

SEXUAL DIMORPHISM, THE BIG-MOTHER HYPOTHESIS, AND ANALYSES OF HORMONES,
MINERALS, AND STABLE ISOTOPES TO DETERMINE REPRODUCTIVE EVENTS IN
BOWHEAD WHALES (*BALAENA MYSTICETUS*)

by

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ABSTRACT

Baleen from bowhead whales (*Balaena mysticetus*) provided an opportunity to study reproduction and sexual dimorphism via analyses of hormones, minerals, and stable-isotopes. Concentration of progesterone in baleen was significantly different in nonpregnant and pregnant females, and correlated with size of fetus in pregnant females. Analyses of progesterone of serial samples (every 1 cm) along the length of baleen from a sexually-mature (16.9 m in length), nonpregnant female were conducted to determine short-term fluctuations of progesterone. Preliminary results indicated that reproductive events could be estimated using concentrations of progesterone in baleen obtained from sexually mature females. Stable-isotope analyses conducted previously were used as a timeline in conjunction with analyses of progesterone to estimate frequency of pregnancy in 15 mature females. Correlation between length of body and estimates of frequency of pregnancy did not support the “big mother” hypothesis. A stronger relationship between age and estimates of frequency of pregnancy indicated that age appears to be a greater contributing factor than length of body in determining frequency of pregnancy. Using data from historical whaling records, comparisons of length of body between males and females of several species of mysticete whales did not support Rensch’s Rule.

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TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGMENTS	iii
LIST OF TABLES	vi
LIST OF FIGURES	vii
CHAPTER 1: SEXUAL DIMORPHISM IN LENGTH OF MYSTICETE WHALES AND AN INVESTIGATION OF RENSCH'S RULE	1
Abstract.....	1
Introduction	2
Materials and Methods	5
Results	9
Discussion.....	10
Literature Cited.....	15
CHAPTER 2: ANALYSIS OF MINERALS AND HORMONES IN BALEEN TO DETERMINE DIFFERENCES BETWEEN PREGNANT AND NONPREGNANT BOWHEAD WHALES (<i>BALAENA MYSTICETUS</i>).....	27
Abstract.....	27
Introduction	27
Materials and Methods	30
Results	34
Discussion.....	36
Literature Cited.....	42
CHAPTER 3: EVALUATION OF THE BIG MOTHER HYPOTHESIS USING ESTIMATIONS OF CALVING RATE IN BOWHEAD WHALES (<i>BALAENA MYSTICETUS</i>) AS DETERMINED BY FLUCTUATIONS IN PROGESTERONE AND STABLE ISOTOPES IN BALEEN.....	57
Abstract.....	57
Introduction	58
Methods	61
Results	66

Discussion.....	67
Literature Cited.....	73
CHAPTER 4: DIFFERENCES AMONG REPRODUCTIVE GROUPS IN CONCENTRATIONS OF TESTOSTERONE AND CORTISOL IN BALEEN OF BOWHEAD WHALES (<i>BALAENA</i> <i>MYSTICETUS</i>).....	
	85
Abstract.....	85
Introduction	86
Methods	88
Results	89
Discussion.....	90
Literature Cited.....	94

LIST OF TABLES

TABLE 1.1 – A comparison of the degree of sexual dimorphism as calculated by the ratio of average length of sexually mature males to average length of sexually mature females in my study and as compiled by Ralls (1976).....26

TABLE 2.1 – Data from dilution series (1:1, 1:2, 1:4) of progesterone in baleen from whale 11B6.....55

TABLE 2.2 – Log concentrations of hormones and calcium from baleen collected from fifteen sexually mature females included in a MANOVA analysis.....55

TABLE 2.3 – Data summary of concentrations of progesterone and concentration of calcium from baleen, fetus lengths, and Julian date of capture collected from 10 pregnant females included in a Pearsons correlational analysis.....56

TABLE 2.4 – *P* values of the two-tailed, Pearsons correlational analysis (*n* = 10) between log concentration of progesterone, log concentration of calcium, log of the length of fetus, and Julian Date.....56

TABLE 3.1 – The 18 sexually mature females used in my study and their lengths (m) measured at time of capture, calving interval as calculated in my study, estimation of age as determined by analyses of lenses in the eye, and data from dissections of ovaries (CL = corpus luteum; CA = corpora albicantia).....84

Table 3.2 – The 7 whales that had data for calving interval, age in years, and ovarian tissue available to estimate age at sexual maturity assuming no spontaneous ovulations.84

TABLE 4.1 – Average concentrations ($\pm SE$) of cortisol and testosterone (pg/g) from the proximal end of baleen of males, nonpregnant females, and pregnant females of bowhead whales (*Balaena mysticetus*).....99

LIST OF FIGURES

Figure 1.1 – Phylogeny adapted from Sasaki et al. (2004) and constructed using R (version 3.0.1). This clade was used to calculate independent contrasts for average length of sexually mature males and females to correct for phylogenetic effects within Mysticeti.....	21
Figure 1.2 – Phylogeny adapted from Slater et al. (2011) and constructed in R (version 3.0.1). This phylogeny contains a more resolved hypothesis between Balaenopteridae and Eschrichtiidae than Sasaki et al. (2004). For branch lengths in millions of years refer see Slater et al. (2011). This clade was used to calculate independent contrasts for average length of sexually mature males and females to correct for phylogenetic effects within Mysticeti.....	22
Figure 1.3 – Histogram of length of blue whales (<i>Balaenoptera musculus</i>) from the International Whaling Commission whale-catch database.....	23
Figure 1.4 – Histogram of whaling data from bowhead whales (<i>Balaena mysticetus</i>)....	24
Figure 1.5 – The least-squares regression of independent contrasts of the log of average lengths of male and female mysticetes compared to a slope of 1	25
Figure 2.1 – Relationship between concentration of calcium in the most recently grown portion of baleen and length of bowhead whales (<i>Balaena mysticetus</i> ; squares = males; circles = nonpregnant females; triangles = pregnant females). The dotted line represents the length at which all bowhead whales should be sexually mature.....	52
Figure 2.2 – Log-concentration of progesterone plotted against log-length of fetus for pregnant female bowhead whales (<i>Balaena mysticetus</i>).....	53
Figure 2.3 – Log-concentration of calcium plotted against log-length of fetus for pregnant female bowhead whales (<i>Balaena mysticetus</i>).....	55
Figure 3.1 – Depiction of serial sampling methods for progesterone and stable isotopes for preliminary analyses on baleen from whale 11B6.....	79
Figure 3.2 – Fluctuations in the concentration of progesterone in the baleen of 00B5 as determined in 2 different assays.....	80
Figure 3.3 – Scatterplot between calving rate and length of whales (in meters) of 15 sexually mature female bowhead whales (<i>Balaena mysticetus</i>).....	81

Figure 3.4 –Scatterplot between age in years and calving rate of 15 sexually mature bowhead whales (*Balaena mysticetus*).....82

Figure 3.5 – Fluctuations in concentrations of progesterone in baleen in a female bowhead whale (*Balaena mysticetus*) identified as 95B8. Samples were taken every 5 cm.....83

Figure 4.1 – Log-concentration of cortisol and length of male bowhead whale (*Balaena mysticetus*).....98

CHAPTER 1: SEXUAL DIMORPHISM IN LENGTH OF MYSTICETE WHALES AND
AN INVESTIGATION OF RENSCH'S RULE

ABSTRACT: Sexual dimorphism may be the result of phylogeny, sexual selection, or natural selection. Mysticete whales are sexually dimorphic in size (mass and length), with females attaining a larger size than males at maturity. This study took advantage of a historic database maintained by the International Whaling Commission and supplemental records to quantify sexual dimorphism of 10 species of mysticete whales. Length of females was significantly greater than length of males in 9 of the 10 species of whales examined. Rensch's Rule states that within a lineage where females are larger than males, the degree of sexual dimorphism will decrease as size of species increases. Log transformations on average length of males and females in each species were compiled and assessed to determine if mysticete whales comply with Rensch's Rule. After a phylogenetic correction calculating independent contrasts of log-transformed lengths using 2 phylogenies, neither analysis showed evidence that mysticete whales conform to Rensch's Rule. These results do not take into account newly formed divisions of species within the mysticete clade or extinct species because the database did not differentiate between these new divisions or contain data from extinct whales. Phylogeny may be a significant contributor to sexual dimorphism among mysticete whales. Sexual dimorphism may be a relic of natural selection or sexual selection from the common ancestor of mysticete whales.

Keywords: Rensch's Rule, sexual dimorphism, Mysticeti, phylogeny

INTRODUCTION

Sexual dimorphism in size is common. In most groups of animals that exhibit sexual dimorphism, females are usually larger than males (e.g., insects, fish, amphibians, and reptiles; Andersson 1994). Yet, in mammals, males are usually larger than females (Alexander et al. 1979; Ralls 1976), which is referred to as male-biased sexual dimorphism. About 45% of orders of mammals have males that are larger than females (Lindenfors et al. 2007). In some groups of mammals, dimorphism in size is exhibited by females being larger than males (referred to as female-biased sexual dimorphism or reversed sexual dimorphism); e.g., flying squirrels (*Glaucomys*), chiropterans (Bornholdt et al. 2008; Myers 1978), mysticete whales, and lagomorphs (Ralls 1976). Theories accounting for differences in size between sexes include phylogeny as proposed by Rensch (1960) and include Rensch's Rule, sexual selection (Szekely et al. 2004), and natural selection (Isaac 2005).

A pattern in sexual dimorphism proposed by Rensch (1950; 1960) predicts that, within a lineage where females are larger than males, the degree of sexual dimorphism will decrease as size of species increases due to scaling of secondary sexual traits. A general analysis concluded mammals that exhibit male-biased sexual dimorphism do conform to Rensch's rule (Lindenfors et al. 2007). However all lineages do not follow this pattern. Lindenfors et al. (2007) concluded that cetaceans are male-biased in sexual dimorphism and do not comply with Rensch's Rule. These analyses did not include data corrected for phylogeny and represented only 10 unidentified species which could have been odontocetes, which are known to exhibit male-biased sexual dimorphism, or mysticetes, which are known to exhibit female-biased sexual dimorphism. A more

detailed look at Mysticeti may show female-biased sexual dimorphism, and a correction for phylogeny may provide evidence for Rensch's Rule.

Few studies have evaluated why females are larger than males in some mammalian taxa. A review of mammals that exhibit female-biased sexual dimorphism and theories were first presented by Ralls (1976). One common explanation, the "big-mother hypothesis," predicts that selection for larger females is due to an advantage in fitness (natural selection). Larger females may have more energy available for reproduction enabling them to give birth to larger or more offspring, produce more or higher-quality milk, or better defend resources (Ralls 1976). Female-biased sexual dimorphism may also be the result of sexual selection by males. Male humpback whales (*Megaptera novaengliae*) select larger females (Pack et al. 2009) and females without a calf (Craig et al. 2000). For selection of increased size of females to exhibit sexual dimorphism, there must be greater selection for females to be larger, or greater selection for males to be smaller (Ralls 1976, 1978; Szekely et al. 2004). This size reduction in males may be manifest by reduced male-male competition (Isaac 2005).

Specific traits have been identified in common to mammals that exhibit sexual dimorphism of size. Low variation in reproductive success of males may be a common trait among mammals that have female-biased sexual dimorphism (Schulte-Hostedde et al. 2004). Size may contribute to sexual dimorphism because reproduction is more energetically costly in larger mammals; e.g., a longer suckling phase increases cost of reproduction (Lindenfors et al. 2007). Likewise, evidence suggests that larger females have an advantage in fecundity by producing larger or more young in some species of mammals. Humpback whales in Alaska were more frequently pregnant later in life

(when they would be presumably larger) and after production of ≥ 1 calf (Straley et al. 1994). In female southern flying squirrels (*Glaucomys volans*), larger females had larger litters and access to more food resources (Fokidis et al. 2007). Sexual dimorphism appears to be influenced by multiple, simultaneous factors that can vary among taxa (Isaac 2005).

The phylogeny of mysticete whales may be an influencing factor on sexual dimorphism. Some aspects of mysticete phylogeny remain unclear. There are 2 suborders within the order Cetacea; Odontoceti (toothed whales) and Mysticeti (baleen whales). Instead of teeth, mysticete whales have baleen, which grows in rows of keratinized plates that extend from a dorsal gumline on the roof of the mouth. There are 4 families within the suborder Mysticeti: Balaenidae, Balaenopteridae, Eschrichtiidae, and Neobalaenidae. There are traditionally a total of 12 species within Mysticeti. The current number of species recognized within Mysticeti is a subject of debate depending on molecular and geographical separation of populations of right whales (*Eubalaena*, *Balaena*), blue whales (*Balaenoptera*), and Bryde's whales (*Balaenoptera*). Phylogenetic relationships among these species also have been the subject of debate, with different theories as to the placing of the single extant species of Eschrichtiidae (*Eschrichtius robustus*) within the Balaenopteridae or as a sister taxon to it (Marx 2011). When compared with extinct species from extant and extinct genera within Mysticeti, *E. robustus* appears to be a sister genus to genera in Balaenopteridae (Marx 2011), which agrees with some phylogenies that were constructed using morphology, mitochondrial DNA, and nuclear DNA (Demere et al. 2008; Sasaki et al. 2005; Steeman et al. 2009).

Other phylogenies have hypothesized that Eschrichtiidae belongs within Balaenopteridae (McGowan et al. 2009; Rychell et al. 2004; Slater et al. 2010).

All mysticete whales exhibit some form of sexual dimorphism of secondary sexual characteristics including weight, length, and length at sexual maturity (Ralls 1976). Females of all mysticetes are longer than males at sexual maturity and as adults. Generally, female mysticetes grow to a greater maximum length than their male counterparts.

The aim of this study is to investigate the relevance of Rensch's Rule, and speculate to the contribution of sexual selection, natural selection, or a combination of these factors in the sexual dimorphism of mysticete whales. The degree of sexual dimorphism in length varies among species of mysticetes, but all species seem to exhibit female-biased sexual dimorphism of length despite their various breeding strategies (Brownell and Ralls 1986).

MATERIALS AND METHODS

The International Whaling Commission maintains a database that contains historical records of whales taken for commercial purposes since the late 1800s from around the world. This database contains species, length, sex, location, and other data collected by commercial whaling crews. Most records were taken in the late 1800s to the 1920s, and include much of the historic populations of whales. This extensive database contained only 1 record for bowhead whales (*Balaena mysticetus*). Data from bowhead whales were taken from yearly reports submitted to the International Whaling Commission. These reports were based on bowhead whales taken by subsistence hunters in coastal villages of northern and western Alaska. Supplemental records were provided

through published studies on whales from the genus *Eubalaena* (Zemsky et al. 1995a, 1995b). Data on length at sexual maturity were taken from primary and secondary sources for individual species (Best and Ruther 1992; Fortune et al. 2012; Nerini et al. 1984; O'hara et al. 2002; Ridgeway and Harrison 1985).

Data from commercial whaling operations were separated by species and sex, and then sorted by length of body. Length when males become sexually mature for each species was used as the minimum length of whales included in analyses. Any whale (male or female) that was less than the length when males become sexually mature was not included in the calculation for sexual dimorphism. A separate one-way ANOVA for each species was used to determine if difference in length between males and females was statistically significant. Sexual dimorphism in length was quantified by dividing average length of females by average length of males (Ralls 1976). Average length of females and males were log-transformed and then a regression analysis was performed to determine significance of the relationship (Lindenfors et al. 2007). Statistical comparisons were made using SPSS software (Green and Salkind 2008).

Before data from databases were analyzed a histogram was made to determine sexual dimorphism of individual species. Many whales, including the blue whale (*Balaenoptera musculus*), Bryde's whale (*Balaenoptera brydei*), fin whale (*Balaenoptera physalus*), gray whale (*Eschrichtius robustus*), humpback whale (*Megaptera noveangliae*), minke whale (*Maegaptera novaeangliae*), and southern right whale (*Eubalaena australis*) showed a bimodal distribution in length of whales captured (Figure 1.3). In species with bimodal data, the first mode corresponded to lengths of calves and the second mode corresponded to lengths of sexually mature individuals. Few or no

juveniles were represented in bimodal data. The other species of mysticetes showed a skewed or normal distribution, such as in the bowhead whale (Figure 1.4), and were not bimodal. Sexual dimorphism is more pronounced in adults than in juveniles or calves. Considering the display of sexual dimorphism and differences among the data between different species, my analyses used a cut-off to exclude sexually immature individuals.

Data was deficient for 1 species of mysticete whale, the pygmy right whale (*Caprera marginata*) which is rare and has not been studied extensively. Most data gathered for pygmy right whales have been taken from stranded, dead whales. There are few documented cases of pygmy right whales being taken during whaling expeditions. Therefore, I did not include pygmy right whales in my analyses.

Log transformations of average length of sexually mature males and females of each species were conducted to satisfy normality and equal variance assumptions (Fokidis et al. 2007). A linear regression was then calculated for the 2 log-transformed variables. Although different ratios have been used to quantify sexual dimorphism, these data do not have spurious relationships and are accurate for independent contrasts and Rensch's Rule (Smith 1999). Data on length of body were readily available as compiled in the International Whaling Commission whale-catch database for species of mysticetes that were hunted commercially.

Multiple studies have been conducted on evolutionary relationships of mysticete whales. Relationships among species and genera of baleen whales are still questioned despite phylogenies that were created using multiple markers of nuclear DNA, mitochondrial DNA, morphology, and characters of both extant and extinct whales (Agnarsson and May-Collado 2008; Bouretel and Muizon 2006; Hatch et al. 2006; Marx

et al. 2011; McGowan et al. 2009; Rychel et al. 2004; Sasaki et al. 2005; Slater et al. 2011; Steeman et al. 2009). The relationship between Balaenidae (3 species) is consistently represented separate from other mysticetes throughout these phylogenetic analyses. I used 2 phylogenies to calculate independent contrasts. The phylogeny from Sasaki et al. (2005) represents a phylogeny where the relationship within Balaenopteridae is an unresolved polytomie (Figure 1.1). Slater et al. (2011) did not have a polytomie within Balaenopteridae and represented a phylogeny with a more resolved hypothesis within the clade (Figure 1.2). Both phylogenies included branch lengths in their phylogenies in millions of years, which are necessary to calculate independent contrasts.

