HEARING AND AGE ESTIMATION IN TWO SPECIES OF ARCTIC WHALE

A dissertation submitted
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by

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# TABLE OF CONTENTS

LIST OF FIGURES........................................................................................................v

LIST OF TABLES.........................................................................................................ix

PREFACE....................................................................................................................x

ACKNOWLEDGEMENTS...............................................................................................xi

CHAPTER I..................................................................................................................1
  Evolution of the cetacean ear.................................................................................4
  Generalized hearing model.................................................................................4
  The outer ear of Cetacea......................................................................................6
  The middle ear of Cetacea...................................................................................8
  The inner ear of Cetacea....................................................................................12
  Communication and hearing in belugas............................................................13
  Communication and hearing in bowheads.........................................................14
  Under water anthropogenic sounds in the Arctic.............................................14
  Age estimation in bowheads.............................................................................16
  Summary of the dissertation.............................................................................17

CHAPTER II...............................................................................................................19
  The spiral ganglion and Rosenthal’s canal of beluga whales
    Introduction..................................................................................................20
    Materials and Methods...............................................................................24
    Results..........................................................................................................39
    Discussion....................................................................................................41
    Conclusions.................................................................................................48
LIST OF FIGURES

Figure 1.1 ........................................................................................................................................... 2
Distribution map of bowhead and beluga whales

Figure 1.2 ........................................................................................................................................... 3
The spiral ganglion and Rosenthal’s canal of beluga and bowhead

Figure 1.3 ........................................................................................................................................... 7
Sound reception pathway for modern land mammals versus odontocetes

Figure 1.4 ........................................................................................................................................... 9
Representative views of the petro-tympanic complex of odontocetes and mysticetes

Figure 2.1 ........................................................................................................................................... 27
Delphinapterus leucas microCT scan

Figure 2.2 ........................................................................................................................................... 28
Delphinapterus leucas AMIRA3-D reconstructions of bony labyrinth and spiral ganglion

Figure 2.3 ........................................................................................................................................... 30
Delphinapterus leucas specimen dissection and preparation

Figure 2.4 ........................................................................................................................................... 32
Delphinapterus leucas petrosal showing anatomy and different stages of dissection

Figure 2.5 ........................................................................................................................................... 34
Delphinapterus leucas cochlear segment map

Figure 2.6 ........................................................................................................................................... 35
Delphinapterus leucas spiral ganglion histological sections of specimen 2010LDL21
Right
Figure 2.7………………………………………………………………………………………..37

*Delphinapterus leucas*, three variables of the spiral ganglion plotted against their cochlear position

Figure 2.8………………………………………………………………………………………..45

Natural logarithm of spiral ganglion counts and basilar membrane length versus body weight for several cetaceans.

Figure 2.9………………………………………………………………………………………..47

*Delphinapterus leucas*, number of ganglion cells in segments of the spiral ganglion against mean histological cross-sectional area of that segment

Figure 3.1………………………………………………………………………………………..54

*Balaena mysticetus* boney anatomy and microCT scan

Figure 3.2………………………………………………………………………………………..55

*Balaena mysticetus* AMIRA3-D reconstructions of cochlea and Rosenthal’s canal

Figure 3.3………………………………………………………………………………………..56

*Balaena mysticetus*, three variables of the spiral ganglion plotted against their position along the cochlea

Figure 3.4………………………………………………………………………………………..58

*Balaena mysticetus* cochlear segment map of 2012B18R

Figure 3.5………………………………………………………………………………………..62

*Balaena mysticetus*, spiral ganglion histological sections specific distances from the base

Figure 3.6………………………………………………………………………………………..63

*Balaena mysticetus*, enlarged histological image of specimen 2010B15R
Balanena mysticetus, number of ganglion cells in segments of the spiral ganglion against mean histological cross-sectional area of that segment

Balaena mysticetus, the tympanic bulla

Balaena mysticetus, baleen isotope signatures and tympanic GLGs for multiple whales

Balaena mysticetus, baleen isotope signature and tympanic GLGs for whale NSB-DWM 2013B18

Balaena mysticetus, baleen isotope signature and tympanic GLGs of whale NSB-DWM 2011B9

Balaena mysticetus, baleen isotope signature and tympanic GLGs of whale NSB-DWM 2013B1

Balaena mysticetus, region 3 microstructure of six tympanic bone slices under brightfield magnification

Balaena mysticetus, region 3 of two whale specimens displaying GLG variability

δ^{13}C signatures of tympanic bone and baleen transects
Figure 4.9.................................................................91

Tympanic bulla GLG counts plotted against four other variables from these same bowhead individuals

Figure 5.1..................................................................100

Three variables of the spiral ganglion plotted against their position along the cochlea

Figure 5.2..................................................................102

Attenuation of sound in fresh and sea water, dB per km and Hz (Denny 1993)

Figure 5.3..................................................................104

Natural logarithm of spiral ganglion counts and basilar membrane length versus natural logarithm of body weight for several cetaceans and terrestrial mammals

Figure 5.4..................................................................108

GLGs in the periosteal region of the tympanic bone of fossil Eocene whale, *Nalacetus*
LIST OF TABLES

Table 2.1........................................................................................................................................26
  Date of sampling, sex, length, and relative age of beluga whales used in this inner ear study

Table 2.2........................................................................................................................................43
  Species, number of cochlear whorls, basilar membrane length (mm) and estimated spiral ganglion neuron number for several odontocete species

Table 3.1........................................................................................................................................65
  Morphometric data and number of neurons in the spiral ganglion for multiple mysticete whales

Table 3.2........................................................................................................................................68
  Biological data on the bowheads used in this inner ear study

Table 4.1........................................................................................................................................79
  Estimated parameters for the von Bertalanffy II growth model of Lubetkin et al. (2012)

Table 4.2.......................................................................................................................................90
  List of bowhead whale specimens used in this age estimation study

Table 5.1.......................................................................................................................................103
  Approximate body weight, basilar membrane length, and number of spiral ganglion neurons in multiple mysticetes, odontocetes, and terrestrial mammals
PREFACE

Chapters I and V of this document are original material written for the purpose of the dissertation. Chapter II was originally published as a research article in the Journal of Morphology in 2015. Contributing authors are Jennifer D. Sensor (myself), R. Suydam, J. C. George, M. C. Liberman, D. Lovano, M. A. Rhaganti, S. Usip, C. J. Vinyard, and J. G. M. Thewissen. J.G.M. Thewissen and I designed the research and wrote the paper, and all contributing authors performed the research and/or analyzed the data. Chapter IV was has been accepted for publication in Marine Mammal Science. Contributing authors are Jennifer D. Sensor (myself), J. C. George, M.T. Clementz, D. M. Lovano, D. A. Waugh, G. H. Givens, R. Suydam, R. Stimmelmayr, and J. G. M. Thewissen. J.G.M. Thewissen and I designed the research, all contributing authors performed the research and/or analyzed that data, and J.G.M Thewissen, M.T. Clementz, G.H. Givens, and I wrote the paper. The data contained in chapter III will be published as part of a larger study at a later date.
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CHAPTER I
INTRODUCTION

The increase of human activity in the marine waters of the Arctic has brought a corresponding increase in anthropogenic sounds and contaminants. These changes, in addition to diminishing ice cover, can have massive effects on the ecology and well-being of the marine species that live in these regions. It is therefore essential for us to understand these effects in order to make the best wildlife management decisions.

Here, I study the ear of two Arctic whale species: the beluga whale (*Delphinapterus leucas*), a toothed whale (odontocete) and high frequency echolocator, and the bowhead whale, (*Balaena mysticetus*) a baleen whale (mysticete) which uses low frequencies for long distance communication. Figure 1.1 shows the distribution of these animals. To study long term hearing effects, I focus on the anatomy of the spiral ganglion and Rosenthal’s canal of the inner ear (Fig. 1.2).

In mammals, hearing is known to decrease with age, and understanding the age of individuals is an important factor in population dynamics studies. Therefore, I also focus on age estimation methods. For belugas, age estimation has been well established using Growth Layer Groups (GLGs) in teeth. In bowheads however, although age estimations methods exist, there are only specific age ranges in which each method is most effective. Specifically, there is a gap in effective age estimation methods between the ages of 10 and 30 years old. Thus, I investigate a new method of age estimation for bowheads using the tympanic bone of the middle ear.
Figure 1.1. Distribution map of bowhead and beluga whales. Beluga distribution is highlighted in red. Bowhead distribution is highlighted in blue. Overlapping ranges are purple. The collection site for beluga whale specimens is Pt. Lay represented with a green star. The collection site for bowhead specimens is Barrow, represented by a yellow star. The studied populations consist of the Bering-Chukchi-Beaufort groups. (Adapted from Jefferson et al. 2008.)
Figure 1.2. The spiral ganglion and Rosenthal’s canal of beluga and bowhead. (A,C,E) views of a beluga’s Rosenthal’s canal and spiral ganglion from a left ear. (B,D,F) views of a bowhead’s Rosenthal’s canal and spiral ganglion from the right ear. (A,B) Amira 3-D reconstruction of the cochlea based on microCT slices, with Rosenthal’s canal highlighted within. The cochlea is blue for beluga and green for bowhead, while Rosenthal’s canal is yellow for beluga and red for bowhead. (C,D) Amira 3-D reconstruction of cochlea, cut virtually to show cross-sectional areas of bony labyrinth and Rosenthal’s canal. (E,F) Histological cross-sections for beluga and bowhead whales, near the mid-modiolar cut. Scale bar applies to E and F.
Evolution of the Cetacean Ear

The order Cetacea, consists of whales, dolphins, and porpoises that evolved from terrestrial artiodactyls (even-toed ungulates) around 50 million years ago (Gingerich et al. 2001, Thewissen et al. 2001, Gingerich 2003, Uhen 2007). This transition from land to water occurred over an evolutionarily short period of less than 10 million years (Thewissen and Williams 2002), and is considered one of the best examples of macroevolutionary change among vertebrates.

Among the sensory systems, the ear has developed a number of unique adaptations to deal with the challenges of an aquatic environment. For example, water is about a thousand times more dense than air and sound travels approximately five times faster in this medium. Additionally, water and soft body tissues have similar acoustic impedances. In a land mammal ear, these issues lead to the inability to detect the direction of a sound source in water, and less effective hearing. This transition from hearing on land to hearing in water has been documented through fossil evidence (Gingerich and Russell 1981, Gingerich et al. 1983, Kumar and Sahni 1986, Oelschläger 1986a, b, 1990, Thewissen and Hussain 1993, Gingerich et al. 1994, Gingerich et al. 1995, Hulbert Jr. et al. 1998, Luo 1998, Luo and Gingerich 1999, Fordyce and de Muizon 2001, Nummela et al. 2004). Here, I will describe the major differences between modern land mammal ears and modern whale ears.

Generalized Hearing Model

In a land mammal, sound waves traveling through the air are received by the outer ear (pinna and external auditory canal). At the end of the external auditory canal, sound waves hit the tympanic membrane causing it to vibrate. Sound vibrations can also be received through bone conduction, as the periotic bone is in close contact or fused with the skull (Nummela et al.
The vibrations are conducted into the middle ear cavity to three small ear bones (ossicles), the malleus, incus, and stapes, which are connected to each other through joints. These bones vibrate and match the impedance of the air to the impedance of the cochlea. During this process, the ossicles amplify the intensity of the sound (Wartzok and Ketten 1999, Nummela et al. 2007). The arm or manubrium of the malleus (the first bone to vibrate) is attached to the tympanic membrane. The stapes, which is the last bone to vibrate rests within the oval window of the snail shaped cochlea and “pumps”, causing waves within the perilymph of the inner ear. The cochlea has three compartments (scala vestibuli, scala media, and scala tympani). The wave initially travels to the apex of cochlea through the scala vestibuli and then back through the same whorls to the round window through the scala tympani. As the perilymph within the scala vestibuli vibrates it sets the endolymph within the scala media into motion. These vibrations are picked up by hair cells (polarized epithelia cells) in the organ of Corti within the scala media, which both detect frequency and amplitude. There are usually three rows of outer hair cells and one row of inner hair cells which sit on the basilar membrane. The basilar membrane plays an important role in frequency detection among mammals. It becomes wider, flatter and more flaccid from base to apex. Highest frequencies are detected basally where the membrane is thick, narrow, and relatively stiff. Low frequencies are detected on the thin, flat, more compliant membrane of the apex. The hair cells transduce the sound in the region in which the sound wave is detected, and are in synaptic contact with afferent and efferent nerve endings. The afferent innervation consists of bipolar cells whose cell bodies reside in the spiral ganglion. The spiral ganglion is housed within a bony canal known as Rosenthal’s canal. The efferent innervation is derived from the olivocochlear system in the brainstem (Spoendlin 1984).
The Outer Ear of Cetacea

All Cetacea lack outer ear pinnae, although some specimens have vestigial pinnae rings (Yamada 1953, Wartzok and Ketten 1999). The outer ear hole is extremely small and virtually or completely closed (Yamada 1953, Reysenbach de Haan 1957, Solntseva 2007). Although present, it is debatable as to whether the external auditory canal is functional (Yamada 1953, Fraser and Purves 1954, Reysenbach de Haan 1957, Dudok Van Heel 1962, Norris 1968, McCormick et al. 1970, Ketten 1997).

A more likely mechanism for sound reception in modern odontocetes is through the lower jaw (Fig. 1.3; Bullock et al. 1968, Norris 1968, McCormick et al. 1970, Norris and Harvey 1974, Brill et al. 1988, Møhl et al. 1999, Cranford et al. 2010). In odontocetes, the mandibular canal is widened and the posterior third is thinned to create a “pan bone,” (Norris 1968, Norris 1980). Biochemically distinct fats fill the canal, and cover the lateral and ventral portions of the mandible (Varanasi and Malins 1971, 1972). These “acoustic fats” have a density close to that of sea water, are in contact with the fused tympanic and periotic bones of the middle ear (petro-tympanic complex), and are thought to act as the preferential pathway for sound (Bullock et al. 1968, Norris 1968, Varanasi and Malins 1971, Brill et al. 1988, Koopman et al. 2006, Zahorodny et al. 2009, Cranford et al. 2010). Other suggested regions of sound reception have been through the soft tissues around the tongue and throat (Cranford et al. 2008). There is also evidence that region near the external auditory meatus may be sensitive to low-frequency sounds (Bullock et al. 1968, Renaud and Popper 1975, Popov and Supin 1990, Popov et al. 2008). This could potentially be due to another body of low impedance fats in this region (Ketten 1994).
Figure 1.3. Sound reception pathway for modern land mammals versus odontocetes. (A) Depiction of ear anatomy for many land mammals, such as artiodactyls (even-toed ungulates). (B) Depiction ear anatomy for odontocetes (toothed whales). (Adapted from Thewissen 2014, used with permission.)
Little is known about the sound reception pathway in mysticetes, but studies show that a fatty sound reception pathways may also exist in at least the Balaenopteridae family (Yamato et al. 2012, Yamato et al. 2014). In homologous regions to odontocetes, these fats also insert themselves onto the petro-tympanic complex and onto the malleus (Yamato et al. 2012). Unlike odontocetes, however, these fats do not reside in the jaw but rather posterior, dorsal, and lateral to the mandibular ramus, ventral to the squamosal bone, and lateral to the petro-tympanic complex. Within the tympanic bulla (described below) lies a secondary fat body, which contacts the inner wall of the tympanic bone and the malleus. Other studies utilizing finite element modeling indicated that bone conduction through skull vibrations is the predominant hearing pathway, although pressure waves through soft tissues may play a role (Cranford and Krysl 2015).

Middle Ear of Cetacea

The petro-tympanic complex holds the inner ear and is made of the fused petrosal (periotic) and tympanic bones of the skull (Fig. 1.4; Yamada 1953, Oelschläger 1986a, b, Nummela 1999, Ketten 2000, Solntseva 2007). It is located in a space known as the peribullar cavity created from expansions of the middle ear spaces (Oelschläger 1986a, Ketten 1991, Wartzok and Ketten 1999). The petrotic bone (petrosal) houses the cochlea and semi-circular canals while the tympanic bone forms a bulla and encloses the middle ear ossicles in an air-filled cavity. This complex has a number features that are hallmarks of Cetacea (Berta et al. 2006). For example, the tympanic bulla is pachyosteoschlerotic (both thick and dense; Thewissen 1994, Luo and Gingerich 1999, Cozzi et al. 2012, Kim et al. 2014). It has an entoglenoid process which serves as an attachment between the tympanic bulla and the squamosal bone of the skull
Figure 1.4. Representative views of the petro-tympanic complex of odontocetes and mysticetes. (A,C,E). Representative views of the left petro-tympanic complex (middle ear) of a odontocete (juvenile harbor porpoise). (B,D,F). Representative views of the left petro-tympanic complex of a mysticete (fetal bowhead whale). (A,B) lateral view, (C,D) medio-ventral view (E,F) anterior view. Anterior (rostral) is labeled for each view. Dorsal and ventral remain at the top and bottom of each view, respectively.
(Luo and Gingerich 1999, O'Leary and Geisler 1999), and the tympanic bone has a thickened medial side, known as the involucrum (Luo 1998, Nummela 2008, Thewissen et al. 2009). The lateral side of the tympanic bone is comparatively thinner and is often referred to as the “tympanic plate.” This is the primary insertion point of the “acoustic fats” found in odontocetes and mysticetes.

