

**ELEMENTAL ANALYSIS OF OTOLITHS AND EYE LENSES IN THE
ASSESSMENT OF STELLER SEA LION DIETS**

by

Joy Ferenbaugh, B.S., M.S.

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Dr. Sandra Diamond

Dr. Richard Strauss

Dr. Nancy McIntyre

Dr. Kevin Pope

Dr. George Cobb

John Borrelli
Dean of the Graduate School

May 2007

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ABSTRACT

Steller sea lions (*Eumetopias jubatus*) have historically ranged along the North Pacific Rim from the coast of California to Japan, but the population has dramatically declined since the 1960s. Research has indicated that nutritional stress is likely to be the main cause of the decline. Scat analysis is the preferred technique for dietary analysis of Steller sea lions, and fish otoliths and eye lenses are routinely recovered from pinniped scat. Fisheries scientists use elemental analysis of otoliths and eye lenses to provide information on fish biology, but marine mammalogists have not incorporated this technique to study prey fish or foraging behavior.

In this dissertation, I examined the use of elemental analysis of prey fish otoliths and eye lenses in dietary studies for Steller sea lions. I first examined the use of otoliths as indicators of total body burdens of metal contaminants in the fish. Then, I assessed the effects of Steller sea lion digestion on the microchemistry of otoliths. Third, I examined the microchemistry of fish eye lenses, the effects of digestion on eye lenses, and their potential use in dietary analysis.

Concentrations of some metals, such as zinc and barium, in undigested otoliths are significantly correlated with concentrations found in homogenized tissues, but several factors affect this relationship, such as fish species, sampling site on the otolith, and the specific metal being analyzed. The degradation of an otolith in the sea lion digestive tract is also likely to affect correlations between otolith and tissue metal concentrations.

Steller sea lion digestion has significant effects on otolith microchemistry.

These effects do not prohibit the use of digested otoliths in species determination for dietary analysis, but they may preclude using otoliths recovered from sea lion scat for fish stock separation, determination of foraging locations, and fish life history analyses.

Eye lenses appear to be resistant to sea lion digestion, and they form sequential growth layers that can be used to age fish. The fibrous structure of the layers may inhibit symmetrical distributions across the lens for some elements, but the distinct elemental distributions across the lens may be useful in distinguishing fish species, discriminating between fish stocks, and tracking fish movements and spatial locations.

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CHAPTER 1

INTRODUCTION

Steller sea lions (*Eumetopias jubatus*) have historically ranged along the North Pacific Rim from the coast of California to Japan. Since the mid 1970s, however, Steller sea lions have shown a significant decrease in population, especially in the Aleutian Islands and Gulf of Alaska (Bickham et al. 1998). Two separate stocks of sea lions were identified in 1996 based on geographic distribution, population dynamics, and genotypic differences in mitochondrial DNA (Bickham et al. 1996). The division at Cape Suckling, Alaska (144° W longitude) separates the population into eastern and western stocks (Loughlin 1997). The western stock was subsequently subdivided into western and Asian stocks in 2005 (Baker et al. 2005). This study was begun prior to the separation of the Asian stock, so in this dissertation I group that stock within the western stock designation. The eastern sea lion stock appears to have started rebounding, but the western stock was reclassified as endangered in 1997 and remains listed as such. Population counts from the western stock indicate that the population declined from about 300,000 animals in the mid-1970s to about 30,000 in 2004 (Trites and Larkin 1996, Fritz and Stinchcomb 2005). Since 2000, Steller sea lion numbers in the western stock have shown considerable variation within their geographic range, making it difficult to speculate on that stock's stability, much less on its recovery (Fritz and Stinchcomb 2005).

Several possibilities have been proposed to explain the decline of the western stock of Steller sea lions. The underlying hypothesis for most of the alternatives is that the primary problem is nutritional stress, which would affect an animal's ability to reproduce

and its likelihood of survival. Possible causes of nutritional stress include: 1) competition with commercial fisheries, 2) contaminants that inhibit nutrient uptake and use, 3) shifts in environmental conditions, 4) disease, and 5) other anthropogenic factors, such as harassment, that cause extra expenditure of energy to flee from the disturbance. Nutritional stress can result from any factor that affects the availability, distribution, diversity, and quality of sea lion prey (Merrick et al. 1997, Rosen and Trites 1999).