Independent contrasts were calculated using the phylogeny from Sasaki et al. (2005) and Slater et al. (2011), and the method of Garland et al. (1992). Independent contrasts were calculated for log of average length of females and log of average length of males as determined from commercial-whaling and subsistence-whaling databases. Regression analysis using least-squares regression was used to determine slope and significance of the regression. A linear-regression analysis also was conducted to determine if there was a significant difference between slopes of regressions from each phylogeny used. Independent contrasts were calculated using the caper package in R (version 3.1.0; R Development Core Team, 2011).

Rensch's Rule is exhibited by a deviation from an isometrical relationship between length of males and females within a lineage. In taxa that exhibit female-biased sexual dimorphism, degree of dimorphism will decrease with an increase in size of body if Rensch's Rule applies. Statistical tests of an isometrical line (slope of 1) will demonstrate to what extent, if any, mysticetes exhibit Rensch's Rule. A least-squares

regression analysis was used to determine if slope of the relationship between log of length of males and females was significantly different from a slope of 1.

RESULTS

Not all species of mysticete whales had lengths of male and female whales that were significantly different ($P < 0.05$). Sample sizes varied from >125,000 blue whales to <100 northern right whales (*Eubalaena glacialis*). The results of one-way ANOVA between lengths of females and males of each individual species varied in significance ($P < 0.050$). All species of mysticetes had a P -value less than 0.001 except for 3 species: gray whales ($P = 0.004$), northern right whales ($P > 0.050$) and common minke whales ($P > 0.050$). All species of mysticetes had females that were, on average, longer than males. The relative average length of female compared to average length of male was greatest in fin whales (1.062) and gray whales (1.062), and was least in common minke whales (1.002) and humpback whales (1.009).

The regression between average length of male and average length of female was statistically significant ($F_{1,9} = 3,149.8$; $P < 0.001$; $r^2 = 0.997$), and the slope was not significantly different from a slope of 1 ($b = 0.966$, $n = 9$, $P = 0.060$). The regression between log of average length of male and log of average length of female was significant ($F_{1,9} = 2,101.8$; $P < 0.001$; $r^2 = 0.996$). Linear regression for independent contrasts of log of average length of male and log of average length of female was significant for the phylogeny of Sasaki et al. (2004; $F_{1,8} = 486.6$; $P < 0.001$; $r^2 = 0.983$), and the linear regression was not significantly different from a slope of 1 ($b = 0.992$, $n = 9$, $P = 0.876$). Linear regression for independent contrasts of log of average length of male and log of average length of female was significant for the phylogeny of Slater et al.

(2011; $F_{1,8} = 424.8$; $P < 0.001$; $r^2 = 0.984$), and the linear regression was not significantly different from a slope of 1 ($b = 0.989$, $n = 9$, $P = 0.847$; (Figure 1.5). The two regression equations from each phylogeny did not have significantly different slopes ($P = 0.868$).

DISCUSSION

Rensch's Rule is not supported by my analyses of mysticete whales. The slope of the relationship between sexual dimorphism in size and total length of mysticetes was not significantly different from a slope of 1 after a phylogenetic correction. Lindenfors et al. (2007) analyzed cetaceans as a group, used data on sexual dimorphism of body mass, and did not account for differences between odontocetes and mysticetes. These results comply with the suggestion that Rensch's Rule is the exception rather than the rule in taxa with female-biased sexual dimorphism (Webb and Freckleton 2007). The conclusions made by Webb and Freckleton did not include an analysis of mammals. This study was the first attempt to determine relationships in mysticete whales separate from other species using length and a phylogenetic correction.

The insignificance of Rensch's rule indicates that the relationship of sexual dimorphism is not simply an allometrical association within a clade. Previous studies have cited natural and sexual selection as explanations for female-biased sexual dimorphism (Colwell 2000; Fairbairn and Preziosi 1994). However, because my analyses did not test for sexual or natural selection I can only speculate to their significance.

Data from my analyses suggest that the effect of sexual dimorphism in length was present in a common ancestor of mysticetes. After correcting for effects of relatedness (phylogenetic independent contrasts), my data showed no significant difference from a slope of 1 (Figure 1.5). This suggests that sexual dimorphism is an ancestral trait.

Evolution of mysticete whales probably was reinforced by some of the causes suggested by Isaac (2005), including reduced male-male competition (as seen in sperm competition of balaenid whales), abundance of resources (filter feeding at the bottom of the food chain), and female-female competition for mates (selection of larger females by male humpback whales). Although Rensch's Rule was not apparent from my data, ancestry does seem to have an effect on sexual dimorphism in length of mysticetes.

The degree of sexual dimorphism among mysticetes may be affected by sexual selection. Mysticete whales have either promiscuous, polygynous, or unknown breeding strategies. Promiscuous species of animals generally have large testes, a trait often associated with sperm competition (Brownell and Ralls 1986). Sperm competition could offer an advantage to smaller males who focus energy on sperm production rather than overall size. Mysticete species of the family Balaenidae (right and bowhead whales) all have large testes for their body size (Brownell and Ralls 1986). Mate preference by males of longer females occurs in humpback whales (Pack et al. 2009), indicating sexual selection. These breeding strategies may also be present in other mysticetes and could be affecting their degree of sexual dimorphism.

Sexual dimorphism may be an effect of feeding strategy of mysticetes. Slater et al. (2011) presented evidence using data from extinct and extant neocete whales and concluded that size niches evolved early in evolutionary history of cetaceans and corresponded with dietary strategies. This would coincide with the conclusion that the effect of sexual dimorphism within mysticetes was most likely an ancestral trait that has been reinforced by sexual and natural selection; perhaps, since divergence of mysticete and odontocete whales, the evolution of baleen, and evolution of filter-feeding. This then

begs the question of whether the ancestor to all cetaceans was sexually dimorphic and if so whether the species was male or female-biased.

Odontocetes and mysticetes have many similarities, but there are some distinct differences. Odontocetes show male-biased sexual dimorphism, have teeth, and generally are smaller than mysticetes. These distinctions may contribute to differences in sexual dimorphism among these sister taxa. An analysis of these two groups, that includes these 3 distinctions, might better reveal the nature and evolution of sexual dimorphism in size of mysticetes specifically and dimorphism of size in cetaceans generally.

The phylogeny used in this study did not have a significant effect on the relationship of sexual dimorphism among species of mysticetes. I used 2 phylogenies that exhibited different relationships among species of Balaenopteridae and Eschrichtidae. The phylogenetic relationships within mysticete whales are still inconclusive. It is unlikely that a more conclusive analysis of the phylogeny of mysticete whales would make a significant difference in my results and conclusion.

Methods employed for calculating sexual dimorphism as mean length of sexually mature males divided by mean length of sexually mature females are comparable to previous methods compiled by Ralls (1976). Of the 7 species that were present in my study and those compiled by Ralls (1976), only 2 were similar. Ralls (1976) cited studies that used smaller datasets and probably were not as accurate as data in this study. The source of most data for my study is compiled by the International Whaling Commission, which continues to check for accuracy in data being gathered and included in their database (Cherry Allison, personal communication).

More species are currently recognized in Mysticeti than were included in my analysis. I could only include species with reliable data. Newly recognized species were not distinguishable at the time data were collected; however, including more data points could possibly change the slope of my regression equations. Gathering data using the same methods (harvesting whales) is currently unfeasible and environmentally irresponsible. Some harvest data are currently being gathered opportunistically (i.e., subsistence hunts of bowhead whales), but few species included in my study are being harvested for commercial purposes because of the moratorium on commercial whaling. Noninvasive methods, such as aerial surveys, are currently being used to collect morphometric data on whales, including the North Atlantic right whale and bowhead whale. If aerial-survey data were expanded to include all species of mysticetes, a more complete analysis, similar to this analysis, would allow for sexual-dimorphic comparisons between historical and extant populations. However, the necessary calibration of harvested whale lengths to aerial surveys would also prove difficult. Aerial surveys are also limited in their ability to determine sex of individual whales in most species and in their abilities to detect individuals below sea ice (Williams et al. 2014).

Mysticete whales have been considerably affected by commercial whaling, including some populations nearing extinction. The over-harvesting of larger individuals of each species has resulted in speculation of some species breeding at younger ages and lengths. Discrepancies in actual sexual maturity and estimates given in the literature would be negligible and would not be expected to affect my overall results or conclusions because the high number of individuals used for this study. Length of males at sexual maturity was used as my cutoff for inclusion of data in analyses. If length of females at

sexual maturity was used as my cutoff, less data would have been available and strength of some data may have been weaker (such as estimates of sexual dimorphism). Length at sexual maturity was also a possible parameter to calculate sexual dimorphism. However, data for length at sexual maturity was not available in many of the species being examined in this study, and only crude estimates of length at sexual maturity were found in the scientific literature for the species of interest.

In summary this study was the first to determine if sexual dimorphism of length of mysticete whales scaled allometrically after a correction for phylogeny, and quantified sexual dimorphism for many of the species of mysticete whales using a large database of historical populations. According to this study, mysticete whales exhibit female-biased sexual dimorphism, and do not comply with Rensch's Rule. Mysticetes appear to be largely ignored by previous analyses of Rensch's Rule (Lindenfors et al. 2007) and must be separated from odontocetes, which mostly exhibit male-biased sexual dimorphism. Future analyses of other sexually dimorphic characters in mysticete whales could use the methods described herein, as applicable, to test for compliance to Rensch's Rule. The underlying factor which accounts for sexual dimorphism within this taxonomic group remains unclear, however it appears a combination of sexual selection, phylogeny, and dietary preference are contributing factors.

Overall, my analyses identified a significant difference in average length of sexually mature males and females of 9 species of mysticete whales. Although Rensch's Rule was not supported, phylogeny may be a contributor to sexual dimorphism in mysticetes. These analyses could be strengthened by inclusion of more species of

mysticetes and possibly a comparison with lengths of male-biased sexually dimorphic odontocetes.

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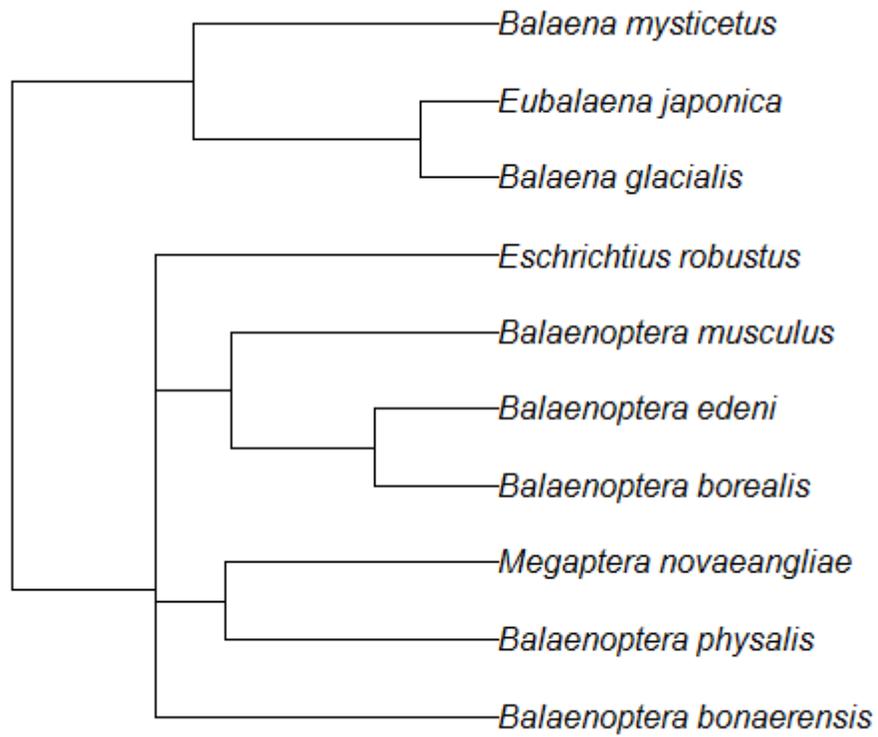


Figure 1.1: Phylogeny adapted from Sasaki et al. (2004) and constructed using R (version 3.0.1). This clade was used to calculate independent contrasts for average length of sexually mature males and females to correct for phylogenetic effects within Mysticeti.

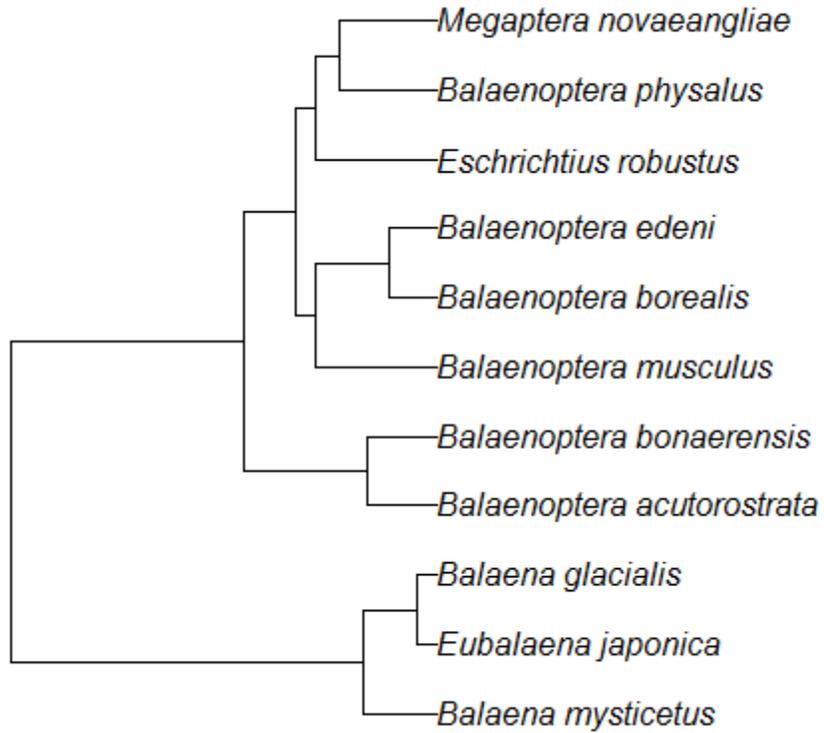


Figure 1.2: Phylogeny adapted from Slater et al. (2011) and constructed in R (version 3.0.1). This phylogeny contains a more resolved hypothesis between Balaenopteridae and Eschrichtiidae than Sasaki et al. (2004). For branch lengths in millions of years refer see Slater et al. (2011). This clade was used to calculate independent contrasts for average length of sexually mature males and females to correct for phylogenetic effects within Mysticeti.

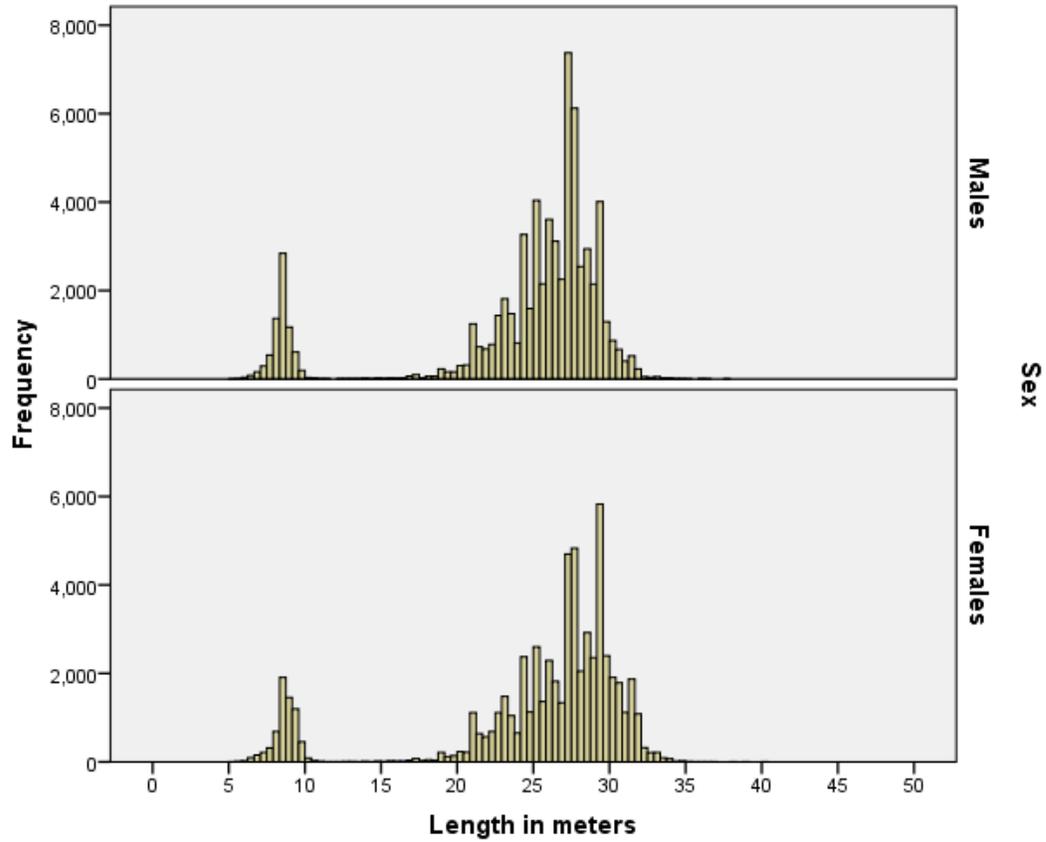


Figure 1.3: A histogram of the distribution of length of blue whales (*Balaenoptera musculus*) from the International Whaling Commission whale-catch database.

