D/L enantiomers of aspartic acid can indicate the age of the whale, by comparing the increase of D enantiomer to baseline amount in a fetus [19]. This aging technique is best used for whales over 20 years old, as whales below 20 years old show great variation in their D/L ratio [19].

Lastly, age can be determined by obtaining the whale's waxy earplug. The earplugs in whales have been shown to have distinct lamina, and when counted provide an estimate of the age of the whale. In the humpback whale there are two dark-colored laminations made per year after birth [12]. The color is most intense toward the earplug's distal end [12]. In the fin and blue whale lamina alternate between light and dark color, and each pair of one light lamina and one dark lamina correlate to one year of the whale's age [14,15]. The light layer has a higher fat content than the dark layer and it is thought that the light layer correlates to the whale's summer months which happen to be the whale's feeding season [14,15]. In contrast, the dark layer is acquired during winter months when food is scarce. In the minke whale, whose earplug is quite soft and whose growth layers are not well formed, it was also found that one growth layer of dark and light represents one year of life [16]. However, because the core of the minke whale plug is soft, different steps must be taken to analyze growth layers. The earplugs were surrounded with gelatin, and then both the earplug and gelatin were hardened with cooling gas [16]. The layers were then stained with alizarin red S and the dark and light layers were counted to estimate the age of the whale.

Before this study was conducted, there was a lack of evidence that bowhead whales produced a waxy earplug that produces lamina that can be counted for aging purposes. However, after our study involving new methods of separation through different solutions and drying techniques, it was determined that the bowhead whale's waxy earplug does indeed have lamina, and these lamina can be separated and counted in order to determine age of the whale. This thesis will focus on the discovery and composition of lamina in bowhead whale waxy earplugs and the methods for separation of those lamina to serve as an indicator of the whale's age.

CHAPTER TWO

Materials and Methods

Chemicals

All chemicals used in this experiment were purchased at reagent grade or better and were stored according to the manufacturing label. The Cortisol Assay (ADI-900-071) kit was purchased from Enzo Life Sciences (Farmingdale, NY).

Earplug Samples

The bowhead whale earplug was obtained from Dr. Hans Thewissen while conducting his research in the North Slope Borough, Barrow Alaska. The left earplug was then sent and stored in Dr. Trumble's lab at -80 °C for 48 hours wrapped in plastic wrap and foil. The plug is Figure 1 is a picture of the whale earplug.
In 2013, this earplug was obtained after a subsistence hunt of the Inuit tribe in Barrow Alaska. The earplug came from an estimated 50-year old female; was 52.8 cm in length and weighed 800g. The plug was assessed for water content by drying 1g subsamples (n = 5) in an oven (VWR) at 40°C until a constant mass was achieved.

Slicing the Earplug

Upon removal from the -80°C freezer, the earplug was sliced longitudinally into three sections to locate the orientation of the lamina. The earplug was sliced with a DeWALT 20" variable speed Scroll Saw (DW788). The second piece that was 8.5 cm wide, 7 cm tall and had a mass of 387g was then further sliced in half medially and labeled. The labeled half section was then sliced in half medially in the same direction to create more labeled sections. After the earplug was sliced, a Ryobi orbital sander (RS290G) was used to smooth the frozen earplug surface to determine if distinct lamina were evident. The laminae were documented through photography via a high-resolution digital camera (12MP) and photographic software (Canon U.S.A). Once the earplug's laminae were revealed (which is shown in figure 2), it was then placed in a series of delaminating solution made of 10% buffered formalin. The earplugs were then dried in a nitrogen filled BelArt desiccator (999320237) for up to two weeks. Once the earplugs were proficiently dried they were ready to be manually separated by tweezer and scalpel (Figure 3). Once each lamina was removed from the plug it was placed in a vial with polytetrafluorethylene caps, filled with nitrogen and then placed in -30 °C.

