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Age Determination of the Sixgill Shark from Hard Parts Using a Series of Traditional and Novel Approaches

By
S. Jeffrey Campbell

Accepted in Partial Completion

Of the Requirements for the Degree

Master of Science

Moheb A. Ghali, Dean of the Graduate School

ADVISORY COMMITTEE

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MASTER’S THESIS

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Age Determination of the Sixgill Shark from Hard Parts Using a Series of Traditional and Novel Approaches

A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

By
S. Jeffrey Campbell
Abstract

Necessary to the management of any species of fish is the ability to determine age in individuals. Age information is used to establish growth rates, longevity, age at maturity, and population age structure, and to predict how population demographics will change over time. For most species of fish, reliable aging techniques have been in use since the early 20th century. Most boney fish are aged by counting bands of calcium phosphate hydroxyapatite that form over time in skeletal hard parts such as otoliths, fin-spines, and scales, which can be used as proxies for age in years. Fishes in the Class Elasmobranchii lack otoliths and have skeletons composed of cartilage, which often do not incorporate enough calcium to enable enumeration by traditional aging methods that rely on light microscopy, in some cases aided by enhancement techniques such as histological staining. For these fishes, alternative methods and aging structures are used to identify and enumerate annual patterns in calcium deposition. The aging structure of choice for most elasmobranches is the vertebral centrum, where bands of calcium are deposited that can be used as proxies for age in years. A few deep-dwelling species of Elasmobranchii, such as the sixgill shark (*Hexanchus griseus*), have as yet defied efforts at age determination, inhibiting efforts to implement science-based management plans.

This study has attempted to identify alternative aging structures that may incorporate seasonally-mediated concentrations of elements or isotopes into cartilage. Once identified, these structures were subjected to two recently developed methods of elemental microanalysis, energy dispersive X-ray spectroscopy (EDAX), and laser ablation, inductively-coupled, plasma mass spectrometry (LA-ICP-MS), in an effort to develop an aging method for *H. griseus*, and potentially other poorly calcified elasmobranchs. In addition, it has also used two traditional, histological staining methods, Von Kossa’s AgNO₃, and Alizarin Red-S and applied them to these non-traditional aging structures.

This study identified regions within the cartilaginous skeleton of *H. griseus* that lay down systematic banding patterns. These patterns were visually detected using AgNO₃ staining enhanced light microscopy, as well as by periodic oscillations in isotopic concentrations for $^{24}\text{Mg}$, $^{88}\text{Sr}$, $^{107}\text{Ag}$, and $^{109}\text{Ag}$ that were detected through the use of LA-ICP-MS. Future
research in this field will need to verify if these bands are deposited on an annual basis. Methodologies must be developed for verification, and validation of these procedures to determine if the band patterns identified by either of these methods have utility for age determination in this and other poorly calcified species of elasmobranchs.
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Introduction

Terminology

The discipline of fish age and growth determination has a long history and a large literature. In reviewing that literature for a chapter in Carrier et al.’s Biology of Sharks and their Relatives (2004), Cailliet and Goldman (2004) found that there was a lack of consistency with which the terminology of the discipline was applied (Cailliet et al. 2006). In the interest of creating some consistency in the use of terms, Cailliet et al. (2006) published *Age and growth studies of chondrichthyan fishes: the need for consistency in terminology, verification, validation, and growth function fitting*. This thesis adheres to the terminology as defined in that article and where it is deemed necessary definitions and notes on usage will be included parenthetically.

Background

The ability to accurately determine age is fundamental to the management of any species of fish (Casselman 1983). Age information is used to determine longevity, mortality, productivity, population status, and to predict how populations will change over time and under specific management scenarios (Ricker 1958; Goldman 2005; Hale et al. 2006). Armed with age information, the fishery manager can begin to formulate a management plan that is scientifically defensible, allows for a sustainable take, and enables managed populations to rebound from unanticipated natural and anthropogenic perturbations.

Most of the fish aging techniques in use for teleost fishes had been developed by 1910 (Ricker 1958). The majority of these techniques rely on the identification and enumeration of band pairs in otoliths, scales, fin-spines or other mineralized structures.
Fishes in the sub-class Elasmobranchii, composed of sharks, skates, and rays, lack most of these aging structures, and thus require alternative techniques (Cailliet and Goldman 2004). Aging methods for most elasmobranchs rely on being able to visually identify and count rings of carbonate or phosphate material in vertebral centra. In the early literature these rings are simply referred to as mineralized or calcified because they were often successfully enhanced with histological stains that were in use for demonstrating calcium (Meloan and Puchtler 1985). It is now known that this calcium usually occurs as calcium carbonate (CaCO$_3$) and calcium phosphate hydroxyapatite (Ca$_{10}$(PO$_4$)$_6$(OH)$_2$), the major mineral constituent of bone, which also occurs in varying concentrations in the cartilage of many elasmobranchs (Egerbacher et al. 2006; Kerr et al. 2006; and Porter et al. 2006).

Growth band deposition in elasmobranchs is thought to result from one or both of the following processes: the first being seasonal oscillations in the availability of environmental factors, and the second is seasonally mediated metabolic shifts that result in differential uptake of environmental factors (Brown and Gruber 1988, Goldman 2005). Thus, fish that live in relatively stable environments, or that have relatively stable annual metabolic regimes may incorporate weaker signals into their hard parts. It is also possible that highly mobile species that engage in long annual migrations may differentially incorporate band constituents as a function of their location within their range, rather than the passage of time.

In some species of elasmobranchs, growth rings in centra are visually apparent without aid of enhancement techniques. In many they are only visible when enhanced through staining, and in a few species of deep-dwelling, poorly calcified elasmobranchs such as the sixgill shark, *Hexanchus griseus*, sevengill shark, *Notorynchus cepedianus*, and the Greenland shark, *Somniosus microcephalus*, there is no known method to reliably identify these rings.
(Ebart 1986; Cailliet 1990; McFarlane et al. 2006). Consequently, management plans for these species, if they exist at all, are not based on a complete understanding of the vital statistics of the species, and are necessarily protective (Greg Bargmann, Washington Department of Fish and Wildlife, personal communication).

My decision to undertake this study was due to the fact that bluntnose sixgill sharks were being taken from the waters of Puget Sound, and the Washington Department of Fish and Wildlife (WDFW) had no management plan in place for them. WDFW provided a Shewmaker grant to develop an age determination method for sixgill sharks, and this provided me with an opportunity to investigate the application of some novel techniques.

The staining methods most often employed in the aging of elasmobranchs are basophilic, meaning the staining chemicals bind to substances with a basic pH. The two most common stains used in the aging of elasmobranchs are Von Kossa’s silver nitrate stain and Alizarin Red S, both of which have been used to demonstrate calcium in bone since the late 1800’s (Gruber and Ingram 2003).

Natural history of the bluntnose sixgill shark

Little is known of the life history of the bluntnose sixgill shark. From bycatch records it is clear that they are sometimes to be found at depths to 2300 m (7500’). Their distribution is world-wide, and the greatest documented length is 482 cm (TL), (15.8’) (Ebart 1986), but video from a dive on August 28, 2006, by a manned-submersible from the University of Hawaii at 1006 M (3300’) recorded an single female in excess of 549 cm (TL), (18’) (Dr. Craig Smith, University of Hawaii, Manoa, personal communication). Stomach contents analysis indicates that they are opportunistic feeders, and has identified the following as prey:

Bluntnose sixgill sharks exhibit ovoviviparity also known as aplacental viviparity, meaning they carry fertilized eggs until they are ready to hatch, but there is no placental connection between the mother and the embryo and nourishment of the embryo is entirely via yolk-sac. They are among the most fecund of shark species with recovered females carrying from 22 to 108 pups, the tendency of near-term females to abort at capture being cited for the disparity (Ebert 1986). In the Puget Sound, Georgia Basin estuary complex, only adult and juvenile females and juvenile males have been observed, leading to the hypothesis that estuaries are used as nursery areas. Females are observed to be sexually mature at lengths greater than 420 cm (TL) (Ebert 1986, Williams et al. 2009). Length/weight regressions have been developed for this species from catch data, and seem to indicate that females show a sharp increase in weight that seems to coincide with the onset of sexual maturity. Embryos are often aborted during capture, but recently aborted, near-term young seem to suggest the length at birth is between 68 and 72 cm (TL) (Ebert 1986).

*Von Kossa’s silver nitrate staining*

The first documented use of silver nitrate as an aid to differentiating tissues in histological investigations occurred in 1844, when it was used to blacken the cell borders of epithelial tissues (Meloan and Puchtler 1985). Von Kossa developed his staining protocols in 1901,
and though they have been somewhat modified, they are still in use today. The method is commonly used to demonstrate the presence of phosphates and carbonates in bone and cartilage which are diagnostic for the presence of osteocytes and chondrocytes respectively. Von Kossa’s staining technique is a two-step reaction. In the first step silver cations bind to phosphate and carbonate deposits, and in the second step the bound silver is reduced to metallic silver through the combination of organic material within the cartilaginous matrix, and exposure to light (Meloan and Puchtler 1985). The process has been described in the literature as depending on the ability of silver cations to be photo-reduced to metallic silver by exposure to a strong light source, ultraviolet most often being used (Meloan and Puchtler 1985). Von Kossa’s staining protocols were first used on an elasmobranch by J. D. Stevens (1975) to highlight age bands in vertebral centra in his work on the blue shark Prionace glauca (Cailliet et al. 1981). Since then the method has been used with success on several other species of elasmobranchs (Cailliet et al. 1982; Thorson and Lacy 1982; Cailliet and Bedford 1983; McFarlane et al. 2002; Goldman 2005).