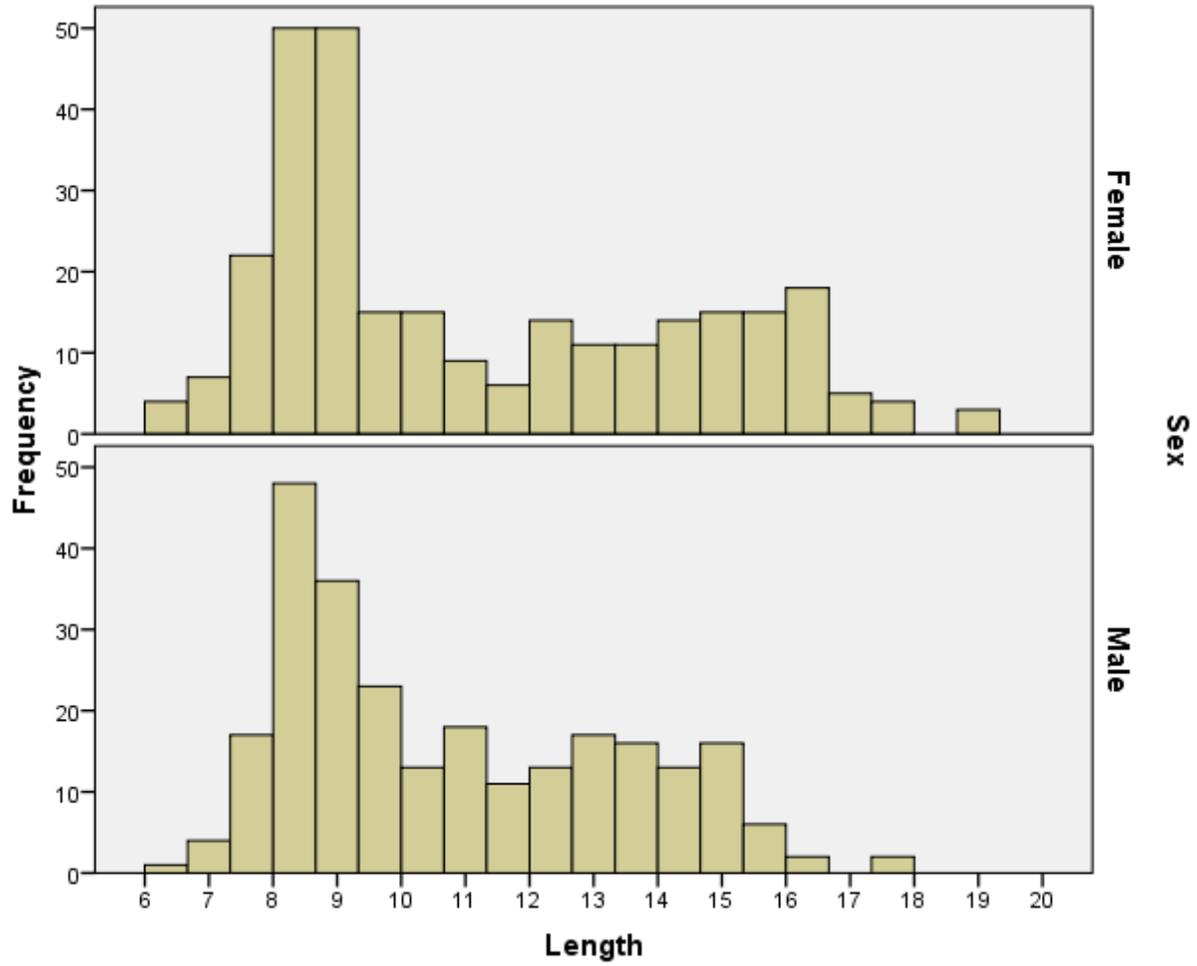


Figure 1.4: Histogram of whaling data from bowhead whales (*Balaena mysticetus*).

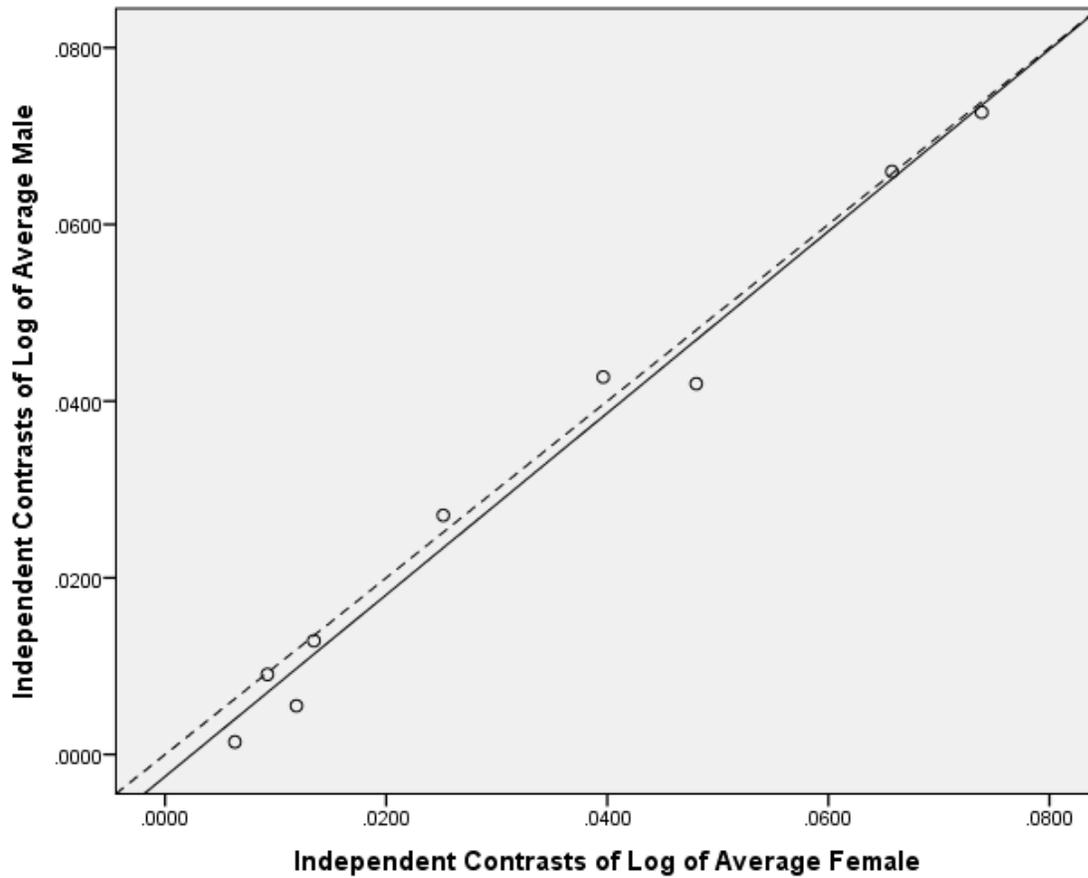


Figure 1.5: The least-squares regression (solid line) of independent contrasts calculated using branch lengths from Slater et al. (2011) of the log of average lengths of male and female mysticetes. The dashed line represents a slope of 1.

Results of this study: average female/average male	Common name	Compiled by Ralls (1976): average female/average male	Sources from Ralls (1976)
1.06+	Gray whale	1.04	Rice and Wolman (1971)
1.05+	Minke whale	1.05+	International Whaling Station (1973); southern Georgia
1.03+	Sei whale	1.06+	International Whaling Station (1959); total Antarctic
1.06+	Fin whale	1.06+	Macintosh and Wheeler (1929)
1.03+	Blue whale	1.06+	Macintosh and Wheeler (1929)
1.03+	Bryde's whale	1.04+	International Whaling Station (1973); USSR
1.01+	Humpback whale	1.07	International Whaling Station (1937); Antarctic pelagic

Table 1.1: A comparison of the degree of sexual dimorphism as calculated by the ratio of average length of sexually mature males to average length of sexually mature females in my study and as compiled by Ralls (1976).

CHAPTER 2:

ANALYSIS OF MINERALS AND HORMONES IN BALEEN TO DETERMINE DIFFERENCES AMONG PREGNANT AND NONPREGNANT BOWHEAD WHALES (*BALAENA MYSTICETUS*)

ABSTRACT: The unique properties of baleen provide an opportunity to study changes in physiology of individual whales over time. Analyses of minerals and hormones on the most recently grown portion of baleen can be attributed to the physiological condition of whales in the recent past. A significant difference was detected between log-transformed concentrations of progesterone in baleen of mature pregnant and nonpregnant females. In addition, a correlation between log-transformed concentrations of progesterone and log-lengths of fetuses were statistically significant and positively correlated. Correlation between concentration of calcium and length of whale was significant. A plot of these data indicated that data from juvenile whales appeared to be a significant contributor to the correlation. After removing whales <14 m in length (sexual maturity occurs at about 13.5 m), the correlation between concentration of calcium and length was no longer significant. Concentration of progesterone appears to be an accurate predictor of pregnancy in bowhead whales and concentration of calcium may provide information on sexual maturity of whales.

INTRODUCTION

A significant proportion of the life history of a species can be explained by behavioral and physiological responses to its environment. Species inhabiting marine environments of the Arctic, including the bowhead whale (*Balaena mysticetus*), are subjected to cold temperatures, ice and its movements, and fluctuations in sunlight that bring with a seasonally fluctuating food supply. The ecosystem occupied by bowhead

whales make them difficult to study via direct observation in the field and their immense size makes traditional laboratory experiments unfeasible. Alternatively, researchers have used several proxies to study marine mammals in the Arctic (Lubetkin et al. 2008, Newsome et al. 2010, Rosa et al. 2007, Rosa et al. 2013, Schell et al. 1989). Bowhead whales have been the subject of extensive study, yet many of their life-history parameters remain unknown. Development and use of proxy studies has increased the ability to examine the ecology and life histories of these whales. The objective of this study is to provide novel methods and baseline data, which will be used in future studies analyzing hormones and minerals in baleen and management of bowhead whales by assessing potential for population growth.

Analyses of stable isotopes in keratin revealed migratory patterns in bats and whales (Cryan et al. 2004; Hirt 2008; Schell et al. 1989). Changes in diet and metabolism of whales will cause a change in isotopes in baleen and other tissues. These fluctuations allow researchers to trace aspects of life histories of whales such as age, trophic position, geographic distribution, and feeding habitat (Hobson and Schell 1998; Hoekstra et al. 2006; Lee et al. 2005; Lubetkin et al. 2008). Still, there has been a paucity of applications of these methods in ecology; in part, due to a lack of preliminary studies. Baleen from bowhead whales offers an ideal model for acquisition of preliminary data because historical samples of baleen are available from the past 40 years and continuous samples are taken in conjunction with Alaskan and Russian Native subsistence hunts. In addition, morphometric measurements are taken during collection of samples of baleen providing necessary baseline data on individual whales.

The unique structure of baleen provides opportunities to detect life-history events that would not be as readily studied in other keratinous tissues. Other than baleen, the only other tissue to be used to infer life-history events over time in bowhead whales has been corpora albicans in ovaries (George et al. 2011). Baleen is produced as an accessory structure made of hard keratin, similar to hair, claws, and nails of other animals, but is much longer and wider, especially in balaenid whales such as the right (*Eubalaena*) and bowhead whales. The length of baleen can reach up to 4 m (Lambertsen et al. 2005), and contain up to 20 years of growth (Schell et al. 1989). As the baleen grows, a continuous record of deposition contains proteins, minerals, hormones, and other biomarkers that can illuminate the physiology and responses of individual whales to their environment over time (Schell 2000; Schell et al. 1989). Although a comparable analysis has not been conducted on any species of whale, analysis of hair of other mammals has revealed correlations between elemental composition of hair and serum (Maugh 1978; Yang et al. 1998; Yukawa et al. 1984). Baleen contains detectable amounts of manganese, iron, copper, and calcium (St. Aubin et al. 1984). Assuming they correlate, analyses of minerals in baleen may provide insights into long-term concentrations in blood, which may fluctuate with life-history events.

Many studies have analyzed hormones of marine mammals, and hormones in baleen of whales has been used in only one other published study (Hunt et al. 2014). Thyroid hormone has been extracted from serum of bowhead whales and may be a biomarker for exposure to toxins (Rosa et al. 2007). Analyses of estrogens, progestins, and androgens have been performed on feces of North Atlantic right whales; concentrations of hormones were associated with gender, pregnancy, and lactation

(Rolland et al. 2005). If detectable in baleen, status of lactation and pregnancy could be valuable in determining reproductive parameters such as age at sexual maturity, age at weaning, cyclicity (Hunt et al. 2014), breeding season, and calving interval.

This study seeks to test for differences in concentrations of minerals and hormones among reproductively active, female bowhead whales. These data will be valuable in assessing fluctuation of hormones along the length of a piece of baleen to determine changes in reproductive states. This study tested the hypotheses that pregnant whales will have lower concentrations of minerals due to allocation of minerals to developing young, and a higher concentration of reproductive hormones (progesterone and estrogen) in baleen compared to nonpregnant whales. Correlations among concentrations of minerals, hormones, morphometric, and reproductive information gathered at the time of harvest also were assessed.

MATERIALS AND METHODS

Baleen from 60 bowhead whales of various lengths, ages, and from both sexes was obtained from the Museum of the North at the University of Alaska Fairbanks and from the North Slope Borough Department of Wildlife in Barrow, Alaska. Only a subset of these samples was used in this study. Baleen of bowhead whales was provided via Alaskan natives and morphometric measurements were taken by researchers prior to butchering. Sexual maturity was determined by length of body; whales >14 m long were considered to be sexually mature. Pregnancy was determined by researchers checking for fetuses, developed corpora lutea, or both; however, some early term pregnancies are difficult to determine and may not have been detected.

Samples of baleen were obtained using a model 114, highspeed, cutter bit (Dremel 300, Mount Prospect, Illinois), and sampled beginning at the proximal end, i.e., the most recently grown portion. For determination of concentrations of minerals, samples of baleen were digested in trace-metal-grade nitric acid in 1.5-mL centrifuge tubes and heated to 100°C for ≥ 30 minutes on a hot plate. After digestion, samples were diluted in water. Standards were made with known amounts of silver, copper, iron, calcium, phosphorous, and zinc with 0.5, 5, 10, 25, and 60 parts/million. Mineral content (calcium, iron, copper, and zinc) was then determined by inductively coupled plasma-optical-emission-spectrometry (ICP-OES; model 7300DV, Perkin Elmer Optima, Waltham, Massachusetts). Silver was used to calculate recovery of minerals, but was highly variable among assays probably due to evaporation that occurred while mixing solutions of silver with the 100°C digest. Standards also were run as samples and a calibration was made every 25 samples. If standards were greater than 10% different than their target then samples were redone. Baleen from 9 whales did not yield accurate results before baleen was no longer available.

Hormones were extracted using anhydrous ether. Ether was mixed with powdered baleen and vortexed in glass test tubes. Samples were then centrifuged at 2,800 rpm for ≥ 8 minutes. The ether and suspended lipid solution was then poured off into another glass test tube. The extraction procedure was repeated. The ether and lipid solution was put under a fume hood and allowed to dry. Samples of extracted hormone were resuspended in a buffer and then mixed with hormone-specific antibodies to create competitive-binding assays. Assays were performed using tritiated radioimmunoassays

to quantify concentrations of hormones using techniques described by Mendonça et al. (1996).

A dilution series was conducted to test the accuracy of extracting and measuring different concentrations of hormones. Extraction of lipids from baleen, were conducted as described. Samples were produced in duplicate and diluted to 1:1, 1:2, and 1:4 solutions. Chi-square analysis was performed using the undiluted sample divided by its respective dilution (1:2 or 1:4) as the expected value and output from radioimmunoassay analyses of diluted samples as the observed value. The dilution series was taken from 4 samples of baleen from different sections of baleen (designated by centimeters from gumline) from whale 11B6. The first sample had a low concentration of progesterone (2.03 ng/g), but still decreased in the 1:2 solution (1.73 ng/g) and 1:4 solution (1.56 ng/g). Because the concentration was so low, it was not included in the chi-square analysis. The other 3 samples showed ratios that were appropriate for their dilution (half dilution for 1:2 solution and quarter dilution for 1:4 solution; see table 2.1) with the biggest difference from expected values being 8% and the lowest difference from expected values being 1%. The chi-square analysis revealed no significant difference in observed and expected values ($\chi^2 = 0.721$, $P > 0.950$). In addition, a one-way ANOVA revealed no significant difference between the curve as determined by radio isotope analysis and the serial dilution of baleen at 146 cm ($F_{1,13} = 0.150$ $P = 0.705$) indicating parallelism similar to findings of Hunt et al (2014).

Efficiency of extraction was determined by comparing recoveries of tritiated-labeled hormones with baleen to recoveries of tritiated-labeled hormones without baleen. For comparison, the extraction procedure was performed in a glass test-tube with baleen

and a solution of tritium-labeled progesterone, and a separate glass test-tube without baleen. After extraction, the solute was suspended in 300 ml of buffer and 50 ml of that suspension was put into a scintillation vial with scintillation fluid and beta emissions were counted. Efficiency of extraction was calculated by dividing average beta emission of solutions extracted from baleen (adjusted for volume) by beta emissions of the solution extracted without baleen. Efficiency of extraction was 94% on average. The 95% confidence interval for average beta emissions of the solution of tritium-labelled hormones mixed with baleen was 1,825-2,303 ($n = 20$). The 95% confidence interval for average beta emissions of the solution of tritium-labelled hormones mixed without baleen was 2,056-2,337. The 95% confidence interval of the efficiency of extraction was 89-98%. Because efficiency of extraction was near 100%, I did not adjust values to account for hormones that may not have been extracted. Although efficiency of extraction was not actually 100%, an adjustment would be applied to all samples equally, and would do little to change results of statistical tests performed or the conclusions drawn herein.