In modern odontocetes, the petro-tympanic complex as a whole is acoustically isolated from the skull in the peribullar cavity (Reysenbach de Haan, 1957, Ketten and Wartzok 1990, Oelschläger, 1990, Ketten 1991, Nummela et al. 2004, Nummela et al. 2007, Cranford et al. 2010). Middle ears are suspended by five or more sets of ligaments (Fraser and Purves 1954, Ketten and Wartzok 1990, Ketten 2000), and surrounded by special membranes (corpus cavernosum and peribullar plexus; Fraser and Purves 1960, Jansen and Jansen 1969, McCormick et al. 1970, Ketten and Wartzok 1990, Nummela 1999, Solntseva 2007). This isolation is important for separating the middle ear components from the rest of the skull, reducing bone conduction in water and therefore increasing sensitivity to sound localization (Nummela 1999, Warzok and Ketten 1999). The membranes may also regulate air pressure by swelling and shrinking as the animal dives (Nummela 1999). In addition, the contact between the periotic and tympanic bones is reduced compared to mysticetes and other mammals.

In mysticetes, the petro-tympanic complex is still surrounded by a peribullar cavity but is not isolated to the degree it is in odontocetes. The petrosal and tympanic are tightly attached to one another through anterior and posterior pedicles (Ekdale et al. 2011). The complex sits tightly between the exoccipital and squamosal bones of the skull by a process made of both tympanic and periotic components (Yamada 1953, Fraser and Purves 1960, Nummela 1999, Ketten 2000, Ekdale et al. 2011).
Within the involucrum of the tympanic bone, many mysticete species display Growth Layer Groups (GLGs) similar to tree rings. For a number of whale species counting these GLGs has been investigated as a means of age estimation (Klevezal and Mitchell 1971, Christensen 1981, Sukhovskaya et al. 1985, Klevezal et al. 1986, Konrádsson and Sigurjónsson 1989, Christensen 1995, Klevezal 1996, Hohn 2009), although some have identified limits to these methods (Larsen and Kapel 1982, 1983, Olsen 2002, Olsen et al. 2003).

Some other types of marine mammals also have adaptations to their middle ear, although none have the same superlatives as cetaceans. For examples, pinnipeds (seals) have a detachment of the petrosal from other skull bones, but this is not as extensive as it is in cetaceans (Repenning 1972). Pinnipeds also display a slightly thickened region of the tympanic. This region, however, is at the attachment with the mastoid bone and is therefore on the lateral side of the complex. In sirenians (manatees and dugongs), the petro-tympanic complex is relatively well insulated from the skull. The tympanic, in this case called an ectotympanic, is inflated and “drop-like” in shape, the primitive condition being deflated (Berta et al. 2006). This “U-shaped” ring is not a bulla as in whales and is tightly adhered to the periotic (Nummela 2008).

The middle ear ossicles of Cetacea vary in size, shape and orientation (Ketten 2000, Nummela et al. 2004, Nummela et al. 2007). Overall, they have a greater mass than land mammals or phocid seals (Nummela et al. 2004, Nummela et al. 2007). The tympanic membrane of odontocetes is thin and folded. It is known as the tympanic ligament. The malleus is fused to the tympanic plate through its anterior process, the process gracilus (Ketten 1991, Nummela 1999, Nummela et al. 2007), and the rest of the ossicular chain is stiffened through ligaments. This likely increases high frequency hearing. In mysticetes, the tympanic membrane is everted, thickened and extended laterally (Reysenbach de Haan 1957, Ketten 1997). The
malleus of mysticetes is also fused to the tympanic plate, but the ossicles are not stiffened as in odontocetes. This likely allows for lower frequency hearing.

The Inner Ear of Cetacea

The cochlea of Cetacea contains the same three scalae as all other mammals (Fig. 1.2) (Ketten 1991). Details of these structures have been described by Wever *et al.* (1971a, b, c, 1972), Ketten (1984), and Ybarz (2012), for odontocetes and by Ketten (1991), Wartzok and Ketten (1999), Ketten (2000), for mysticetes. Solntseva (1990, 2007) also describes the development of the inner ear for both clades.

The odontocete cochlea differs from other mammals in hypertrophied cochlear duct structures with hypercellularity of basilar membrane support cells and an increased number of spiral ganglion cells (Wever *et al.* 1971a, b, c, 1972, Ketten 1984, Ketten and Wartzok 1990). Mysticetes cochlear ducts are known to be larger with a lesser degree of hypercellularity than odontocetes, although this may due in part to most mysticete material being gathered through strandings (Ketten 2000).

Similar to other mammals, frequency detection within Cetacea is largely the result of the stiffness and mass of the basilar membrane along the length of the cochlea (Wever 1971b; Wartzok and Ketten 1999). This is best dictated by a thickness to width ratio over the cochlear length (Ketten and Warzok 1990, Ketten 1991). High frequency specialists such as odontocetes have a comparatively high thickness/width ratio in the basal whorl. Low frequency hearers, such as mysticetes, having a low thickness/width ratio in the apex. Cochlear length is correlated to body size, but not to frequency (Ketten 1994). This is primarily because length and thickness of the basilar membrane do not co-vary in marine mammals. Thus, length does not predict marine
mammal hearing ranges accurately, as it does for land mammals. Bony spiral laminae, which help support the basilar membrane, also make the membrane stiffer (Ketten 1992). All cetaceans have an inner (primary) bony spiral lamina, but odontocetes, have a secondary (outer) spiral lamina attached to the outside wall of the cochlea. The length of the secondary spiral lamina differs among species, but always correlates to their peak hearing frequencies (Ketten and Wartzok 1990).

The secondary spiral lamina is present in mysticetes too, but it disappears within the first half turn. It is narrow and does not support or provide rigidity to the basilar membrane as in odontocetes (Fleischer 1976, Ketten 1991, 1992). These traits seem to be indicative of low frequency and potentially infrasonic hearing.

*Communication and Hearing in Belugas*

Beluga whales are odontocetes with one of the most diverse vocal assortments of any marine mammal (O’Corry-Crowe 2009). They are often referred to as the “sea canary” due to their vocal abilities, producing around 50 different emotive and communicative call types within the frequency range of 0.1 to 12kHz (O’Corry-Crowe 2009). Echolocation frequencies are much higher. Au et al. (1988) found peak frequencies to be variable depending on environment with peak frequencies from 40-60kHz and from 100-120kHz. Most audiograms for wild Alaskan belugas indicate high sensitivity between approximately 30 kHz to 100 kHz. This is based on auditory evoked potentials (Klishin et al. 2000, Castellote 2014), but behavioral audiograms (on captive individuals) are consistent with this (Finneran et al. 2005).
Communication and Hearing in Bowheads

Audiograms for bowheads have not yet been possible. It is suspected that their hearing range is in the lower frequencies due to their inner and middle ear morphologies (Ketten and Wartzok 1990, Ketten 1991, Parks et al. 2007, Ekdale and Racicot 2014). Ketten (1994) estimated the hearing range to be approximately between 0 and 35kHz based on morphometric and stiffness estimates of the basilar membrane. Acoustic studies on bowhead calls and songs as well as behavioral investigations, indicate that bowheads produce sounds from about 0.02kHz to a maximum of 5kHz (Ljungblad et al. 1982, Clark and Johnson 1984, Cummings and Holliday 1987, Würsig and Clark 1993, Stafford et al. 2008, Clark et al. 2015, Johnson et al. 2015) and use these sounds for communication and possibly for navigation (Ellison et al. 1987, George et al. 1989, Johnson et al. 2015).

Underwater anthropogenic sounds in the Arctic

Anthropogenic sound in the arctic and its effect on marine mammals have been reviewed by several authors (Richardson et al. 1995, Stafford 2013, Moore et al. 2012). It is important to note when referring to underwater sound intensities, that the reference pressure is dB re 1 μPa. For gross comparison with air sound (measured at dB SPL or dB re 20 μPa), the underwater sound pressure must be reduced by 61.5 dB (61.5 dB re 1 μPa in water = 0 dB re 20 μPa in air). In the arctic the sources of anthropogenic sound are primarily seismic air gun surveys, drilling for natural resources, and an increased number of ships both commercial and noncommercial. Each of these generally produce low frequency sounds primarily under 1,000kHz. Seismic air guns typically produce impulses between 10-500Hz (Hildebrand 2009, Stafford 2013) with source levels of up to 260 dB re 1 μPa at 1m (Hildebrand 2009). This low frequency and high
amplitude allows sound travel over 1,000km away from the source (IWC 2007, Thode et al. 2010, Weilgart et al. 2013). These pulses can be produced from 4 to 40 s intervals for hours or days (Blackwell et al. 2007, Stafford 2013) and surveys can last over a period of months (Blackwell et al. 2007). Drilling for natural resources primarily produces sounds under 1,000 Hz with a source level of approximately 185 dB re 1 µPa at 1m (Stafford 2013), although some sounds may reach approximately 10,000Hz. Drilling occurs continuously during the spring and summer months when much of the sea ice has melted. Finally, ship traffic is another major source of sound in the Arctic, primarily produced from their propulsion system. Ship traffic includes icebreakers, tankers, cargo ships, tow and tug and vessels, research vessels and personal vessels used nearshore for hunting or fishing (Stafford 2013). With the reduction of sea ice, the number of navigable Arctic shipping routes is expected to increase by mid-century (Smith and Stephenson 2013). Larger vessels typically produce low frequency sounds less than 1,000Hz at a source level of about 171-193 dB re 1 µPa at 1m (Richardson et al. 1995, Hildebrand 2009). The smaller boats however, may produce higher frequency noise than the larger vessels, from approximately 1-5kHz with sources levels at 150-180 dB re 1 µPa at 1m (Hildebrand 2009). Overall, an increase of speed, for both larger and smaller vessels, produces more noise.

The observed behavioral responses to anthropogenic sounds for bowheads have included decreased surfacing as well as time spent on the surface (Würsig et al. 1985), avoidance of the sound source (Richardson et al 1999), and changes in vocalizations (Blackwell et al. 2007). Belugas, have also shown a number of varying responses including changes in vocalizations, swimming speed, surfacing and breathing, diving patterns, and group composition (National Research Council 2003). Regardless of the species, most studies to date have concentrated on the short-term effects of anthropogenic noise. In addition, the noise levels required for hearing loss
is still uncertain (Weigart et al. 2013). Thus, there is a need for understanding the longer term behavioral, anatomical, and physiological effects of anthropogenic noise on marine mammals of the arctic.

*Age Estimation in Bowheads*

Several age estimation methods are available for bowheads, but they only cover limited parts of their lifespan. For whales under 60 years old, growth models utilizing baleen length (Lubetkin et al. 2008, 2012) or isotope oscillations within baleen plates can be used (Schell et al. 1989a, Schell and Saupe 1993). For sexually mature females (older than roughly 25 year), numbers of corpora albicantia can be counted (George et al. 2011, Tarpley et al. 2016). Body length also gives a coarse indication of age (George 2009, Lubetkin et al. 2012). Alpha-crystalline in the eye lens can be studied (George et al. 1999, Rosa et al. 2004), but there is uncertainty regarding estimated enantiomer (D:L) ratio at birth and racemization rate, and the analysis is costly. A critical age range for which most of these methods are imprecise or inadequate is between about 10 to 30 years. Several researchers have documented incremental growth layers in the tympanic bullae of other baleen whale species and have proposed counting the growth layer groups (GLGs) as a method of age estimation, with each GLG representing one year (Klevezal and Mitchell 1971, Christensen 1981, Sukhovskaya et al. 1985, Klevezal et al. 1986, Konrádsson and Sigurjónsson 1989, Christensen 1995, Klevezal 1996, Hohn 2009). Readability may vary, however (Larsen and Kapel 1982, 1983, Olsen 2002, Olsen et al. 2003). GLGs have also been identified in the tympanic bulla of bowhead whales. An important aim of my study is to determine whether counting GLGs in tympanic bullae is a reliable method for age estimation in bowheads, specifically between 10 and 30 years of age.
Summary of dissertation

This dissertation provides baseline information about the inner ear of beluga and bowhead whales, focusing on cell number and density of the spiral ganglion and the cross-sectional area of Rosenthal’s canal along the length of the cochlea. Cochlear morphometric information is provided. The spiral ganglion is investigated for signs of trauma, as neural degeneration in the spiral ganglion is not immediate upon sound exposure (as it is in other cochlear regions) allowing documentation of long term damage to the inner ear.

I also present a new method of age estimation for bowhead whales through counting GLGs within the periosteal region of the involucrum of the tympanic bulla. I characterize the different parts of the involucrum isotopically and histologically. The results are compared to life history parameters and to estimation methods that use baleen isotopes and baleen length.

Chapter II investigates the spiral ganglion, Rosenthal’s canal, and cochlear morphometrics of the beluga whale. Results show that absolute spiral ganglion cell counts vary along the length of the cochlea. Rosenthal’s canal cross-sectional area also varies, having two local maxima. Cochlear morphometrics indicate that although the cochlear shape of belugas is similar to other odontocetes it does not fit well into traditionally established categories. Histological assessment and neuron counts do not suggest evidence of auditory damage to beluga ears.

Chapter III investigates the spiral ganglion, Rosenthal’s canal, and cochlear morphometrics of the bowhead whale. Results show that absolute spiral ganglion cell counts do not vary significantly along the length of the cochlea. Conversely, Rosenthal’s canal cross-sectional does vary, reducing in size near the apical portion of the apical whorl. A drop in mean cell number in upper basal whorl and apical whorl of the Rosenthal’s canal for two specimens
along with histological evidence may indicate auditory damage in these whales. Cochlear morphometrics for bowheads fit well into the category of mysticete cochleas.

Chapter IV describes the microstructure of the involucrum of the tympanic bone of bowhead whales, with a focus on using the GLGs in the periosteal region to estimate age. Different parts of the involucrum are characterized histologically and isotopically. Comparing age estimates using GLGs to number of isotopic oscillations in a baleen plate, length of the baleen plate, age estimates based on baleen length, and total whale length indicate that counting GLGs is a reliable method for individuals 20 years of age or younger. Bone histology of the involucrum records events that are not found in other anatomical samples, and thus can be a window into the life history of the individual.
CHAPTER II

THE SPIRAL GANGLION AND ROSENTHAL’S CANAL IN BELUGA WHALES

ABSTRACT

With the increase of human activity and corresponding increase in anthropogenic sounds in marine waters of the Arctic, it is necessary to understand its effect on the hearing of marine wildlife. We have conducted a baseline study on the spiral ganglion and Rosenthal’s canal of the cochlea in beluga whales (*Delphinapterus leucas*) as an initial assessment of auditory anatomy and health. We present morphometric data on the length of the cochlea, number of whorls, neuron densities along its length, Rosenthal’s canal length and cross-sectional area, and show some histological results. In belugas, Rosenthal’s canal is not a cylinder of equal cross-sectional area, but its cross-section is greatest near the apex of the basal whorl. We found systematic variation in the numbers of neurons along the length of the spiral ganglion, indicating that neurons are not dispersed evenly in Rosenthal’s canal. These results provide data on functionally important structural parameters of the beluga ear. We observed no signs of acoustic trauma in our sample of beluga whales.

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INTRODUCTION

The ear of the mammalian order Cetacea (whales, dolphins, and porpoises) is adapted for underwater hearing, and a range of frequency specializations occurs among different cetaceans. Mysticetes (baleen whales) use low-frequency sounds for long-distance communication and spatial orientation, whereas odontocetes, (toothed whales, which includes dolphins and porpoises) use high frequencies to echolocate and have complex communication repertoires (Wartzok and Ketten 1999). The anatomy of these vastly different auditory specializations is intrinsically interesting but it is difficult to study hearing in cetaceans. Many cetaceans are large, hard to take samples from in the wild, and husbandry is difficult or impossible. In addition, there are legal (U.S. and international) and ethical issues surrounding the acquisition of fresh samples. However, some arctic cetaceans are harvested by Alaskan Native populations under subsistence-hunting provisions of the law, and necropsy material provides a unique source of fresh samples. Here, we use this material to study the cochlear morphology of wild belugas (*Delphinapterus leucas*) as a baseline for future studies on hearing in cetaceans. The increase of human activity and corresponding increase in anthropogenic sounds in the Arctic is known to affect arctic marine wildlife in the short term (Stocker 2011), but our study is interested in permanent, long-term damage. Establishing a baseline for healthy beluga ear anatomy can provide insights into the impact of potential future acoustic exposures.

Beluga whales live in the subarctic and Arctic often near the edge of the frozen ocean. Some populations migrate north and south with the annual waxing and waning of the ice. They have one of the largest vocal repertoires within odontocetes (Bel’kovitch and Sh’ekotov 1990) Belugas produce around 50 different emotive and communicative call types within the frequency range of 0.1 to 12 kHz (O’Corry-Crowe 2009) and are also capable of vocal learning and
imitation (Vergara and Barrett-Lennard 2008). Their echolocation frequencies are much higher; Au et al. (1988) found peak echolocation frequencies to be variable but with a primary high near 40 kHz. Most audiograms for wild Alaskan beluga indicate high sensitivity between approximately 30 kHz to 100 kHz. This is based on auditory evoked potentials (Klishin et al. 2000, Castellote et al. 2014), but behavioral audiograms (on captive individuals) are consistent with this (Finneran et al. 2005).