The understanding that calcium in cartilage occurs as calcium phosphate hydroxyapatite is important because investigations into the chemistry of Von Kossa’s technique by Meloan and Puchtler (1985) have shown that Von Kossa only regarded a yellow coloration as demonstrating the presence of calcium phosphate hydroxyapatite (Meloan and Puchtler 1985). Furthermore, Von Kossa understood that the dark brown or black coloration obtained in the presence of intense light indicates calcium salts, or “calcium deposits in tissues contain[ing] organic material that can reduce silver” (Meloan and Puchtler 1985) but not necessarily calcium phosphate as an indicator of ossification. Interestingly, the darkening of
proteins or other organic material requires a strong light, while the formation of the silver phosphate that demonstrates for bone does not (Meloan and Puchtler 1985).

In every instance where I have seen Von Kossa’s technique described in the elasmobranch aging literature, strong light has been described as necessary to the reduction of calcium, and for the deposition of metallic silver (Stevens 1975; Cailliet et al. 1982; Thorson and Lacy 1982; Cailliet and Bedford 1983; McFarlane et al. 2002; Goldman 2005). Thus it would appear that many studies that have used this technique, and interpreted the resulting dark brown or black deposits as evidence of osteocytes, have potentially been in error.

McFarlane et al. (2002) used a modified Von Kossa’s technique on the neural arches of the bluntnose sixgill shark, Hexanchus griseus in 1994, and reported that they were able to demonstrate bands of calcium in the neural arches of 9 of the 10 sharks used in the study and that the number of bands observed increased as the total length of the shark increased. Goldman et al. (2005) reported that McFarlane was involved in on-going attempts to refine the use of silver nitrate staining on the neural arches of Bluntnose sixgill sharks and other poorly calcified species, but to date, nothing further has been published on these techniques. I believe that, as of this writing, the current study represents the only attempt to reproduce the work of McFarlane et al. on the neural arches of Bluntnose sixgill sharks.

Alizarin red S staining

Alizarin is a dye extracted from the root of plants of the genus Rubia in the madder family. It is believed to have been used as early as 3000 B.C., and was used by the ancient Egyptians for coloring textiles (Puchtler et al. 1968; Travis 1994). Artificial Alizarin was synthesized in 1869, reducing production costs and increasing commercial availability
Alizarin was first used as a histochemical reagent for the demonstration of calcium by Lieberkhün in 1874, who found that it dyed calcium phosphate, but not calcium carbonate (Puchtler et al. 1968). Alizarin red S is the common name given to sodium Alizarin sulphonate (C₁₄H₁₇O₇SNa). It is formed by the sulphonation of Alizarin and has been in common use as a reagent for the demonstration of calcium since the early twentieth century (McGee-Russell 1957).

Alizarin red S reacts with the Ca²⁺ cation to form a salt in one of three ways: the calcium cation can form ionic bonds with either the 1- or 2-OH⁻ groups, or it can form a bond with the sulfonic acid group. Alizarin red S will demonstrate simple Ca²⁺, and calcium carbonate, but “cannot be depended on to visualize primary calcium phosphate” (Puchtler et al. 1968). The technique is also very sensitive to pH and has been demonstrated to only be effective at pH greater than 8.5, with the most intense staining occurring at pH 12.0 (Puchtler et al. 1968).

Alizarin red S was first investigated as a tool for age determination in elasmobranchs by LaMarca (1966). LaMarca used a modification of Williams’ (1941) method to enhance calcified annuli in vertebral centra of the sand tiger shark, Charcharias Taurus. He noted that the method offered the advantages that it worked well on dried samples that had not been fixed and it did not require the sectioning of vertebrae, something that can be challenging with large, well mineralized specimens (LaMarca 1966).

Several other authors have used Alizarin red S successfully with other species of sharks including: lemon sharks, Negaprion brevirostris (Gruber and Strout 1983, Brown and Gruber
1988), smooth-hound shark *Mustelus mustelus* (Goosen and Smale 1997), and the silky shark *Carcharhinus falciformis* (Oshitani et al. 2003).

*Microanalytical Techniques*

Technological developments that enable special resolution of microanalysis across discrete transects have made possible several new approaches that may have utility in determining ages from tissue samples of poorly calcified sharks. Two of these new technologies, energy dispersive X-ray spectrometry (EDAX,) and laser ablation, inductively-coupled, plasma mass spectrometry (LA-ICP-MS,) rely on the use of novel sampling methods and can produce spatially resolved elemental analysis along discrete transects. Given this ability, it is natural to apply these technologies to the problem of detecting fluctuations in isotopic, or elemental concentrations, across transects on hard parts extracted from sample fish in an attempt to determine age.

*EDAX*

Energy dispersive X-ray spectrometry (EDAX) also known as electron microprobe analysis, is a non-destructive, semi-quantitative method for enumerating the species of elements present, their relative abundance, and their location within samples. EDAX works by firing an electron beam at the surface of a sample, causing it to emit X-rays that have frequencies that are unique to the analyte. The X-rays pass through a collimator and then to a detector. The current produced by the detector is amplified, and its frequency is correlated with the frequency of the appropriate analyte identity, higher frequencies correlating with higher atomic weights (Jones and Geen 1977). Method detection limits for EDAX are on the order of one part per thousand, which is relatively poor when compared to LA-ICP-MS, and
the method is also comparatively time-consuming (Jones and Geen 1977; Cailliet and Radtke 1987).

X-ray spectrometry was first used to age an elasmobranch by Jones and Geen (1977). In this study X-ray spectrometry was used to explore the spatial distribution of seasonally mediated bands of calcium and phosphorus in the vertebral centra of the spiny dogfish, *Squalus acantbias*, from the Strait of Georgia. The resulting output showed peaks in the concentrations of these elements that correlated closely with age estimates derived from both age/length regressions for Strait of Georgia populations of *S. acantbias*, and visual counts of dorsal fin spine circuli. X-ray spectrometry was also used by Cailliet and Radtke (1987) to verify the existence of higher calcium and phosphorus concentrations at the sites of visually detectable growth bands in a tropical near-shore shark, the grey reef shark, *Carcharhinus amblyrhynchos*, and in a pelagic shark, the common thresher shark, *Alopias vulpinus*. These experiments indicated that spatially resolved peaks in concentration of both elements, as demonstrated by X-ray spectrometry, were in “perfect agreement with the number, and spacing of bands” observed by light microscopy for both of the species in the study (Cailliet and Radtke 1987).

**LA-ICP-MS**

Laser ablation, inductively-coupled, plasma mass spectrometry (LA-ICP-MS) is a highly sensitive (method detection limit of <1 μg/kg for most isotopes) (Durrant and Ward 2005) minimally destructive, semi-quantitative method of chemical analysis that is appropriate for sampling for isotopic presence or absence and relative concentration along transects of hard-suraced samples.
LA-ICP-MS works by using a laser to ablate a portion of the sample to be analyzed. The width of transect to be sampled is controlled by the width of the laser beam doing the sampling, and the depth of the sample is determined by the energy density of the laser. Material sampled by the laser is carried in a stream of inert gas, usually argon or helium, into the plasma torch, where it is nebulized. The nebulized material is then carried by the gas stream into the quadrupole, where it is accelerated into the mass spectrometer (Anonymous 2005). Because of its extreme sensitivity, its ability to detect multiple analytes simultaneously, to spatially resolve analyte concentrations, and to continuously sample along discrete transects, LA-ICP-MS is the analytical method of choice for applications where compositional changes across space can be used as proxies for the occurrence of discrete events along a temporal continuum. Disciplines that take advantage of this ability include geology, materials science, and most recently, biology (Durant and Ward 2005).

In 2005, Brenkman et al. (2007) used LA-ICP-MS on the otoliths of bull trout *Salvelinus confluentus* from the Hoh Rover in Washington State to determine the age of first seaward migration. By correlating high Sr/Ca ratios (the study’s proxy for residence in the marine environment) with location in the otoliths they were able to determine age at first outmigration. They were also able to discern that outmigration timing was variable, and occurred at ages from 3 to 6 years, with 88% of all fish in the study having their first outmigration to marine waters in their third or fourth growth year. This methodology also revealed that 75% of all fish in the study had migrated between fresh and marine waters multiple times and that 3 out of the 105 fish in the study outmigrated from the Hoh River and reentered fresh water in another stream system. Brenkman et al. (2007) were able to identify unique isotopic signatures for each of the streams in the study region and correlate these with
the results of LA-ICP-MS otolith chemistry analysis for individual fish in the study (Brenkman et al. 2007). These findings allowed them to determine not only the timing of outmigration but also the streams to which the fish returned.

The first published reference to the use of LA-ICP-MS in age determination of elasmobranchs is reported by Hale et al. (2006,) who used it on sagittal sections of vertebral centra from the round stingray, *Urobatis halleri*. The method produced good correlations with age estimates obtained from visual analysis of round stingrays that were less than five years old, but agreement between visually-derived age estimates and LA-ICP-MS-derived age estimates became less well defined in older fish. The analysis performed by Hale et al. (2006) relied on the superimposition of a graph of the LA-ICP-MS output in integrated counts, which had been normalized to strontium, on a picture of the ablated transect. This allowed for a visual confirmation of the correlations between spatially-resolved LA-ICP-MS scans for selected isotopes and visually detectable age-bands in vertebral centra.