Cortisol Assay Technique

To begin, samples were vortexed for 30 seconds each along with 1 ml of phosphate buffered saline. The samples then had 3 ml of diethyl ether added to them and then vortexed for another 30 seconds. The samples were then centrifuged at 3000 rpm for 10 minutes and frozen -20 °C for 2 hours. After freezing the ether layer was decanted and evaporated under nitrogen. 2 ml of toluene was then added to the remaining waxy residue and vortexed for 45-60 seconds. Samples were then dried in nitrogen until all the solvent evaporated. The remaining residue after the evaporation was reconstituted with assay buffer until a desired dilution was achieved. Samples were then run in Enzo Life Science Cortisol Assay to determine cortisol concentrations for each hormone discovered. Samples were verified using high-throughput and extremely sensitive ultra-performance liquid chromatography/tandem mass spectrometry (UPLC-MS-MS) instrumentation.

CHAPTER THREE

Results

Slicing Results

When the bowhead whale earplug was sliced laterally and then horizontally distinct lamina were seen. Sanding on the surface of the earplug created a much more defined image of the distinct laminae. The laminae were found to be very thin and did not lay in a strictly linear fashion. When placed into the 10% formalin bath the layers were separated by the water in the solution and preserved by the formalin. Once dried the layers could be tweezed apart and counted. In total 65 distinct layers were counted for the bowhead whale earplug. Extrapolating from this information this female bowhead whale is estimated to be 65 years old, one year for each layer separated.

Water Composition

The Bowhead whale earplug had a high water composition (Percentage = 18% ± 5%)

Cortisol Recovery

A Cortisol Assay was performed on each layer sampled to determine cortisol concentration. Cortisol concentrations from the samples ranged from 0.21 to 38.7 ng/g (Mean = 1.87 ± 5.02 ng/g). These differing concentrations in cortisol further indicate that each layer contains and preserves unique amounts of cortisol. Cortisol composition per layer can be seen in Figure 4.

CHAPTER FOUR

Discussion

Discussion

Through our investigation of the bowhead whale earplug, we determined that distinct lamina were evident and could be used to estimate the age of the Bowhead whale. It has been previously thought that bowhead whales did not have earplugs with distinct lamina because of their sheer size, or because of the gelatinous nature of the plug itself.

This study has revealed there are distinct laminae within the bowhead whale earplug, however, the orientation of the lamina are not similar to other baleen species and do not lay in a linear fashion as seen previously. Possible reasoning behind this kind of layering lies in the earplug's relatively high water composition. Because of the water concentration in the plug (18% ± 5%) the layers can be much more fluid, and much less defined. The swirling nature of the lamina in parts makes it seems as if the motion of the animal and the fluidity of the plug create a much more erratic fashion for laying lamina. In order to maintain the lamina orientation the earplug was cut into 5 cm blocks. However, while the lamina may not lay in what would be considered an expected fashion, counting the layers does in fact yield the age of the whale. After counting the layers separated from this plug, this female bowhead has been aged at 65 years old. The variation in the cortisol count can the bolster the hypothesis that individual lamina were extracted. Because each layer has a significantly different cortisol concentration this indicates that each layer is a different year. Each year would bring different stressors to the whale such as injury, lack of food, or puberty, thus different levels of cortisol [15]. This age is extremely feasible based on the little information known on the whale, as well as the typical lifespan of bowhead whales reaching as old as 150-200 years old [5,8].

By aging a whale through its earplug much more can be extrapolated in order to create a full lifetime profile. Not only does the earplug indicate the whale's age through the distinct lamina, but also the composition of each layer can give an idea of what the whale encountered each year. Hormone assay can show peak levels of stress as discussed earlier, which can indicate that the animal may have gotten injured or faced a food shortage. They can also indicate when the whale may have reached puberty, as well as (for females) when the whale could have given birth.

Along with analyzing hormones, contaminant composition can also be recovered from each layer. Toxins in the plug can indicate the exposure to natural and anthropogenic chemicals. This has broad practical use in terms of conservation efforts. It can be inferred from the sample if certain toxins or chemicals are getting into the oceans, and thus into the whale's system. Because of the lamina nature of the plug, it can be estimated closely as to which year each toxin has been in the water. Thus if companies are invested in conservation efforts, their efforts can be analyzed through the whale's earplug. If there is an oil spill or if a company discovers that it has been polluting the ocean with toxic waste, the efforts to manage and eradicate the pollution can be monitored.