Only baleen from individuals >13 m in length were included in analyses of hormones; sexually maturity in bowhead whales is attained at 13-14 m in length (Koski et al. 1993; Nerini et al. 1984; O'hara et al. 2002). Correlation analysis of minerals with length of whales contained data from immature and sexually mature whales.

Statistical analyses were conducted using SPSS software (Green and Salkind 2008). A multivariate ANOVA (MANOVA) was conducted using reproductive groups (nonpregnant female and pregnant female) as the independent grouping variable and log-concentrations of progesterone, estrogen, and calcium as independent variables. To reduce Type I error, a Bonferonni procedure was used to adjust α to $0.05/3 = 0.017$. Not

all samples had values for all variables in the regression analysis; thus, reducing the total number of samples that could be analyzed ($n = 15$; 6 pregnant females and 9 nonpregnant females; see table 2.2). Posthoc one-way ANOVAs of log-concentrations of progesterone, estrogen, and calcium were done as a follow-up to the MANOVA test.

Because of limitations on the amount of baleen collected, not all samples were analyzed for all hormones. For hormones that were not included in MANOVA, univariate statistics, including one-way ANOVAs and correlations, were used.

For pregnant females, Pearson's R correlations were used to test for associations between length of fetus, Julian date, concentration of calcium, and concentration of progesterone (Table 2.3). Log-transformations of data were conducted on length of fetus, concentration of calcium, and concentration of progesterone before statistical analyses. A Bonferroni adjustment to alpha was applied to test for significant correlations.

RESULTS

Results of the one-way MANOVA using reproductive groups (nonpregnant female and pregnant female) as the independent grouping variable and log-concentrations of progesterone, estrogen, and calcium as independent variables indicated significant differences between nonpregnant females and pregnant females (Wilk's $\lambda = 0.531$, $F_{1,14} = 3.533$, $P = 0.048$). The multivariate η^2 based on Wilk's λ was strong at 0.469. As a follow-up test, one-way ANOVA was conducted on log-concentrations of progesterone, log-concentrations of estrogen, and log-concentrations of calcium. The ANOVA for log-concentration of progesterone was significantly greater in pregnant females than nonpregnant females ($F_{1,14} = 8.596$, $P = 0.004$). The ANOVA for log-concentration of

estrogen was not significant ($F_{1,14} = 4.796$, $P = 0.779$) as was the ANOVA for log-concentration of calcium ($F_{1,14} = 4.416$, $P = 0.486$).

To determine differences in mineral concentrations in baleen among bowhead whales of different lengths ($n = 51$; including immature whales) baleen was analyzed for 5 minerals (calcium, phosphorus, iron, zinc, and copper). Only concentrations of calcium and zinc were high enough for statistical analyses. Correlation between concentrations of calcium and zinc was positive and statistically significant ($r = 0.333$, $P = 0.024$).

Correlation between length of whale and concentration of calcium was significant ($r = -0.452$, $P = 0.001$). A plot of these data (Figure 2.1) indicated that immature whales were contributing most to the correlation. After removing whales < 14 m in length (sexual maturity occurs at about 13.5 m), the correlation between concentration of calcium and length was no longer significant ($P = 0.793$).

To determine correlates with reproductive and seasonal data a Pearson correlation analysis was conducted. Log-transformed length of fetus, log-transformed concentrations of calcium, log-transformed concentrations of progesterone, and Julian date revealed significant correlations with a Bonferroni adjustment of α of $0.05/4 = 0.013$ (Table 2.3; $n = 10$). A summary of correlations can be found in Table 2.4. Log-concentrations of progesterone and log-lengths of fetuses were positively correlated and significant (Figure 2.2; $r = 0.843$, $P = 0.002$). Similarly, log-concentration of calcium and log-concentration of fetal length was positively correlated and significant (Figure 2.3; $r = 0.838$, $P = 0.002$). Log-concentration of progesterone and log-concentration of calcium were not significantly correlated ($r = 0.410$, $P = 0.046$), and Julian date was not significantly correlated with log-concentration of progesterone ($r = 0.683$, $P = 0.030$), with log-length

of fetus ($r = 0.480$, $P = 0.161$), or with log-concentration of calcium ($r = 0.526$, $P = 0.118$). Julian date and log-concentrations of fetuses was positive and not significant ($r = 0.488$, $P = 0.064$).

DISCUSSION

Calcium in baleen could be an indicator of sexual maturity in bowhead whales. Because of their higher density of fat, tenderness of meat, and relative ease in landing and butchering immature whales are taken preferentially by native whalers. Traditional hunters have commented that these young whales, called *ingutuks*, also have thick bones (J. C. George personal communication). My results indicate that immature whales have a higher concentration of calcium in baleen than whales of reproductive length. Higher concentration of calcium in baleen of smaller whales and thicker bones as perceived from this anecdotal traditional observation may coincide with increased concentrations of calcium in serum. Observed increases and decreases of calcium in hair and serum has been observed in humans including women with osteoporosis (Afridi et al. 2012) and pregnant women (Hambridge and Droegmueller 1974; Huang et al. 1999). Decrease in concentrations of calcium in baleen of pregnant females may be associated with allocating of minerals to a fetus or nursing calf, as in other mammals. Although it has been determined that milk of bowhead whales is high in protein and fat, concentrations of minerals in milk have not been determined (Harms 1993). An elevated concentration of calcium in baleen is more likely indicative of a growing whale. Growth rates of bowhead whales are greatest before sexual maturity (George et al. 1999). Growth of bowhead whales would require an elevated input in circulating calcium for growth of bones. This concentration of calcium would decrease as the whale increases in length, decreases their

rate of growth, and attains sexual maturity. Concentrations of calcium in baleen could be used as an indicator of age or length in immature bowhead whales, but larger sample sizes would be needed to determine more specific concentrations and the changes that occur with growth.

Concentration of calcium in baleen adds to the current understanding between length, age, and sexual maturity in bowhead whales. Size has been more commonly used than age to determine sexual maturity in female bowhead whales (Koski et al. 1993; Nerini et al. 1984). Determining age of adult bowhead whales by size has been difficult because their rates of growth can differ after they reach sexual maturity (Lubetkin et al. 2008; Rosa et al. 2013). Female bowhead whales become sexually mature when they are about 13-14 m long (George et al. 2011; Koski et al. 1993), with a mean age at sexual maturity for females estimated at 25.86 years ($SE = 5.868$; Rosa et al. 2013). Baleen has been used to estimate age of individuals, but is accurate only on whales that have not reached sexual maturity (Lubetkin et al. 2008). Age has been estimated in female bowhead whales that are sexually mature by counting corpora albicantia in the ovaries (George et al. 2011). A procedure that determines age based on dextrorotatory:levorotatory ratios of aspartic acid in the lens of the eye (aspartic acid racemization) is the most accurate method for determining age of bowhead whales, but requires an uncontaminated lens (George et al. 1999; Rosa et al. 2013). Comparisons between the 3 methods of determining age of whales (growth rates of baleen, counts of corpora albicantia, and aspartic-acid racemization in lenses) have provided similar estimates of age (Rosa et al. 2013). Concentration of calcium in baleen may be another valuable indicator of age at sexual maturity in bowhead whales.

My results are similar to studies assessing mineral concentrations in keratinous tissues of pregnant women (Hambridge and Droegmueller 1974; Huang et al. 1999), maybe due to similarities in lengths of gestation between whales and humans. Bowhead whales have a gestation of about 14 months (Nerini et al. 1984; Reese et al. 2001) and a lactation of about 9-12 months (Nerini et al. 1984). As humans shift nutritional and metabolic needs during pregnancy, there is a shift in concentrations of minerals within hair and serum, including isotopes of nitrogen (Fuller et al. 2004) and elemental concentrations of zinc (Dang and Jaiswal 1983; Hambridge et al. 1974; Vir et al. 1981), calcium (Huang et al. 1999), iron (Hambridge et al. 1978), and copper (Huang et al. 1999). Concentrations of calcium in baleen appear to have significance in understanding reproductive condition of bowhead whales. Concentrations of calcium in baleen appear to decrease with maturity of a fetus in pregnant females and may be an indicator of late-term pregnancies. However, concentrations of calcium were not significantly different in baleen of pregnant and nonpregnant females in the MANOVA analysis. A one-way ANOVA of only concentrations of calcium between pregnant and nonpregnant females ($n = 21$) also detected no significant difference (see chapter 3). A study that includes a larger sample size focused exclusively on minerals is needed to determine if this relationship is consistently significant.

Of variables analyzed in my study, log-concentrations of progesterone in baleen of bowhead whales appear to be the best predictor of pregnancy. Log-concentration of progesterone had the lowest P -value in the MANOVA ($P = 0.004$). This was expected because an increase in concentration of circulating progesterone is vital to maturation of the ovum, zygote, and progression of pregnancy in marine mammals (Pomeroy 2011).

Analyses of progesterone in other tissues of bowhead whales similarly showed elevated levels in pregnant compared to nonpregnant females (Kellar et al. 2013). My correlational evidence between length of fetus and concentration of progesterone concurred with my expectation of progesterone as an indicator of pregnancy. Along the length of baleen sampled, concentrations of progesterone appear to be able to be used to determine pregnancies. Such fluctuations in progesterone could be used to estimate calving interval of bowhead whales, which has been hard to accurately detect in baleen whales (Best 1990; George et al. 2011; Kraus et al. 1986; Rugh et al. 1992).

The results herein warrant the continued use of tissues to understand reproductive physiology of marine mammals. Opportunistic studies that included analyses of hormones have proved valuable in assessment of marine mammals. Analysis of progesterone in serum of captive beluga whales (*Delphinapterus leucas*) and killer whales (*Orcinus orca*) was used to study cyclicity and age at sexual maturity of females (Katsumata 2010). A significant difference was detected in levels of progesterone in serum of pregnant and nonpregnant North Atlantic minke whales (*Balaenoptera acutorostrata*), and there also was an increase of progesterone in blood of females during the breeding season (Kjeld et al. 2004). In a similar study measuring progesterone in blubber samples of minke whales, pregnant females had concentrations of progesterone almost 60 times greater than nonpregnant females (Mansour et al. 2002). In mammals, progesterone is an important reproductive hormone in maturation of ovaries and development of a fetus (Pomeroy 2011). These results add support the use of progesterone as an indicator of reproductive events in research on marine mammals.

Bowhead whales inhabit an ecosystem going through dramatic changes in sea ice. Their Arctic range is also of significant economic value, which has led to exploration and extraction of oil and gas in the Arctic Ocean. Effects of changes on health and movement patterns of bowhead whales and other Arctic species are yet to be determined (Citta et al. 2012), but may add stress to populations as the ocean experiences more human-caused changes. Using the methods presented in this paper, long-term measurements of stress could potentially be evaluated by analyzing corticosteroids in serial samples of keratinous tissues. Cortisol in baleen may be a valuable indicator of stress; specifically, of bowhead whales in association with environmental perturbations (Hunt et al. 2014).

The significant correlations between calcium and progesterone in baleen of bowhead whales coincide with the assumed physiology of sexually mature females. Progesterone in nonpregnant females appears to increase as they increase concentrations of minerals. Reproductive hormones of marine mammals, including mysticete whales, are assumed to fluctuate similarly to other mammals (Pomeroy 2011). The detection of correlations of calcium and progesterone in baleen in this study are consistent with this assumption. In mammals, secretion of progesterone by the corpus luteum decreases after a female has weaned her offspring. Because of regulations of the International Whaling Commission, females are taken only if they do not have a calf present. The probability that nonpregnant females used in this study had calves is low. However, sexually mature females >14 m in length probably had a calf within a few years prior to their death. Pregnancy and lactation may have caused a strain on reserves of minerals, including calcium, used to facilitate growth of the fetus and calf (Kovacs 2005; Marie et al. 1986).

In addition, secretion of progesterone by the corpus luteum decreases significantly post-partum and post-weaning (Pomeroy 2011).

Baleen is probably much like blubber in its assimilation of hormones from the bloodstream with a lag in assimilation of hormones in serum (Kellat et al. 2013). Because data for fetal length were not available for all individuals used in my study, some error could be attributed to the lag between an increase in serum concentrations and assimilation into baleen. Additional support of this lag was evident in a strong relationship between concentration of progesterone and calcium in baleen with length of fetus. Serial sampling along the length of baleen will better indicate pregnancies; especially, if the interval is small enough to include concentrations for pregnancies that are later in term.

Contrary to my research, previous studies have revealed a significant correlation between fetal lengths and Julian dates (Reese et al. 2001). This may be due to my smaller sample ($n = 10$) compared to Reese et al. ($n = 24$; 2001). Despite my small sample, correlations between log-concentrations of progesterone and calcium had strong, positive, and significant correlations with log-length of fetus. The correlation with progesterone is as expected because the concentration of progesterone in bowhead whales was among the highest recorded in serum samples of a cetacean (Kellar et al. 2013), and concentrations of progesterone are assumed to increase with development of a fetus as in other cetaceans and mammals (Pomeroy 2011). The correlation with calcium was somewhat unexpected because the physiological response to pregnancy was unknown. It appears that during pregnancy circulating calcium increases which is similar to humans (Kalkwarf et al. 1996). This probably is due to mobilization of calcium from bones via

degradation of bone tissue by osteoclasts, increase in absorption of calcium as found in humans (Cross et al. 1995), or both.

Hormonal and mineral analyses of baleen of lactating females could provide valuable insights into fluctuations of mineral and reproductive hormones post-partum that would add to the conclusions of this study. Lactating individuals are underrepresented in collections of baleen because of restrictions by the International Whaling Commission that prohibits taking whales sighted with a calf, although this does still occur by mistake. Harvesting mothers with calves should be met with caution because of the endangered status of the bowhead whale and the possible damage such harvest could have on recovery of populations. The Beaufort-Chukchi-Bering Sea stock of bowhead whales has shown increases in their population since monitoring began, and probably would be healthy enough to allow limited harvesting of females with a calf for scientific purposes.

Results of my study could be supported by testing other hormones in bowhead whales, other baleen whales, or in other species of vertebrates with different keratinous tissues. Prolactin or oxytocin in baleen or other keratinous tissues may be an indicator of lactation and weaning. Levels of testosterone in baleen of males could help test theories of reproduction, such as whether males are sexually active all year (Brownell and Ralls 1986). Results of my research are encouraging for future studies that examine baleen to assess reproductive events in lives of bowhead and other baleen whales.

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FIGURES AND TABLES

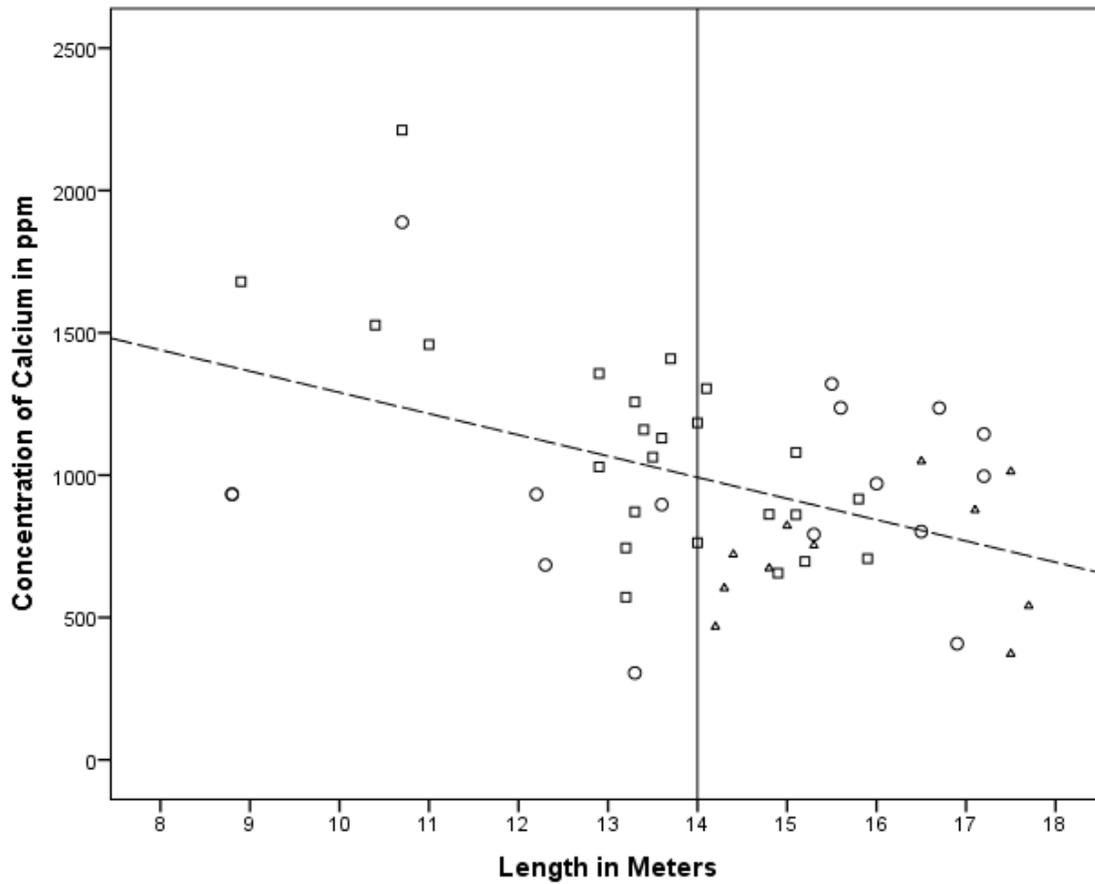


Figure 2.1: Relationship between concentration of calcium in the most recently grown portion of baleen and length of bowhead whales (*Balaena mysticetus*; squares = males; circles = nonpregnant females; triangles = pregnant females). The *solid* line represents the length at which all bowhead whales should be sexually mature, and the *dashed* line represents the linear least-squares regression line of all whales $y = -74.6x + 2040$