*Other Emerging Technologies*

Finally, there are two additional technologies that hold promise for age determination in poorly calcified species of elasmobranchs. Both of these technologies have the potential to provide “absolute age” and are being considered for their potential to validate other methods (validation being a confirmation of the closeness of the estimated quantity to its true value (Panfili et al. 2002, cited by Cailliet et al. 2006)).

*Disequilibrium analysis*

Disequilibrium analysis of radioisotopes, primarily using the ratio of $^{210}$Pb to $^{226}$Ra, has been used to validate age estimates derived from growth increment counts (Andrews et al.
1999). It relies on knowledge of the relatively stable ratio of occurrence of these isotopes through time, the rates at which they are assimilated and integrated into living tissues, and the rate at which $^{226}\text{Ra}$ decays to $^{210}\text{Pb}$ (Burton et al. 1999; Andrews et al. 1999). The main problem in the application of disequilibrium analysis to the aging of poorly calcified elasmobranchs is that the target isotopes have the potential to be mobilized by the colloidal fluid component of the extra-cellular matrix of the cartilage. Most studies attempting to determine age examine the spatial arrangement of age markers within an aging structure, thus this property of cartilage will probably confound the application of disequilibrium analysis to poorly calcified elasmobranchs (A. H. Andrews, Pacific Center for Shark Research, personal communication). I am not aware of any studies that have attempted to use radioisotope disequilibrium analysis as an aging method for elasmobranchs.

**Bomb carbon**

One other micro-analytical technique examines the change in $^{14}\text{C}$ values across an aging structure to estimate age or validate age estimates derived from other, indeterminate methods (methods that have proven precise, but not always accurate). The bomb-carbon method, also known as the radio-carbon method, relies on the rapid increase of radiocarbon ($^{14}\text{C}$) that occurred in the world’s oceans as a result of atmospheric testing of thermo-nuclear devices during the 1950s and 1960s (Goldman 2005; Ardizzone et al. 2006; Andrews et al. 2007).

To use this method a reference chronology must be constructed to establish a time series for the rate of uptake of $^{14}\text{C}$ for the sample collection area (Kalish 1993). To be effective, reference chronologies should provide information about the post “bomb-bump” $^{14}\text{C}$ decay curve for as large a part of the pre-asymptotic decay time series as possible (Craig Kastelle, NOAA Fisheries Biologist, Sand Point lab, personal communication).
Reference chronologies are constructed by analyzing samples of carbon-fixing organisms for which aging methods exist. Correlations are developed for the quantity of $^{14}$C within hard (non-labile) structures, and the known age represented by the structure being analyzed. Early studies using this method relied on reference chronologies derived from hard corals (Kalish 1993; Campana 1997). The method was slow to gain acceptance by fisheries biologists because for arctic and temperate species, the closest reference corals could be thousands of km distant (Piner and Wischniowski 2004).

Reference chronologies are best constructed from carbon fixed in biota within the study area and are preferably developed from a conspecific (Campana 1997; Piner and Wischniowski 2004). This is because the rate at which atmospheric carbon, primarily as CO$_2$, is assimilated into the water column is dependent on local physical conditions, i.e. seasonality of winds and currents and the depth of the mixed layer, and species may differ in the rates at which they incorporate $^{14}$C into their bony parts (Campana 1997; Piner and Wischniowski 2004). The bomb-carbon method is considered a determinate method, meaning it is capable of returning an accurate absolute age. Thus, it has gained acceptance as an aging method and a validation technique for other methods among fish aging scientists, as more and more reference chronologies become available.

Kerr et al. (2006) used the bomb-carbon technique on the white shark, Carcharodon carcharias, in an attempt to evaluate the utility of $^{14}$C age validation for a wide-ranging species with a complex life history. They found the method was confounded by the fact that the fish in their study had uncharacteristic timing of $\Delta^{14}$C values, which differed from the closest reference chronologies in the region. In other words, the reference chronologies they were developing for the white sharks as their study progressed were not synchronous with
reference chronologies developed from five other species local to the study region. They put forth three possible explanations for this discrepancy:

1) Ages derived from superimposing the $\Delta^{14}$C plot of the sample on the $\Delta^{14}$C plot of the reference chronology were greater than those estimated from visual counts of growth bands.

2) Metabolic reworking of the vertebral collagen may have reduced the $^{14}$C signal more in older parts of samples being analyzed. This would also have produced a delayed $\Delta^{14}$C time series.

3) Depletion of the $\Delta^{14}$C plot levels were due to depleted dietary sources of carbon. This could result from significant portions of the diet coming from deep water. It is known that $^{14}$C become attenuated with depth (Broeker and Peng 1982, cited in Kerr et al. 2006, Druffel and Williams 1990, cited in Kerr et al. 2006).

Systematic bias in aging accuracy from visual counts of growth bands was determined to be insignificant (Kerr et al. 2006), and it has been established that calcium phosphate hydroxyapatite, the main mineral constituent of the mineralized portion of shark cartilage, is quite conservative, meaning it does not tend to remobilize once it is fixed in cartilage (Campana et al. 2002).

This points up a few of the limitations for this technique. It is most effective for fish that inhabit the mixed layer because the lag for assimilation of $^{14}$C from atmospheric CO$_2$ is much shorter for organisms in the mixed zone (Kerr et al. 2006). It also requires that reference chronologies be established from fish that were born between the early 1950s and the mid-1960s when atmospheric tests of thermonuclear devices were being conducted by the U.S. and Russia. (Kerr et al. 2004; Campana et al. 2008).
Research Objectives

The objective of this study was to develop a method for age determination in the poorly calcified bluntnose sixgill shark. To this end I examined the potential of two traditional histological methods, Von Kossa’s silver nitrate staining, and Alizarin red S staining on non-traditional aging structures. I also evaluated two microanalytical techniques, EDAX and LA-ICP-MS as methods for detecting systematic concentrations of elements and isotopes in non-traditional aging structures.
Methods

Background

The cartilage of the bluntnose sixgill shark is similar in texture to watermelon rind or *Nereocystis* stipe in that it is firm and resilient with a Shore durometer between 45 and 50. It is moist and flexible, has a high percentage of interstitial aqueous hyaluronic acid, and can easily be cut with a knife. What is referred to in the literature as mineralized cartilage is structurally and functionally very similar to bone. Mineralization within the cartilage of the bluntnose sixgill shark is only found in low concentrations. Thus the search for these deposits would likely need to be undertaken on a fine-scale using chemical reagents to enhance their detectability. Vertebral centra from the broadnose sixgill shark had been extensively examined by Ridewood (1921), Ebert (1986) and Macfarlane et al. (2002), and being composed of fibrocartilage seemed too labile to hold a spatially resolvable long-term record. Therefore, I examined other skeletal structures for compositional clues that might indicate age.

Selection of aging structures

After cleaning all of the aging structures and seeing how poorly calcified they were, I surveyed other hard parts with potential to be used as aging structures. My criteria were; 1) they needed to have a large enough cross section to provide for reasonable band separation; 2) they should be composed of hyaline cartilage which is known to calcify in response to stress; and 3) they should be the site of large muscle attachments which would create stress.

The survey dissections revealed that the cartilaginous structures with the greatest cross-sectional areas were jaws, chondrocrania, and pectoral girdles, all of which had greater cross-
sectional areas than thoracic vertebrae. Jaw structures were rejected, as the rate of calcium phosphate hydroxyapatite mobilization for the production of teeth was assumed to be great enough as to make the recording of any long-term information in that region unlikely (Jones and Geen 1976; Kerr et al. 2006.) Chondrocrania were rejected (although one was retained for further study) because of their proximity to the jaws, and because they lacked an obvious longitudinal axis across which to cut sections. This left the pectoral girdle and the mesopterygia, both of which were relatively large and had obvious axes.

The collection of aging structures used in this study were loaned to me by WDFW were initially composed solely of sections of vertebral column from ten adult and sub-adult sharks and 88 whole near-term pups. During the course of the study WDFW came into possession of three more sub-adult sharks that were collected from strandings. These provided the opportunity to obtain other structures for examination. From these last three sharks I extracted pectoral girdles and from the last two I collected mesopterygia.

The pectoral girdle has a very large cross-section, and is a piece of hyaline cartilage that forms the site of attachment for the pectoral fins and several large muscle groups. These include the hypobranchials (composed of the common coracoarcuals, the coracomandibular, the coracohyoids, and the coracobranchials) anteriorly, and the ventral longitudinal bundle posteriorly. The hypobranchials originate at the coracoids bar, and insert on the lower jaw and on the gill arches, performing various functions in connection with opening the mouth, swallowing, and expanding the gill arches (Gilbert 1973.) The ventral longitudinal bundles are long axial muscles, composed of bundles of myomeres, which run the length of the shark on the ventral side. They provide support for the coelomic cavity and are used in locomotion.
Potential structures where large muscle groups attach include the coracoid bar of the pectoral girdle. This area has a roughly T-shaped cross section as a result of being the site of attachment for several large muscle groups that are involved in feeding, undulatory motion of the body trunk, and the control of the pectoral fins. The development of the large cross-sectional area of the pectoral girdle at the coracoid bars (Figure 24) was thought to be a result of the forces imposed by the attachment of both of these large muscle groups.