The purpose of this paper is to document the morphology of the beluga cochlea, in particular those features that are important in hearing. Such anatomical studies on wild animals can complement functional studies on captive specimens. Laboratory studies on acoustic trauma often focus on the organ of Corti, since it transduces sound. However, because our samples came from individuals that were hunted, organs of Corti are not usable for our study for the following reason. Acoustic trauma to the organ of Corti can be immediate upon exposure (e.g. Ohlemiller et al. 2000, Wang et al. 2002, Abrashkin et al. 2006, Henderson et al. 2008), and blast damage to the ear has been documented in detail in domesticated animals (Roberto et al. 1989). Instead of the organ of Corti, we study the spiral ganglion and Rosenthal’s canal in order to gain insight in the functional parameters of the cochlea and to provide a baseline for beluga hearing during life. Neurons in the spiral ganglion are susceptible to cell death when their peripheral connections with inner and outer hair cells (within the organ of Corti) die (Hurley et al. 2007, Henderson et al. 2008). This neural degeneration is not immediate. Spiral ganglion damage arises slowly; neuron death can be documented based on histological data no sooner than several weeks after the death of the hair cells (Lurie et al. 1944, Fredelius 1988, Fredelius et al. 1988), although minor signs of damage (e.g., vacuoles in the ganglion) can be seen within several hours after exposure (Wang et al. 2002).
One objective of our study is to estimate the total number of neurons in the spiral ganglion, and we compare absolute counts of spiral ganglion cells along the cochlea to density estimates of these cells. Neuron counts in the spiral ganglion have been estimated in a variety of ways. Firbas et al. (1970) explored variation in the spiral ganglion in the guinea pig on regular tangential sections, using two methods. First, these authors counted numbers of nuclei of all neurons in all sections and reported these with a mathematical correction factor. Second, they calculated cell densities, as opposed to cell numbers, per whorl of the cochlea. They justified the use of densities on the basis of three considerations: it is less expensive since not all sections are counted, it is less in need of mathematical corrections, and it avoids the need to determine exact borders between whorls. Most modern studies have used only densities at the mid-modiolar sections to evaluate the health of the ganglion cell population of the spiral ganglion (Dazert et al. 1996, Ruijven et al. 2004, Shepherd et al. 2005, Agterberg et al. 2010). Others have determined neuron densities along Rosenthal’s canal and used this to calculate an estimate of neuron numbers (Wicke and Firbas 1970, Keithley and Feldman 1979, Schuknecht 1993). Keithley and Feldman (1979) indicated that this would correct for oblique sectioning. When only density estimates are used to evaluate the spiral ganglion over its length, however, information regarding changes in cross-sectional area of Rosenthal’s canal is lost. Johnson et al. (2011) showed that larger canal cross-sections match areas of higher neuron numbers in mice, resulting in similar neuron density numbers in spite of higher neuron numbers. Consistent with this, Richter et al. (2011) found that, in gerbils, neuron numbers in the spiral ganglion as well as Rosenthal’s canal cross-sectional area increase toward the cochlear apex and that in ontogeny packing densities of neurons change.
There is some evidence that cross-sectional areas of Rosenthal’s canal and numbers of spiral ganglion neurons along the length of the cochlea are variable in cetaceans too. Wever et al. (1971c) found that neuron densities drop toward the apex of the cochlea in the dolphin *Tursiops truncatus*. Broadly speaking, the spiral ganglion cells innervate adjacent parts of the hair cell bands, although the spatial correlation is complicated at the ends of the spiral, where the peripheral axons of spiral ganglion cells fan out to reach their most apical and basal targets. Thus, over much of the spiral ganglion, cell distribution can be used to evaluate innervation density and thereby infer limits on the frequency resolving power of the inner ear in different parts of the hearing range.

There has been some research on the cochlea of belugas. Solntseva (2007) published a number of images of the developing beluga labyrinth, and Ketten (1997) listed in a table that the spiral ganglion of belugas has a neuron density of 3557 cells/mm, with an overall total estimate of 149,386 cochlear ganglion cells. There are several estimates of fiber number in the statoacoustic nerve (CN VIII) of belugas. Morgane and Jacobs (1972) estimated 171,100, whereas Jansen and Jansen (1969) estimated 210,000. However, the statoacoustic nerve not only includes fibers from afferent spiral ganglion neurons, but also efferent fibers to the cochlea, as well as fibers to and from the vestibular organ. Gao and Zhou (1991) compared the composition of the statoacoustic nerve of the cetaceans Baiji (*Lipotes vexillifer*) and finless porpoise (*Neophocaena phocaenoides*). They found that both the ratio of spiral ganglion neuron count to cochlear fibers (*Lipotes*, 0.69; *Neophocaena*, 0.94) and the ratio of cochlear fibers to total statoacoustic nerve fibers (*Lipotes*, 0.75; *Neophocaena* 0.98) varied greatly between these species. The ratio of cochlear fibers to total statoacoustic nerve fibers in *Tursiops* is 0.6 (Morgane and
Jacobs, 1972). This indicates that it is unwise to estimate spiral ganglion counts in beluga based on assumptions about the fiber composition of this nerve in other cetaceans.

The other objectives of this study are to report on cell densities along the cochlea, cochlear length, Rosenthal’s canal length, number of cochlear whorls, and show histological results to establish a baseline in beluga cochlear morphology.

MATERIALS AND METHODS

Specimen Acquisition

Subsistence hunting of beluga whales takes place in the village of Point Lay, Alaska, and health-related sampling of these whales occurs three to twenty-four hours after death and is overseen by the Department of Wildlife Management, North-Slope Borough, Alaska. Samples were acquired under NOAA-NMFS permit 814-1899-03. Belugas are protected under several U.S. laws, and this makes acquisition of all types of samples under controlled conditions impossible, since samples can only be collected after subsistence harvest. Methods commonly used to study the inner ear in laboratory, domestic, or even most wild animals cannot be used here, and we modified these to adapt to the conditions present.

To remove a periotic bone from the skull, we approach the ear ventrally, removing parts of the hyoid arch. This exposes the ventral side of the tympanic bulla. Rongeurs are used to open the middle ear cavity, and the tympanic, malleus, and incus are removed. The stapes is firmly attached in the oval window and is not dislodged in this process. The periotic is only loosely attached to the skull on its caudal side, and is unattached by bone otherwise. We use a scalpel to resect soft-tissue connections rostral, lateral, and medial, and then use rongeurs to extract the periotic. The periotic is immediately submerged in 2.5% glutaraldehyde (a common
fixative for the organ of Corti, Wang et al. 2002), and post-fixed for at least 3 weeks. After fixation, specimens are stored in 1x PBS.

Specimens are catalogued in the collection of the North Slope Borough, Department of Wildlife management. Specimens numbers indicate the year caught (e.g., 2010), a locality and species acronym (LDL, Point Lay, Delphinapterus leucas), and a rank order number (e.g., 10), yielding 2010LDL10. Table 2.1 provides information about the date of sampling, sex, length and relative ages of the animals from which samples were collected for this study.

MicroCT Scanning

We scan each periotic using a Scanco Medical vivaCT-75 scanner. Images are acquired at 70 kVp, 114 μA, and 200-ms integration time. Voxel size is 41μm. We export scans in TIFF format to a work station and use Amira 5.4.1 to build three-dimensional images using the segmentation feature of Amira to image the relevant structures: the bony labyrinth and within it, Rosenthal’s canal (Fig. 2.1). Approximately 400 scans are used to build the complete bony labyrinth of one beluga. We use the reconstructed images in Amira to visualize the number of whorls in the cochlea and Rosenthal’s canal (Fig. 2.2). Length measurements are taken in three dimensions on the Amira cochlear reconstruction to determine the specific length of these structures. This allows us to express positions along the cochlea as a percentage of total cochlear length.
<table>
<thead>
<tr>
<th>ID #</th>
<th>Date</th>
<th>Sex</th>
<th>Length (cm)</th>
<th>Age</th>
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</thead>
<tbody>
<tr>
<td>2010LDL8</td>
<td>30-Jun-10</td>
<td>Male</td>
<td>356</td>
<td>Adult</td>
</tr>
<tr>
<td>2010LDL11</td>
<td>30-Jun-10</td>
<td>Female</td>
<td>329</td>
<td>Adult</td>
</tr>
<tr>
<td>2010LDL12</td>
<td>30-Jun-10</td>
<td>Male</td>
<td>318</td>
<td>Adult</td>
</tr>
<tr>
<td>2010LDL20</td>
<td>30-Jun-10</td>
<td>Male</td>
<td>334</td>
<td>Adult</td>
</tr>
<tr>
<td>2010LDL21</td>
<td>30-Jun-10</td>
<td>Male</td>
<td>364</td>
<td>Adult</td>
</tr>
<tr>
<td>2012LDL4</td>
<td>9-Jul-12</td>
<td>Female</td>
<td>279</td>
<td>Subadult</td>
</tr>
<tr>
<td>2012LDL5</td>
<td>9-Jul-12</td>
<td>Male</td>
<td>335</td>
<td>Adult</td>
</tr>
<tr>
<td>2012LDL7</td>
<td>9-Jul-12</td>
<td>Female</td>
<td>280</td>
<td>Subadult</td>
</tr>
<tr>
<td>2012LDL9</td>
<td>9-Jul-12</td>
<td>Female</td>
<td>317</td>
<td>Subadult</td>
</tr>
<tr>
<td>2013LDL7</td>
<td>4-Jul-13</td>
<td>Female</td>
<td>368</td>
<td>Adult</td>
</tr>
<tr>
<td>2013LDL13</td>
<td>4-Jul-13</td>
<td>Male</td>
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<td>Adult</td>
</tr>
<tr>
<td>2013LDL15F</td>
<td>4-Jul-13</td>
<td>Female</td>
<td>153</td>
<td>Near-term fetus</td>
</tr>
</tbody>
</table>

Table 2.1. Date of sampling, sex, length, and relative age of beluga whales used in this inner ear study. Standard length—straight line measurement from snout to fork in the tail. Relative age based on color (white = adult; gray-white = subadult).
Figure 2.1. *Delphinapterus leucas* microCT scan. (A) Representative midmodiolar microCT scan beluga whale 2012LDL7 Left. The accessory ossicle is an enlarged part of tympanic. (B) Enlarged view of Rosenthal’s canal in the basal whorl of this section.
Figure 2.2. *Delphinapterus leucas* AMIRA3-D reconstructions of bony labyrinth and spiral ganglion. Based on microCT slices, the bony labyrinth is in blue, with Rosenthal’s canal highlighted in yellow in some. Apical view (A, C, E, G), other representative views (B, D, F, H). Specimens used: 2010LDL11 left (A-B), 2013LDL8 right (C-D), 2012LDL4 left (E-F), and 2012LDL5 right (G-H). (I) AMIRA 3-D reconstruction of cochlea of 2010LDL11 left, cut virtually to show how cross-sectional areas of Rosenthal’s canal were determined. Scale bar between C and E is for views A-H. Scale bar next to I is for view I.
Rosenthal’s Canal Assessment

We determine cross-sectional area of Rosenthal’s canal using the apical view of the Amira 3D-reconstructed image in nine or ten locations along the cochlea, more or less equally-spaced. Each cross-sectional area is measured from an image produced by the ‘surface cut’ feature of Amira. Using the rotate tool, we position the ‘surface cut’ to be at a right angle to the canal’s curvature (Fig. 2.2I). After a cut is made on the image by using the clip button, we create a new surface and visualize it using the ‘surface view’ feature. We then toggle off ‘surface cut’ and ‘surface view,’ hiding all parts of the cochlea that were not in the surface cut. Using the ‘draw’ button, we circle regions that we do not measure and remove these. We once again create a new surface on the appropriate cross-section and measure this surface area. Such measurements of cross-sectional areas based on CT-scans are more accurate than similar measurements on histological sections, because we avoided artifacts (shrinkage) associated with histological processing. We determined cross-sectional areas of Rosenthal’s canal for eight beluga cochleas.

Spiral Ganglion Dissection and Histology

Given that beluga cochleas are larger than those of most laboratory animals, we evaluated several methods of processing them (Fig. 2.3). Methods used in studies of small, perfused mammals (Wang et al. 2002, Ruijven et al. 2004) cannot be used to study our large specimens that could only be acquired after death. Hence, our specimens are immersion fixated.

For most specimens, we remove some of the bone with a Foredom microdrill to speed up the decalcification process. We immerse specimens in 10% EDTA (pH 7.5) for three to six
**Figure 2.3.** *Delphinapterus leucas* specimen dissection and preparation. (A) Apical view of part of the cochlea during dissection; (B) Mid-modiolar section of cochlea, where entire specimen was embedded in Araldite resin and stained with Eosin; (C) Mid-modiolar section of cochlea, where entire specimen was paraffin embedded and stained with Hematoxylin and Eosin; (D) Excised specimen of organ of Corti and spiral ganglion stained with Hematoxylin and Eosin; (E) Enlarged area of spiral ganglion from D. Scale bar in center is for all images.
weeks, renewing the solution after the first two weeks, testing occasionally for level of
decalcification. This level of decalcification preserves the structural coherence of the cochlea,
while making extraction of the organ of Corti and spiral ganglion possible. Subsequently, we
use one of three methods, method three is the preferred method at present.

In the first method, we embedded some cochleas in araldite resin without initial removal
of bone and cut them at 12μm on a rotary microtome, staining them with eosin. These specimens
preserve the internal anatomy, such as basilar and Reissner’s membranes, well (Fig. 2.3B). Hair
cells cannot be seen, either because of processing damage or as a result of blast damage at the
time of death. Roberto et al. (1989) noted that exposure to gun shots at close range can result in
immediate detachment of the organ of Corti, and indeed fragments of this organ are commonly
found as debris in the scala media of our specimens.

In the second method, after decalcification, we embedded other entire cochleas in
paraffin, cut them at 7 microns on a rotary microtome and stained with Hematoxylin and Eosin.
These display damage to the organ of Corti and often ruptured basilar and Reissner’s membranes
(Fig. 2.3C). Paraffin-embedded specimens do show well preserved cells in the spiral ganglion
(Fig. 2.3E), allowing precise counting of spiral ganglion neurons over the entire length of the
cochlea.

Most of our data were acquired using a third method. This method is modified from the
dissection method of Johnsson and Hawkins (1975) for humans. For these specimens, after
fixation but before decalcification, we flush 0.1% thionin through the scalae via the round and
oval windows (Fig. 2.4), so that the cochlea can be seen through the bone during excess bone
removal later on.
Figure 2.4. *Delphinapterus leucas* petrosal showing anatomy and different stages of dissection. (A) Bony specimen; (B) Specimen after thionin flush and excess bone removal; (C) Decalcified specimen with some bone removed and scala exposed; (D) Partially dissected specimen. Scale bar is for all images. Specimen 2013LDL left.
Some specimens were flushed with 3% OsO₄ (Fig. 2.3A), but we found that this does not enhance resolution of the eventual sections. We then use a Foredom microdrill to remove excess bone, and provide access the scala vestibuli. Then we decalcify the specimen using the method described above.

After decalcification, we dissect the organ of Corti and spiral ganglion under a dissection microscope using iris scissors and scalpels to remove excess decalcified bone. This opens the scala vestibuli. We remove pieces of the spiral ganglion with its adenexa, starting apically, and proceeding to the base. In some specimens only the spiral ganglion and Rosenthal’s canal are extracted, whereas in others spiral lamina and stria are also included in the excised sample (Fig. 2.3D). The size of individual segment is mostly determined by the degree of curvature of the cochlea and our ability to handle small pieces of tissue with forceps. We map the position and size of each segment on a 3D-image that is based on the CT-scans and we refer to this diagram as the segment map (Fig. 2.5). Segments are dehydrated and embedded in paraffin, and the spiral ganglion segments are cut at right angles to the long axis of the ganglion, creating cross-sections. Sections are 7μm thick and are stained with hematoxylin and eosin. No thionin remains at this stage, but some spiral ganglia retain their OsO₄ staining (Fig. 2.6).

**Stereology**

We use stereologic methods utilizing StereoInvestigator software (MicroBrightField, Williston, VT, USA, version 10) to estimate the number of neurons in each segment of spiral ganglion, and use that estimate to assess neuron density of each cochlear segment. Although presumed Type I and Type II spiral ganglion cell bodies can be identified based on size and cytologic traits in cross section (Thomsen 1966, Kellerhals et al. 1967, Spoendlin 1972, Merck et al. 1977),
Figure 2.5. *Delphinapterus leucas* cochlear segment map. AMIRA 3D reconstruction of bony labyrinth (blue) and Rosenthal’s canal (yellow) based on approximately 400 microCT scans: The size and location of Rosenthal’s canal samples that were dissected out of this specimen are indicated by numbers. Each of these samples is a segment of Rosenthal’s canal that is histologically processed and counted. H- Helicoirea. Map based on sample 2012LDL7 left.
Figure 2.6. *Delphinapterus leucas* spiral ganglion histological sections of specimen 2010LDL21 Right: Samples taken (A) 6% from base of spiral ganglion; (B) 19% from base; (C) 42% from base; (D) 67% from base; (E) 84% from base; (F) 89% from base. M indicates modiolar side, S indicates spiral lamina side. Scale bar for all images.
differentiation among these falls outside of the scope of our research and we did not distinguish between these.

The set up includes an Olympus Bx-51 photomicroscope equipped with a Ludl XY motorized state, Heidenhain z-axis encoder, a digital camera, and a flat panel monitor to project images. Within a section, Rosenthal’s canal is traced and all cells within it are counted using a computer assisted fractionator sampling scheme to count the nuclei. Neurons are counted at 40x magnification when the nucleus (diameter of 12.5μm) is in focus, using a Counting Frame Width (X and Y) of 150μm and a Sampling Grid (X and Y) of 150μm. We do not use guard zones or the optical dissector of StereoInvestigator, because our sections are thin (7μm). The section evaluation interval is usually five (sometimes four or six), meaning we count every fifth (fourth or sixth) section. We usually count a total of five sections (sometimes four or six, once just three) to make up a data point in Figure 2.7B and C. The variation in evaluation interval or total number of sections counted resulted from our rejection of sections that were damaged due to processing and the number of sections available per slide.