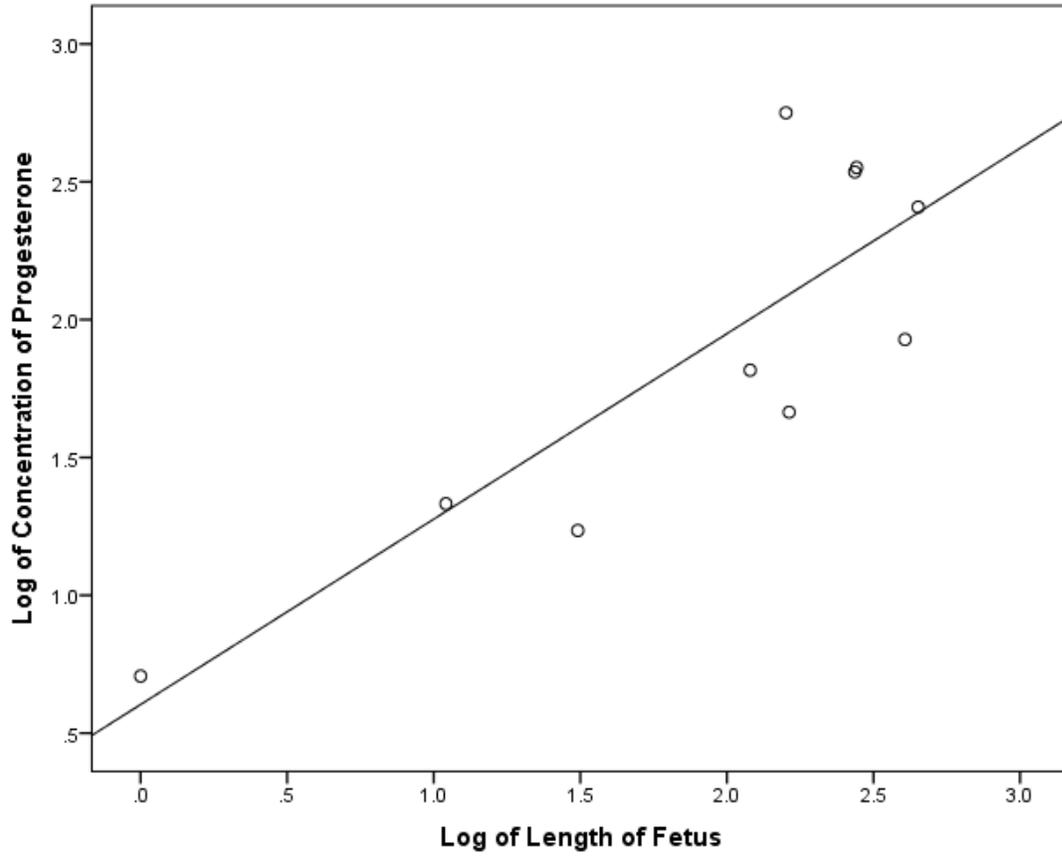


Figure 2.2: Log-concentration of progesterone plotted against log-length of fetus for pregnant female bowhead whales (*Balaena mysticetus*). The solid line represents the least-squares linear regression line $y = 0.67x + 0.6$.

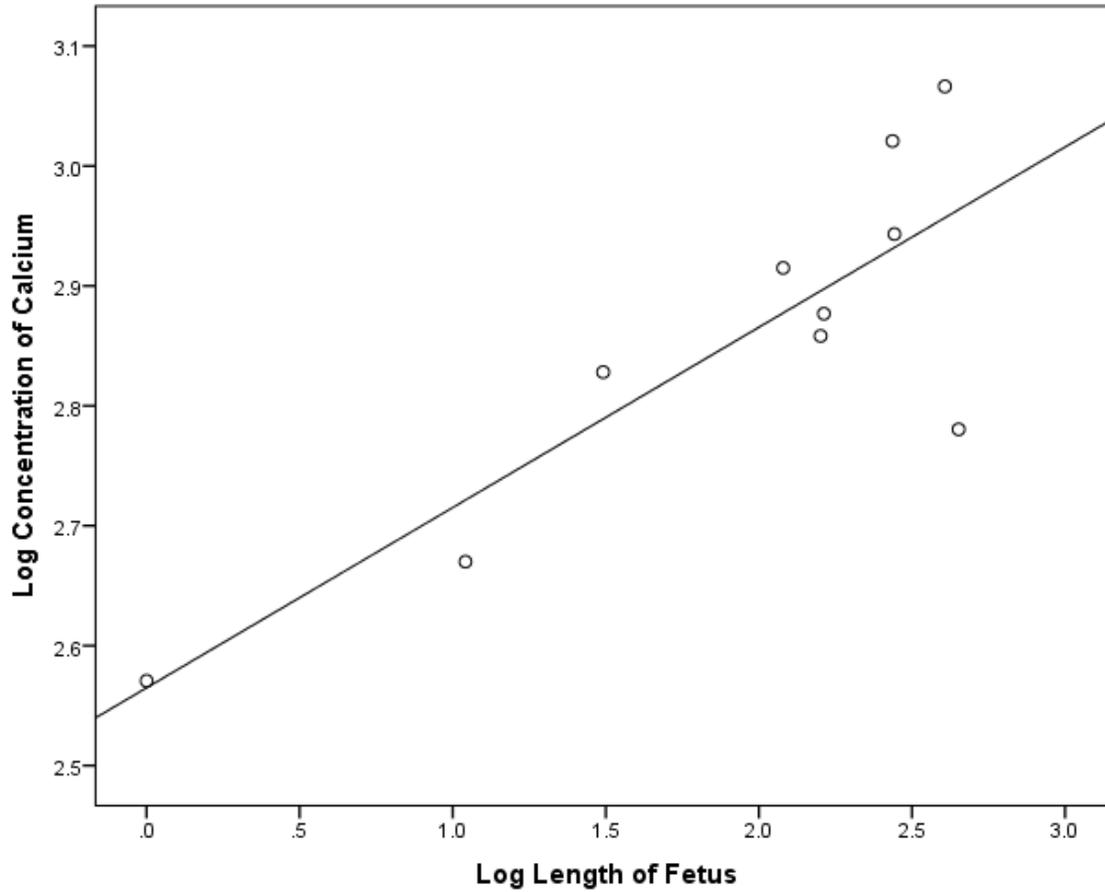


Figure 2.3: Log-concentration of calcium plotted against log-length of fetus for pregnant female bowhead whales (*Balaena mysticetus*). The line represents the linear least-squares regression line $y = 0.15x + 2.56$.

Sample cm from 11B6	Dilution	ng/ml	Observed dilution	Expected dilution
245	1:1	2.03		
245	1:2	1.73	0.86	0.50
245	1:4	1.56	0.90	0.25
246	1:1	26.16		
246	1:2	13.83	0.53	0.50
246	1:4	5.27	0.20	0.25
247	1:1	13.54		
247	1:2	6.25	0.46	0.50
247	1:4	3.26	0.24	0.25
248	1:1	14.49		
248	1:2	6.82	0.47	0.50
248	1:4	4.77	0.33	0.25

Table 2.1: Data from dilution series of progesterone in baleen from whale 11B6. Expected dilutions were calculated by taking the ng/ml of the 1:1 dilution and dividing by 2 for the 1:2 dilutions and dividing by 4 for the 1:4 dilutions.

ID	Reproduction	Log P	Log E	Log Ca
05B21	NP	1.24	1.09	2.97
07WW2	NP	1.43	1.38	2.9
07WW4	NP	1.7	0.15	2.99
10S3	NP	0.9	1.33	3.12
10S4	NP	1.75	0.34	2.48
11B6	NP	0.8	0.5	2.61
86KK2	NP	0.98	0.68	3
87B4	NP	1.61	0.58	3.09
87B6	NP	0.79	0.01	2.94
88B11	NP	1.69	1.37	3.09
88G2	NP	1.23	0.48	2.9
07B12	P	1.63	1.1	2.83
07B16	P	2.75	0.67	2.86
07B9	P	2.41	0.87	2.78
09KK1	P	1.66	0.58	2.88
10S1	P	1.82	0.01	2.91

Table 2.2: Baleen collected from fifteen sexually mature females identified above were included in the MANOVA analysis. Log P is the log concentration of progesterone in nanograms per gram of baleen, Log E is the log concentration of estrogen in pictograms per gram of baleen, Log Ca is the log concentration of calcium in parts per million.

ID	Log P	Log Ca	Log FL	Julian Date
04B4	1.33	2.67	1.04	133
05S5	2.54	3.02	2.44	333
05S6	2.55	2.94	2.44	333
07B12	1.24	2.83	1.49	144
07B9	2.41	2.78	2.65	137
09KK1	1.66	2.88	2.21	257
10S1	1.82	2.91	2.08	119
87B5	1.93	3.07	2.61	166
07B16	2.75	2.86	2.2	280
11B3	0.71	2.57	0	126

Table 2.3: Baleen collected from 10 pregnant females identified above was included in a Pearsons correlational analysis. Log P is the log concentration of progesterone in nanograms per gram of baleen, log Ca is the log concentration of calcium in parts per million, log FL is the log of the length of fetus measured after the whale was harvested, Julian date is the day of the year the whale was harvested.

	Log P	Log Ca	Log FL	Julian Date
Log P		0.046	0.002*	0.030
Log Ca	0.046		0.002*	0.118
Log FL	0.002*	0.002*		0.161
Julian Date	0.030	0.118	0.161	

Table 2.4: *P* values of the two-tailed, Pearsons correlational analysis ($n = 10$). Log P is the log concentration of progesterone in nanograms per gram of baleen, log Ca is the log concentration of calcium in parts per million, log FL is the log of the length of fetus measured after the whale was harvested, Julian date is the day of the year the whale was harvested. * Denotes significance at the Bonferroni adjusted α of 0.013.

CHAPTER 3

EVALUATION OF THE BIG MOTHER HYPOTHESIS USING ESTIMATIONS OF CALVING INTERVAL IN BOWHEAD WHALES (*BALAENA MYSTICETUS*) AS DETERMINED BY FLUCTUATIONS IN PROGESTERONE AND STABLE ISOTOPES IN BALEEN

ABSTRACT: Baleen of bowhead whales (*Balaena mysticetus*) provides an opportunity to study and evaluate physiological events over time. Previous studies showed a significant difference in concentrations of progesterone in baleen of nonpregnant and pregnant females. Serial samples of baleen from a mature female bowhead whale were analyzed for concentrations of progesterone using competitive-binding assays of tritium-labeled progesterone. Oscillations of stable isotopes in baleen were used to determine yearly rates of growth of baleen. Concentrations of progesterone were determined from a serial sampling of baleen from 18 sexually mature females. Calving interval was determined by counting peaks of progesterone and dividing by number of years of growth as determined by analysis of stable isotopes. Average calving interval was 1 pregnancy every 4.05 years. My data somewhat support evidence of the big mother hypothesis in bowhead whales; however, age of females (versus length) seems to be a contributing factor to calving interval in mature bowhead whales suggesting that the experienced mother hypothesis is more relevant than the big mother hypothesis.

Keywords: big mother hypothesis, sexual dimorphism, bowhead whales, rate of pregnancy, calving interval

INTRODUCTION

Advantages of sexual dimorphism in species of baleen whales remain unclear (Isaac 2005; Ralls 1976). Sexual dimorphism in size exists in bowhead whales (*Balaena mysticetus*) with females averaging greater length and body weight than males of a similar age (Koski et al 1993; Nerini et al. 1984). One theory explaining female-biased sexual dimorphism is the “big mother hypothesis” (Ralls 1976): being a larger mother would provide a reproductive advantage by increasing available energy and nutrition that the mother can pass to her offspring through lactation, maternal care, or both, therefore, increasing offspring survivorship.

There is a paucity of data on quality of motherhood and advantages in fecundity (Isaac 2005) and the ability to test the big mother hypothesis has proven difficult. However, with advent of DNA technology, assessments of paternity and maternity have provided relevant data to test this hypothesis (Ward et al. 2014). Allocation of resources by females to their young has been difficult to assess; however, number of pregnancies provides a measurement indicative of reproductive success. Relative fitness has also been evaluated using litter or clutch size, and number of offspring has been observed in size of clutch (rodents, egg-laying animals). The novel method used in my study does not require DNA, but has provided an assessment of number of pregnancies over time by sexually mature, female bowhead whales.

Knowledge of reproductive biology of bowhead whales has relied heavily on proxy studies and opportunistic measurements taken in cooperation with subsistence hunts carried out by the Inupiat Eskimos of Alaska. Collections and analyses of baleen taken from subsistence hunts have documented migratory events (Schell et al.1989),

feeding (Schell et al. 1989), age (Lubetkin et al. 2008), and differences in reproductive hormones (Chapters 2 and 4). This study builds on previous analyses of baleen and other tissues in bowhead whales and other mammals (Koren et al. 2002; Liu et al. 1988; Mansour et al. 2002). In addition this study incorporates novel data from baleen to estimate calving interval in female bowhead whales.

It is estimated that female bowhead whales do not become sexually mature until they are >20 years old and reach a length of 13.5 m (Lubetkin et al. 2008; Rosa et al. 2013). It is thought that their large size is required to maintain growth of a fetus and maintenance of a large calf after parturition via lactation (Ralls 1976). To give birth to such a large neonate, multiple seasons of feeding prior to pregnancy may be required to provide enough fat (energy stores) to maintain pregnancy and lactation. The period between births is referred to as “resting;” however, females are more accurately recovering from energy depletion after weaning their young and preparing for another pregnancy. One advantage larger females may have is more energy and reserves in their blubber (Oftedal 2000) and mineral reserves in their bones (Prange et al. 1979) making them better able to recover from pregnancy and lactation and prepare for another pregnancy. If the big mother hypothesis is accurate it would predict a larger mother may have a reduced resting period and calving interval on average compared to a smaller mother.

Bowhead whales probably have a calving interval similar or slightly lesser than closely related species (Koski et al. 1993). Right whales (*Eubalaena*) and bowhead whales have similar morphology of feeding structures, including size of baleen. Bowhead whales have larger heads in proportion to their bodies and they inhabit higher

latitudes than right whales (*Eubalaena*; Mcleod et al. 1993). Because the Arctic environment the bowhead whale occupies is colder, they spend more energy on thermoregulation, which involves building up larger fat reserves for insulation, energy, and other functions. Calves of bowhead whales have a slower rate of growth (1.5 cm/day; Koski et al. 1993) compared to southern right whales (*Eubalaena australis*; 3.5 cm/day, H. Whitehead and R. Payne in litt.; 2.9 cm/day, Best and Ruther 1989). Southern and northern right whales have calving intervals that are 3.0-3.6 years (Bannister 1990; Best 1990; Payne et al. 1990) and 3.0-3.1 years (Hamilton and Mayo 1990; Kraus et al. 1986), respectively. Assuming more energy is required for maintenance in the Arctic than sub-Arctic habitats, a lesser proportion of total energy would be available for reproduction in bowhead whales. However, this could be offset by differences in prey abundance, as the Arctic Ocean is significantly higher in primary production in the summer months (Huston and Wolverson 2009). The colder environment could induce a longer calving interval and reduced frequency of pregnancy in bowhead whales compared to right whales.

Estimates of calving interval and calving interval over the life of bowhead whales have been calculated by using counts of corpora albicantia in ovaries (George et al. 2011). Analyses of stable isotopes have revealed that oscillations of isotopes of carbon and nitrogen in baleen of bowhead whales are associated with changes in diet due to their migrations to and from the Bering and Beaufort seas (Schell et al. 1989), and these oscillations could be used to give an estimate of time with other samples of baleen.

This study used results established in previous studies to and novel data to provide another estimate of calving interval in bowhead whales. The aims of this study

were 1) to estimate calving intervals in sexually mature female bowhead whales over a shorter length of time (< 20 years) using stable isotopes and concentrations of progesterone in baleen and 2) to assess the “big mother” hypothesis by testing for a correlation in length of females, age, counts of corpora albicantia, and calving interval.

If my data support the big mother hypothesis mothers greater in length will have a lesser calving interval (i.e., more calves per time period). Because size, counts of corpora albicantia, and age are associated in adult females (George et al. 2011), I expected each to be negatively associated with calving interval.

MATERIALS AND METHODS

During the past >30 years, the North Slope Borough Department of Wildlife Management in Barrow, Alaska, has archived baleen from bowhead whales taken by whaling communities of Alaska. Samples of baleen assessed herein were obtained from the Museum of the North at the University of Alaska Fairbanks and from the North Slope Borough Department of Wildlife in Barrow, Alaska (National Marine Fisheries Service permit number 814-1899-03).

Using a high-speed, cutter bit (Dremel 300 model 114, Mount Prospect, Illinois), samples of baleen were obtained beginning at the proximal end; i.e., the most recently grown portion and then serially taken toward the distal end. Samples of baleen were measured between 0.030 and 0.070 grams and put in glass test tubes. Hormones were extracted using anhydrous ether. Ether was mixed with powdered baleen and vortexed in glass test tubes. Samples were then centrifuged at 2,800 rpm for ≥ 8 minutes. The ether and suspended lipid solution was then poured into another glass test tube. The extraction procedure was repeated and the ether and lipid solution were placed in a fume hood and

allowed to dry. Samples of extracted hormone were resuspended in a buffer and mixed with hormone-specific antibodies to create competitive-binding assays. Assays were performed using tritiated radioimmunoassays to quantify concentrations of hormones using techniques described by Mendonça et al. (1996). All samples were run in duplicate with a full standard curve in each assay, non-specific binding assays were run in duplicate, and blank assays were run in quadruplicate. Assays of samples that did not show up on the curve or did not show peaks any changes in concentration of progesterone were rerun using more baleen (baleen from 9 whales). Any sample that was too low to be detected on the curve after being rerun was counted as 0.