Given that all of these muscle groups attach at the coracoid bar, it met my criteria as a candidate structure for recording life history events in deposits of calcium or possibly other minerals. I also selected sections of mesopterygia for staining, as this structure lays very close to the exterior of the shark, and thus may be susceptible to banding resulting from seasonal fluctuations of mineral availability in ambient water.

This gave provided pectoral girdle samples from three sharks, mesopterygia samples from two of these three sharks, and neural arches from all three sharks were included as well, to see if the results of MacFarlane et al. (2002) could be reproduced with AgNO$_3$ staining, or confirmed by any of the other methods (Table 12).

*Von Kossa’s AgNO$_3$ stain on neural arch, pectoral girdle & mesopterygium*

Prior to staining cleaned aging structures were bisected at the desired location. The portion to be stained was immersed in the staining solution, removed and rinsed in distilled H$_2$O and sectioned. Successive sections were cut until an area with the desired degree of staining was encountered. The selected section was then soaked in a stop bath of sodium thiosulphate, to halt further development of the stain, and examined under a dissecting microscope for banding.
Sample preparation

Initial attempts were made to clean aging structures using a dermestid beetle colony (Gburski 2005). It soon became evident however, that the beetles were not differentiating between soft tissue and cartilage, and the aging structures were being compromised.

For the remainder of the study I used a 6% sodium hypochlorite solution for its simplicity and effectiveness at removing soft tissue (Radtke’ 1983; Schwartz 1983, Officer et al. 1996, Gburski 2005; Piercy et al. 2006). All aging structures were cleaned by first removing as much of the soft tissue as possible with a sharp knife and scalpel.

Structures were then soaked in a 6% sodium hypochlorite solution (common bleach) for 3 to 15 minutes, and any remaining muscle tissue and fascia were removed with a stiff nylon bristle brush. Structures were cleaned down to the perichondrium, which was left intact (Figure 1).

Excessive soaking in the hypochlorite solution can result in decalcification, so care was taken to keep these soak times to a minimum.

After the soft tissue was removed, aging structures were rinsed in distilled water for several minutes, air dried under a vent-hood, and vacuum-sealed in FoodSaver brand plastic food storage pouches. They were then placed in the freezer to await staining and sectioning.

Fig. 1 A section of vertebral column after cleaning.
Preservation by freezing was chosen over fixation in formaldehyde because formalin breaks down to formic acid which will dissolve calcium deposits (LaMarca 1966).

\textbf{AgNO}_3 \textbf{staining}

The basis for the silver nitrate staining in this study was Von Kossa’s protocols as described by McFarlane et al. (2002) and Goldman (2005,) with a few minor modifications.

Prior to staining the vacuum pouches used to store the aging structures were removed from the freezer and allowed to sit in room temperature (25°C) tap water until they were completely thawed (25-35 minutes). The region to be sampled was then roughly cut with a fillet knife, and the desired half of the bisected aging structure was set aside to await staining.

A 1\% AgNO\textsubscript{3} solution was prepared in a clear glass beaker using distilled water. The beaker was then positioned over a UV light source (Mineralamp, model UVGI-25, at \( \lambda = 315\sim280 \text{ nm} \)) so that the cut face would be exposed to the UVB and thus photo-reduced. Initial soak times in this solution were 1-3 minutes, as outlined by Goldman (2005,) but at this duration of soak no staining was noted. Soak times were gradually increased until some darkening was seen in the region of cortical calcification (15-30 minutes.) Samples were then removed from the AgNO\textsubscript{3} solution, and placed in a 3.5\% acetic acid stop bath to prevent overdevelopment, which can occlude the ultra-structure of the cartilage in the region of cortical calcification as noted by Egerbacher et al. (2006).

Age structures were then rinsed in distilled water and sections cut at approximately 1.5 mm until a region with moderate staining was reached. Two or three sections from this
region were again rinsed in distilled water and placed under the dissecting scope and examined for banding. Once a section had been examined, it was placed on a glass slide, labeled, vacuum-sealed in a plastic pouch and returned to the freezer for future reference.

**Sectioning**

I did not have access to the industry standard, an Isomet slow-speed saw for sectioning, so I initially attempted to use a lapidary to perform this task. It soon became apparent that this technique was not appropriate for sectioning blunt nose sixgill sharks cartilage, as it is so poorly mineralized that a wet-saw was not needed. It also occurred to me that sawing through the aging structure would have a tendency to homogenize the surface, potentially confounding future spatial analyses utilizing microsampling, such as EDAX, and LA-ICP-MS.

Slicing, as opposed to sawing samples, would likely provide a cleaner surface for analysis, and I used a Hobart series 2000, 12” commercial deli meat slicer for this purpose. After moving the platen closer to the blade and truing the blade with a grinder in a tool rest on a South Bend turret lathe, this proved to be an excellent tool for repeatedly cutting serial sections in the 1.0 -3.0 mm thickness range.

**Alizarin Red S Staining**

Alizarin red S has been demonstrated to be quite sensitive to pH. Puchtler et al. (1969) in a comprehensive review of the literature and chemistry regarding Alizarin and Alizarin red S staining for calcium, noted that intense staining of calcium deposits only occurs when the staining is done around pH 12. However, the staining protocols observed by this study were those published by Dr. John A. Kiernan of the Department of
Anatomy and Cell Biology at The University of Western Ontario London, Canada, and available on the Immunohistochemistry world web-site (IHC 2008). In these protocols, we are told to adjust pH of the staining reaction to pH to 4.1~4.3. Other aspects of the staining protocols are substantially similar, so it is difficult to make a determination as to the results of the Alizarin red S staining conducted in this study, without further research.

Sample preparation

Samples were prepared in the same manner as for the silver nitrate staining described above.

Sectioning

Samples of neural arch, mesopterygium, and pectoral girdle were sectioned at approximately 1.0 mm thickness prior to staining using the Hobart series 2000, 12” deli meat slicer.

Alizarin red S staining

A staining solution was made by adding 2 g Alizarin red S powder to 100 ml distilled H2O. Solution pH was then adjusted from pH 2.91 to pH 4.25 by the addition of two drops ammonium hydroxide (NH4OH). Sections were initially soaked in the staining solution for two minutes, but this time was later increased to five minutes as no staining was observed. Excess stain was blotted from the surface of the sections, and sections were then dehydrated by being dipped in a 1:1 solution of xylene and acetone 20 times as per the protocols described at the Immunohistochemistry World on-line site (ihcworld 2008). Sections were then cleared in 100% xylene and allowed to dry prior to mounting on glass slides using an acrylic mounting medium.
Slides were examined under a dissecting scope and then under a compound scope to determine if staining of microscopic structures was occurring. Slides with stained samples were then sealed in FoodSaver brand vacuum bags and placed in the freezer for future reference.

**EDAX microprobe analysis of neural arches**

This study used a Vega TS 5136MM scanning electron microscope fitted with an EDAX energy dispersive X-ray spectrometer, running Genesis Spectrum software, V.5.2.

**Sample preparation**

Neural arch sections were prepared for energy dispersive X-ray spectroscopy by cutting sections approximately 2 mm thick using a scalpel. These were dried through three ethanol baths, 80%, 90%, and 100%. They were then sandwiched between two glass microscope slides and placed under a vent hood to dry for a period of four days (Figure 2). This pressing technique was used to ensure a relatively smooth surface on the dried specimen.

**EDAX analysis**

Dried, sectioned samples of neural arch were then glued to 12 mm aluminum stubs using cyanoacrylate adhesive. The samples were then grounded to the stub with a piece of self-adhesive copper sheet, and placed into a Quorum SC7640, gold-palladium sputter-coater. After sputter-coating with gold, the stubs were fitted to the SEM stage, the stage chamber closed and vented, and the SEM was then operated in EDAX mode according to protocols described in the Western Washington University Geology Department internal
document: SEM/EDAX Basics for Geologists. The samples were surveyed by running a manual grid pattern to search for regions of relatively high calcium concentration.

**LA-ICP-MS Analysis of Neural Arches, Pectoral Girdle, and Mesopterygia**

This study utilized a VG Elemental, PQ Ex Cell laser ablation inductively-coupled mass spectrometer with a UP-266 Macro laser from New Wave Research, calibrated to a NIST 616 glass standard.

**Sample preparation**

Eight aging structures from three sharks were cleaned and sectioned as above for AgNO₃ staining. The samples sections used for analysis were from shark: HG08-901 (2-pectoral girdle, 2-mesopterygium, and 2-neural arch), HG07-104 (2-pectoral girdle, and 2-neural arch), and HG08-02 (2-pectoral girdle, 2-mesopterygium, and 2-neural arch). There was no mesopterygium for HG08-02 in the WDFW collection (Table 13). This resulted in a set of 16 samples to be used for LA-ICP-MS analysis. Sections were cut at 1 mm thick from aging structures, and duplicates were all placed on their slides with the same side down, so that the surfaces being sampled represented sections 1 mm apart in the pre-dried aging structure.
Sample drying

Sections to be analyzed by LA-ICP-MS were run through three ethanol baths of 70%, 80%, and 90%. They were placed between two 50.8 mm X 76.2 mm glass slides and clamped with a series of four medium-sized binder clips (Figure 2). This prevented the sections from curling as they dried and preserved the smooth surface necessary for maintaining laser focus in the LA-ICP-MS.