We use the ‘Estimated Population using Mean Section Thickness from Sites with Counts’ of StereoInvestigator to estimate neuron numbers per spiral ganglion subsegment. This method estimates the number of cells based on the operator (JDS) counting neurons in a number of ‘counting sites’ (squares outlined on the screen) chosen by the program for each histological section. Section thickness is evaluated by the operator setting the top and bottom of each section at every fifth counting site as prompted by the program. These measured thicknesses are then averaged across the counted histological sections to give a ‘mean measured thickness value.’ ‘Counting sites’ that do not contain marked objects are not included in this estimate.
Figure 2.7. *Delphinapterus leucas*, three variables of the spiral ganglion plotted against their cochlear position. X-axis, in percent of its length. (A) Cross-sectional areas of Rosenthal’s canal based on CT data as analyzed by AMIRA; (B) Neuron numbers of the spiral ganglion, as determined for each cochlear segment based on counting multiple (usually five) histological sections; (C) Neuron densities of the spiral ganglion, calculated based on the data in B with cross-sectional areas of the histological sections that yielded those data. Specimen numbers for belugas omit the acronym LDL (see materials and methods for numbering convention).
This estimation process yields an approximation for the total number of neurons over the
subsegment of the spiral ganglion segment studied. Usually each subsegment is $175\mu m$ in length
(when every fifth section is counted). We apply the Abercrombie correction as described by
Konigsmark (1970) to avoid error due to cell splitting, following procedures used before for
other cetaceans (Wever et al. 1971c and 1972). This gives us a final neuron number estimate for
that sub-segment.

To standardize neuron estimates, we divided this number by subsegment length ($175 \mu m$
in the above example). As such, we assume that the neurons per $\mu m$ in each subsegment of the
cochlea to be representative of the segment. We plot these estimates (neurons per $\mu m$) against
the midpoint of each cochlear segment from base to apex (Fig. 2.7B). For example, if a cochlear
segment was taken from 50 to 70% of cochlear length, its data point was plotted at 60%. Figure
2.7B thus shows estimates of neuron counts per cochlear segment and is not affected by cross-
sectional area of Rosenthal’s canal.

To estimate neuron density along the length of the cochlea, we take the estimated neuron
counts within specific subsegments (as described above) and divide them by the total volume
($\mu m^3$) of that subsegment. Volumes are estimated in StereoInvestigator using the area of
Rosenthal’s canal, which is outlined by the operator, and the length of the subsegment. We plot
these density estimates for each segment versus percent length along the cochlea from basal to
apical (Fig. 2.7C).

Finally, we estimate the total neuronal count for the entire spiral ganglion based on
calculated neuron count per linear micrometer for each subsegment. These neuron counts have
to be multiplied by segment length to yield an estimate of a total neuron count per segment. We
determined segment length based on measurements of the 3D-reconstructed CT-scans. Cell
counts are based on histological images whereas the segment map is based on CT-scans before decalcification. Therefore, tissue shrinkage has to be taken into account in order for the total number of ganglion cells to be estimated. For one specimen (2013LDL15F, the left cochlea of a full-term fetus), we measure spiral ganglion segment length of the ten excised preserved dissection specimens before and after histological processing. Post processing segment length is on average 88% (0.88) of its preprocessed length, with a standard deviation of 0.05. Using this, we estimate the post-processing length of each spiral ganglion segment by multiplying segment lengths before decalcification by 0.88. This yields the length of the post-processing segment. We multiply the number of neurons per µm within that segment (found above) by the post-processed segment length to estimate the total number of neurons per segment. We then add all segment estimates together to obtain an overall neuron count for Rosenthal’s canal.

RESULTS

Cochlear Morphometrics

We measured the length of the basilar membrane at the inner secondary spiral lamina, as well as Rosenthal’s canal using the reconstructed 3-D images in Amira. We determined that the cochlea of a beluga has 1.75 to 2 cochlear whorls, whereas Rosenthal’s canal is shorter: between 1.5 and 1.75 whorls. The mean length of Rosenthal’s canal is 37.2 mm (SD = 2.1 mm; n = 10, 2010LDL8 Left, 2010LDL8 Right, 2010LDL12 Right, 2010LDL11 Left, 2012LDL5 Right, 2012LDL9 Left, 2012LDL4 Right, 2012LDL7 Left, 2012LDL4 Left, and 2013LDL7 Left; Table 1). As would be expected, this is significantly shorter than the length of the basilar membrane (e.g. 2010LDL7 Left: 47.3mm).
Rosenthal’s Canal

Figure 2.7A shows the variation in Rosenthal’s canal cross-sectional area along its length. The cross-sectional surface area of Rosenthal’s canal changes significantly and non-linearly over the length of the cochlea with the largest areas found in the middle segment of the canal (Quadratic regression: $F_{2,77} = 40.022, p < 0.001$). Analysis of variance for percent length and individual ears further supports the interpretation of curvilinearity, and indicates that the cross-sectional area of Rosenthal’s canal changes significantly along its length within individual ears (two-way ANOVA: $F_{47,25} = 11.54, p < 0.001$). There are also significant differences among individual ears (two-way ANOVA: $F_{7,25} = 13.93, p < 0.001$) regardless of the position along the cochlea.

Neuron Numbers of the Spiral Ganglion

Based on three specimens (2010LDL11 Left, 2012LDL7 Left, 2012LDL9 Left), we estimate that the mean total number of neurons in Rosenthal’s canal of beluga whales is 234,504 (SD=18,416). We also study distribution of neurons along the length of the cochlea, expressing ‘length’ as a percent along the cochlear spiral, shown as the x-axis in Figure 2.7. We assess neural numbers in two different ways, as simple counts and as densities (Fig. 2.7B, C). We tested linear and quadratic models and found that, for simple counts, the quadratic regression $y = -0.001 \times \text{length}^2 + 0.091 \times \text{length} + 5.261$ shows a better fit than a linear model based on comparisons using AIC (Akaike Information Criterion), as a measure of relative quality of models for a given dataset, although it explains only a small fraction of the total variation in neuron counts (Quadratic regression: $F_{2,60} = 3.51, p = 0.036, R^2 = 0.11$). This indicates that the overall number of neurons per linear µm along the length of the cochlea more closely follows a
curvilinear pattern than a straight line (Fig. 2.7B). Standardized regression coefficients indicate that both length and length$^2$ are weighted more than individuals, meaning that no single individual is driving the observed trend in neuron counts.

Figure 2.7C shows the variation of neuron density calculated by dividing neuron count by volume (as determined in StereoInvestigator). Neuron density here is defined as the number of neurons of a segment of the spiral ganglion divided by Rosenthal’s canal volume for that segment (as determined from the histological sections).

Histology of the Spiral Ganglion

Figure 2.6 shows representative histological sections of the spiral ganglion throughout the length of the cochlea of several individuals. Histological fixes and stains provide clear images, and neurons are easily counted. Neurons and their nuclei are easily visible as large cells, surrounded by small nuclei of accessory cells, and there are no features related to necrosis. Our method allows for the study of full or near cross-sections of the cochlea, in all regions. Neurons are distributed more or less evenly throughout each of the Rosenthal’s canal sections, although there are fewer cell bodies peripherally, near the habenula perforata. This is the area where the intraganglionic spiral bundle is located.

DISCUSSION

Cochlear morphometrics

Ketten (1984, 1991) distinguished two morphological types of odontocete cochleas: Type I cochleas are more-or-less flat and have fewer than 2 full whorls, while Type II cochleas are more cone shaped and have more than 2 full whorls. Wartzok and Ketten (1999) linked these
to frequency specializations, stating that Type I species have peak hearing spectra above 100 kHz, and Type II have spectra below 80 kHz. The cochlea of the beluga (Fig. 2.2) is a loose spiral with less than two whorls that do not overlap, a shape characteristic of most non-delphinid odontocetes (Fleischer 1976, Ketten 1984). Beluga auditory evoked potentials suggest optimum sensitivity between 30 and 100 kHz (Klishin et al. 2000, Castellote et al. 2014). Thus, belugas are morphologically similar to Type I species, but functionally to Type II species, showing that intermediates between the Type I and Type II extremes occur.

Wever (1971b and 1972) calculated basilar membrane length for several delphinids based on sectioned specimens, reconstructing them using Guild’s method (1921). Ketten (1984, 1992) reported basilar membrane length for several cetaceans as “length of scalae” (Table 8 of Ketten 1984). Ketten (1984, 1992) proposed that the length of the scalae and basilar membrane increase proportionately with body size in cetaceans. Our data are consistent with that hypothesis (Table 2.2).

*Rosenthal’s Canal*

Assessment of the size of Rosenthal’s canal generally takes place in conjunction with studies that count spiral ganglion neurons in order to study neuron densities (Wicke and Firbas 1970, Keithley and Feldman 1979, Schuknecht, 1993, Dazert *et al.* 1996, Ruijven *et al.* 2004, Shepherd *et al.* 2005, Agterberg *et al.* 2010). In our view, the number of neurons that serves a segment of the cochlear spiral is more important for hearing than how densely those neurons are packed. Johnson *et al.* (2011) showed that larger canal cross-sections match areas of higher neuron numbers in mice. This implies that two areas that have similar neuron densities may actually have very different neuron counts, and that studying densities would hide the spike in
<table>
<thead>
<tr>
<th>Species</th>
<th>Number of whorls (turns)</th>
<th>Basilar membrane length (mm)</th>
<th>Estimated total neuron number</th>
<th>Author(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Delphinapterus leucas</em></td>
<td>1.75-2</td>
<td>47.3</td>
<td>234504</td>
<td>This study</td>
</tr>
<tr>
<td><em>Monodon monoceros</em></td>
<td>2-2.25</td>
<td>-</td>
<td>-</td>
<td>1,2</td>
</tr>
<tr>
<td><em>Globicephala melaena</em></td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td><em>Delphinus delphis</em></td>
<td>2.5</td>
<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td><em>Lagenorhynchus obliquidens</em></td>
<td>1.75-2</td>
<td>29.5</td>
<td>50412</td>
<td>5</td>
</tr>
<tr>
<td><em>Lagenorhynchus albirostris</em></td>
<td>2.25</td>
<td>34.9</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td><em>Stenella attenuata</em></td>
<td>2.5</td>
<td>36.9</td>
<td>82506</td>
<td>4</td>
</tr>
<tr>
<td><em>Stenella coeruleoalba</em></td>
<td>2.5</td>
<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td><em>Stenella longirostris</em></td>
<td>2.5</td>
<td>-</td>
<td>-</td>
<td>4</td>
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<tr>
<td><em>Tursiops truncatus</em></td>
<td>2-2.25</td>
<td>38.5</td>
<td>95004</td>
<td>6</td>
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<tr>
<td><em>Phocoena phocoena</em></td>
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<td>25.9</td>
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<td><em>Physeter catodon</em></td>
<td>1.75-2</td>
<td>54</td>
<td>-</td>
<td>1 (number of whorls); 7 (basilar membrane)</td>
</tr>
<tr>
<td><em>Inia geoffrensis</em></td>
<td>1.5</td>
<td>38</td>
<td>-</td>
<td>7</td>
</tr>
</tbody>
</table>

*Table 2.2* Species, number of cochlear whorls, basilar membrane length (mm) and estimated spiral ganglion neuron number for several odontocete species. *Delphinapterus* data is from this study. (1) Hyrtl 1845; (2) Pilleri *et al.* 1987; (3) Reysenbach de Haan 1957; (4) Ketten 1984; (5) Wever *et al.* 1972; (6) Wever *et al.* 1971a, b, c; (7) Ketten 1992.
neuron counts. We tested this for beluga (Fig. 2.7A). Consistent with the observations for rodents (Johnson et al. 2011, Richter et al. 2011), our results indicate that the cross-sectional area of Rosenthal’s canal of belugas varies along its length. This variation follows a similar path of increase and decrease in different individuals. Visually (Fig. 2.7A), it appears that cross-sectional area of Rosenthal’s canal peaks near 20% to 70% of the length of the cochlea. In addition, the absolute cross-sectional area at any point along the length of the cochlea varies greatly between individuals, in some regions by as much as 50%.

**Neuron Numbers of the Spiral Ganglion**

Table 2.2 compares estimates of total ganglion cell populations (neuron counts) for several odontocete species and uses only published data with well-documented estimation methods. Figure 2.8A shows that, in general, spiral ganglion number increases with body size, with the important exception that the Pacific white-sided dolphin, *Lagenorhynchus obliquidens*, has far fewer neurons than expected for its size. Consistent with its low neuron counts, *L. obliquidens* also has the shortest basilar membrane among the included delphinids (*Tursiops* and *Stenella*, Fig. 2.8B). Belugas also have relatively short basilar membranes given their size. It is not clear whether these minor differences are significant with regard to hearing, and it is possible that different estimation methods introduced some spurious effects in these results. Wever et al. (1971c) found that the neuron count of the dolphin *Tursiops truncatus* spiral ganglion is not constant along the length of the cochlea, consistent with recent findings in rodents (Johnson et al. 2011, Richter et al. 2011). For beluga, the data points of Figure 2.7B significantly fit a quadratic curve. Age has been shown to affect neuron counts in other mammals (Schuknecht, 1993, Keithley and Feldman 1979, Richter et al. 2011).
Figure 2.8. Natural logarithm of spiral ganglion counts and basilar membrane length versus body weight for several cetaceans. (A) spiral ganglion counts (B) basilar membrane length. Cetaceans labeled as follows: *Delphinapterus leucas* (D, beluga), *Inia geoffrensis* (I, river dolphin), *Lagenorhynchus albirostris* (La, dolphin), *Lagenorhynchus obliquidens* (Lo, dolphin), *Phocoena phocoena* (Pp, porpoise), *Physeter catodon* (Pc, sperm whale), *Stenella attenuata* (S, dolphin), *Tursiops truncatus* (T, dolphin). See Table 2.2 for weight data (from Jefferson et al. 2008) plotted against two cochlear variables.
The two youngest individuals in our sample (based on their grey-white skin color; Jefferson et al. 2008) are 2012LDL4 and 2012LDL7, whereas 2010LDL21 is white and therefore older (Brodie et al. 2013). Data points for the two young individuals are low within the data envelop of Figure 2.7B, whereas the older whale is high in that distribution, the opposite of what would be expected if age induced hearing loss was present.

Given that Figure 2.7A is similar in shape, we suggest that cross-sectional diameter of Rosenthal’s canal and cell counts are correlated. In Figure 2.9, we plotted spiral ganglion numbers against mean histological cross-sectional areas of Rosenthal’s canal within the same subsegment, using the same data collected for Figure 2.7B and C. This figure shows that there is a clear correlation between Rosenthal’s canal cross-sectional area, and number of neurons. This would mean that density (the quotient of neuron number and cross-sectional area) does not capture functional differences in variation of neuron numbers serving different segments of the cochlea. To evaluate this effect, we calculated neuron densities (Fig. 2.7C). Density estimates are useful when comparing similar areas of the cochlea among comparable animals (such as animals of the same age with different levels of hearing damage), but we are interested in variability along the cochlea of individuals. The variation in Rosenthal’s canal cross-sectional area may introduce a significant effect.

Histology of the Spiral Ganglion

Damage to the spiral ganglion can be the result of acoustic trauma: exposure of hair cells to high intensity sounds (Schuknecht 1993, Hurley et al. 2007, Henderson et al. 2008). When such exposure takes place in a limited frequency range, damage to the hair cells and associated spiral ganglion cells is often limited to a specific part of the cochlea, and neuron densities in this
Figure 2.9. *Delphinapterus leucas*, number of ganglion cells in segments of the spiral ganglion against mean histological cross-sectional area of that segment. Number of ganglion cells in segments (x-axis), mean histological cross-sectional area (µm$^2$) of that segment (y-axis). Neuron number data from Figure 7B, area data as determined by StereoInvestigator.
area will be lower than in adjacent, healthy areas. Histologically, we observed no areas of low spiral ganglion cells (Fig. 2.6), and this supports the qualitative findings on the cell counts (Fig. 2.7B). This is consistent with the interpretation that, using our method, no spiral ganglion damage in this group of belugas can be detected.

CONCLUSIONS

We studied a sample of wild-caught, healthy beluga whales, and gathered baseline information on cochlea and spiral ganglion. Beluga cochlear shape is similar to that of other odontocetes, but does not fit well in the traditionally distinguished categories of odontocete cochleas as defined by Ketten (1984) and Wartzok and Ketten (1999). We studied Rosenthal’s canal cross-sectional areas and found these to vary along the cochlea, having two local maxima. Similarly, spiral ganglion neuron counts vary in different segments of the cochlea. We studied simple neuron counts, as well as densities (counts/cross-sectional areas). In our opinion, studying simple neuron counts along with cross-sectional areas of Rosenthal’s canal captures some aspects of functional variation of the spiral ganglion along the cochlea, whereas neuron density measures do not in this cetacean.

Histological assessment and neuron cell counts suggest that there is no evidence for auditory damage to the spiral ganglion of these beluga ears.
Chapter III

SPIRAL GANGLION AND ROSENTHAL’S CANAL IN A LOW-FREQUENCY BALEEN WHALE: THE BOWHEAD WHALE

ABSTRACT

We conducted a study of the spiral ganglion and Rosenthal’s canal of the cochlea in bowhead whale (Balaena mysticetus) as an initial assessment of auditory anatomy and health. We present morphometric data on number of cochlear whorls, neuron densities along the length of the spiral ganglion, and Rosenthal’s canal length and cross-sectional area. We also show some histological results. In bowheads, Rosenthal’s canal cross-sectional area varies along its length from base to apex, reducing in diameter near the apex. We did not find systematic variation in the numbers of neurons throughout the canal. Two of the ears studied showed potential signs of hearing damage due to an explosion, both in histology and reduced cell count in the upper basal and apical regions of the cochlea. These results provide data on functionally important structural parameters of the bowhead ear and indicate the need for further investigations into the hearing health of these animals.
INTRODUCTION

In general, odontocete cetaceans (toothed whales) use their ears to receive echoes from high-frequency echolocating signals that they emit, whereas their sistergroup, mysticete cetaceans (baleen whales), use their ears to receive low frequency sounds that are often used for intraspecific communication (Edds-Walton 1997). Both of these cetacean suborders are derived from an ancestor that had relatively unspecialized ears (Mourlam and Orliac 2017). Mysticetes are difficult to impossible to keep in captivity, and physiological experiments with wild individuals are technically nearly impossible. As a result, a number of authors have studied the inner ear of baleen whale morphologically (Norris and Leatherwood 1981, Ketten 1992, Ekdale and Racicot 2014), and some have attempted to correlate specific morphologies to low frequency sound reception (Ketten 1991, Wartzok and Ketten, 1999, Parks et al. 2007, Ekdale and Racicot 2014).