For preliminary studies, a full piece of baleen was donated by the North Slope Borough Department of Wildlife. The baleen was from a female bowhead whale 11B6 harvested in 2011 at Barrow that was not pregnant but sexually mature based on length of body (16.9 m).

Baleen from 11B6 was sampled at 1-cm intervals and analyzed for progesterone and sampled at 3-cm intervals and analyzed for stable isotopes of carbon (Figure 3.1). Total growth (in years) and average growth of baleen per year was calculated by counting oscillations of changes in ratios of carbon-13 to carbon-12 (1 oscillation = 1 year) and dividing by number of centimeters of growth per oscillation. Peaks of progesterone (< 5-fold increase in concentration of progesterone) were associated with a sloping increase above basal levels in the centimeters of baleen before the peak and sloping decrease in the centimeters of baleen after the peak, hereafter referred to as length of increase of progesterone. Multiple lengths of increase of progesterone were measured by observing

peaks of progesterone and subtracting the centimeter of the initial increase from the centimeter of the final decrease of progesterone.

Using an average of oscillations of stable isotopes as an estimate of time, average rate of growth of baleen for the sections samples was calculated for 11B6. Length of progesterone increase, in centimeters, was divided by average rate of growth of baleen, in centimeters per year, to estimate duration (in years) of progesterone increase during pregnancies of 11B6. Average length of increase of progesterone was then calculated for all lengths of increase of progesterone in baleen from 11B6.

From 73 to 294 cm in length, 11B6 contained 16.7 years of growth, calculated in oscillations of stable isotopes of carbon and nitrogen, and 5 peaks of progesterone. Considering peaks of progesterone as pregnancies, 11B6 had a pregnancy every 3.3 years. Average rate of growth of baleen per year as determined by oscillation of carbon and nitrogen isotopes was 13.2 cm. Average length of increase of progesterone was 18 cm (range = 12-28 cm). Average time associated with average length of increase of progesterone, as estimated by oscillations of stable isotopes, was 16.3 months.

To determine an indication of pregnancy from baleen of bowhead whales, statistical analyses were conducted on the concentration of progesterone and calcium from 1 sample from the proximal end of baleen of pregnant and nonpregnant females. Previous studies revealed that length of fetus was correlated with concentrations of calcium and progesterone in baleen (chapter 1). A one-way ANOVA was used to determine if there was a significant difference between concentration of progesterone in baleen of pregnant and nonpregnant females and to determine a relative difference between the two groups. 3 pregnant females that had fetuses that were <31 cm long were

excluded from the analysis. These 3 pregnant females had the lowest concentration of calcium and progesterone among pregnant females and calculated concentrations probably were indicative of concentrations of serum prior to pregnancy.

The one-way ANOVA between concentration of calcium in baleen of pregnant and nonpregnant females was not statistically significant ($F_{1,20} = 0.97$, $P = 0.340$). Removing pregnant females with small fetuses also did not make a difference ($F_{1,17} = 0.19$, $P = 0.670$). The one-way ANOVA between concentration of progesterone in baleen of pregnant and nonpregnant females was statistically significant ($F_{1,22} = 8.40$, $P = 0.001$); however, the error associated with reproductive groups violated the equality-of-variance assumption as indicated by a significant Levene's test for equality of variances ($F_{1,21} = 26.9$, $P < 0.001$). Removing pregnant females with small fetuses also was significant ($F_{1,19} = 16.7$, $P = 0.001$). Average concentration of progesterone in baleen of nonpregnant and pregnant females was 20.0 ng/g ($SE = 32.8$) and 232.2 ng/g ($SE = 40.1$), respectively. Pregnant females had concentrations of progesterone in baleen > 10 times nonpregnant females on average. Differences in concentrations of progesterone associated with pregnant and nonpregnant periods along the length of the baleen of individual females were used to estimate calving interval.

Concentration of progesterone was determined from a serial sampling at every 5 cm along the length of baleen taken from 18 sexually mature females. Based on the pilot work done by sampling baleen from 11B6 and differences in concentrations of progesterone in baleen of pregnant and nonpregnant females the following procedure was used to determine pregnancies in baleen of other mature females. To account for inter-assay variation, a pregnancy along the baleen was determined as a relative increase above

basal levels that covered ≥ 10 cm (the shortest increase for 11B6) with a peak >5 -times basal levels (accounting for the difference between average concentrations of progesterone in baleen of pregnant and nonpregnant females). Stable isotopes were used as a timeline for frequency of pregnancies as indicated by fluctuations in progesterone. Analyses of stable isotopes had been conducted previously (Lubetkin et al. 2008; Schell et al. 1989). For baleen that did not have data available for stable isotopes (2 samples), oscillations of stable isotopes of a female similar in age and size was used as an estimate of time. Calving interval was calculated by taking the number of oscillations of stable isotopes and dividing by the number of peaks of progesterone.

One whale identified as 00B5 had progesterone concentrations calculated in two different assays. One assay was done for centimeters 5 to 220 and another assay was done for centimeters 225 to 285. A plot of the data appears to show differences among the two assays in basal levels and peaks (Figure 3.2). The average concentrations of progesterone of the basal levels (excluding peaks) were significantly different according to a t-test ($F_{45} = 0.192$ $P < 0.001$). Pregnancies, however, were still calculated by adjusting for relative increases in progesterone from basal levels in each assay, and thus the interassay variation did not change the interpretation of the results.

A one-tailed Pearson-R correlation analysis using calculated calving intervals, counts of corpora albicantia from George et al. (2011), length, and estimates of age taken from George et al. (1999) and Rosa et al. (2013), was conducted to evaluate the big mother hypothesis. A Bonferroni adjustment was applied to α by dividing 0.05 by the number of variables $0.05/3 = 0.02$. A significant, negative relationship between time and calving interval was expected in support of the big mother hypothesis.

RESULTS

Average estimate of calving interval as estimated by the method described herein was 1 pregnancy every 4.05 years ($SD = 0.67$, $n = 15$). Average body length of female whales was 16.2 m ($SD = 1.1$, $n = 18$). Average estimate of age was 57.5 years ($SD = 25.7$, $n = 10$).

The calculated calving intervals from this study were significantly correlated with data gathered by researchers through measurements of reproductive and other tissues. Calving interval was associated negatively with length, but was not significant ($r = -0.27$ $P = 0.170$; Figure 3.3). Calving interval was associated negatively and significantly with age that ranged from 27 to 123 years ($r = -0.84$ $P = 0.001$; Figure 3.4) and counts of corpora albicantia ($r = -0.86$ $P = 0.007$). Length and age were associated positively with each other, but were not significant ($r = 0.45$ $P = 0.070$). Length and counts of corpora albicantia also were associated positively, but not significant ($r = 0.40$ $P = 0.140$). Counts of corpora albicantia were associated positively with age and significant ($r = 0.99$ $P < 0.001$). Concentration of progesterone and Julian date revealed no significant correlation ($P = 0.884$, $n = 9$).

Female 86WW2 was especially large for her age. This female was the second longest of all females at 17.7 m, but was younger (34 years old) and had only 5 corpora albicantia. This female had a significant effect on the correlation between length and calving interval. After removing data from 86WW2, the correlational analysis for length was not significantly correlated with calving interval ($r = -0.532$ $P = 0.025$), age ($r = 0.62$ $P = 0.020$), and counts of corpora albicantia ($r = 0.652$ $P = 0.040$).

DISCUSSION

The pilot work conducted on baleen from 11B6 was used to determine the appropriate interval for sampling and the relative increase of progesterone that would indicate a pregnancy. The shortest length of baleen of whale 11B6 associated with an increase in progesterone, which was attributed to a pregnancy, was 12 cm. Therefore, intervals of 5 cm along baleen were considered an appropriate interval for sampling and for detecting pregnancies. However, these data were taken from only 1 whale and variation in progesterone among whales was not assessed. Some pregnancies could have been undetected, but considering average length of the increase in progesterone was 18 cm in 11B6, this was unlikely.

Estimates of duration associated with an increase in progesterone in 11B6 (16.3 months) was longer than the gestation period proposed for bowhead whales (13-14 months) by Koski et al. (1993). This may be due to inaccuracies in estimation of gestation in previous studies. However, it is more likely that an increase in progesterone is also associated with lactation, which would extend the time that progesterone levels are elevated above nonpregnant levels. The half-life of progesterone in circulating blood appears to be brief (as in minutes) as demonstrated in humans (Short and Eton 1959) and mares (*Equus caballus*; Ganjam et al. 1975), however the half-life of progesterone may be different in bowhead whales. Conducting a similar study analyzing concentrations of oxytocin and prolactin in association with progesterone could provide data specific to lactation. Information on lactation in bowhead whales is scarce because subsistence-hunting regulations discourage taking females with calves.

The variance in concentrations of progesterone among reproductive females was great in both pregnant and nonpregnant females probably due to the lag between the reproductive state of the individual and the concentrations of hormones in baleen. Variance was also expected because not all females were in the same stage of pregnancy. Size of calf was correlated with concentration of progesterone in baleen (Chapter 2) indicating that progesterone increases as the fetus grows. Females in my study had variable lengths of fetuses. Likewise, concentrations of progesterone in nonpregnant females probably included various stages of follicular development which would be accompanied by various levels of reproductive hormones. Subsequently, I conducted a Pearson-R correlation analysis on concentration of progesterone and Julian date that revealed no significant correlation ($P = 0.884$, $n = 9$). However, previous studies with larger samples have detected significant correlations with size of fetus and Julian date (Reese et al. 2001).

Estimates of average calving interval in this study are higher than previous studies that divided number of corpora albicantia by age of whales (George et al. 2011). Samples in this study were not taken at random. I took samples from a variety of sizes and ages of females from museum specimens that were 14.6-18 m long, and 27-123 years of age. Samples in this study more than likely do not approximate the proportion of lengths and ages of females in the actual population, but instead reflect the biases in whaling practices. However, a significant correlation between age and calving interval was detected, and between age and length (with outlier removed). If ages and lengths of females in the current population could be determined, calving interval estimated by my results could be used in evaluating other long-term trends in populations. These data

could add relevance to studies that identify whales via photographs where length can be measured without mortality (Best 1990). Accuracy of this estimate could be increased by repeating this procedure on baleen from as many sexually mature females as available.

The big mother hypothesis was not supported by my data. The big mother hypothesis states that larger mothers have a reproductive advantage due to their greater acquisition of energy (Ralls 1976). The quality of rearing young by mother bowhead whales (i.e. body condition of mother/calves) was not able to be assessed; and, quantity of calves as indicated by calving interval did not significantly correlate with size. Length of females was the only parameter that was used to assess size, however there are other variables that were not available that could have also been used such as mass, girth, blubber thickness, etc. The size and location of these whales makes many of these measurements difficult to impossible to measure and were unavailable for analysis (Koski et al. 1993; Nerini et al. 1984).

Young females are generally smaller in size than older females, however 86WW2 was considerable long (17.7 m) for her age (34 years old), and the ovary data suggest she had ovulated at least 5 times (1 corpus luteum and 4 corpora albicantia). Her estimated calving interval was once every 5.17 years. Bowhead whales will stretch in length as they are being pulled onto shore. Possible explanations for why this whale was long in length for her age are only speculative but could be that 86WW2 was stretched while be pulled ashore more so than other whales before researchers could make measurements. Length appears to not be a good indicator of age after sexual maturity (Lubetkin et al. 2012), and growth appears to stop around 40-50 years old (George et al. 1999). The data

collected and estimated from whale 86WW2 provide further evidence of variation in length and reproduction in females.

Estimates of calving interval in this study correlated strongly with estimates of age ($P < 0.001$) more so than estimates of length ($P = 0.170$). Thus, an adjustment of the big mother hypothesis might be the “old mother” or “experienced mother” hypothesis; which is that an older mother can bear young more frequently and thus has a fitness advantage to younger mothers. Furthermore, length did not correlate significantly when an abnormally large and young whale (86WW2) was taken out of the analysis. These results may be in part because size and age are correlated in female bowhead whales, with the relationship being most significant in immature whales (George et al. 1999, Lubetkin et al. 2008, Rosa et al. 2013). Older females are likely to have young at a higher rate than younger females.

Studies have been limited in their ability to assess quality of pregnancies per offspring of bowhead whales, unlike humpback whales (Pack et al. 2009), because of their environment. Any associations between reproductive success of males and size would currently be speculative. Genetic analyses may be able to quantify number of offspring from individual males and females within the population of bowhead whales and some familial associations have been identified (Givens et al. 2010). A larger-scale genetic analysis would be better able to evaluate reproductive success of males and females in quantity of offspring and determine if size or age correlates with this reproductive success. This could add evidence to the big mother, experienced mother, or both hypotheses, as well as provide data for reproductive success in males.

Accuracy of detecting pregnancies in baleen was verified by Kellar et al. (2013), and an abnormal pattern of concentrations of progesterone in whale 95B8.

Concentrations of progesterone were significantly different in pregnant and nonpregnant bowhead whales in serum and blubber (Kellar et al. 2013). Concentrations of hormones in hair, other keratinous tissues (Liu et al. 1988), and feces (Rolland et al. 2005), have been used to determine pregnancy in other mammals. Calculations of calving interval in my study were done prior to comparing them with results from dissections of ovaries (Table 3.1) to avoid bias.

Whale 95B8 showed a pattern in concentrations of progesterone that was unique to any other whale that was analyzed and may be indicative of a spontaneous ovulation. Whale 95B8 was pregnant at time of harvest and concentrations of progesterone in the first 25 cm of baleen reflect a high to low concentration of progesterone as expected (Figure 3.5). The concentration of progesterone decreased after the initial pregnancy with a slight increase in concentration at 70 cm, and then a persistent low concentration of progesterone near 0, but still present. Data collected on ovaries of this whale coincide with these fluctuations in concentrations of progesterone in the baleen. The ovary had 1 corpus luteum and 1 corpus albicans (George et al. 2011). The secretion of progesterone during pregnancy by the corpus luteum would account for the significant increase in concentration of progesterone until 25 cm. The second slight increase could have been an ovulation that occurred a few years previous. The increase was not high enough or long enough to indicate a pregnancy at 5-times basal levels, but may represent an ovulation that did not result in a pregnancy. Bowhead whales appear to have spontaneous ovulations (Koski et al 1993), but this has been hard to verify (R. Tarpley

personal communication). The pattern of progesterone in 95B8 appears to add evidence to this theory and concurs with data collected from ovarian tissue (George et al. 2011).

Estimates of calving interval found in this study, with estimates of age and ovarian data give further evidence of bowhead whales having spontaneous ovulations. The estimates of age, counts of ovarian scars, and estimates of calving interval determined in this study can be used to estimate the age at which a whale sexually matured; assuming every ovulation represents a pregnancy and a constant calving interval. For example, whale 86WW2 had a calving interval of once every 5.17 years, was estimated to be 34 years old, and had evidence of 5 ovulations (1 corpus luteum and 4 corpora albicantia). If each of these ovulations resulted in a pregnancy and she had a constant calving interval of once every 5.17 years, she would have had her first calf 26 years previously when she was 8.15 years old (a scenario that is not impossible). It may be the estimate of her age is somewhat younger than her actual age, but is more likely that she had spontaneous ovulations that did not result in a pregnancy. The estimated ages of first pregnancy, using the same assumptions and data, of all females with data available in this study, also results in an impossible or highly unlikely estimated age of first pregnancy (Table 3.2).

Novel data from this study collaborates with previous data taken on length and age of bowhead whales. Whale 95B8 had evidence of only 1 pregnancy and was 15.2 m long; 1 m longer than estimated length at sexual maturity. Whale 95B8 was also estimated to be 27 years of age. 95B8 had probably just recently sexually matured and was killed during its first pregnancy. Length appears to be the best indicator of sexual maturity in bowhead whales, but this may be due to inabilities to determine accurate

estimates of age, until recently. Bowhead whales appear to become sexually mature at about 25 years of age (Lubetkin et al. 2008; Rosa et al. 2013). Data from all 3 sources (age, length, and progesterone concentration in baleen) confirm that this whale was sexually mature and had only recently (most likely within the past few years) become mature.

Data from this study have added valuable information that can be used in the management of bowhead whales and warrants continued analyses of hormones in baleen (Hunt et al. 2014). Implementing conservation efforts and practicing beneficial management of endangered species are contingent on obtaining accurate information specific to species or populations. Sheldon et al. (2001) evaluated the federal listing of 5 populations of bowhead whales as endangered; however, they noted that additional data including life-history information would be necessary to more effectively declassify these populations under the Endangered Species Act. The results of this and future studies could be applied to current estimates of growth in the populations of bowhead whales based on the age and size of females.

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FIGURES AND TABLES

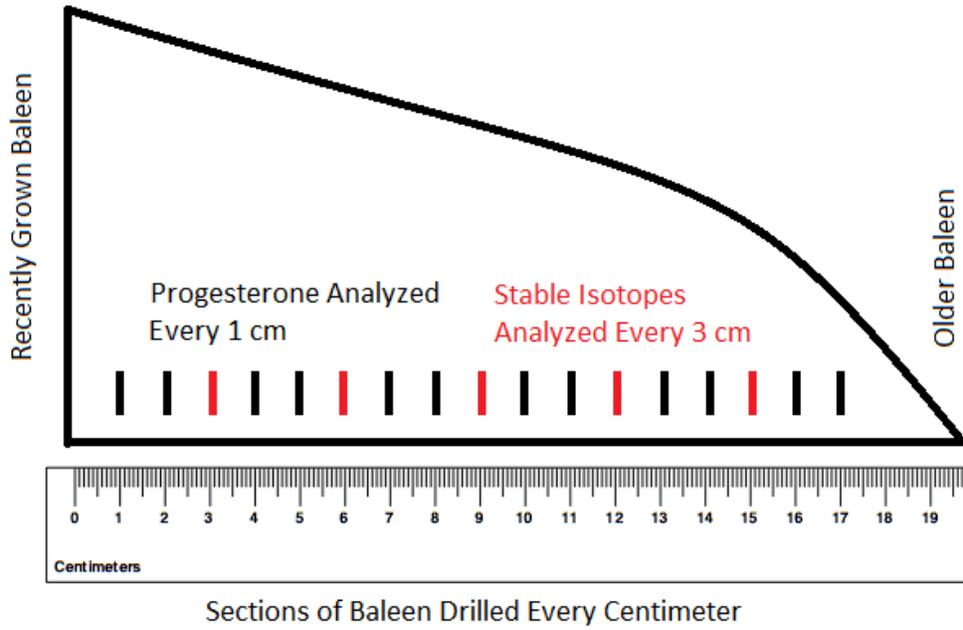


Figure 3.1: Depiction of serial sampling methods for progesterone and stable isotopes for preliminary analyses on baleen from whale 11B6.