Research indicates that changes in prey diversity or prey availability could be responsible for the decline in the western stock of Steller sea lions. Studies conducted during the 1990s on dietary differences between the two stocks showed that Atka mackerel (*Pleurogrammus monopterygius*) and walleye pollock (*Theragra chalcogramma*) were the most common prey species eaten by Steller sea lions in the western stock (Merrick et al. 1997), whereas sea lions in the eastern stock consumed significant amounts of pollock, herring (*Clupea pallasii*), sandlance (*Ammodytes hexapterus*), and salmon (*Oncorhynchus* spp.). Merrick et al. (1997) suggested that the sea lion diet was very different before the onset of the decline, and this change in diet could have had a significant effect on sea lion survival and reproduction. Rosen and Trites (1999) showed that captive sea lions were unable to maintain their body mass when they were fed a low-energy diet (squid), despite being fed ad libitum. Sea lion metabolism was depressed in response to these conditions, and the study results suggested that juvenile sea lions in particular might have difficulty when faced with a change in their prey base. Changes in prey could be a result of fundamental changes in the ecosystem caused by removal of species and their replacement with others due to

fishing and whaling, contamination, other anthropogenic factors, or because of shifts in oceanic conditions resulting from changes in global climate regimes.

The hypothesis favored by the National Marine Fisheries Service (NMFS) to explain nutritional stress is competition with fisheries. Several studies indicated that commercial fisheries trawl in the same areas where sea lions forage and that the same species are being taken by both fishermen and sea lions (Ferrero and Fritz 1994, Loughlin and Merrick 1988). In the mid-1990s, the areas of the greatest sea lion declines showed sustained high catches of pollock and other groundfish (North Pacific Fisheries Management Council 1995a, Ferrero and Fritz 1994). These catches came from areas that were within 20 miles of sea lion rookeries and may have represented a substantial take of potential prey fish (North Pacific Fisheries Management Council 1995b). The initial management strategy taken by NMFS to stop the decline of Steller sea lions was to limit fishing spatially and temporally in ways that closed off critical sea lion habitat to fishing during the seasons when sea lions are likely to use the areas. These measures included closing waters within a 20-mile radius of any sea lion haulout sites to fisheries that target pollock, Pacific cod (*Gadus macrocephalus*), and Atka mackerel. Fisheries management actions also included establishment of no-fishing zones for groundfish within three miles of rookeries and spreading out catches of key fish species in time and space. Although this strategy may have been responsible for the eastern stock rebound, it does not appear to have had any beneficial effects on western stock numbers (Fritz and Stinchcomb 2005). Information on Steller sea lion foraging locations and shifts in foraging patterns would help with regulatory measures and allow fisheries to remain operational in waters that are unused by the sea lions.

Dietary analyses could also provide information on seasonal shifts in fish species consumed by sea lions, allowing for specific fisheries to be closed during seasons when the target fish species constitutes a large proportion of sea lion diets. Scat analysis is the preferred technique for examining the diets of protected Steller sea lions (NMFS 2000) because it is non-invasive. Scat is collected from rookeries and haulouts, preferably when sea lions are foraging at sea. The importance of non-invasive techniques for studying the Steller sea lions increased in May 2006 when the District Court in Washington D.C. ruled that the Environmental Assessment prepared by NMFS was not sufficient to address the impacts of permitted research on Steller sea lions and the environment. The Court directed NMFS to prepare a full Environmental Impact Statement (EIS) before any research, other than “hands off” activities, would be allowed to continue. At present, the EIS is in draft format, and the moratorium on Steller sea lion research continues. Techniques that do not require direct contact with wild sea lions, such as analyzing otoliths (fish ear bones), eye lenses, and other fish remnants recovered from scat, are the only means presently available to continue research into the problem of nutritional stress.