Bowhead whales are baleen whales that spend their entire lives near the edge of the sea ice in arctic and subarctic waters, and Ketten (1994) estimated the hearing range to be approximately between 0 and 35kHz based on morphometric and stiffness estimates of the basilar membrane. Parks et al. (2007), estimated a hearing range of 10Hz to 22kHz for the north atlantic right whale (*Eubalaena glacialis*), which is closely related to bowhead (Würsig et al. 2017), based on measurements of the basilar membrane pitch, dimensions, and basal turn ratio. Such estimates, in general, have been consistent with the frequencies that bowheads produce (Ljungblad et al. 1982, Clark and Johnson 1984, Clark et al. 1986; Cummings and Holliday 1987, Würsig and Clark 1993, Stafford 2008, Clark 2015).

Increased human activities in the polar seas have increased noise pollution and may significantly alter the auditory landscape of the ocean (Hildebrand 2009, Stafford 2013, Weilgart
In order to monitor the impact of these activities on future bowhead populations, it is important that detailed descriptions of the functional parts of the bowhead ear are made.

In general, it is difficult to amass large baleen whale samples that preserve soft anatomical organs. However, bowhead whales are an exception to this rule, because, each year, a small number of bowheads is captured by Alaskan natives (Inupiat eskimos), as part of an indigenous exemption to the endangered species act. In this paper, we undertake a study of inner ear morphology that is similar to our earlier work on beluga whales (Sensor et al. 2015). We focus on the spiral ganglion (the first afferent ganglion for sound), and Rosenthal’s canal (the canal in which the spiral ganglion is housed). Of particular interest is the distribution of neurons in the ganglion. This distribution relates to frequencies perceived, and records evidence of past acoustic trauma (Lurie et al. 1944, Fredelius 1988, Fredelius et al. 1988, Kujawa and Liberman 2006, Kujawa and Liberman 2009), since areas where neurons have died can be identified morphologically.

The process of cell death after acoustic trauma is well documented. Whereas initial overstimulation of the hair cells of the organ of Corti may damage these cells permanently, the damage to the spiral afferent ganglion cells related to these hair cells is not immediate. Instead, such ganglion cells die later. This lag period may last between a few days, and a few weeks of the sound exposure allowing us to document hearing loss that occurred at previous point(s) in time. Additionally, Kujawa and Liberman (2009) have shown in mice that although hair cells may repair after sound exposure, delayed degeneration of the cochlear nerve may still take place.
MATERIALS AND METHODS

Sensor et al. (2015) designed a procedure to study spiral ganglion morphology in beluga whales and we follow this method with minor modifications. The bowhead cochlea differs from that of belugas in having more whorls, being more tightly wound, and being approximately twice as large, but the essence of the method used is similar. We summarize it here.

Specimen Acquisition

Bowhead whales are harvested in Spring and Fall by Inupiat Alaskan natives in Barrow, Alaska, when the whales migrate between the Bering and Beaufort Sea (George et al. 2004, George 2009), as permitted under the Endangered Species Act and the Marine Mammal Protection Act as part of native Alaskan subsistence hunting. Periotic bones are collected by us in collaboration with scientists from the Department of Wildlife Management of the North Slope Borough, Barrow, Alaska (NSB-DWM) under NOAA-NMFS permit # 814-1899-03. Specimens are catalogued in the collection of the North Slope Borough, Department of Wildlife management (NSB-DWM). Specimen numbers indicate year caught (e.g. 2012), a locality (Barrow), rank order number (e.g. 15), and side of the body (“L” indicating left) yielding 2012B15L. Periotic bones are extracted from the heads of the whales using hand tools and are immediately immersed in 2.5% glutaraldehyde, within 24 hours of the death of the individual. Specimens are fixed for at least 3 weeks before processing.

MicroCT Scanning

We use a Scanco Medical vivaCT-75 scanner to scan each periotic at 70 kVp, 114 μA, 200-ms integration time, and voxel size of 41μm. Using Amira 6.1.1 we produce 3D
reconstructions of the bony labyrinth cochlea and Rosenthal’s canal (Fig. 3.1, 3.2). These reconstructions are used to guide subsequent dissections and to acquire metric data for each individual whale. This allows us to express positions along the length of Rosenthal’s canal as a percentage of total length and determine cross sectional area measurements of Rosenthal’s canal in various regions (Fig. 3.3). The reconstructions are also used to determine cochlear length, and number of turns (whorls) found in the cochlea and Rosenthal’s canal. Basilar membrane length is measured at the primary spiral lamina. These values are more accurate than similar measurements on microscopic preparations that may have shrunk considerably due to histological processing.

*Spiral Ganglion Dissection and Histology*

Dissection methods are adapted from human autopsy techniques (Johnsson and Hawkins 1975). After fixation, we flush a histological stain (usually 0.1% thionin or 3% OsO₄) through the scalae via the round and oval windows after removal of the stapes. This allows for visualization of the bony labyrinth during subsequent processing. The promontorium of bowhead whales is considerably thicker and more variable morphologically than that of belugas. Some excess bone is removed using a Foredom microdrill, taking care not to damage the stained scalae. Specimens are then immersed in 10% EDTA (pH 7.5) for three to six weeks for decalcification, where progress of decalcification is tested occasionally using CT.

Dissection takes place under a dissection microscope with camera, using iris scissors, scalpels, and forceps to remove the decalcified bone and expose the scalae. Dissection commences at the apex of the cochlea, and triangular, pie-shaped slices that include Rosenthal’s canal, organ of Corti and stria are removed and mapped on a 3D reconstruction for the specimen.
Figure 3.1. *Balaena mysticetus* boney anatomy and microCT scan. (A) Representative midmodiolar cut of a dry boney labyrinth of bowhead whale 2014B17R. (B) Representative modiolar microCT scan of bowhead whale 2012B6R. (C) Enlarged view of Rosenthal’s canal in the basal whorl of this section.
Figure 3.2. *Balaena mysticetus* AMIRA3-D reconstructions of cochlea and Rosenthal’s canal. Based on microCT scans, the bony labyrinth in pink and Rosenthal’s canal in blue. Apical view (A, B, C, D), other representative views (E, F, G, H). Specimens used: 2012B15L (A,B,E,F), 2012B17R (C,D,G,H). (I) AMIRA 3-D reconstruction of cochlea of 2012B17R, cut virtually to show how cross-sectional areas of Rosenthal’s canal were determined. Scale bar below H is for views A-H. Scale bar next to I is for view I.
Figure 3.3. *Balaena mysticetus*, three variables of the spiral ganglion plotted against their position along the cochlea. X-axis in percent of cochlear length. (A) Cross-sectional areas of Rosenthal’s canal based on CT data as analyzed by AMIRA. (B) Neuron numbers of the spiral ganglion, as determined for each cochlear segment based on counting multiple (usually five) histological sections. (C) Neuron densities of the spiral ganglion, calculated based on the data in B with the volume of the region in which the cell counts were taken.
This produces a segment map (Fig. 3.4). Individual segments then are processed histologically; they pass through dehydration series, are embedding in paraffin, and cut at 7 μm at right angles to the long axis of the ganglion, creating cross-sections. Sections are stained with hematoxylin and eosin. No thionin remains at this stage, but some spiral ganglia retain their OsO₄ staining.

**Stereology**

We use StereoInvestigator software (MicroBrightField, Williston, VT, USA, version 11) to estimate the number of neurons (Including both Type I and Type II neurons) in each segment of spiral ganglion, and use that estimate to assess neuron density of each cochlear segment.

Within a section, Rosenthal’s canal is traced and all cells within it are counted using a computer assisted fractionator sampling scheme to count the nuclei. Neurons are counted at 40x magnification when the nucleus (average diameter of 9.18 μm) is in focus, using a Counting Frame Width (X and Y) of 150μm and a Sampling Grid (X and Y) of 150μm. We do not use guard zones or the optical dissector of StereoInvestigator because our sections are thin (7μm). We counted every fifth section, or occasionally, every fourth or sixth section if the fifth section was damaged during processing. Each data point is composed of data from a total of five sections (Fig. 3.2B). In a few cases, fewer than five sections were preserved sufficiently well to be counted, the least number of slices counted per segment was 3. We use the ‘Estimated Population using Mean Section Thickness from Sites with Counts’ of StereoInvestigator to estimate neuron numbers for the portion of the dissected segment that was counted. We call this a “subsegment.” This estimation process yields an approximate total number of neurons over the subsegment of the spiral ganglion studied. Usually each subsegment is 175μm in length (when every fifth section is counted). We apply the Abercrombie correction as described by
Figure 3.4. *Balaena mysticetus* cochlear segment map of 2012B18R. Map is based on AMIRA 3D reconstruction of bony labyrinth. The size and location of Rosenthal’s canal samples that were dissected out of this specimen are indicated by numbers. Each of these samples is a segment of Rosenthal’s canal that is histologically processed and counted. H- Helicotrema.
Konigsmark (1970) to avoid error due to cell splitting, following procedures used before for other cetaceans (Wever et al. 1971c and 1972, Parks et al. 2007). This gives us a final neuron number estimate for that sub-segment. To standardize neuron estimates, we divided this number by subsegment length (175 µm in the above example). As such, we assume that the neurons per µm in each subsegment of the cochlea to be representative of the entire segment.

We plot these estimates (neurons per µm) against the midpoint of each cochlear segment from base to apex (Figure 3.3B). For example, if a cochlear segment covered the area from 30 to 40% of cochlear length, its data point is plotted at 35%.

To estimate neuron density along the length of the cochlea, we divided estimated neuron counts of a subsegment by the total volume (µm$^3$) of that subsegment. Volumes are estimated in StereoInvestigator using the area of Rosenthal’s canal of the pertinent histological sections. We plot these density estimates for each segment against percent length of the cochlea (Fig. 3.3C).

Finally, we estimate the total neuronal count for the entire spiral ganglion based on calculated neuron counts per linear micrometer for each subsegment. These neuron counts are multiplied by segment length to yield an estimate of a total neuron count per segment. We determined segment length based on measurements of the 3D-reconstructed CT-scans.

RESULTS

Cochlear Morphometrics

**Rosenthal’s Canal**

Figure 3.3A displays results for cross-sectional area of Rosenthal’s canal over its percent length. For analysis, Rosenthal’s canal was split into sections that related to each quarter turn of Rosenthal’s canal. (1 = 0%-23%, 2 = 24%-70%, 3 = 70%-89%, 4 = 90%-100%). Mixed model analysis (fixed factor = each quarter whorl, random factor = ear) of each quarter whorl with Tukey’s post-hoc test ($p < 0.05$) indicated Rosenthal’s canal cross-sectional area changes significantly within the upper portion of the apical whorl (above 90% percent of the total length $F_{3, 22.487} = 5.547, p < 0.05$). There was no effect between region of Rosenthal’s canal and individual ear, meaning this trend was found regardless of specimen ($F_{21,40} = 2.914, p < 0.05$). Ears differed significantly from one another ($F_{8,23.504} = 3.274, p < 0.05$), but this result does not appear to be driven by any individual.

**Neuron Numbers of the Spiral Ganglion**

We report neuron numbers as counts and as densities distributed over the length of Rosenthal’s canal. Figure 3.3B displays the number of neurons per linear micrometer for the segments of the spiral ganglion over the length of Rosenthal’s canal. Rosenthal’s canal was again split into four sections that related to each quarter turn of Rosenthal’s canal. Mixed model analysis (fixed factor = each quarter whorl, random factor = ear), indicates mean cell number does not differ significantly between each quarter whorl ($F_{3,36.702} = 1.05, p>0.05$). This trend was again true regardless of ear (no interaction between quarter whorl and specimen; $F_{21,39} = 1.067, p > 0.05$), and ears differed significantly from one another ($F_{9,25.8} = 5.514, p < 0.001$). Upon inspection of the specimens it appears two (2010B15R and 2011B9L) have relatively lower cell counts in the upper portion of the basal whorl and the apical whorl. When these two specimens
are removed from the analysis, ears no longer differ significantly from one another ($F_{7,19,121} = 2.369, p = 0.64$). Figure 3.3C shows neuron density over the length of Rosenthal’s canal.

Based on three specimens (2011B9R, 2012B17R, 2012B18R), we estimate that the mean total numbers of neurons within the spiral ganglion is approximately 152,672 (SD = 45,888). The mean number of cells per linear micrometer is 4.7 (SE = 0.19), and the mean cell density is 1.67E-05 (SE = 9.21E-07).

**Histology of the Spiral Ganglion**

Figure 3.5 shows histological sections from representative regions of the spiral ganglion for 4 ears. Generally, as was found in the beluga (Sensor *et al.* 2015), there are fewer cell bodies in the regions near the modiolus. Additionally, for some specimens, certain regions of the spiral ganglion display dispersed neurons, and glial cells (Fig. 3.6). These regions often coincide with lower cell counts (Fig. 3.3). Some areas also display perikaryal shrinkage, similar to the effects of drug induced hearing damage (Ruijven *et al.* 2004, Kong *et al.* 2010). This is likely due to a delay in fixation. When present, this occurred throughout the cochlea, and could not be isolated to a particular region.

**Discussion**

*Cochlear Morphometrics*

The cochlea of the bowhead is a tight, cone-shaped, equiangular, spiral with 2.25 whorls. This is consistent with what Norris and Leatherwood (1981) and Ketten (1994) have described for
Figure 3.5. *Balaena mysticetus*, spiral ganglion histological sections specific distances from the base. Specimens: 2010B15R at (A) 37%, (B) 81%; 2011B9L at (C) 63%, (D) 97%; 2011B8R (E) 58%, (F) 79%; 2012B17R (G) 52%, (H) 83%. M indicates modiolar side, SL indicates spiral lamina side. Arrowheads indicate regions of perikaryal shrinkage. Scale bar for all images.
Figure 3.6. *Balaena mysticetus*, enlarged histological image of specimen 2010B15R. (A) 37% of the length of the spiral ganglion from the base. (B) 81% of the length of the spiral ganglion from the base.
bowheads as well as what has been described for other mysticetes (Ketten 1992, 1997, Parks 2007, Ekdale and Racicot 2014). Our average basilar membrane length measurements were shorter (43.4 mm), than what has been previously reported (61.3 mm; Norris and Leatherwood 1981). This difference may be due to the data collection method. In their analysis, Norris and Leatherwood extracted the basilar membrane, mounted and photographed the specimen, and measured the length and width on a micrograph. Table 3.1 provides morphometric data on various mysticetes along with estimated total number of spiral ganglion cells. Ketten (1984, 1992) proposed that the length of the scalae and basilar membrane increase proportionately with body size in cetaceans. Generally, this trend is less apparent in mysticetes than it is in odontocetes (Sensor et al. 2015).

Increased curvature of the cochlea, such as what is seen in bowheads, has been shown to enhance sensitivity to low frequency sounds in land and aquatic mammals (Manoussaki et al. 2006, 2008). Low frequency hearing limits in ground dwelling mammals have also been correlated to the product of basilar membrane length and cochlear turns (West 1985). If this is true for whales, it would suggest a low frequency hearing range for bowheads. Thickness to width ratio of the basilar membrane and length of secondary spiral lamina are linked to frequency perception in mammals (Ketten and Wartzok 1990, Ketten 1991, 1994, Wartzok and Ketten 1999). Bowheads have a low thickness to width ratio (Norris and Leatherwood 1981, Ketten 1994) which further supports the hypothesis of lower frequency hearing ranges.
<table>
<thead>
<tr>
<th>Species</th>
<th>Common name</th>
<th>Weight (kg)</th>
<th>Whorls (turns)</th>
<th>Basilar Membrane Length (mm)</th>
<th>Total Number of Spiral Ganglion Cells</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Balaenoptera acutorostrata</em></td>
<td>Minke whale</td>
<td>9,200 (max)</td>
<td>2.25</td>
<td>55</td>
<td>-</td>
<td>1,6</td>
</tr>
<tr>
<td><em>Balaena mysticetus</em></td>
<td>Bowhead</td>
<td>90,000</td>
<td>2.25</td>
<td>41.2</td>
<td>152,672</td>
<td>5,6</td>
</tr>
<tr>
<td><em>Balaenoptera physalus</em></td>
<td>Fin whale</td>
<td>&lt;120,000</td>
<td>2.5</td>
<td>64.7</td>
<td>134,098</td>
<td>4,6</td>
</tr>
<tr>
<td><em>Eubalaena glacialis</em></td>
<td>Right Whale</td>
<td>90,000</td>
<td>2.5</td>
<td>49.5</td>
<td>-</td>
<td>1,6</td>
</tr>
<tr>
<td><em>Magaptera novaeangliae</em></td>
<td>Humpback whale</td>
<td>&gt;40,000</td>
<td>2.5</td>
<td>54</td>
<td>156,374</td>
<td>1,6</td>
</tr>
</tbody>
</table>

*Table 3.1:* Morphometric data and number of neurons in the spiral ganglion for multiple mysticete whales. Data complied from: 1 = Ketten 1992; 2 = Norris and Leatherwood 1981; 3 = Parks et al. 2007; 4 = Wartzok and Ketten 1999; 5 = This study; 6 = Jefferson et al. 2008 (for weight data).
Rosenthal’s Canal

Most studies of Rosenthal’s canal take place in conjunction with studies of spiral ganglion cell density (Wicke and Firbas, 1970; Keithley and Feldman, 1979; Schuknecht, 1993; Dazert et al., 1996; Ruijven et al., 2004; Shepherd et al., 2005; Agterberg et al., 2010). Johnson et al. (2011) showed that, in mice, larger cross-sectional areas of Rosenthal’s canal match areas of higher neuron numbers. This results in similar densities across the cochlea despite increased cell number in certain regions. In order to more specifically investigate fluctuations in cell number and Rosenthal’s canal cross-section along the length of the cochlea, we chose to study cell number, Rosenthal’s canal cross-sectional area and density separately. In our view neuron number is a better indicator of hearing than how densely packed the cells are. Consistent with the observations for rodents (Johnson et al., 2011; Richter et al., 2011), our results indicate that the cross-sectional area of Rosenthal’s canal varies over its length for bowheads, similar to what was shown by Johnson et al. (2011) for mice and in Sensor et al. (2015) for belugas. For all individuals, Rosenthal’s canal decreased in the upper portion of the apical whorl.