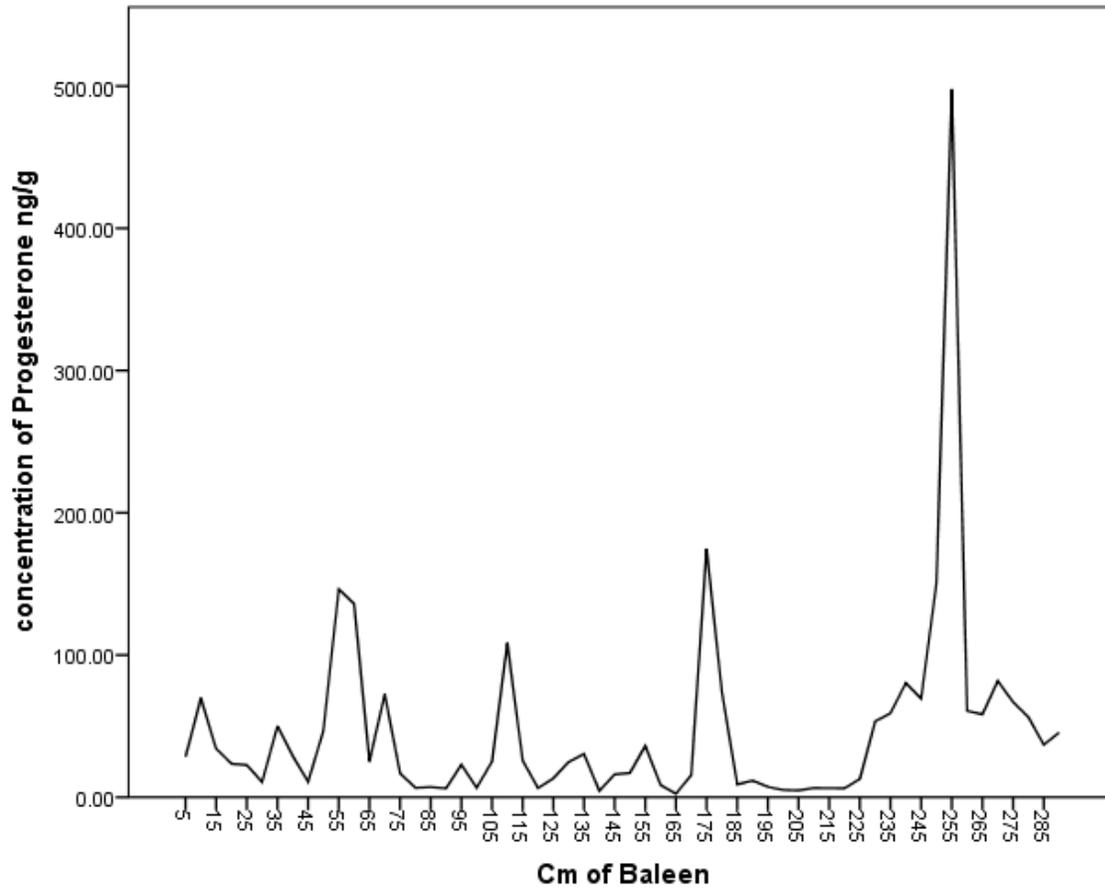


Figure 3.2: Fluctuations in the concentration of progesterone in the baleen of 00B5 as determined in 2 different assays. The first assay was constituted baleen from centimeter marks 5-225 and the second assay constituted baleen from centimeter marks 230-285.

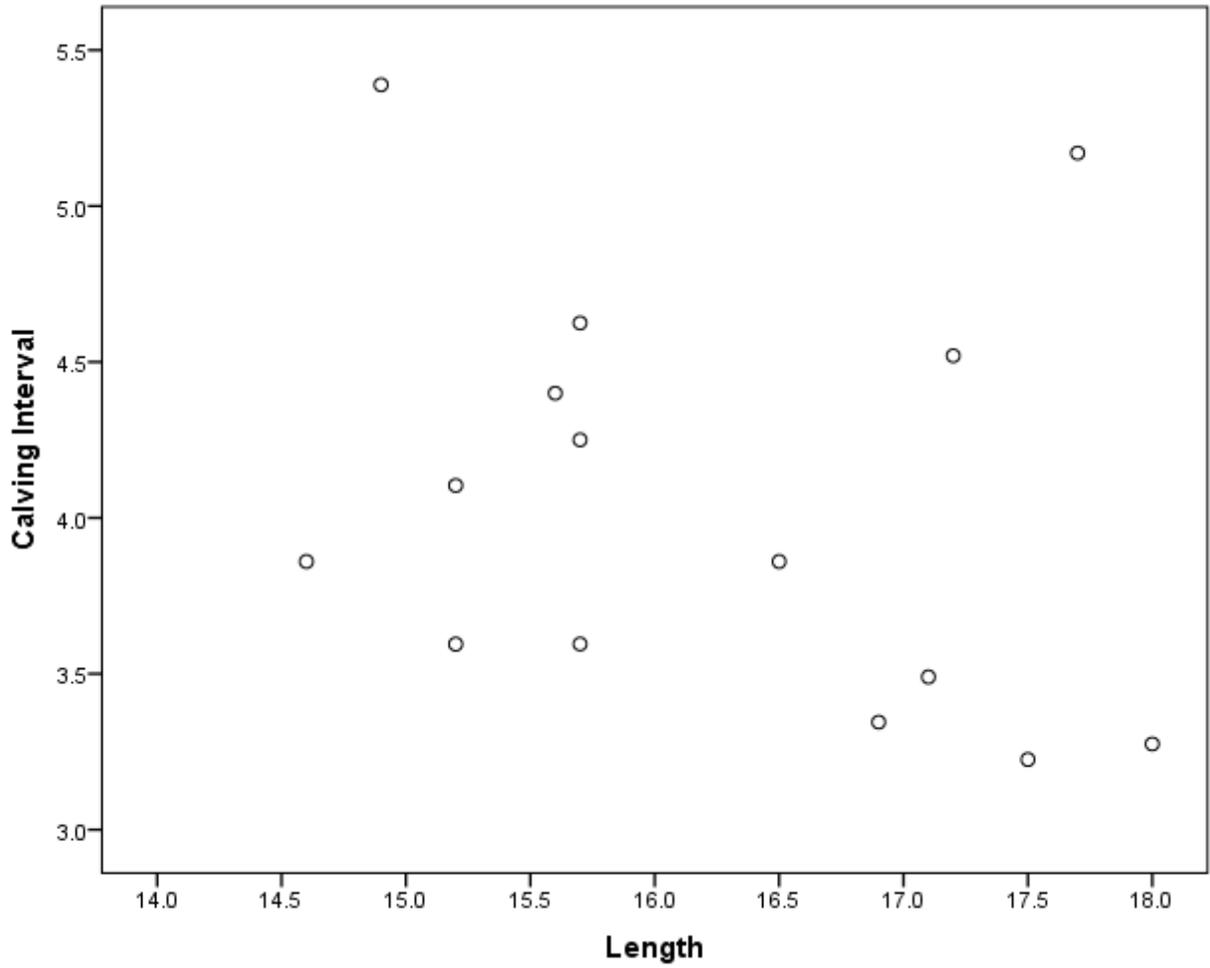


Figure 3.3: Scatterplot between calving interval and length of whales (in meters) of 15 sexually mature female bowhead whales (*Balaena mysticetus*).

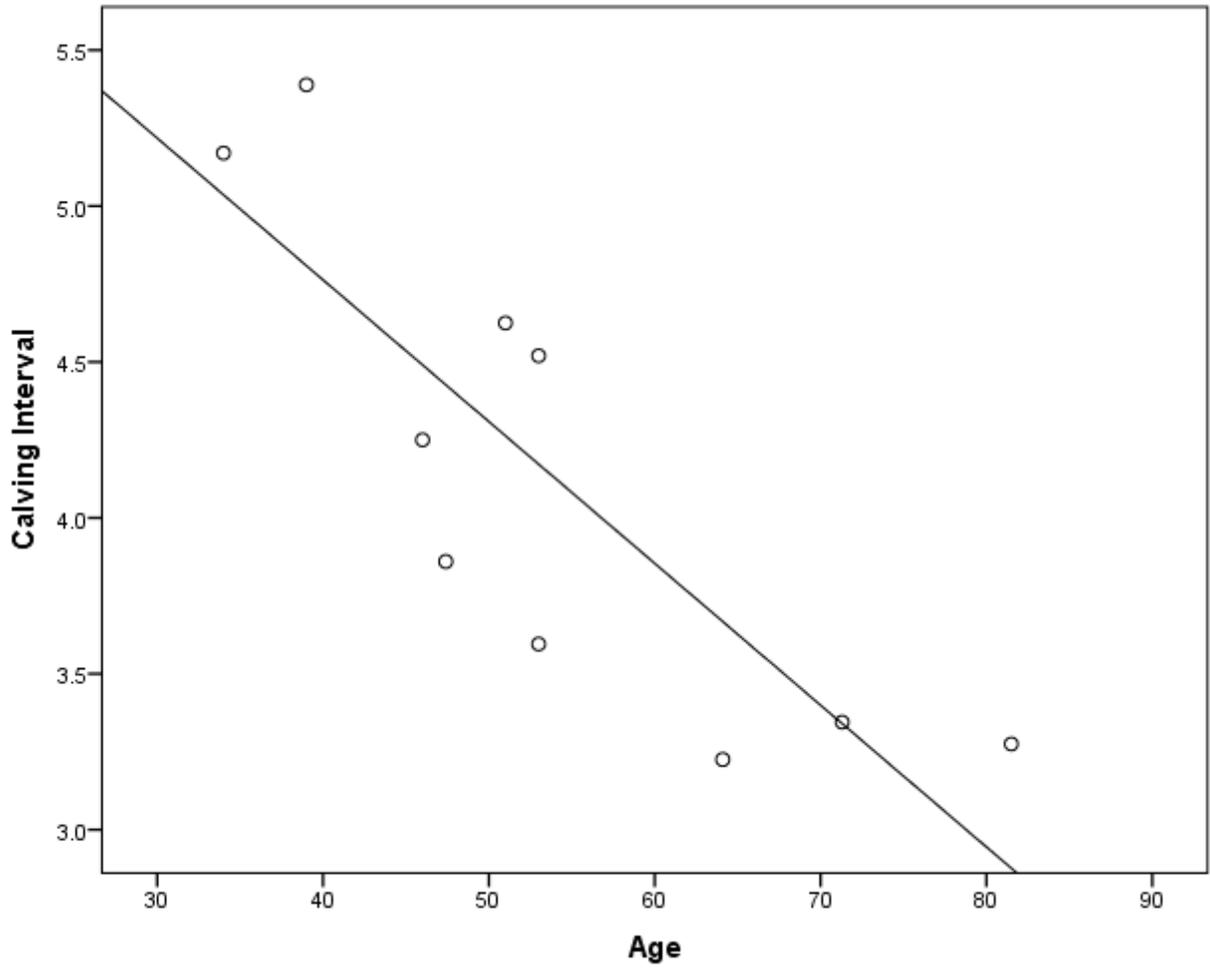


Figure 3.4: Scatterplot between age in years and calving interval of 15 sexually mature bowhead whales (*Balaena mysticetus*).

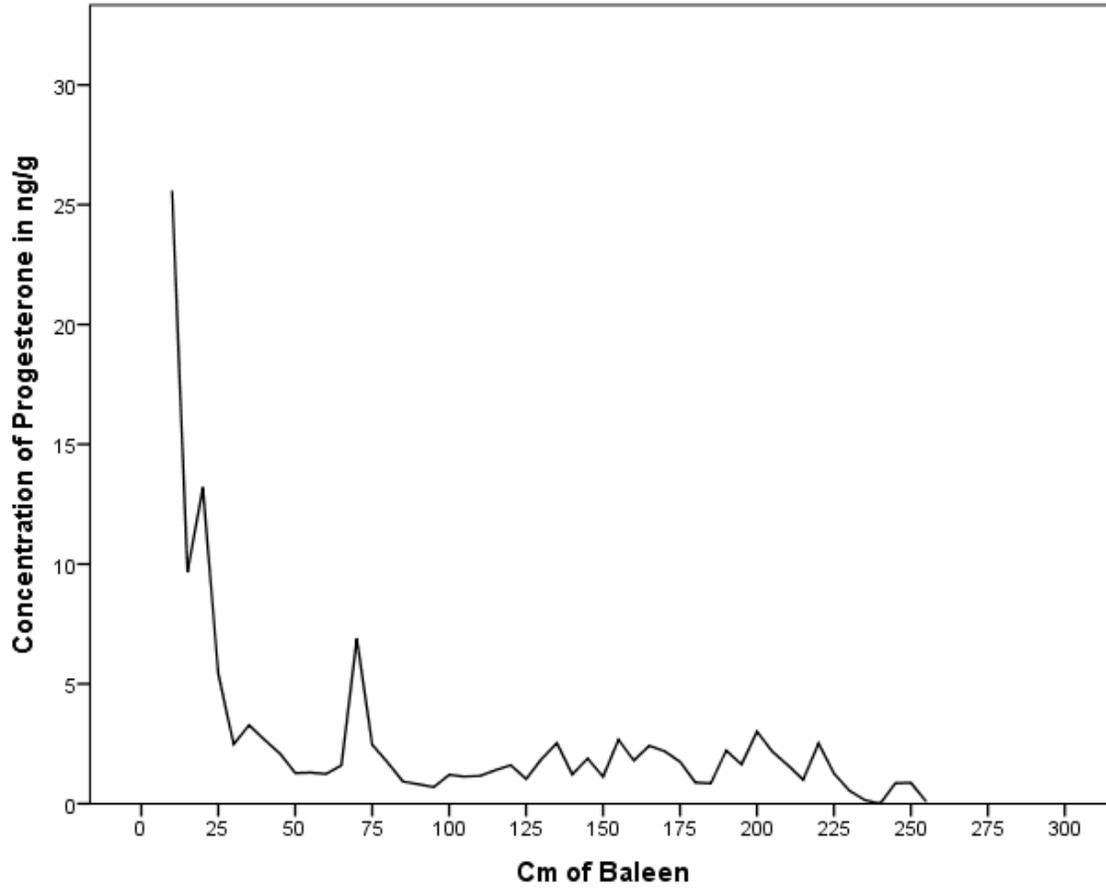


Figure 3.5: Fluctuations in concentrations of progesterone in baleen in a female bowhead whale (*Balaena mysticetus*) identified as 95B8. Samples were taken every 5 cm.

Bowhead whale	Length in meters	Calving interval in number of years per pregnancy	Age in years (George et al. 1999, Rosa et al. 2013)	Ovarian tissue (George et al. 2011)
00B5	17.5	3.23	64.1	1 CL and 21 CA
05S5	16.5	3.86	47.4	
86KK2	17.2	4.52	53	0 CL and 12 CA
87B5	15.7	4.63	51	1 CL and 11 CA
87B6	15.7	3.60	53	0 CL and 12 CA
88B11	15.6	4.4		
88G1	15.7	4.25	46	1 CL and 9 CA
88KK1	14.9	5.39	39	1 CL and 6 CA
89B3	16.9	No peaks detected	123	0 CL and 41 CA
90G4	15.2	4.10		
95B8	15.2	Only 1 pregnancy	27	1 CL and 1 CA
11B6	16.9	3.09	71.3	
86WW2	17.7	5.17	34	1 CL and 4 CA
89B7	14.6	3.86		
87N1	15.2	3.60		
87B4	16.8	No peaks detected		
05S6	17.1	3.49		1 CL and 22 CA
05S7	18	3.28	81.5	

Table 3.1: The 18 sexually mature females used in my study and their lengths (m) measured at time of capture, calving interval as calculated in this study, estimation of age as determined by analyses of lenses in the eye, and data from dissections of ovaries (CL = corpus luteum; CA = corpora albicantia).

Bowhead Whale	Calving interval in number of years per pregnancy	Age in years (George et al. 1999, Rosa et al. 2013)	Ovulations (George et al. 2011)	Estimated age at sexual maturity assuming no spontaneous ovulations and constant calving interval
00B5	3.23	64	22	-6.85
86KK2	4.52	53	12	-1.24
87B5	4.63	51	12	-4.50
87B6	3.60	53	12	9.86
88G1	4.25	46	10	3.50
88KK1	5.39	39	7	1.28
86WW2	5.17	34	5	8.15

Table 3.2: The 7 whales that had data for calving interval, age in years, and ovarian tissue available to estimate age at sexual maturity assuming no spontaneous ovulations and constant calving interval.