Elemental analysis

Sample mounts were fabricated to interface with the vacuum chamber of the LA-ICP-MS. These mounts were constructed from Green Diamond brand 50 mm plastic cover lenses (model SP-1G,) attached to indexing stubs milled down to 25 mm O.D. from 1” PVC pipe. Dried samples were mounted to the lenses using cyanoacrylate adhesive.

Operating parameters for the LA-ICP-MS were determined for maximum sensitivity to the group of selected analytes, and were set as follows:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beam (spot size)</td>
<td>20 µm</td>
</tr>
<tr>
<td>Pass speed</td>
<td>1 µm s⁻¹</td>
</tr>
<tr>
<td>Frequency</td>
<td>10 Hz</td>
</tr>
<tr>
<td>Energy density</td>
<td>12-13 J (cm²⁻¹)</td>
</tr>
</tbody>
</table>

Sensitivity was determined to be adequate, as calibration runs were detecting known concentrations of scant isotopes (¹³⁷⁹Ba and ²³⁸U) at concentrations < 1 µg/kg.
Because the length of time required for analysis is directly related to the number of analytes being counted, it was necessary to reduce the list of analytes to those that occurred in differing concentrations in light bands and dark bands. To produce a list of desired analytes, the instrument was programmed to collect data for all possible analytes while a transect was sampled along a light band and again while a transect was sampled along an adjacent dark band. The resulting data sets were compared for analytes whose concentrations varied between light and dark bands in these two transects. Analytes whose concentrations were similar in both transects were removed from the list of analytes in subsequent transects. This resulted in data collection for the following list of analytes: $^{24}\text{Mg}$, $^{27}\text{Al}$, $^{31}\text{P}$, $^{43}\text{Ca}$, $^{44}\text{Ca}$, $^{55.9}\text{Mn}$, $^{87.9}\text{Sr}$, $^{106.9}\text{Ag}$, $^{108.9}\text{Ag}$, $^{117.9}\text{Sn}$, $^{137.9}\text{Ba}$, $^{202}\text{Hg}$, $^{208}\text{Pb}$, $^{238}\text{U}$.

Data Analysis

Output from the LA-ICP-MS is in integrated counts per second (ICPS). Because of the extreme sensitivity of this instrument and the fine-scale compositional variability of the cartilage being sampled, the output appeared quite noisy. However, while this excess information is not noise in the traditional sense, meaning it does convey real information about the composition of the sample, it is not helpful in identifying seasonally mediated changes in cartilage composition. Therefore a smoothing and filtering technique developed by Sinclair et al. (1998) was used to remove these fine-scale variations in the data (See Figure 3 for a comparison of smoothed vs. unsmoothed data). The smoothing and filtering technique first applies an 11-point running median to the data and then applies an 11-point running average to the median transformed data (Sinclair et al. 1998).
This was effective at removing micro-deposit signals from the data and retaining signal from regions of isotopic concentration that were greater than the background. In an attempt to follow the methods used by Hale et al. (2006) output was initially normalized to an isotope that showed very little fluctuation over any of the initial test transects. In this case that isotope was $^{27}$Al. Normalization is often used with LA-ICP-MS data and is thought to be necessary to remove artifacts that result from variations in laser ablation efficiency and topographical effects. These artifacts are easily identified, as they will occur across all species in the analysis at rates that are relative to local concentrations. However, after searching the data for these characteristic concentration spikes in $^{27}$Al and seeing nothing that could be attributed to either variation in laser efficiency, or topographical effects, normalization was deemed unnecessary, and abandoned.

Two methods of analysis were used to determine the usefulness of LA-ICP-MS for predicting age from elemental analysis of cartilage. One method, initially used by Hale et al. (2006) was to superimposed a smoothed plot of isotopic intensity on a photomicrograph of the aging structure at the site of the ablated transect. This method allows a quick check to see if peaks in isotopic abundance coincide with visually detectable banding in the aging structure. The other method was to look for correlations between isotopes within transects to see if there were pairs of isotopes that co-varied consistently, and then to look for patterns in the concentrations of these isotopes across the ablated transects.
Visual analysis

The transformed data for each transect were plotted against time, and plots were superimposed on photomicrographs of the transect from which the data were derived. The resulting compound images were examined for correspondence between concentration peaks and visible banding for each of the isotopes of interest (Figures 6 - 19).

Multiple transects from the same structure within the same shark were visually compared to see if banding was uniform within the structure at different locations. Transects from different structures within the same shark were compared for homogeneity, and transects from the same structure across different sharks were compared to see if the number of bands was correlated with structure, rather than age.

Correlation analysis

The numerical output of the LA-ICP-MS was also subjected to a series of linear correlation analyses as an exploratory method in an attempt to discover patterns of co-occurrence of analytes. The initial correlations examined were for all fourteen isotopes from each transect. Examination of the initial correlation plots showed a strong break occurring between pairs of analytes with correlation $R^2$ values above 0.90, and those with correlation $R^2$ values below 0.78. Thus, I decided to conduct additional analysis with that group of analytes with strong linear correlation (those with correlation $R^2$ values greater than 0.90). Those analytes for which there were few strong correlations ($R^2 < 0.90$) were removed from future analyses (Table 9).
Correlation matrices were then plotted using the remaining analytes: $^{24}\text{Mg}$, $^{43}\text{Ca}$, $^{44}\text{Ca}$, and $^{88}\text{Sr}$ for all transects across samples of the same structure from the same shark (Tables 1 - 8). In this last set of correlations I looked for similarities in patterns of isotopic concentration at different transects within the same aging structure. Once identified, pairs of plots for co-occurring analytes were superimposed on photomicrographs of the aging structure for visual confirmation of co-occurrence, and to see if there were correlations between visual bands and isotopic concentrations (Figures 4 & 5).
Results

Von Kossa's AgNO₃ stain on pectoral girdle & mesopterygia

The first section stained with silver nitrate was from the pectoral girdle of sample HG07-104, a 289.6 cm (TL) male. This sample was sectioned at the coracoid bar and stained according to the protocols described in the methods section of this paper. Initial inspection under a dissecting scope and a compound scope revealed no banding in any of the samples stained that day. The stained sections were placed on glass slides, sealed in vacuum pouches, and frozen. Three days later, I found that banding was visible on one of the stained pieces of pectoral girdle. Depending on where the count was done on this section there were between 20 and 26 bands (Figure 22). The patterns of bands were similar to those often seen on the otoliths of boney fishes in that they were relatively fine, followed the outside contour of the aging structure, and contained some bands that became discontinuous, like the features known as “checks” in otoliths. However, after that initial success, I stained another 32 samples of pectoral girdle and mesopterygium before once again discovering banding, this time on a section of mesopterygium from sample HG08-02, a 207.3 cm (TL) male. The mesopterygium from this shark clearly showed eight bands (Figure 23) and it was not necessary to place it in the freezer before the banding became apparent.

No discernible banding was observed in any of the other 32 samples stained.

Alizarin red S staining

No staining was observed in any of the sixteen stains attempted. In general, the process produced a uniform, featureless pink hue across the entirety of the samples being stained.
EDAX microprobe analysis of neural arches

Two neural arches were examined with the EDAX microprobe. In three hours of manually scanning these samples on a grid pattern one particle of calcium was observed as a discrete, cubical structure containing high concentrations of carbon, oxygen, and calcium (Figures 20 and 21). This single deposit of calcium may have become lodged in the neural arch while it was being sectioned on the lapidary saw. No other deposits of calcium were observed, and I abandoned the EDAX as a screening tool for this study.

LA-ICP-MS analysis of pectoral girdles, mesopterygia, and neural arches

The output of the LA-ICP-MS was subjected to both a correlation analysis, and a visual analysis.

Correlation analysis

The numerical output from the LA-ICP-MS was subjected to a two-part correlation analysis. The first analysis used a 14 X 14 matrix to identify correlations among all analytes at each transect surveyed. The second analysis looked for correlations among selected analytes, within all transects on samples taken from the same age structures (Tables 2 - 9).

In the first analysis of 33 transects, each of which was configured to detect 14 analytes, 89 out of 3234 correlations had $R^2$ values > 0.90. The analytes with the greatest number of strong correlations were $^{24}\text{Mg}$, $^{43}\text{Ca}$, $^{44}\text{Ca}$, and $^{88}\text{Sr}$ (Table 9). These four analytes formed correlations with $R^2$ values above 0.90 as $^{3}\text{Ca}/^{88}\text{Sr}$ (79.8% of all correlations with $R^2$>0.90), $^{88}\text{Sr}/^{24}\text{Mg}$ (72.7% of all correlations with $R^2$>0.90), and $^{24}\text{Mg}/^{8}\text{Ca}$ (63.4% of all correlations with $R^2$>0.90). The next highest correlation with an $R^2$ value above 0.90 was for $^{3}\text{Ca}/^{31}\text{P}$
(12.1% of all correlations with $R^2 > 0.90$). All of these selected correlations occurred in six of the eight aging structures used in this study; three pectoral girdles, two mesopterygia, and one neural arch.