Otoliths and eye lenses often pass through the sea lion digestive system relatively intact, and physical examinations of fish bones, including otoliths, and other fish remnants are employed to determine dietary content. Unfortunately, there are inherent biases in diet analysis using only physical examinations of bones recovered from scat. The number of small fish in pinniped diets tends to be underestimated based on counts of otoliths recovered from scat (Dellinger and Trillmich 1988, Tollit et al. 1997), and captive feeding studies have shown that prey size, meal size, and the size of the pinniped

affect the percentage recovery of otoliths (Tollit et al. 2003). Two methods have been used to offset errors associated with physical analysis: numerical correction factors (Bowen 2000) and the all-structure technique, where diet analysis is undertaken using all of the hard prey remains present in the scat (Tollit et al. 2003). Although these methods reduce the error of diet reconstruction, they have certain limitations. Numerical correction factors are not always available for key species and do not account for double-counting of a single fish based on multiple remains found in scat (Bowen 2000). Both methods are partially limited by the inability to assign extremely digested otoliths and bones to a known prey species (Bowen 2000, Tollit et al. 2003). This occurs most often for gadoids, flatfish, salmonids, and rockfish (Sinclair and Zeppelin 2002).

Otoliths and eye lenses incorporate chemicals from the environment, including heavy metals and other pollutants based on fish species, environmental concentrations, environmental conditions, and metabolic factors (Figure 1.1). For example, sand gobies (*Pomatoschistus minutus*) and sole (*Solea solea*) accumulated lead and mercury in their otoliths in direct proportion to the environmental concentration, whereas plaice (*Pleuronectes platessa*) did not (Geffen et al. 1998). The elements incorporated into otoliths and eye lenses create unique chemical signatures (called elemental fingerprints), which can provide insight into the movements and life history of an individual fish. One example is strontium (Sr), a naturally occurring element that has the same oxidation potential as calcium (Ca) and may substitute for it in fish hard parts. Strontium uptake and incorporation into otoliths is dependent on temperature (Radtke et al. 1990) and/or salinity (Secor and Rooker 2000). Otolith concentrations of barium (Ba) show a negative correlation with Sr and may also be a useful tracer of fish residency in different water

masses (Thorrold et al. 1997). Fish eye lenses also incorporate Sr and other metals (Dove 1999). Differences in the concentration of rubidium (Rb) in fish eye lenses have been correlated with depth of capture (Kingsford and Gillanders 2000), and Sr and mercury (Hg) concentrations helped to differentiate between locations along the coast of New South Wales (Dove and Kingsford 1998). The differential incorporation of elements due to temperature, salinity, and other factors means that otoliths, and potentially eye lenses, contain the record of an individual fish's movements and location throughout its life history.

Recently, several new techniques that allow detection of chemicals even at trace amounts have been used for elemental analysis of otoliths and eye lenses. One of the most powerful techniques is inductively coupled plasma mass spectrometry (ICPMS). ICPMS can take two forms, a solution-based approach for the analysis of whole materials, and a laser ablation form, which can sample specific locations from prepared materials. In solution-based ICPMS, the sample is prepared by dissolving the materials in acid, whereas the laser-ablation method (LA-ICPMS) uses a high-powered laser beam focused on a small part of the sample material, vaporizing it and sweeping it into a plasma, which is then analyzed by a mass spectrometer. These techniques have been used by fisheries scientists to age fish, discriminate between fish stocks, and describe the environmental conditions experienced by fish at different times during their lives (Campana 1990, Radtke et al. 1990, Campana et al. 1994). LA-ICPMS has also been used to measure heavy metal concentrations in otoliths (Geffen et al. 1998) and to identify stock structure in larval and juvenile walleye pollock (Severin et al. 2001).

Thus, fisheries scientists routinely use elemental analyses of otoliths, and have begun to use elemental analyses of eye lenses, to investigate several aspects of fish biology and fish life histories. Applying this analytical technique to dietary analyses of Steller sea lions, using otoliths and eye lenses obtained from scat, can potentially give information on diet content, minimum age of prey fish when consumed, contaminant loads in prey fish, and the location of prey fish residence, which could indicate sea lion foraging grounds. However, to date, marine mammalogists have not used elemental analysis of bones or eye lenses recovered from scat in their analyses on pinniped diets, including Steller sea lions.