CHAPTER 4

DIFFERENCES AMONG REPRODUCTIVE GROUPS IN CONCENTRATIONS OF TESTOSTERONE AND CORTISOL IN BALEEN OF BOWHEAD WHALES (*BALAENA MYSTICETUS*)

ABSTRACT: Reproduction in bowhead whales has proved difficult to observe because of the inaccessibility of their Arctic habitat. Methods for measuring chronic stress in bowhead whales has also proven difficult because of the difficulty in obtaining samples without causing significant stress in individuals. Analysis of hormones in baleen may be a suitable method for understanding fluctuations in reproductive and stress hormones and could be used to identify peaks of reproduction and variance among whales. This preliminary study compared concentrations of cortisol and testosterone in baleen among reproductively mature groups of bowhead whales (*Balaena mysticetus*; males, pregnant females, and nonpregnant females). To assess possible differences among male bowhead whales, concentrations of cortisol and testosterone in baleen were analyzed for correlations of length and Julian date. One-way ANOVAs of log-concentration of cortisol and testosterone revealed significant differences among reproductive groups. A Tukey-Kramer post-hoc test revealed significant differences between males and nonpregnant females for both concentrations of testosterone and cortisol. No significant correlation was detected between log-concentration of testosterone, log-concentration of cortisol, Julian date, and length. These samples were taken in months outside of the proposed mating season, thus testosterone and cortisol concentrations may be more variable during that time.

INTRODUCTION

Parameters of reproduction of bowhead whales (*Balaena mysticetus*) have been difficult to fully assess due to lack of access to these whales during much of the year. Research analyzing tissues of both male and female bowhead whales has provided much of what is known about reproduction in this species (Brownell and Ralls 1980; George et al. 2011; Kellar et al. 2013; Koski et al. 1993; Nerini et al. 1984, O'Hara et al. 2002). Analyses of baleen have provided valuable reproductive information (Chapter 2; Chapter 3). Concentrations of testosterone in baleen have not been analyzed. However, cortisol and progesterone have been assessed in baleen (Hunt et al. 2014; Chapter 2, 3), as well as in blood, urine, and blubber of bowhead whales (Kellar et al. 2013). This study is the first to analyze reproductive hormones specific to male bowhead whales from baleen in an attempt to understand the reproductive ecology of males.

The reproductive success of bowhead whales may correlate with variations in testosterone. Sexual dimorphism in bowhead whales (*Balaena mysticetus*) is reversed from what is seen in other non-mysticete species, with females being larger on average than males (Koski et al. 1993; Nerini et al. 1984; Chapter 1). All mysticete whales exhibit female-biased sexual dimorphism in length (Ralls 1976; Chapter 1); however, the reasons why remain unclear. Current theories include phylogeny such as Rensch's Rule (Chapter 1), the big-mother hypothesis (Chapter 3), a lack of selection by females for size, or a combination of these. An often overlooked, but possible, contributing factor might be a lack of reproductive advantage related to size of males. For their size, bowhead whales have large testes and a long penis, which indicates sperm competition and a promiscuous breeding strategy (Brownell and Ralls 1986). Physical aggression

among males to compete for mates is probably minimal, but mating behavior has been difficult to observe and distinguish from playing behavior (Koski et al. 1993). An elevated level of testosterone in a male bowhead whale may have a fitness advantage via an increase in production of sperm as opposed to aggression and territoriality as with other reproductive strategies.

The differences between concentrations of reproductive hormones in male and female bowhead whales may be more predictable than corticosteroids. Males naturally have higher concentrations of testosterone than females as congruent with mammalian physiology (Pomeroy 2011). Concentrations of corticosteroid has been shown to vary with stress, age, reproduction, and development in different tissues of mammalian species (Hunt et al. 2006; Keay et al. 2006; Reeder and Kramer 2005). Among adult bowhead whales, females have more physiological demands tied to reproduction (and possible stress) than males because of pregnancy, lactation and rearing of young.

Concentrations of cortisol fluctuate with age and sex in other species of mammals, but a profile consistent with chronic stress has yet to be determined in bowhead whales. Baleen provides a unique opportunity to assess changes in cortisol levels over time and fluctuations of cortisol throughout migration, feeding, and/or breeding seasons. Differences among male and female groups would provide valuable baseline data for future assessment of cortisol in baleen.

The aims of this study were to quantify differences in concentrations of testosterone and cortisol in baleen of sexually mature bowhead whales, and to determine if differences in concentrations of hormones in baleen among males correlated with size.

If size does not provide a reproductive or other competitive advantage among males then I expect size will not correlate with concentrations of testosterone or cortisol in baleen.

MATERIALS AND METHODS

During the past >30 years, the North Slope Borough Department of Wildlife Management in Barrow, Alaska, has archived baleen from bowhead whales taken by whaling communities of Alaska. Samples of baleen I assessed were obtained from the Museum of the North at the University of Alaska Fairbanks and from the North Slope Borough Department of Wildlife in Barrow, Alaska. Samples of baleen were taken from whales that were sexually mature as indicated by length of body (>13 m in length).

Samples of baleen were obtained using a model 114, highspeed, cutter bit (Dremel 300, Mount Prospect, Illinois), and sampled at the proximal end, i.e., the most recently grown portion (Hunt et al. 2014). The mass of drilled baleen was measured and recorded. All samples were within 0.03 and 0.05 g in mass. No samples of baleen were taken from other parts of the baleen. Baleen from 27 whales greater than 13m in length were analyzed for testosterone (9 males, 7 nonpregnant females, and 11 pregnant females). Baleen from 28 whales was analyzed for cortisol (10 males, 7 nonpregnant females and 11 pregnant females).

Hormones were extracted using anhydrous ether. Ether was mixed with powdered baleen and vortexed in glass test tubes. Samples were then centrifuged a 2,800 rpm for ≥ 8 minutes. The ether and suspended lipid solution was then poured off into another glass test tube. The extraction procedure was repeated and ether and lipid solution was placed under a fume hood and allowed to dry. Samples of extracted hormone were resuspended in a buffer in duplicate and mixed with hormone-specific

antibodies to create competitive-binding assays. Resuspended extracts were assayed with cortisol-specific antibodies using an enzyme-immunoglobulin assay kit (Item 500360, Caymen Chemical, Ann Arbor, Michigan). For assays of testosterone, the same extraction procedure was followed using an enzyme-immunoglobulin assay kit with testosterone-specific antibodies (Item 582701, Cayman Chemical, Ann Arbor, Michigan). After samples and antibodies were allowed to equilibrate, concentrations of hormones were determined using light spectroscopy and comparing to known concentrations of hormones. Blanks were run in duplicate with each assay. All samples in each assay (cortisol and testosterone) were between 10% and 90% binding except one sample in the cortisol assay which had binding of 97% and was not used in the statistical analyses.

Data were analyzed using SPSS statistical software (Green and Salkind 2008). Two separate one-way ANOVAs were conducted using log-concentration of cortisol and log-concentration of testosterone as the dependent variables and reproductive groups as the independent variable. The 3 reproductive groups were pregnant females, nonpregnant females, and males. Statistical significance were adjusted using a Bonferroni correction of $\alpha = 0.025$. A Tukey-Kramer adjustment was made for all post-hoc comparisons. Pearson-*r* correlation analysis between log-concentration of testosterone, log-concentration of cortisol, Julian date, and length of whales was used to determine if there were significant relationships among these variables.

RESULTS

Males had significantly higher concentration for both testosterone and cortisol on average than females. Average concentration of testosterone for males was 11,601.7 pg/g ($SD = 12,620.0$, $n = 9$) for nonpregnant females 1885.3 pg/g ($SD = 2,128.5$, $n = 7$), and

pregnant females 8,070.6 ($SD = 13,966.2$, $n = 11$). Average concentration of cortisol for males was 452.3 pg/g ($SD = 320.1$, $n = 10$) for nonpregnant females 80.9 pg/g ($SD = 88.1$, $n = 7$), and pregnant females 197.8 ($SD = 159.0$, $n = 11$).

The one-way ANOVA for log-concentration of cortisol among reproductive groups was significant ($F_{2,27} = 7.87$, $P = 0.002$). A Tukey-Kramer post-hoc test revealed significant differences between males and nonpregnant females ($P = 0.002$), but there was neither a significant difference between males and pregnant females ($P = 0.200$) nor a significant difference between nonpregnant and pregnant females ($P = 0.055$).

The one-way ANOVA assessing log-concentration of cortisol among reproductive groups was significant ($F_{2,31} = 4.63$, $P = 0.018$). A Tukey-Kramer post-hoc test revealed significant differences between males and nonpregnant females ($P = 0.014$), but there was neither a significant difference between males and pregnant females ($P = 0.140$) nor between nonpregnant and pregnant females ($P = 0.290$).

No significant correlation was detected between log-concentration of testosterone, log-concentration of cortisol, Julian date, and length of body ($n = 8$). The correlation with the lowest P -value was between log-concentration of cortisol and length (Figure 4.1; $r = -0.47$, $P = 0.170$).

DISCUSSION

As expected, males showed evidence of significantly higher concentration of testosterone than females; especially, nonpregnant females, but the difference was not as much as expected. This may be because samples did not coincide with the mating season. Testes of bowhead whale are large and production of sperm may be continuous, although fluctuations in production of sperm are unknown. A serial sampling of

testosterone in baleen may show fluctuations that correspond to the mating season and provide more insight into when testosterone peaks and indicate possible times of the year when production of sperm is highest.

Pregnant females had an average concentration of testosterone that was greater than nonpregnant females although it was statistically insignificant. Concentrations of testosterone in serum increases in humans during pregnancy, but not until later in pregnancy; the source of the increase is unknown (Bammann et al. 1980). A larger sample that includes late-term, pregnant females as a subgroup may show significant differences from nonpregnant and early term, pregnant females in concentration of testosterone.

Baleen may provide valuable long-term data on fluctuations of cortisol in bowhead whales. Corticosteroids, including cortisol, can be important indicators of stress, health, and age. Results of this study showed significant differences between reproductive groups, similar to differences detected among age groups in hair of humans (Dettenborn et al. 2012). Cortisol in blood of bowhead whales has not been analyzed due to the stressful conditions that whales are put under before samples of blood are taken during subsistence hunts. Incorporation of hormones from circulating blood into baleen occurs over long periods as indicated by rate of growth of baleen (Lubetkin et al. 2008) and probably reflects long-term changes in concentrations of hormones rather than daily or hourly fluctuations. Thus, concentrations of cortisol in baleen are more likely an indicator of chronic rather than acute stress.

Males showed evidence of more stress than females, especially nonpregnant females contradictory to findings by Hunt et al. (2014). Males had the highest average

concentration of cortisol in their baleen (Table 4.1). Analyses of fecal glucocorticoids of North Atlantic right whales (*Eubalaena glacialis*) were significantly greater in pregnant females and males than nonpregnant females and immature whales (Hunt et al. 2006). However, concentration of cortisol in baleen may be more of an indicator of age than sex among sexually mature bowhead whales. Significant correlations between cortisol and age have been detected in humans (Larsson et al. 2009; van Cauter et al. 1996). Studies that have determined age of bowhead whales have revealed that adult males are older on average than females, and the oldest individuals that have been aged were also male (George et al. 1999; Rosa et al. 2013). A sufficient number of whales of known age needed to test if age was a confounding factor in this study was not available, but would be of interest for future studies.

Comparisons between concentrations of cortisol in baleen from this study and Hunt et al. (2014) showed similar results. Conflicts between the studies can be attributed to the number of individuals and the sexual maturity of the samples. The results of this study used a larger sample size ($n = 27$) and excluded baleen from immature whales. Hunt et al. used a smaller sample size ($n = 16$) only 8 of which were sexually mature and included only 1 mature male. Similar methods were used for the drilling and pulverizing of baleen (Dremel), extraction method (lipid solvent and centrifuge), and measuring of steroid hormones (enzyme immunoassays) in both studies.

The results of this study were consistent with what would be expected in a population where males are older than females if cortisol concentrations increase with age (difference in sex aside). However, conclusions based on correlational evidence would require more samples (including serial samples from the same baleen plates) and

more data (including age and measurements of testes). Hunt et al. did a serial analysis of cortisol on 1 mature male, and showed some variation in concentrations of cortisol in baleen at 0 cm, 10 cm, 20 cm, and 30 cm. A smaller interval and samples from more whales would probably better reveal fluctuations in testosterone among male bowhead whales. Results of this study are consistent with previous assumptions and data on males (Brownell and Ralls 1986; Koski et al. 1993; Nerini et al. 1984) and warrant continued research comparing concentrations of hormones in baleen within and among individual male bowhead whales.

Although males showed a higher concentration of log-concentration of cortisol and testosterone in baleen than nonpregnant females, there were no significant correlations of males among the log concentration of hormones, Julian date, and length. These results may also be due to sampling outside the predicted mating season. My results are consistent with previous studies that revealed no seasonal difference in concentration of Leydig and Sertoli cells in testes of bowhead whales (Miller et al. 2002), and no seasonal difference in size of testes (O'Hara et al. 2002), but these samples were also taken outside of the predicted mating season. Two possible explanations for these results may be first, concentration of testosterone, and probably production of sperm, is not being produced in large quantities outside of the mating season, or second, concentration of testosterone, and probably production of sperm, is being produced at a constant rate through all seasons. A serial sample of baleen that corresponds with different times of the year would be useful in determining seasonal fluctuations in testosterone and other hormones.

If there is no selection by females or reproductive advantage for length of males, I would expect no correlation in size of testes or testosterone with length of males. Females showed a correlation between calving rate, size, and age (Chapter 3). Males may also exhibit a similar correlation as females among sexually mature males and testosterone, if samples were analyzed within the mating season. More data on age of males and concentration of testosterone, especially during baleen grown during the mating season, would be necessary to test for such correlations.

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FIGURES AND TABLES

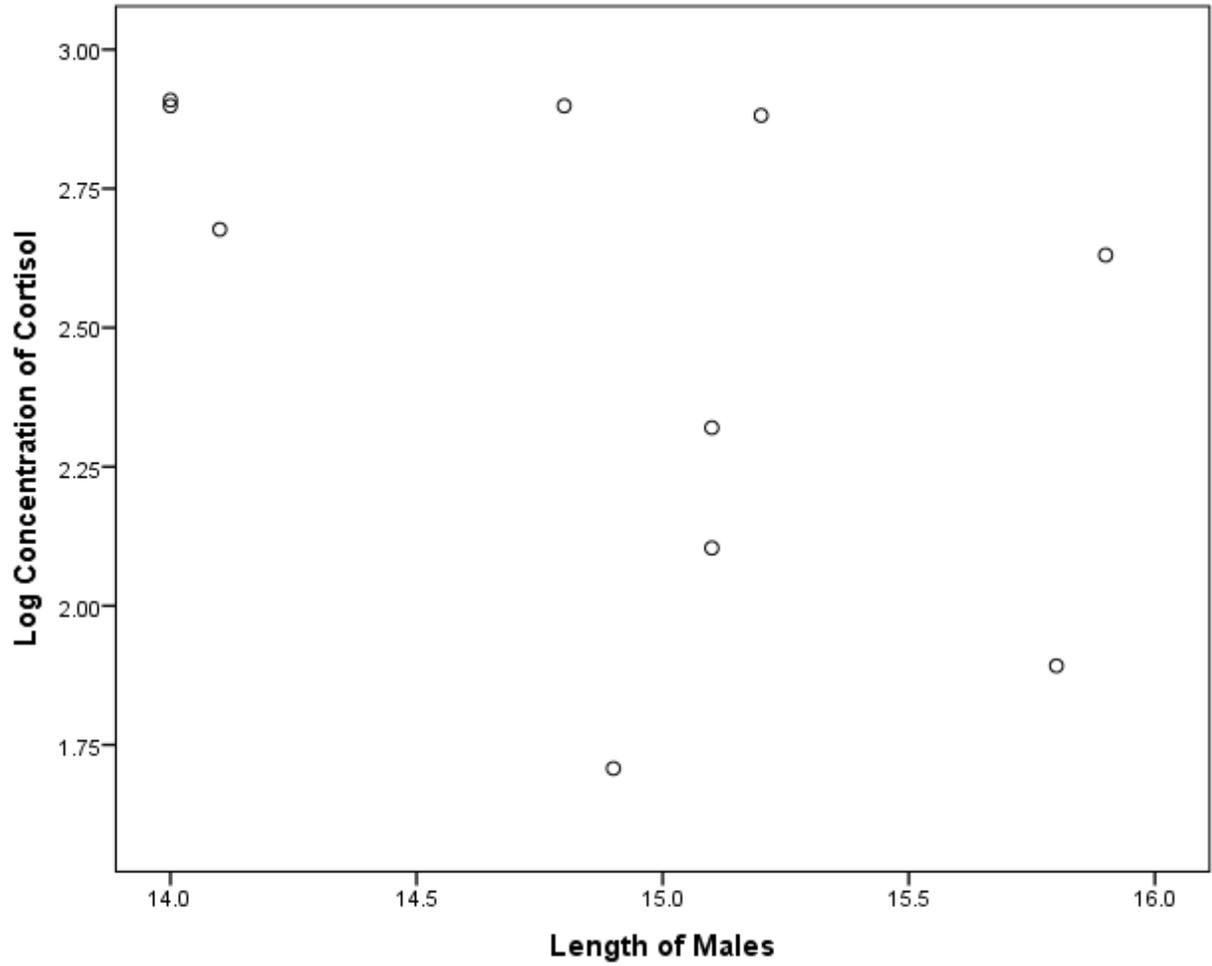


Figure 4.1: Log-concentration of cortisol and length of male bowhead whale (*Balaena mysticetus*).

Reproductive Group	Average Concentration of Cortisol (pg/g)	SE	Average Concentration of Testosterone (pg/g)	SE
Male	453	31.8	10,501	3,414
Nonpregnant Female	81	8.8	1,885	4,080
Pregnant Female	356	55.4	6,346	2,699

Table 4.1: Average concentrations ($\pm SE$) of cortisol and testosterone (pg/g) from the proximal end of baleen of males, nonpregnant females, and pregnant females of bowhead whales (*Balaena mysticetus*).