In the second analysis, only $^{24}\text{Mg}$, $^{43}\text{Ca}$, $^{44}\text{Ca}$, and $^{88}\text{Sr}$ were examined. These correlation matrices were constructed using data from all of the transects that were sampled from each aging structure. In most cases this was four transects, two each from two replicate samples. In one of the initial samples, a second transect was not sampled on one of the samples.
Table 1. Percent of occurrence for correlations with $R^2$ values > 0.90. Shaded rows contain analytes that were retained for further analysis.

<table>
<thead>
<tr>
<th>Specie</th>
<th>Specie</th>
<th>Corr Occurances Of Total</th>
<th>% Occ.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{40}$Ca $^{88}$Sr</td>
<td>Pos</td>
<td>26 / 33</td>
<td>78.79%</td>
</tr>
<tr>
<td>$^{88}$Sr</td>
<td>$^{40}$Mg</td>
<td>Pos</td>
<td>24 / 33</td>
</tr>
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<tr>
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Visual analysis

This visual method of analysis could potentially produce a set of 462 compound images. However, there was a lack of visually discernible banding in many of the samples, and the quality of the photomicrographs produced by the camera in the LA-ICP-MS stage chamber was poor enough to make some of the fainter bands impossible to see in some images. In an attempt to produce viable images of the transects where photo quality was an issue, I re-imaged several of the sample sections under a phase contrast compound microscope. However, as many of the samples had developed cracks and curled away from the slides by the time I realize they needed to be re-photographed. This reduced the number of useful samples to the 252 compound images for which there were reasonably clear photographs. These images were evaluated for visible banding, and for correlations between plots of isotopic intensity and band structures. From this analysis, I created three groups of samples: those with no apparent correlations, those where slight visual correlations were seen between isotopic concentration and structure in the cartilage, and those with stronger visual correlations between isotopic concentration and band structures in the cartilage.

This last category contained 84 compound images from six transects across five different sample sections. Pairs of isotopes showing correlations with R² values > 0.90, were ²⁴Mg, ¹⁰⁷Ag, and ¹⁰⁹Ag. For these three isotopes, 66.7% of the scans for which pictures with good visual banding existed showed correlations between the isotope and the visual banding. This was followed by ⁸⁸Sr, for which 50% of these correlations existed. The rest of these correlations were 33% per isotope or less (Tables 10 and 11).
Discussion

Selection of aging structures

The selection of an aging structure for most elasmobranchs has not been a challenge. The majority of these fish produce band-pairs of calcium-based mineralization in their vertebral centra, and the primary challenge to the aging scientist has been to make these bands apparent. In the case of the bluntnosed sixgill shark, and several other poorly calcified sharks, the vertebral centrum is not an appropriate aging structure, as it is composed of fibrocartilage, and completely uncalcified. Therefore, one of the primary challenges of this study has been the identification of alternative aging structures. MacFarlane et al. (2002) took this approach in their work on the bluntnose sixgill shark, and concluded that the neural arches were a better potential aging structure than vertebral centra. Being unable to reproduce the work of MacFarlane et al. (2002), it was necessary to look for yet another candidate structure, and this is what drove me to consider the pectoral girdle, and the mesopterygium.

Sectioning of aging structures

The industry standard for sectioning aging structures from elasmobranchs has been the Isomet slow speed saw. This saw uses two diamond blades separated by a spacer that is the thickness of the desired section, and can be operated either wet, or dry. Early attempts in this study to use a diamond blade resulted in contamination, and surface homogenization. This was seen as a problem that was intrinsic to a sectioning technique that grinds through the materials, as opposed to slicing through it. My solution was to use a deli-meat slicer, and this was quite satisfactory for use on the relatively un-calcified cartilage of the bluntnose sixgill
shark. The stage of these slicers is controlled by a screw advance that is similar to the micrometer adjustment on the Isomet saw, though the adjustment mechanism is somewhat coarser than the Isomet. Minor adjustments were made to bring the platen closer to the blade, and the blade angle was also reduced from the stock 15 degrees to a finer 12.5 degrees. With these modifications, the Hobart series 2000 meat slicer proved to be a reliable tool for cutting repeatable sections in the 1 – 3 mm thickness range.

Calcification processes

Early studies of shark cartilage for use as aging structures made the assumption that calcification processes in elasmobranchs were the same as those in mammals (Egerbacher et al. 2006). Calcification of cartilage in mammalian infants occurs as an ontogenic process, where metaplasia, the replacement of one cell type with a tougher cell type in response to stress, transforms embryonic cartilage into bone. It also occurs in older individuals as a degenerative process mediated by stress and trauma, and the onset of arthritis (Ridewood 1921; Applegate 1967; Egerbacher et al. 2006). Calcification in the extra-cellular matrix of mammalian cartilage is a natural part of the aging process and precedes, and initiates the destruction of the cartilage (Egerbacher et al. 2006). Consequently the extent of metaplasia and the degree of calcification in mammalian skeletal remains is often used to determine age at time of death.

In elasmobranchs however, it is thought that calcification of the cartilaginous matrix proceeds not as a dystrophic process where the parent cell is destroyed and then replaced with a new type of cell, but as “direct metaplasia” where normal, healthy adult cells convert from one cellular type to another, without the destruction of the parent cell occurring first.
(Egerbacher et al. 2006). This direct metaplasia was observed in a study published as The Mineralization of Hyaline Cartilage in the Small-Spotted Dogfish, Scyliorhinus canicula L. by Egerbacher et al. (2006), in which the region where direct metaplasia occurs, the “rindenverkalkung”, or region of cortical mineralization, is described as follows:

“Cortical mineralisation: In tissue sections, a 15 to 45 μm wide band of morphologically altered cartilage matrix was identified equivalent to the dense lines observed in roentgenograms (Fig. 1b). This layer of altered matrix was spread out parallel to the cartilage surface, being constantly separated from the perichondrium by a 20 to 30 μm thick layer of non-mineralised cartilage. The external half of this interposed layer of cartilage, just beneath the perichondrium, appeared as subperichondrial cartilage with flattened chondrocytes or chondroblasts; the internal half, next to the mineralised zone, represented inconspicuous hyaline cartilage (Egerbacher et al. 2006).”

This description correlates very well with the banding patterns that I was able to develop using the silver nitrate staining technique on both pectoral girdle and mesopterygium (Figures 22 & 23), with the exception of scale. In the silver nitrate-stained sections of bluntnose sixgill shark cartilage, the width of this interposed layer of cartilage, just beneath the perichondrium was in the range of 0.80 to 1.2 mm. However, the size disparity between the small-spotted dogfish in the study of Egerbacher et al. (29-35 cm), and the sixgill sharks in this study (207-290cm) may be reflected in banding pattern width.

Silver nitrate staining

It is unclear why this method was so hard to reproduce. The obvious variables to control are solution concentration, and soak time, and every attempt was made to be precise in measuring these quantities. The one variable that I did not control was temperature. During the period when I was conducting staining sessions, my lab ambient temperature was routinely above 25°C, and this may have caused the rates of reactions in the staining process to accelerate to the point where they could not be controlled.
The silver nitrate staining did produce more bands on the larger of the two fish which does not counter indicate silver nitrate staining as a potential aging method.

While I was not able to consistently reproduce these results, they did serve to identify locations within the pectoral girdle and mesopterygium with the potential to form age-related banding. Knowledge of these locations later informed my selection of transect sites for analyses performed with the LA-ICP-MS.

Alizarin red S staining

Staining with Alizarin red S produced no visible banding in any of the three structures tested in this study. There are two obvious explanations for this: either the calcium in sixgill shark cartilage exists in very diffuse concentrations, or it occurs as primary calcium phosphate, which as noted by Puchtler et al. (1968) is not demonstrated by Alizarin red S.

Microanalytical techniques

EDAX has previously been applied to the problem of shark aging by Jones and Geen (1977), and by Cailliet and Radke (1987). Both of these studies found EDAX to predict age estimates that were verified by those derived through visual band counts in the spiny dogfish, Squalus acanthias (Jones and Geen 1976), the grey reef shark, Carcharhinus amblyrhynchos, and the common thresher shark, Alopias vulpinus (Cailliet and Radtke 1987). All of these are species for which visual band counts have been validated as an aging method.

The fact that the EDAX did not detect more than a single deposit of calcium in neural arch samples from the bluntnose sixgill shark is evidence that calcium deposits within the neural
arches of the bluntnose sixgill are absent, or below the method detection limit of the instrument.

The single detection of calcium encountered in scanning neural arches with the EDAX may have been a crystal of some compound containing calcium, carbon, and oxygen that was deposited onto the sample while it was being sectioned on the lapidary saw.

LA-ICP-MS applied to sections of pectoral girdle and mesopterygium from the bluntnose sixgill shark has shown that there are regular oscillations in elemental concentration, and that the elements involved in these oscillations are regularly, and systematically correlated. To my knowledge these structures has not previously been used in aging studies, and this study has shown that they hold promise for use as age structures in studies of poorly calcified species of elasmobranchs. The crux here, as with any newly proposed aging technique, is in devising a method to validate ages predicted using the new method.

Visual analysis

Two obvious problems exist with visual analysis; the first is that trying to correlate spatially resolved isotopic signals with visually discernable banding on a species for which visually discernable banding is rare necessarily reduced the sample size and, consequently, the power of the analysis. The other problem is the poor image quality of photomicrographs produced by the camera in the LA-ICP-MS stage chamber. Although this camera is intended to record information on the location of transects relative to other major topographical features, its lack of resolution was an unforeseen problem.