Neuron Numbers and Spiral Ganglion

Figure 3.3A shows that overall, spiral ganglion number per linear micrometer does not change significantly over the length of the spiral ganglion. However, it appears that two specimens (2010B15R and 2011B9L) have a reduced count in the upper basal and apical whorl of Rosenthal’s canal relative to other individuals. Significant variation between individual ears was indicated in a mixed model analysis, but when these two specimens were removed the variation was no longer significant.
Age has been shown to affect spiral ganglion counts in other mammals (Schuknecht 1993, Keithley and Feldman 1979, Richter et al. 2011). Although ears 2010B15R and 2011B9L were from older individuals in our sample (Table 3.2), they are relatively young considering bowheads can live to be nearly 200 years old (George 2009). In addition, both left and right ears from whale 2011B9 are represented in the graph. The right ear of this animal displays cell counts that are much higher than the left. Finally, specimen 2013B1, one of the oldest whales in our study (estimated to be 76 years old based on baleen length), displays spiral ganglion counts that appear to fall somewhere in the midrange of the data envelope. It does not display the same pattern of reduced cell number as 2010B15R and 2011B9L. These data conflict with an age related hearing loss hypothesis. Finally, age related hearing loss is known to most seriously affect the basal end of the cochlea where high frequencies are transduced (Keithley and Feldman 1979, Dazert et al. 1996), if the reduction in cell number were due to age related hearing loss, we should see reduced cell count, in the more basilar regions as well.

Figure 3.3C displays spiral ganglion cell density along the length of Rosenthal’s canal. Given that the cross-sectional area of Rosenthal’s canal is shown to change over its length while cell count does not, it appears that cell number and the cross-sectional area of Rosenthal’s canal are not tightly correlated. This is different from what was found in mice (Johnson et al. 2011) and belugas (Sensor et al. 2015). Figure 3.7, displaying cells per linear micrometer for all ears plotted against surface area of Rosenthal’s canal. This figure shows no correlation between Rosenthal’s canal cross-sectional area and number of neurons. This means that cell density estimates along the length of the cochlea may misrepresent neural processing power. Density estimates are useful when comparing similar areas of the cochlea among comparable animals, but we are interested in variability along the cochlea of individuals.
<table>
<thead>
<tr>
<th>Whale ID</th>
<th>Sex</th>
<th>Whale L (m)</th>
<th>Longest baleen (cm)</th>
<th>Whale age based on longest baleen plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>2010B15</td>
<td>F</td>
<td>12.5</td>
<td>267</td>
<td>32</td>
</tr>
<tr>
<td>2010B18</td>
<td>F</td>
<td>9.1</td>
<td>164</td>
<td>6</td>
</tr>
<tr>
<td>2010B20</td>
<td>M</td>
<td>7.8</td>
<td>85</td>
<td>1.5</td>
</tr>
<tr>
<td>2011B3</td>
<td>F</td>
<td>17.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2011B8</td>
<td>F</td>
<td>8.4</td>
<td>131</td>
<td>3</td>
</tr>
<tr>
<td>2011B9</td>
<td>F</td>
<td>12.5</td>
<td>235</td>
<td>22</td>
</tr>
<tr>
<td>2012B15</td>
<td>M</td>
<td>8.4</td>
<td>85</td>
<td>1.5</td>
</tr>
<tr>
<td>2012B17</td>
<td>F</td>
<td>10.8</td>
<td>217</td>
<td>17.7</td>
</tr>
<tr>
<td>2012B18</td>
<td>F</td>
<td>9.4</td>
<td>155</td>
<td>5</td>
</tr>
<tr>
<td>2013B1</td>
<td>F</td>
<td>16.5</td>
<td>342</td>
<td>76</td>
</tr>
</tbody>
</table>

*Table 3.2: Biological data on the bowheads used in this inner ear study. Estimated age based on baleen plate length employs the two-stage von Bertalanffy II model fit by Lubetkin et al. 2012.*
Figure 3.7. *Balanena mysticetus*, number of ganglion cells in segments of the spiral ganglion against mean histological cross-sectional area of that segment. Number of ganglion cells in segments of the spiral ganglion on the x-axis, mean histological cross-sectional area (µm²) of that segment on the y-axis. Neuron number data from Figure 3B, area data as determined by StereoInvestigator and seen in Figure 3A.
Histology of the Spiral Ganglion

Exposure of the cochlea to high intensity sounds may injure hair cells and associated spiral ganglion neurons. If this exposure takes place within a specific frequency range, damage will be limited to only a part of the cochlea. When examining the spiral ganglion this damage will manifest as reduced cell count. Histological images (Fig. 3.5, 3.6) of various regions of the spiral ganglion for multiple bowhead ears show that specific ears have areas of dispersed neurons and glial cells compared to adjacent regions. These areas of dispersed cells, histologically resemble regions of hearing loss in rodents (Keithley and Feldman 1979, Dazert et al. 1996, Shepherd et al. 2005, Agterberg et al. 2008, Agterberg et al. 2010, Perez and Bao 2011). Not surprisingly, these coincide with areas of reduced cell counts for these specific ears. This loss spiral ganglion neurons is consistent with damage due to acoustic insult.

CONCLUSIONS

We studied the inner ear of wild-caught bowhead whales collected over multiple years, and gathered information about the cochlea, spiral ganglion and Rosenthal’s canal. Cochlear shape coincides with what is known for mysticetes. Rosenthal’s canal cross-sectional area, cells per linear micrometer and cell density were measured over the length of Rosenthal’s canal. Rosenthal’s canal cross-sectional area was shown to decrease in size near the apex in all individuals, however, number of spiral ganglion cells was not shown to significantly differ over length. Because of this, we believe that studying neuron counts and cross-sectional area separately may capture more aspects of functional variation than studying cell densities along the length of Rosenthal’s canal alone.
Histological evidence as well as a curious drop in cell count for two of the ears in our study, may suggest hearing damage for some individuals. Further histological assessment, and more samples with similar spiral ganglion distributions may be needed to confirm this hypothesis.
Chapter IV

AGE ESTIMATION IN BOWHEAD WHALES USING TYMPANIC BULLA HISTOLOGY AND BALEEN ISOTOPES

ABSTRACT

Tympanic bullae and baleen plates from bowhead whales of the Western Arctic population were examined. Growth layer groups (GLGs) in the involucrum of the tympanic bone were used to estimate age of the whales, and compared to stable isotope signatures along transects of baleen plates and the involucrum. The involucrum of the tympanic bone consists of three regions that form in utero, during nursing in the first year, and during the first decades of life, respectively. Life history events such as annual migration are recorded in the bowhead tympanic bulla. It is likely that bone growth in the bowhead tympanic occurs during periods of high food intake, while slow or arrested growth occurs during periods of low food intake. Comparisons between numbers of GLGs in the tympanic, number of isotopic oscillations in a baleen plate, length of the baleen plate, and total whale length show correlation coefficients as high as 0.97. The tympanic GLG method is particularly useful for estimating the age of whales up to 20 years old.

INTRODUCTION

Estimating the age of individual bowhead whales (*Balaena mysticetus*) is important in the management of existing populations. Several age estimation methods are available, but only cover limited parts of the bowhead lifespan. Growth models utilize baleen length (Lubetkin *et al.* 2008, 2012), isotope oscillations within baleen plates (Schell *et al.* 1989a, Schell and Saupe 1993), numbers of corpora albicantia in sexually mature females (George *et al.* 2011, Tarpley *et al.* 2016), and enantiomer ratios in the alpha-crystalline of the eye lens (George *et al.* 1999, Rosa *et al.* 2004). A critical age range for which most of these methods are imprecise or inadequate is between 10 to 30 yr.

Whereas growth layer groups (GLGs) in the dentin of odontocete teeth are often easily readable for age estimation (Perrin and Myrick 1980, Hohn 2009), this is not usually the case for GLGs in the bones of whales (Larsen and Kapel 1982, 1983, Olsen 2002, Olsen *et al.* 2003). One exception to this may be the tympanic bullae of baleen whales (Klevezal and Mitchell 1971, Christensen 1981, Sukhovskaya *et al.* 1985, Klevezal *et al.* 1986, Konráðsson and Sigurjónsson 1989, Christensen 1995, Klevezal 1996, Hohn 2009). Similarly, Marmontel *et al.* (1996) used histology of the periotic bone of manatees to estimate age. These authors showed that the number of periotic GLGs correlates well with the age of the animal, with the best accuracy for manatees up to 10-15 years of age.

Here, we investigate the microstructure of the involucrum part of the tympanic bone in a sample of the Western Arctic population of bowhead whales. We focus on growth layer groups (GLGs), as defined by Perrin and Myrick 1980 for marine mammals, in the periosteal region of tympanic bullae and assess their potential to estimate age. In addition, we characterize the
different parts of the involucrum histologically and isotopically. We compare results from the involucrum with data on baleen plate length and number of isotopic oscillations in the baleen.

In the Western Arctic population, baleen stable isotope values record the fall migration of the whales from the Beaufort Sea to the Chukchi and eventually the Bering Sea where they winter (Saupe et al. 1989, Schell et al. 1989a, b, Schell and Saupe 1993). Life history events, such as nursing and weaning are also recorded by stable isotopes. In addition, birth is recorded morphologically in the baleen, since the direction of plate growth changes. This produces a neonatal notch in the labial side of the baleen plate (Zenitani and Kato 2010).

MATERIALS AND METHODS

Inupiat subsistence hunters in Barrow, Alaska, harvest a small number of bowhead whales during the annual migration in Spring and Fall (Suydam and George 2004). Data and samples are gathered by scientists from the Department of Wildlife Management of the North Slope Borough, Barrow, Alaska (NSB-DWM) under NOAA-NMFS permit # 17350-01. We studied tympanic bullae and the longest baleen plate. All specimens (baleen, sectioned slices of the involucrum, and thin sections of the involucrum) will be deposited in the collections of the NSB-DWM.

We cut rostral and caudal slices in the coronal plane of the involucrum of the bulla with an approximate thickness of 3 mm. The involucrum and its periosteal region are thickest in the caudal slices, and this region is the focus of our study (Fig. 4.1A, B). For two specimens (2014B11, 2014B14) a simple bone core was taken from the caudal involucrum, and slices were prepared from it.
Figure 4.1. *Balaena mysticetus*, the tympanic bulla. (A) Tympanic bulla of bowhead whale NSB-DWM 2015B2 with caudal involucrum slice removed. (B) Anterior view of involucrum slice taken from NSB-DWM 2015B2. (C) Microstructure of transect of involucrum from a fetal bowhead NSB-DWM 2014B6F, showing that only region 1 is present. (D) Microstructure of transect of involucrum from bowhead whale NSB-DWM 2012B5, showing regions 1 and 2. This animal is estimated to be one year old based on baleen length and single baleen δ¹³C oscillation. (E) Microstructure of transect of involucrum from bowhead whale NSB-DWM 2013B18 estimated to be about 10 years of age using GLGs. Red arrowheads indicate borders of regions. Two mm scale bar refers to C-E.
Bone slices were embedded in Buehler EpoThin epoxy resin, polished on one side, and mounted (polished side down) with either epoxy or Loctite 349 (a UV curing adhesive). Slices were then ground down to approximately 50 to 100 micrometers thickness and polished. We used 2x and 4x objectives under brightfield lighting with an Olympus BX40 microscope to take photos.

We decalcified one bone slice for one week in formic acid, embedded it in paraffin and stained it with hematoxylin and eosin, following a method modified from Christensen (1981) and Marmontel et al. (1996). Although this method helped to distinguish GLGs close to the periosteal edge, deep GLGs became diffuse and unreadable.

In describing the histology, we use the term ‘growth layer group’ (GLG) defined as “a repeating or semi-repeating pattern of adjacent groups of incremental growth layers” (Perrin and Myrick 1980, p. 49). Incremental growth layer’ is defined as a “discernible layer occurring parallel to the formative surface of a hard tissue (dentine, cementum, bone) which shows any contrast with adjacent layers” (p.49). ‘Accessory layers’ are defined as “any single layered component of a growth layer group which is discernible from adjacent layers in a hard tissue” (p.48). We also occasionally use bone histological terms defined by Huttenlocker et al. (2013) as they are used in the broader field of hard tissue histology (see also Francillon-Vieillot et al. 1990, de Ricqlès et al. 1991, Currey 2003).

For two bullae specimens (NSB-DWM 2012B5, 2013B6), samples were collected for $\delta^{13}$C analysis of structural carbonate within the bone mineral (bioapatite). Approximately 6-9 mg of bone powder was drilled at 1 mm intervals along a transect starting at the center of the involucrum to the ventromedial edge. Since the incremental growth layers in the periosteal region are thin, collection of samples sufficiently large for isotopic analysis could not be
accomplished for all GLGs. Bone samples were taken using Carpenter Microsystems 2 software with a 600 µm drill bit. Sample sites were 5-7 mm long and were 600-1500 µm deep. Approximately 0.6 mg of bone powder from each sample was dissolved in 100% phosphoric acid at 25°C and the resulting CO₂ was analyzed. Stable isotope analysis of bone mineral employed a ThermoFisher GasBench II coupled to a continuous flow ThermoFisher Delta Plus XP Isotope Ratio Mass Spectrometer (IRMS). Repeated analysis of internal lab standards (NIST SRM 1486, NIST SRM 120c) and international standards established an instrument precision (1 σ) of < 0.1‰ for δ¹³C values.

Baleen plate lengths were measured and powdered samples were taken every centimeter from the most proximal end (embedded in the palate) to the most distal (protruding into the oral cavity), along the labial edge using a Foredom microdrill. A number of authors have executed δ¹³C and δ¹⁵N analyses on baleen (Schell et al. 1989a, b, Schell 1992, Withrow et al. 1992, Hobson and Schell 1998, Lee et al. 2005, Lubetkin et al. 2008) and we follow their methods. Dry baleen powder samples were weighed in tin capsules (~1 mg) and combusted using a Costech 4010 Elemental Analyzer coupled to a continuous flow ThermoFisher Delta Plus XP IRMS. Instrument precision (1 σ) was determined as < 0.1‰ for δ¹³C and δ¹⁵N values through repeated measurement of internal lab standards (acetanilide, glutamic acid, keratin) and international standards. All δ¹³C and δ¹⁵N isotope values are reported relative to VPDB (Vienna Pee Dee Belemnite) scale and AIR scale respectively, in standard delta notation: δ¹³C or δ¹⁵N (‰) = (R_sample/R_standard - 1) X 1000 where R is the ratio $^{13}$C/$^{12}$C or $^{15}$N/$^{14}$N.

Counting the number of annual isotopic oscillations along a baleen plate with a neonatal notch provides a method of age estimation. In older animals that lack a neonatal notch, however,
counting annual isotopic oscillations provides only a minimum age estimate, since part of the baleen has worn off.

Baleen plate length correlates well with age for certain age ranges of male and female bowheads (Lubetkin et al. 2008, 2012). Lubetkin et al. (2012) fit a sex-specific von Bertalanffy II growth model with a juvenile growth spurt which yielded the predictive model \( A = c_0 + c_1 \ln \left\{ \frac{c_2}{c_3 - L} \right\} \). Where \( A \) is age, \( L \) is the (longest) baleen plate length, and the estimated parameters \((c_0-c_3)\) are given in Table 4.1. We estimated relationships between the number of GLGs and baleen length, estimated whale age, and number of stable \( \delta^{13} \)C isotope cycles using simple linear regression. A log-log fit was used when appropriate to account for nonlinearity and/or to stabilize variance.

RESULTS

Tympanic Bone Structure

Three roughly concentric regions can be distinguished on a slice of the tympanic bulla (Fig. 4.1B-E), referred to as regions 1 through 3 (Fig. 4.1B-E). Region 1 covers most of the cross-sectional area. Region 2 is external to region 1 on all sides, and is thickest on the ventromedial side of the involucrum. Region 3 is a narrow band along the periosteal surface, external to region 2. On the ventromedial side of the slice, the bulla forms a gnarled crest with many infoldings. In this area, region 3 is thicker than anywhere else and displays the best developed GLGs.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Small Bowhead Longest Plate ≤ 1.81 m</th>
<th>Large Male Bowhead Longest Plate &gt; 1.81 m</th>
<th>Large Female Bowhead Longest Plate &gt; 1.81m</th>
</tr>
</thead>
<tbody>
<tr>
<td>c₀</td>
<td>0</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>c₁</td>
<td>3.1075</td>
<td>26.4135</td>
<td>37.3516</td>
</tr>
<tr>
<td>c₂</td>
<td>166.56</td>
<td>136.5578</td>
<td>193.1078</td>
</tr>
<tr>
<td>c₃</td>
<td>187.82</td>
<td>317.71</td>
<td>374.26</td>
</tr>
</tbody>
</table>

Table 4.1. Estimated parameters for the von Bertalanffy II growth model of Lubetkin et al. (2012).
On the cut slice, region 1 is the darkest in color. On thin-section in coronal view, it consists primarily of woven bone with a largely reticular pattern of vasculature (fibrolamellar bone; Fig. 4.1C-E). Vessels are oriented predominantly dorso-medial in postnatal individuals (Fig. 4.1D-E). Primary osteons occur in region 1, and this region lacks Haversian systems proper, with no cement line or evidence of resorption around the osteons. Some specimens, in addition to their reticular vasculature, have radial vessels that cross the interface between regions 1 and 2 (NSB-DWM 2012B5, 2013B6, 13B15, 2014B11, 2014B14, and 2015B24). Additionally, specimen NSB-DWM 2015B2 has some radial vessels throughout region 1. Region 1 is the only region present in the tympanic bone of a fetus, and is spongy in appearance, being poorly mineralized (Fig. 4.1C; NSB-DWM 20146F).