Further, the bowhead whale earplug can be used along with other matrices to get an even more comprehensive profile. Using fecal glucocorticoid analysis stress can also be correlated the earplug. High levels of these hormone metabolites may indicate the animal is stressed. This was seen in the whales in the Bay of Fundy, Canada. Ship traffic in the area caused whales in that area a significant amount of stress. Whales experienced behavioral changes, and began to signal to each other differently, because of the ship traffic creating the same frequency of sound as the whales. As soon as the ship traffic was halted then the whales showed a significant drop in stress-related hormone metabolite concentration [17]. These finding can be made even more conclusive if verified against the cortisol levels in the whale's earplug. If there is a spike in cortisol concentration in the same layer at the same year where there is a high level of fecal glucocorticoids then they both indicate the whale experienced stress in that year. Analysis of the plug can also verify other aging matrices such as the baleen plate studies, analyzing feces, as well as comparing the plug to the whale's accumulation of corpora albicantia as mentioned earlier.

Conclusions

Distinct laminae were found in the bowhead whale earplug disproving earlier thoughts against their existence. By using a new and unique method these layers could be separated and analyzed individually to indicate the age of the whale and begin to give a comprehensive lifetime profile for the whale. These layers were also run through a cortisol assay, which indicated that each layer had a variant cortisol concentration, further indicating that each lamina corresponds to a year in the life of the whale. Hormone and toxin concentration analysis can also later be run to give a more complete lifetime profile. Lifetime profiles can be used to better understand these magnificent creatures and the impact that the environment has on their physiology and behavior. These profiles can also be used to provide insight on how to better conservation efforts and protect not only the bowhead whale, but other marine species in their environment.

FIGURES

Figure 1. An aerial view of the Bowhead whale left earplug

Figure 2. Earplug section after it was sanded down

Figure 3. Dried earplug section with separated lamina

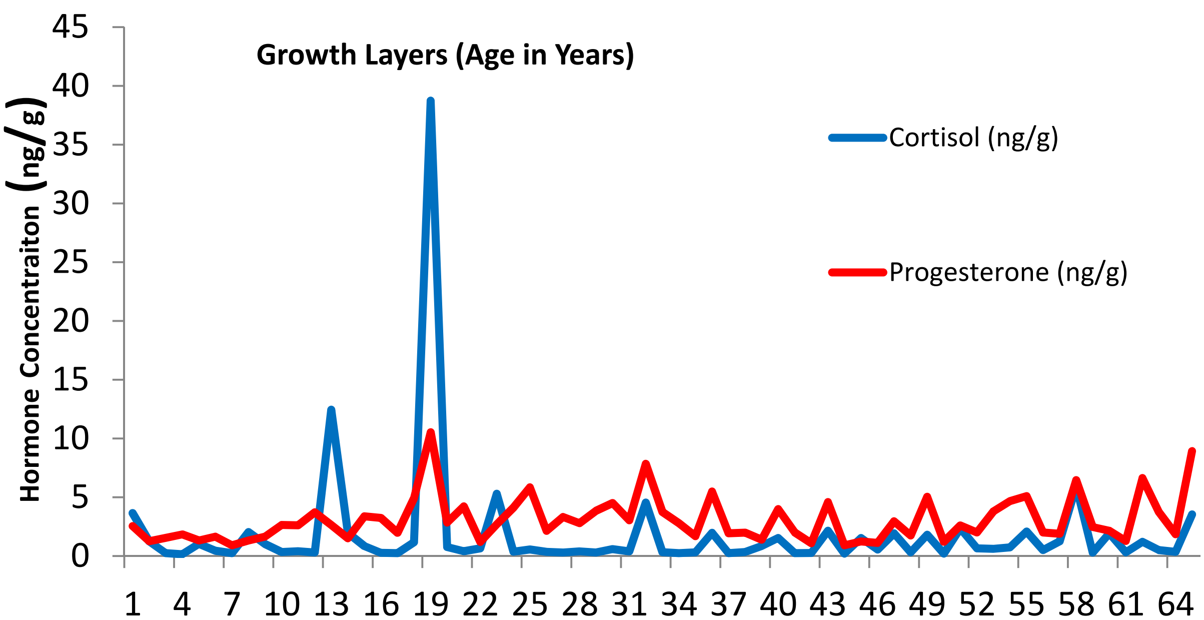

Figure 4. Graph depicting hormone concentration per each lamina. Red is progesterone and blue is cortisol.

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