The possibility of deriving age predictions from any kind of analysis relies on the ability to procure enough validated age information to evaluate the rate of agreement between
validated ages and ages predicted by the proposed method of prediction. It also requires that an objective method for determining which of the peaks in the analysis are significant. Hale et al. (2006) used Microcal Softwares, Origin™ program configured such that “peaks were identified using prescribed parameters within the software that included a minimum height of 3% of the total amplitude of the data in the range and a minimum width of 3% of the total number of points in the data range.” These parameters worked fairly well when the predicted year counts were summed across replicates. In that study the visual banding was pronounced enough that there was little ambiguity as to the correlations in fish less than five years old, but the correlations were less obvious in older fish. At the time of that study, visual band counts had not been validated as an aging method for Urobatis halleri, but two years later Hale and Lowe (2008) did validate visual band counts using captive rearing and oxytetracycline (OTC) injection, a method that leaves a permanent mark in the cartilage shortly after injection. I explored methods of graphically interpreting the peaks but was not able to eliminate some of the subjective aspects of interpretation.

Correlation analysis

The second type of analysis applied to the LA-ICP-MS output involved a series of correlation matrices used as an exploratory tool to look for patterns in the data. The significance of identifying correlations between analytes in an aging structure is that it indicates there is a real signal. Co-occurring concentrations of analytes are likely related to either metabolic oscillations over time, or differential availability of an analyte in the environment. There were very strong correlations for most of the selected analytes in every transect, but only three strong correlations occurred in different transects of the same
structure. The fact that these correlation are abundant within a given transect indicates that the metabolic uptake trajectory is similar for the co-occurring analytes. The lack of correlations across different transects on the same aging structure is also noteworthy, but not surprising. This is because transects vary in length, and also because start and stop points for transects were identified visually, so that two transects sampled on two different sections of the same aging structure probably did not start and stop at the same point. Furthermore, the size of an aging structure changes along its length, so the same features on two transects from different sections of the same structure will occur at different scales. This has the potential to confound correlations that might have existed across different transects on the same aging structure.
Conclusion

The problem of estimating age in the bluntnose sixgill sharks has been tackled by few researchers over the years, but the problems associate with this undertaking are well understood. Traditional aging methods applied to elasmobranchs rely on the ability to identify, enhance, and enumerate bands of differential mineralization within cartilaginous structures, but the cartilage of the bluntnose sixgill sharks is so poorly mineralized, that these bands, if they exist at all are difficult to discern.

The ultimate goal of any fish age and growth study is to be able to build a validated length/age regression that can readily be used to gain an understanding of the population-age structure of a stock from easily collected length data. Among the myriad problems associated with developing such a regression for a fish like the bluntnose sixgill shark are: difficulty in finding and enumerating a signal that can be used as a proxy for age in years, verifying that the signal can be reliably found and repeatedly enumerated within acceptable error limits, and devising a method that allows the age predicted from the signal to be validated. Like a three-legged stool, all three of these elements must be in place before we can comfortably lean on any conclusions. The current study may have moved closer to the solution of the first two of these problems, but until the difficulties associated with validation are overcome, these efforts are merely suppositional.

This study has accomplished three things relative to its objective; first, through the use of Von Kossa’s silver nitrate technique it has identified a region on two skeletal structures in the bluntnose sixgill shark where banding does form. Second, through the use of LA-ICP-MS it
has identified a suite of isotopes that co-occur in these regions, show systematic fluctuations across these regions, and that have potential as age markers for micro-analysis. And third, it has identified two methods, Von Kossa’s silver nitrate technique, and LA-ICP-MS, that have potential utility in enumerating those bands.

Of the histological methods employed in this study, only Von Kossa’s silver nitrate staining technique showed promise, while the use of Alizarin red S was not supported as an aging tool for the bluntnose sixgill shark. My limited success at replicating stains with Von Kossa’s technique at once indicated its promise, even while the reasons for its limited success remained a mystery. Others have reported these problems as well (MacFarlane et al. 2002), but if it can be made to work occasionally, I believe that a better understanding of the process involved will eventually lead to its becoming repeatable. Because of the relative ease with which staining can be accomplished and the small amount of preparation required, it is worth taking the time to try and remove the barriers to repeatability with this technique, as it may eventually have application in aging poorly calcified sharks.

Regarding the microanalytical techniques, LA-ICP-MS may also eventually be useful in the aging of poorly calcified sharks, but there will need to be another aging method developed to independently validate the results obtained through LA-ICP-MS analysis. LA-ICP-MS is still somewhat exotic, but it is becoming more available with time. Its extreme sensitivity and ability to spatially resolve relative concentrations of elements across transects combine to make it a likely candidate for success in aging poorly calcified sharks.

The need for a method to validate any age predicted by LA-ICP-MS, Von Kossa’s silver nitrate staining technique, or indeed any new technique, cannot be overstated. Banding has
correlated well with age in so many elasmobranchs, that the temptation exists to bypass validation. However, knowing of species like the Pacific Angel shark, *Squatina californica*, and the little skate, *Leucoraja erinacea*, that produce banding that does not predict well for age (Goldman 2005, Natanson and Cailliet 1990), informs us that we cannot afford to assume that all observed banding is appropriate for the derivation of aging estimates.
Suggestions for Future Research

*Bomb carbon dating*

A study to look at the potential for bomb-carbon analysis to establish absolute age in the bluntnose sixgill shark could provide us with a valuable tool for validating other aging methods like LA-ICP-MS, or visual analysis. It does not require resources beyond those available at most research universities, and would make a good graduate study. The crux would probably lay in identifying, or establishing a suitable reference chronology for a region co-located with the shark sampling area.

*Aspartic acid racemization*

Aspartic acid is an amino acid that naturally occurs in equal proportions of two (D and L) enantiomeric forms. However, in living organisms, they are produced, exclusively as the L enantiomer, and this exclusivity is maintained metabolically as long as the organism is alive (George et al. 1999). After death, the conversion of the L enantiomer to the D enantiomer proceeds at a known, temperature-dependant rate. Tooth enamel and the eye lenses are both metabolically inactive, and thus the ratio of L to D enantiomers in them is proceeding toward equilibrium from the time of birth. Thus if the L/D ratio at birth is known, and the average tooth or lens temperature since birth can be ascertained, this rate of racemization can be used as an aging technique.

While this technique was applied to bowhead whales in 1999 (George et al.), I have read nothing of its application to the problem of aging sharks, for which it may have some utility.
Non-lethal sampling techniques.

If a transect can be identified within the cartilage of the bluntnose sixgill shark where age information can be reliably found, it may be possible to use a large Jamshidi biopsy needle to remove a sample for aging without having to sacrifice the animal. Aside from the obvious advantage of preserving the animal, it may also have utility for repeatedly sampling the same individual over time, thus enabling us to build a growth time-series, and gain an understanding of how these aging structures change with age and growth.
Figure 3. Plot of raw (above) and smoothed (below) spatial isotope signal from the LA-ICP-MS output superimposed on a photomicrograph of the ablated transect. Data is from shark HG08-901, transect HG13B. Structure is pectoral girdle.
Figure 4. Visual confirmation of the strong correlation between the smoothed spatial isotope signals for $^{24}\text{Mg}$ and $^{44}\text{Ca}$. 

Transect HG8B from the pectoral girdle of HG08-02

Visual check of correlation analysis performed in JMP 8.0.2.2

$R^2 = 0.988$
Transect HG8B from the pectoral girdle of HG08-02

Visual check of correlation analysis performed in JMP 8.0.2.2

\[ R^2 = 0.989 \]