On a cut slide, region 2 is white to yellow in color (Fig. 4.1B). It consists predominately of primary woven bone with reticular and radial vasculature (fibrolamellar bone). Most of the longer vessels extend parallel to each other and curve in a ventro-medial direction, as opposed to the general dorso-medial direction of vessels in region 1. Primary osteons occur within region 2, but as in region 1, there is no evidence of remodeling.

Region 3 consists of incremental growth layers that constitute Growth Layer Groups (GLGs). These are subsequent dark and light/translucent layers (Fig. 4.2-4.7). Accessory layers may occur within a GLG (white boxes in Fig. 4.2, 4.3, 4.5, 4.6; NSB-DWM 2013B1, 2013B6, 2013B18, 2014B14, 2015B16, and 2015B24). These are dissimilar from one GLG to another, and are not cyclic.

The dark incremental growth layers of a GLG are thicker than the translucent growth layers, except near the periosteal surface in some older individuals, when total GLG thickness decreases. Histologically, growth layers may consist of slow growing lamellar bone.
Figure 4.2. *Balaena mysticetus*, baleen isotope signatures and tympanic GLGs for multiple whales. (A, C, E, G, I) $\delta^{13}C$ (black) and $\delta^{15}N$ (green) values from baleen keratin taken along the total length of the plate from distal (left) to proximal (right). Yellow zones within the plots indicate isotope values of prenatally formed baleen (as indicated by the neonatal notch), white indicates the nursing period, blue indicates post-weaning period. (B, D, F, H, J) Details of region 2 and 3 microstructure of tympanic bullae from A, C, E, G, I, respectively. Yellow arrowheads in histological images indicate boundaries between GLGs, 2 mm scale bar refers to all these images. Boxes in D refer to insets and show accessory layers, 1 mm scale bar refers to insets only (A, B) NSB-DWM 2012B5. (C, D) NSB-DWM 2013B6. (E, F) NSB-DWM 2013B15. (G, H) NSB-DWM 2013B19, NSB-DWM 2013B5.
Figure 4.3. *Balaena mysticetus*, baleen isotope signature and tympanic GLGs for whale NSB-DWM 2013B18. (A) δ¹³C values of baleen keratin covering the entire length of the plate from distal (left) to proximal (right), this baleen only preserves a record of the independent feeding period. (B) Region 3 microstructure of tympanic bulla of the same whale. Yellow arrowheads indicate boundaries between GLGs. A white box indicates presence of accessory layers.
Figure 4.4. *Balaena mysticetus*, baleen isotope signature and tympanic GLGs of whale NSB-DWM 2011B9. (A) δ¹³C values from baleen keratin along the entire length of the plate from distal (left) to proximal (right), this baleen only preserves a record of the independent feeding period. (B) Region 3 microstructure of tympanic bullae of the same whale. Blue arrowheads mark the boundary of the first and last GLG as well as every 5ᵗʰ GLG.
Figure 4.5. *Balaena mysticetus*, baleen isotope signature and tympanic GLGs of whale NSB-DWM 2013B1. (A) Region 3 microstructure of the tympanic bone. Yellow box is enlarged in B. (B) Enlarged view of GLGs magnified from A, yellow arrowheads indicate boundaries between GLGs, and white box marks area of accessory lines. (C) $\delta^{13}$C values of baleen keratin along the entire length of the plate from distal (left) to proximal (right) of this whale.
Figure 4.6. *Balaena mysticetus*, region 3 microstructure of six tympanic bone slices under brightfield magnification. (A) NSB-DWM 2014B11. (B) NSB-DWM 2015B2. (C) NSB-DWM 2014B14. (D) NSB-DWM 2015B24. (E) NSB-DWM 2015B16. (F) NSB-DWM 2015B17. Yellow arrowheads indicate boundaries between GLGs, and blue arrowheads are placed on the boundary of the first and last GLG and every 5th GLG. A white box marks accessory lines.
Figure 4.7. *Balaena mysticetus*, region 3 of two whale specimens displaying GLG variability. (A) Low magnification image of region 3 of the tympanic bone of NSB-DWM 2013B18. GLGs are distinctive on the left side of the image but less so on the right in the black box. (B) Low magnification image of region 3 in NSB-DWM 2013B1. Yellow arrows indicate presence of secondary osteons and areas of resorption.
(Fig. 4.2, 4.4, 4.6; NSB-DWM 2011B9, 2013B5, 2014B11, 2014B14, 2015B16, 2015B17, 2015B19, and 2015B24), woven bone with reticular vasculature (Fig. 4.2, 4.3; NSB-DWM 2013B15 and 2013B18) or a combination of the two (Fig. 4.2, 4.6; NSB-DWM 2013B6 and 2015B2).

Radial and longitudinal vessels can occur in region 3, and these intersect with the GLGs at a variety of angles. Some specimens display only radial vessels (Fig. 4.2, 4.3; NSB-DWM 2013B5, 2013B6, and 2013B18) in the dorsal portion of a tympanic slice, while others display both types of vasculature (Fig. 4.4, 4.6; NSB-DWM 2011B9, 2014B11, 2014B14, 2015B2, 2015B16, 2015B17, and 2015B24). Small secondary osteons occur in some of the older whales (Fig. 4.7; NSB-DWM 2013B1, 2015B2, 2015B17, and 2015B24).

**Stable Isotopes**

Isotopic analyses show that baleen $\delta^{13}$C and $\delta^{15}$N values oscillate along the length of the plate (Fig. 4.2-4.5). The neonatal notch of the baleen plate is a morphological indicator of the timing of birth, and can be used to calibrate isotope signals around birth. Baleen growth in the first year (which includes the nursing period) is approximately 75cm (George 2016). In our specimens, at length between 62 and 92 cm we observed a drop in $\delta^{15}$N values (Fig. 4.2), and this drop is statistically significant for different specimens (NSB-DWM 2013B15: white area of Fig 2E: $1 \sigma = 0.26\%$, blue area: $1 \sigma = 0.69\%$, $F = 6.593$, $P < 0.0001$; NSB-DWM 2013B6: white area of Fig. 1.2B: $1 \sigma = 0.25\%$, blue area: $1 \sigma = 0.49\%$, $F = 3.844$, $P < 0.0001$). We also determined $\delta^{13}$C composition for transects through region 1 and 2 of two involucra (Fig. 4.8), in order to compare these with baleen isotope signals and baleen morphology (neonatal notch).
Figure 4.8. $\delta^{13}C$ signatures of tympanic bone and baleen transects. $\delta^{13}C$ values of bone apatite (red line) and baleen keratin (black line). Bone samples were taken along a transect of an involucrum slice from the center of region 1 of the involucrum (left) to the border between regions 1 and 2 (right). Baleen samples were taken along a transect the total length of the plate from distal (left) to proximal (right). Yellow area marks prenatally formed tissue, white indicates the nursing period, blue indicates the post-weaning period. (A) NSB-DWM 2012B5. (B) NSB-DWM 2013B6.
Life History Data

Table 4.2 compiles biological data with age estimates for the individuals studied. Figure 4.9 explores regression models for these data. Figure 4.9A shows a linear regression of whale length on GLGs. The slope and intercepts are 0.264 (SE=0.014) and 8.018 (SE=0.207), respectively, with $R^2 = 0.97$. Figure 4.9B plots baleen plate length in a log-log regression on GLG number. Since baleen plate length does not grow linearly with whale age, we fitted these data to a non-linear curve. The relationship between estimated age (based on baleen length) and number of GLGs is essentially linear (Fig. 4.9C), but a log-log fit was required to stabilize variance. The slope and intercept estimates for such a fit are 1.097 (SE=0.074) and 0.168 (SE=0.173) respectively, with $R^2 = 0.95$. Figure 4.9D shows a linear regression of the number of stable $\delta^{13}C$ cycles in baleen plate GLGs. The slope and intercept estimates are 0.659 (SE=0.072) and 1.961 (SE=1.059) respectively, with $R^2 = 0.94$. For each of these four models, we tested sex as a potential additional predictor, but this factor was not statistically significant in any model.

DISCUSSION

The bone histology of regions 1 through 3 displays a pattern that indicates that bone growth in the bowhead tympanic is similar to that in other mammals (Huttenlocker et al. 2013, Woodward et al. 2013). Fibrolamellar bone, as found in region 1 and 2 indicates fast growth. This type of bone is also located in the cortex of long bones of land mammals, a region comparable to region 2 of the bowhead tympanic. In contrast, the periosteal surface of mammalian long bones is similar to region 3 of the bowhead tympanic and consists of slow-growing, parallel-fibered or lamellar bone. With age, bone remodels, but the rate for this process varies (Klevezal 1996). We see some evidence of remodeling in bowhead in the form of
<table>
<thead>
<tr>
<th>Whale ID</th>
<th>Season</th>
<th>Sex</th>
<th>Whale L (m)</th>
<th>Longest baleen (cm)</th>
<th>Age based on longest baleen length</th>
<th># of tympani c GLGs</th>
<th>Age based on baleen stable isotope cycles</th>
<th>Neonatal notch present</th>
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<tr>
<td>2011B9</td>
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<td>F</td>
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<td>235</td>
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<td>F</td>
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<td>F</td>
<td>16.46</td>
<td>342</td>
<td>76.8</td>
<td>31</td>
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<tr>
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<td>M</td>
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<td>195</td>
<td>12.8</td>
<td>7.5</td>
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<td>2013B6</td>
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<td>F</td>
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<td>4.1</td>
<td>4.5</td>
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<tr>
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<td>M</td>
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<td>152</td>
<td>4.8</td>
<td>2.5</td>
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<td>211</td>
<td>16.2</td>
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<tr>
<td>2014B6F</td>
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<td>NA</td>
<td>NA</td>
<td>none</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>fetus</td>
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<tr>
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<td>M</td>
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<td>273</td>
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<tr>
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<td>34.5</td>
<td>24</td>
<td>24</td>
<td>no</td>
</tr>
</tbody>
</table>

*Table 4.2. List of bowhead whale specimens used in this age estimation study.*
Figure 4.9. Tympanic bulla GLG counts plotted against four other variables from these same bowhead individuals. (A) Whale length (in m) fitted to a linear regression line. (B) Longest baleen plate length (in cm) fitted to a log-log regression curve. (C) Age estimated based on longest baleen plate (for model used, see text) fitted to a log-log regression. (D) Number of δ¹³C isotope cycles in the longest baleen plate fitted to a linear regression line.
secondary osteons (NSB-DWM 2013B1, 2015B2, 2015B17, 2015B24; Fig. 5-7), but only in older individuals. Remodeling is also slow to occur in the periotics of manatees (Marmontel et al. 1996) and other mysticetes (Klevezal 1996). This indicates that much of the structure of bowhead tympanic has remained unchanged since it was formed, and thus can form a record of the life of the individual. Our data indicate that the three morphological regions that we identified were formed during different parts of the life history of the animal.

Region 1 of the involucrum is deposited during the fetal period, as evidenced by NSB-DWM 2014B6F (Fig. 4.1C), a fetal specimen in which only region 1 of the tympanic is present. Fertilization in bowheads occurs around March (George et al. 2016), and this whale was caught in early October, hence region 1 represents approximately 6-7 months of bone formation. Interestingly, baleen formation begins only a few months before birth (Thewissen et al. 2017), and none was present in this specimen when it(s mother) was caught. Therefore, the isotopic record of the bone in region 1 of the involucrum represents a longer period of fetal life than the baleen plate does (Fig. 4.8). The border of the yellow and white area in Figure 4.8, marks the position where the neonatal notch was located on the baleen plate.

Region 2 of the bulla is deposited during the nursing period. This is evidenced by specimen NSB-DWM 2012B5 which displays only regions 1 and 2 (Fig. 4.1D, 4.2B), and is approximately one-year-old based on the baleen length. Bowheads nurse for approximately nine months (George et al. 2016), and consistent with this, baleen of this individual displays only a single isotopic oscillation (Fig. 4.2A and Table 4.2). The isotopic signal ends when the whale was caught in the Chukchi Sea, likely around weaning. The seasonal oscillations that are evident in the isotope record of the baleen of independently-feeding individuals (Shell et al. 1989a, b) are dampened in nursing whales due to the buffering effect of the mother’s milk, the carbon and
nitrogen of which are sourced from diet and body tissue stores (Newsome et al. 2010). This effect also occurs in isotope records of dentin in belugas (Matthews and Ferguson 2015). We interpret the drop in $\delta^{15}$N values of baleen as the end of nursing, and this occurs when the plate is between 62-92 cm long in our specimens (Fig. 4.2; NSB-DWM 2013B6, 2013B15, and 2015B19; nursing period with white background, independent feeding in blue).

Figure 4.8 shows the calibration of isotope records for baleen and region 1 and 2 of the tympanic bone. The neonatal notch is calibrated to the end of region 1 (mentioned above) and the drop in isotope values of baleen is calibrated to the end of region 2. When both bone and baleen signals end suddenly these are calibrated because this is when the whale was harvested (Fig. 4.8A). Bone of the tympanic bulla does not grow at the same rate as baleen, but both record specific points in the life history of bowheads. The $\delta^{13}$C values recorded from bone mineral of regions 1 and 2 of the tympanic bulla of NSB-DWM 2012B5 match those of its baleen, but this is not the case in 2013B6 (Fig. 4.8). The reason for this is unclear.

Region 3 of the tympanic is only found in whales older than one year, and it represents slow periosteal growth with growth layer groups (GLGs). This pattern has also been observed in the tympanic bone of fin, minke and gray whales (Klevezal and Mitchell 1971, Christensen 1981, Klevezal et al. 1986, Christensen 1995, Klevezal 1996). The physiological reason why GLGs form is unclear, but the process has been studied in a variety of non-cetacean mammals. Köhler et al. (2012) showed that, in some ruminant species, formation of incremental growth layers is the result of cyclical endocrine and physiological changes that are related to the energetically unfavorable season. When feeding is limited, incremental growth layers are thinner. Huttenlocker et al. (2013) indicated that thick incremental growth layers reflect a period of robust growth, whereas thin ones indicate slow or ceased growth.
Castanet et al. (2004) proposed a different mechanism for GLG formation in the bones of the small arboreal primate *Microcebus*, showing conclusively that GLG formation was dependent on photoperiod during the year. Physiological variables that affect GLG formation are also known to be affected by photoperiod in arctic and subarctic artiodactyls, although these may be modulated by other environmental factors (Suttie and Webster 1995, Pösö 2003, Piccione et al. 2009).

Bowheads go through periods of robust feeding during summer in the Beaufort and Chukchi Seas and opportunistic and limited feeding during winter in the Bering Sea (George 2009). Thicker, dark colored incremental growth layers would be formed in summer, and thinner, light-colored layers in winter. Christensen (1981) came to similar conclusions for minke whale bullae based on results from teeth of seals and odontocetes. Thus, bowhead GLGs would be formed by a process that reflects seasonality and this process is probably not tightly tied to photoperiod, unlike *Microcebus*. This should not be a surprise given that the longest continuous light period in the Beaufort Sea lasts for more than two months, and that winters in the Bering Sea have nights that last more than 21 hours.

To assess the validity of GLG counts in bowhead tympanics as a means of age estimation, we regressed body length, baleen length, and age estimates based on baleen length against the number of GLGs (Fig. 4.9A-D). Overall, the correlation between body length and GLG numbers is strong and linear (Fig. 4.9A). However, bowhead body length has been shown to plateau after weaning for much of the middle of the first decade of life (George et al. 2016), and this signal is not seen in our data. It is possible that a subtle growth trend is not recovered due to our small sample size, but other explanations are also possible. For instance, limited body growth and limited tympanic bone growth may co-occur. An extended period of low resource
intake, such as the growth hiatus experienced in young bowheads, may result in a loss of annual resolution of growth patterns in the tympanic for that time in some individuals.

Baleen plate length increases with age, but the increment of annual growth slows (Fig. 4.9B) (Schell and Saupe 1993, Lubetkin et al. 2008, Lubetkin et al. 2012, George et al. 2016), and a power function models the relation between GLG counts and baleen length reasonably well (Fig. 4.9B). Lubetkin et al. (2012) designed a model for relating baleen length to age (Y-axis in Fig. 4.9C), and the outcome of this function correlates relatively closely to GLG counts, where the slope of 1 is evidence that one GLG accumulates each year. Indeed, age estimates based on the Lubetkin et al. (2012) model are similar to those of GLG counts (Table 4.2) in most individuals. A substantial difference occurs in NSB-DWM 2013B5 and 2014B14, where the Lubetkin et al. (2012) model predicts ages approximately twice of the number of GLGs. We hypothesize that these two whales experienced some lean years with absence of GLG formation, or with GLGs so thin that they cannot be recognized.

Figure 4.9D shows that a positive linear relationship between baleen isotope oscillations and GLGs exists, however, the data are limited and the estimated slope differs from 1. This is partly caused by wear of the baleen, where entire annual migration records are lost in older whales. The match between tympanic GLG numbers and isotope cycles is also not perfect for young individuals either. Here, it appears, again, that particulars of an individual’s life history matter. Animals may be born in different seas, may migrate at different times, may wean earlier or later, and may spend more or less time in isotopically different areas of their range (Moore and Reeves 1993, Citta et al. 2015). For instance, NSB-DWM 2013B15 (Fig. 4.2E) shows two poorly resolved tympanic GLGs. These do not match the number of baleen isotopic oscillations which suggests two years of independent feeding, plus one year of nursing. The absence of clear
GLGs may suggest that this whale did not experience the normal physiological stress that underlies the deposition of a thin incremental growth layer. Overall, Figure 4.9 indicates that GLGs provide reasonable estimates for age of individual whales, although they are not absolute and infallible age gauges.

Deposition of recognizable GLGs is influenced by unique events in the lives of bowhead individuals, and, for older whales, GLGs become thin and indistinguishable from one another (Fig. 4.7B). This limits the use of GLGs for estimating age to younger individuals. A further complication in distinguishing GLGs is that they do not form homogenously along the periosteal edge of the involucrum (Sukhovskaya et al. 1985, Klevezal 1996), and may become diffuse in some areas (Fig. 4.7A). Non-uniformity could be due in part to bone remodeling (arrowheads in Fig. 4.7B). Another issue is the distinction between GLGs and accessory layers (white boxes in Fig. 4.2D, 4.3B, 4.5A, 4.6C-E; Hohn 2009). Discussed by Larsen and Kapel (1982) and Olsen (2002), such accessory layers also occur in other tissues, such as teeth. Accessory layers may record particular transitory but important events in the life of the animal (Klevezal 1996, Hohn 2009), and mimic or overprint cyclical, annual patterns.

CONCLUSIONS

Tympanic GLGs can be used to estimate the age of bowhead whales, and we believe that this method is reliable up to ages of approximately 20 years, somewhat reliable for ages between 20 and 30, but not reliable for older bowhead whales. This is broadly consistent with findings on the efficacy of this method in balaenopterid whales. As such, GLG counts are a useful addition to age estimation methods already available for bowheads. They hold particular promise for
estimating the age of specimens where only limited samples are available, such as fossils or stranded specimens.

More important in our view is that tympanic GLGs are a window into the life history of the individual, and that histology of the tympanic bone records events that are not recorded in other anatomical samples. The recognition that region 1 and 2 of the tympanic are formed, respectively, in the fetal and nursing period, is an example thereof. Other life history parameters that could affect GLGs are feeding and migration. Thus, the GLGs in the tympanic of bowhead whales are more similar to a diary than to a calendar recording the individual’s life.
Chapter V

CONCLUDING REMARKS

The objective of this dissertation is to provide baseline information about the inner ear of beluga (*Delphinapterus leucas*) and bowhead whales (*Balaena mysticetus*), while exploring the auditory health of these species. Additionally, it is to provide a new method of age estimation for bowhead whales.

With the increase in human activity in the arctic and a projected increase in coming years (Moore *et al.* 2012, Stafford 2013), it is important to evaluate the health and well-being of whale populations in order to provide evidence to guide the best management decisions. From a hearing research prospective, although data on behavioral and physiological responses for whales exist (reviews by Richardson *et al.* 1995, National Research Council 2003, Weilgart 2007, Stafford 2013, Weilgart 2013), the data are insufficient to appropriately predict how anthropogenic sounds impact the hearing of marine mammals long term. In order to gain a better understanding, anatomical studies are necessary. Along the same vein, age estimation is important to help us properly assess the data on hearing as well as provide information for population dynamics studies.

Chapter II focuses on the beluga whale and uses 3-D reconstructions and histology to present Rosenthal’s canal length and cross-sectional area, absolute neuron counts and neuronal densities along the length of Rosenthal’s canal, and cellular visualization. The cochlea is a loose spiral with less than two-whorls that do not overlap, which is characteristic of non-delphinid
odontocetes (Fleischer 1976, Ketten 1984). Results show that both absolute spiral ganglion cell counts and Rosenthal’s canal cross-sectional area vary along the length of the cochlea, with Rosenthal’s canal having two local maxima. Neither cell counts nor histology suggested areas of reduced cell number and thus no spiral ganglion damage was found in this group of belugas.

Chapter III focuses on the bowhead whale and also uses 3-D reconstructions and histology to present Rosenthal’s canal length and cross-sectional area, absolute neuron counts and neuronal densities along the length of Rosenthal’s canal, and cellular visualization. A bowhead cochlea is a tight spiral, with greater than 2 whorls which partially overlap. This is characteristic of a mysticete species. Results indicate Rosenthal’s canal cross-sectional area varies along the length of the cochlea, with reduced cross-sectional area near the apex. Absolute spiral ganglion cell number did not vary significantly. Two specimens visually appeared to have a reduced cell count in the upper basal and apical whorls. This along with histological evidence indicates no firm conclusion for potential hearing damage for bowheads.

Integrating results from chapters II and III, the overall findings for the number of spiral ganglion cells in the beluga and bowhead inner ear (Fig. 5.1) coincide with the idea that mysticetes do not have the same degree of hyper-cellularity as odontocetes (Wartzok and Ketten 1999). Belugas were found to have an estimated overall cell count of 234,504 (SD = 18,416), with an estimated 152,672 (SD =45,888) cells for bowheads. Given that belugas and bowheads have overlapping habitat ranges and all study subjects covered a variety of ages, it is interesting that only one of these species in this study showed visible drops in cell count. One explanation is that these species differ behaviorally when exposed to anthropogenic sounds (National Research Council 2003). Both bowheads and belugas display a number of behavioral changes when exposed to loud sounds which include avoidance and altering vocalizations.
Figure 5.1. Three variables of the spiral ganglion plotted against their position along the cochlea (x-axis, in percent of its length). (A,B,C) *Delphinapterus leucas*; (D,E,F) *Balaena mysticetus*. (A,D) Cross-sectional areas of Rosenthal’s canal based on CT data as analyzed by AMIRA. (B,E) Neuron numbers of the spiral ganglion, as determined for each cochlear segment based on counting multiple (usually five) histological sections. (C,F) Neuron densities of the spiral ganglion, calculated based on the data in B and E with the volume of the region in which the cell counts were taken.
However, belugas have been observed to flee up to 80km from icebreaker noise (LGL and Greeneridge 1986, Finley et al. 1990). The reason may due to the whales’ partial confinement in heavy ice during spring migration (when sound propagation is high), as well as lack of prior exposure to ship sounds. Finley et al. (1990) observed that although, belugas may flee at sound levels of 94 and 105dB re 1 µPa, they return a day or two later when icebreaker noise is louder at 120dB re 1 µPa. Additionally, belugas summering in the Beaufort Sea display long range avoidance of seismic air gun surveys (Miller et al. 2005). Bowheads, on the other hand, display some level of avoidance, but seem to be much more tolerant of anthropogenic noise. For example, during migration, when only one eastward lead is available, will pass through sound fields with projected sounds up to 131dB re 1µ Pa (Richardson et al. 1991). In addition, bowheads will still summer in the Beaufort Sea despite the occurrence of seismic surveys near this region at the same time (Richardson et al. 1987). Behavioral evidence is not enough to determine, however, if fewer whales return each year or if the same whales are returning. A second explanation for why bowheads and belugas show differing cell counts could be the frequencies that each species of whale receives. Many of the anthropogenic sounds such as seismic exploration with air guns, sea floor mapping, resource extraction, and ships all produce sounds in the lower frequency ranges (10Hz -10,000Hz or less; Stafford 2013). This would likely affect bowhead hearing more than beluga and lower frequencies can travel a much greater distance. Figure 5.2 shows attenuation profiles for sound in both freshwater and sea water (Denny 1993). Finally, it is possible that the differences seen in cell counts and histology could simply be natural variation.

Table 5.1 provides the weights, basilar membrane lengths, and number of spiral ganglion neurons within the cochlea of several mysticete, odontocete, and terrestrial species. Figure 5.3
Figure 5.2. Attenuation of sound in fresh and sea water, dB per km and Hz (Denny 1993; Fig. 10.9).
<table>
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<th>Common name</th>
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<th>Estimated number of spiral ganglion cells</th>
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<td>55</td>
<td></td>
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<tr>
<td><em>Balaena mysticetus</em></td>
<td>Bowhead</td>
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<td>41.2</td>
<td>152,672</td>
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</tr>
<tr>
<td><em>Balaenoptera physalus</em></td>
<td>Fin whale</td>
<td>&lt;120,000</td>
<td>64.7</td>
<td>134,098</td>
<td>Wartzok and Ketten 1999</td>
</tr>
<tr>
<td><em>Eubalaena glacialis</em></td>
<td>Right Whale</td>
<td>90,000 kg</td>
<td>49.5</td>
<td>-</td>
<td>Ketten 1992</td>
</tr>
<tr>
<td><em>Megaptera novaeangliae</em></td>
<td>Humpback whale</td>
<td>&gt;40,000</td>
<td>54</td>
<td>156,374</td>
<td>Ketten 1992</td>
</tr>
<tr>
<td><em>Delphinapterus leucas</em></td>
<td>Beluga</td>
<td>1600</td>
<td>47.3</td>
<td>234504</td>
<td>This study</td>
</tr>
<tr>
<td><em>Lagenorhynchus obliquidens</em></td>
<td>Pacific white-sided dolphin</td>
<td>198</td>
<td>29.5</td>
<td>50412</td>
<td>Wever et al. 1972</td>
</tr>
<tr>
<td><em>Lagenorhynchus albirostris</em></td>
<td>White-beaked dolphin</td>
<td>350</td>
<td>34.9</td>
<td></td>
<td>Ketten 1984</td>
</tr>
<tr>
<td><em>Stenella attenuata</em></td>
<td>Pantropical spotted dolphin</td>
<td>119</td>
<td>36.9</td>
<td>82506</td>
<td>Ketten 1984</td>
</tr>
<tr>
<td><em>Tursiops truncatus</em></td>
<td>Common bottle nosed dolphin</td>
<td>650</td>
<td>38.5</td>
<td>95004</td>
<td>Wever et al. 1971 b,c</td>
</tr>
<tr>
<td><em>Phocoena phocoena</em></td>
<td>Harbour porpoise</td>
<td>70</td>
<td>25.9</td>
<td>66933</td>
<td>Ketten 1984</td>
</tr>
<tr>
<td><em>Physeter catodon</em></td>
<td>Sperm whale</td>
<td>57000</td>
<td>54</td>
<td>-</td>
<td>Ketten 1992</td>
</tr>
<tr>
<td><em>Inia geoffrensis</em></td>
<td>Amazon river dolphin</td>
<td>207</td>
<td>38</td>
<td>-</td>
<td>Ketten 1992</td>
</tr>
<tr>
<td><em>Rhinolophus ferrumequinum</em></td>
<td>Greater Horseshoe bat</td>
<td>0.034</td>
<td>16.1</td>
<td>15,953</td>
<td>Wartzok and Ketten 1999</td>
</tr>
<tr>
<td><em>Pteronotus parnellii</em></td>
<td>Mustached bat</td>
<td>0.02</td>
<td>14</td>
<td>12800</td>
<td>Wartzok and Ketten 1999</td>
</tr>
<tr>
<td><em>Cavia porcellus</em></td>
<td>Guinea pig</td>
<td>1</td>
<td>19</td>
<td>24011</td>
<td>Wartzok and Ketten 1999</td>
</tr>
<tr>
<td><em>Felis domesticus</em></td>
<td>Cat</td>
<td>3.26</td>
<td>28</td>
<td>51755</td>
<td>Wartzok and Ketten 1999</td>
</tr>
<tr>
<td><em>Homo sapiens</em></td>
<td>Human</td>
<td>60</td>
<td>32.1</td>
<td>30500</td>
<td>Wartzok and Ketten 1999</td>
</tr>
<tr>
<td><em>Mus musculus</em></td>
<td>Mouse (CBA/JCr)</td>
<td>0.0205</td>
<td>5.9</td>
<td>8626</td>
<td>Johnson et al. 2011</td>
</tr>
</tbody>
</table>

Table 5.1. Approximate body weight, basilar membrane length, and number of spiral ganglion neurons in multiple, mysticetes, odontocetes, and terrestrial mammals. Weight data for cetaceans (Jefferson et al. 2008), horseshoe bat (Koopman 1994), mustached bat (Nowak et al. 1994), guinea pig, cat, and human (Eisenberg 1981), and mouse (Johnson et al. 2011).
Figure 5.3 Natural logarithm of spiral ganglion counts and basilar membrane length versus natural logarithm of body weight for several cetaceans and terrestrial mammals. (A) spiral ganglion counts (B) basilar membrane length. Cetaceans labeled as follows: *Balaenoptera acutorostrata* (Ba, Minke), *Balaena mysticetus* (Bm, Bowhead), *Balaenoptera physalus* (Bp, Fin), *Cavia porcellus* (C, Guinea pig), *Delphinapterus leucas* (D, beluga), *Eubalaena glacialis* (E, right), *Felis domesticus* (F, cat), *Homo sapien* (H, human), *Inia geoffrensis* (I, river dolphin), *Lagenorhynchus albirostris* (La, dolphin), *Lagenorhynchus obliquidens* (Lo, dolphin), *Magaptera novaeangliae* (Mn, Humpback), *Mus Musculus* (Mm, mouse), *Phocoena phocoena* (Pp, porpoise), *Physeter catodon* (Pc, sperm whale), *Pterontus purnelli* (P, bat), *Rhinolophus ferrumequinum* (R, bat), *Stenella attenuata* (S, dolphin), *Tursiops truncatus* (T, dolphin).
compares the ln of basilar membrane length and ln of overall spiral ganglion number to the ln of overall body weight. Similar to what was found in odontocetes (Sensor et al. 2015), basilar membrane length generally increases with body size for both terrestrial mammals and cetaceans. Overall, however, it appears that two odontocete species, the harbor porpoise (*Phocoena phocoena*) and the pacific white sided dolphin (*Lagenorhynchus obliquidens*), have shorter basilar membrane lengths, than other mammals of similar size. In fact, they have shorter basilar membrane lengths than a much smaller mammal, the domestic cat, *Felis domesticus*. Along the same vein, three mysticete species, the sperm whale (*Physeter catodon*), the right whale (*Eubalaena glacialis*) and the bowhead whale (*Balaena mysticetus*) all have shorter basilar membrane lengths than what would be expected for their size.

Overall, the number of spiral ganglion neurons throughout the cochlea also generally increased with body size. Consistent with basilar membrane length the pacific white sided dolphin has a lower spiral ganglion neuron count than the other delphinid species in the graph. Interestingly, humans (*Homo sapiens*) have a lower spiral ganglion cell count than some other smaller mammals with shorter basilar membranes. Additionally, the fin whale (*Balaenoptera physalus*), has a lower spiral ganglion neuron count than would be expected given its size and basilar membrane length. The bowhead whale, on the other hand, despite having a shorter than expected basilar membrane length, seems to follow the general trend in regard to spiral ganglion neuron number. It is not clear if these differences among odontocetes or mysticetes are significant to hearing. In addition, different estimation methods among authors may introduce variation into these results.

Chapter IV focuses on the bowhead whale and utilizes bone histology, stable isotopes, and baleen and body measurements to investigate whether growth layer groups (GLGs) in the
periosteal region of the tympanic bone correlate with age. GLGs show correlation coefficients as high as 0.97 when compared to number of isotopic oscillations in the baleen plate (relating to number of migrations), baleen plate length, age estimation based on plate length, and total whale length. Tympanic GLGs are a reliable method of age estimation for bowheads up to approximately 20 years of age, and somewhat reliable for age estimation up to 30 years of age. A parallel investigation of bone histology with baleen and bone stable isotope signatures indicated that the tympanic bone, including the growth layer groups, may be a window into the life history of the individual. The tympanic bone records fetal growth, growth while nursing, and times of high and low food intake, which are often linked to time of year and migration cycle.

CONTRIBUTIONS TO THE FIELD

This thesis provides baseline information about the inner ear anatomy and health of wild caught beluga and bowhead whales. With the difficulty of obtaining fresh samples from the wild, these studies will provide the field with a unique source of information. Additionally, a new method of age estimation has been established for bowhead whales. Although several methods of age estimation exist, they only cover limited parts of the bowhead lifespan. In particular there is a need for estimation between the ages of 10 and 30 years of age. Counting GLGs provides an inexpensive way to estimate the age of individuals reliably up to about 20 years.

FUTURE DIRECTIONS

Nearly all studies that investigate the effects of anthropogenic sounds on marine mammals focus on short-term responses (National Research Council 2003). Therefore, further
research on the anatomy of the inner ear is necessary to explore long-term effects.

Investigations may include a longitudinal study of the spiral ganglion for bowhead and belugas over time, and analysis of the organ of Corti if stranding specimens become available. (We cannot study the organ of Corti on current specimens as it may be damaged during death.)

Age estimation techniques can be expanded to investigate other species of whales in which baleen in short, and thus will likely not hold a long term record of life, or missing, such as in fossil specimens (Fig. 5.4).
Figure 5.4 GLGs in the periosteal region of the tympanic bone of fossil Eocene whale, *Nalacetus* (specimen: HGSP 96384).
REFERENCES


Finley, K. J., G. W. Miller, R. A. Davis and C. R. Greene. 1990. Reactions of belugas (Delphinapterus leucas) and narwhals (Monodon monoceros) to ice-breaking ships in the Canadian high arctic. Canadian Bulletin of Fisheries and Aquatic Science 224:97-117.