**Figure 5.** Visual confirmation of the strong correlation between the smoothed spatial isotope signals for $^{88}$Sr and $^{24}$Mg.
Figure 6. Smoothed spatial isotope signal for $^{24}\text{Mg}$, superimposed on a photomicrograph of the transect from which it was obtained. Data are from shark HG08-901, transect HG13B. Age structure is the pectoral girdle.
Figure 7. Smoothed spatial isotope signal for $^{27}$Al, superimposed on a photomicrograph of the transect from which it was obtained. Data are from shark HG08-901, transect HG13B. Age structure is the pectoral girdle.
Figure 8. Smoothed spatial isotope signal for $^{31}$P, superimposed on a photomicrograph of the transect from which it was obtained. Data are from shark HG08-901, transect HG13B. Age structure is the pectoral girdle.
**Figure 9.** Smoothed spatial isotope signal for $^{43}\text{Ca}$, superimposed on a photomicrograph of the transect from which it was obtained. Data are from shark HG08-901, transect HG13B. Age structure is the pectoral girdle.
Figure 10. Smoothed spatial isotope signal for $^{44}$Ca, superimposed on a photomicrograph of the transect from which it was obtained. Data are from shark HG08-901, transect HG13B. Age structure is the pectoral girdle.
Figure 11. Smoothed spatial isotope signal for $^{56}\text{Fe}$, superimposed on a photomicrograph of the transect from which it was obtained. Data are from shark HG08-901, transect HG13B. Age structure is the pectoral girdle.
Figure 12. Smoothed spatial isotope signal for $^{88}$Sr, superimposed on a photomicrograph of the transect from which it was obtained. Data are from shark HG08-901, transect HG13B. Age structure is the pectoral girdle.
**Figure 13.** Smoothed spatial isotope signal for $^{107}$Ag, superimposed on a photomicrograph of the transect from which it was obtained. Data are from shark HG08-901, transect HG13B. Age structure is the pectoral girdle.
**Figure 14.** Smoothed spatial isotope signal for $^{109}$Ag, superimposed on a photomicrograph of the transect from which it was obtained. Data are from shark HG08-901, transect HG13B. Age structure is the pectoral girdle.
Figure 15. Smoothed spatial isotope signal for $^{118}$Sn, superimposed on a photomicrograph of the transect from which it was obtained. Data are from shark HG08-901, transect HG13B. Age structure is the pectoral girdle.
Figure 16. Smoothed spatial isotope signal for $^{138}$Ba, superimposed on a photomicrograph of the transect from which it was obtained. Data are from shark HG08-901, transect HG13B. Age structure is the pectoral girdle.
Figure 17. Smoothed spatial isotope signal for $^{202}\text{Hg}$, superimposed on a photomicrograph of the transect from which it was obtained. Data are from shark HG08-901, transect HG13B. Age structure is the pectoral girdle.
Figure 18. Smoothed spatial isotope signal for $^{208}\text{Pb}$, superimposed on a photomicrograph of the transect from which it was obtained. Data are from shark HG08-901, transect HG13B. Age structure is the pectoral girdle.
Figure 19. Smoothed spatial isotope signal for $^{238}$U, superimposed on a photomicrograph of the transect from which it was obtained. Data are from shark HG08-901, transect HG13B. Age structure is the pectoral girdle.
Figure 20. Display on the EDAX microprobe showing the location on the neural arch being analyzed.
Figure 21. Display on the EDAX microprobe showing the calcium spike with the calcareous structure centered in the red box at the upper left.
Figure 22. Section of pectoral girdle stained with silver nitrate to reveal sub-perichondrial banding. Sample is from a 289.6 cm male recovered at Gig Harbor, WA.
Figure 23. Section of mesopterygium stained with silver nitrate to reveal eight sub-perichondrial bands. Sample is from a 207 cm male recovered at Bremerton, WA.
**Figure 24.** A section of the pectoral girdle at the coracoid bar. Sample is from HG07-104, a 289.6 cm male recovered at Gig Harbor, WA.
Table 2. Correlation matrix composed from three transects across two different sections of pectoral girdle from the same shark (HG07-104). Yellowed boxes show correlations with $R^2 > 0.90$ for different isotopes within the same transect (suffix indicates transect, 1A, 1B, 2A, etc…). Red X’s indicate the association of $^{43}$Ca with $^{44}$Ca from the same transects, which is expected and non-remarkable.
Table 3. Correlation matrix composed from four transects; two each across two different sections of neural arch from the same shark (HG07-104). Yellowed boxes show correlations with $R^2 > 0.90$ for different isotopes within the same transect (suffix indicates transect, 5A, 5B, etc...). Red X’s indicate the association of $^{43}$Ca with $^{44}$Ca from the same transects, which is expected and non-remarkable.
Table 4. Correlation matrix composed from four transects; two each across two different sections of pectoral girdle from the same shark (HG08-02). Yellowed boxes show correlations with $R^2 > 0.90$ for different isotopes within the same transect (suffix indicates transect, 7A, 7B, etc…). Red X’s indicate the association of $^{43}$Ca with $^{44}$Ca from the same transects, which is expected and non-remarkable.
Table 5. Correlation matrix composed from four transects; two each across two different sections of mesopterygium from the same shark (HG08-02). Yellowed boxes show correlations with $R^2 > 0.90$ for different isotopes within the same transect (suffix indicates transect, 5A, 5B, etc…). Red X’s indicate the association of $^{43}\text{Ca}$ with $^{44}\text{Ca}$ from the same transects, which is expected and non-remarkable.
Table 6. Correlation matrix composed from four transects; two each across two different sections of neural arch from the same shark (HG08-02). Yellowed boxes show correlations with $R^2 > 0.90$ for different isotopes within the same transect (suffix indicates transect, 11A, 11B, etc…). Red X’s indicate the association of $^{43}\text{Ca}$ with $^{44}\text{Ca}$ from the same transects, which is expected and non-remarkable.
Table 7. Correlation matrix composed from four transects; two each across two different sections of pectoral girdle from the same shark (HG08-901). Yellowed boxes show correlations with $R^2 > 0.90$ for different isotopes within the same transect (suffix indicates transect, 13A, 14B, etc…). Red X’s indicate the association of $^{43}\text{Ca}$ with $^{44}\text{Ca}$ from the same transects, which is expected and non-remarkable.
Table 8. Correlation matrix composed from three transects of mesopterygium from the same shark (HG08-901). Yellowed boxes show correlations with $R^2 > 0.90$ for different isotopes within the same transect (suffix indicates transect, 13A, 14B, etc…). Red X’s indicate the association of $^{43}\text{Ca}$ with $^{44}\text{Ca}$ from the same transects, which is expected and non-remarkable.
**Table 9.** Correlation matrix composed from four transects; two each across two different sections of neural arch from the same shark (HG08-901). Yellowed boxes show correlations with $R^2 > 0.90$ for different isotopes within the same transect (suffix indicates transect, 11A, 11B, etc…). Red X’s indicate the association of $^{43}$Ca with $^{44}$Ca from the same transects, which is expected and non-remarkable.
Table 10. Matrix for quantifying subjective analysis of transects for which good visual correlations existed, and the isotopes that were best at predicting those correlations. Highlighted transects indicate good visual correlations for which picture quality was adequate for further analysis.

<table>
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<th>Photo quality</th>
<th>Visual band/peak correlations</th>
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</tr>
<tr>
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<tr>
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<td>24Mg, 31P 43Ca, 44Ca, 56Fe, 88Sr, 107Ag, 109Ag, 118Sn, 138Ba, 202Hg, 208Pb</td>
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<tr>
<td>HG 14</td>
<td>B HG '08-901</td>
<td>Pectoral girdle</td>
<td>Fair, faint banding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HG 15</td>
<td>A HG '08-901</td>
<td>Mesopterygium</td>
<td>Fair, no banding, but some structure</td>
<td></td>
<td>24Mg, 31P 43Ca, 44Ca, 88Sr, 107Ag, 109Ag, 118Sn, 202Hg</td>
</tr>
<tr>
<td>HG 16</td>
<td>A HG '08-901</td>
<td>Mesopterygium</td>
<td>Fair, faint banding</td>
<td>31P, 56Fe, 107Ag, 109Ag, 118Sn, 138Ba, 208Pb</td>
<td></td>
</tr>
</tbody>
</table>
**Table 11.** Breakdown of all analytes used in initial analysis and the percentage of strong correlations seen within them.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Percent Involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>24Mg</td>
<td>67%</td>
</tr>
<tr>
<td>107Ag</td>
<td>67%</td>
</tr>
<tr>
<td>109Ag</td>
<td>67%</td>
</tr>
<tr>
<td>88Sr</td>
<td>50%</td>
</tr>
<tr>
<td>31P</td>
<td>33%</td>
</tr>
<tr>
<td>43Ca</td>
<td>33%</td>
</tr>
<tr>
<td>44Ca</td>
<td>33%</td>
</tr>
<tr>
<td>56Fe</td>
<td>33%</td>
</tr>
<tr>
<td>118Sn</td>
<td>33%</td>
</tr>
<tr>
<td>138Ba</td>
<td>33%</td>
</tr>
<tr>
<td>208Pb</td>
<td>33%</td>
</tr>
<tr>
<td>202Hg</td>
<td>17%</td>
</tr>
</tbody>
</table>
Table 12. Breakdown showing aging structures and the percentage of strong correlations in analytes that were seen on them.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Percent Involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pectoral girdle</td>
<td>50.00%</td>
</tr>
<tr>
<td>Mesopterygium</td>
<td>33.30%</td>
</tr>
<tr>
<td>Neural arch</td>
<td>16.70%</td>
</tr>
<tr>
<td>Shark ID</td>
<td>Sex</td>
</tr>
<tr>
<td>----------</td>
<td>---------</td>
</tr>
<tr>
<td>HG 07-104</td>
<td>Male</td>
</tr>
<tr>
<td>HG 07-104</td>
<td>Male</td>
</tr>
<tr>
<td>HG 07-104</td>
<td>Male</td>
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<tr>
<td>HG 07-104</td>
<td>Male</td>
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<td>Male</td>
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</tr>
<tr>
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</tr>
<tr>
<td>HG 08-02</td>
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<tr>
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<tr>
<td>HG 08-02</td>
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<td>Unknown</td>
</tr>
<tr>
<td>HG 08-0901</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

**Table 13.** Breakdown showing the relationship of sample sections and individual transects to fish used in this study.
References Cited


Campana, S.E., J.M. Casselman, and C.M. Jones. 2008. Bomb radiocarbon chronologies in the arctic, with implications for the age validation of lake trout (Salvelinus namaycush) and other arctic species. Canadian Journal of Fisheries and Aquatic Science 65:733-743.


Gburski, C.M. 2005. Aging procedures for big skate (Raja binoculata), longnose skate (Raja rhina), Alaska skate (Bathyraja parmifera), Aleutian skate (Bathyraja aleutica) and Bering skate (Bathyraja interrupta) at the Alaska Fisheries Science Center. Age and Growth Program, internal document, Alaska Fisheries Science Center, Seattle.