In this dissertation, I address some of the potential uses of elemental analysis via ICPMS in dietary studies for Steller sea lions. This research includes three separate investigations, each formatted for submission to different peer-reviewed journals. My first inquiry examines the potential for using otoliths as indicators of total body burdens of metal contaminants in prey fish. Otoliths may incorporate metals in a way that allows them to be used as accurate indicators of fish total body burdens. If this is the case, I can estimate contaminant loads ingested by Steller sea lions from the fish they eat based on otoliths recovered from scat. My second inquiry addresses the effects of Steller sea lion digestion on the microchemistry of otoliths. Is enough of the original elemental fingerprint retained to allow for species discrimination and analyses of life histories, locations, and movements? Third, I examine the use of eye lenses recovered from scat for dietary analysis. Eye lenses are recovered more often than any other hard structure, but little research has been done using eye lenses as indicators of environmental conditions or fish life history. Finally, there is a discussion of the results of the three

studies and how they fit in with ongoing research on nutritional stress and the potential causes of the decline in the western Steller sea lion stock.

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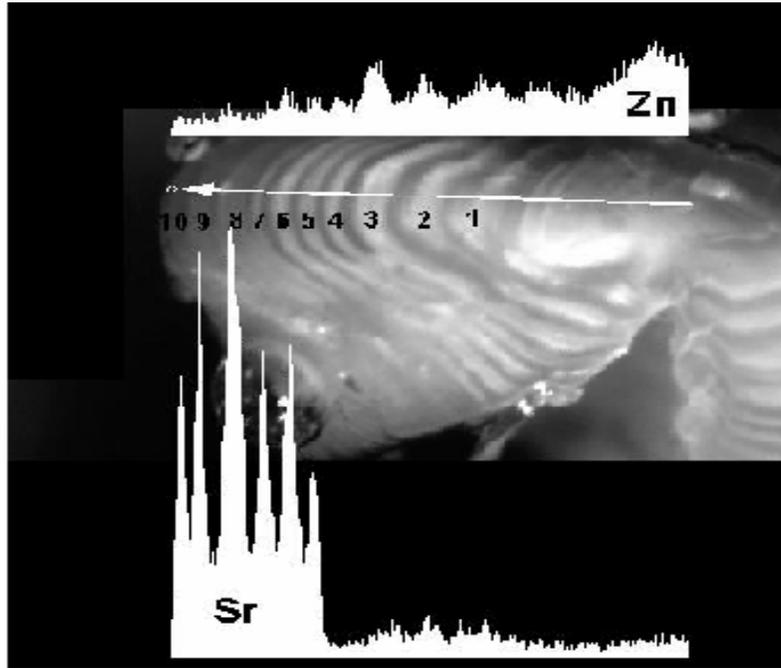


Figure 1.1: Zinc and strontium scans superimposed on a reflected light optical image of an anadromous charr otolith. The high Sr in year 5 probably marks movement into a marine environment. Picture created by Norman Halden and used with permission (www.umanitoba.ca/faculties/science/geological_sciences/faculty/halden/enviro.html).

CHAPTER II

DETERMINING METALS INGESTED BY STELLER SEA LIONS (*EUMETOPIAS JUBATUS*) FROM PREY OTOLITHS COLLECTED FROM SCAT

Introduction

The Steller sea lion (*Eumetopias jubatus*) population along the North Pacific Rim has been declining since the 1960s (Bickham et al. 1998). In 1990, both the eastern and western stocks in Alaska were listed as threatened under the Endangered Species Act. The western stock was reclassified as endangered in 1997 and had declined to 30,000 animals by 2004 (Fritz and Stinchcomb 2005). Although nutritional stress is thought to be the primary cause of the decrease in abundance, other factors that may contribute to the decline are also causes for concern, such as the effects of environmental toxins or increased contaminant loads that can impair sea lion endocrine, reproductive, and immune system functions (Ross et al. 2003).

Metals are one type of contaminant that affects wildlife, and wildlife in the Arctic region often shows significant metal burdens (Mallory et al. 2004). Unfortunately, the mechanisms of metal accumulation in marine mammals, as well as metal toxicity, are not well-understood (Woshner et al. 2001). In addition, metal contaminants in the marine environment tend to form complex compounds, and exposure to combinations of pollutants can have unexpected consequences (Krishnan and Brodeur 1991). Although high concentrations of metals can cause acute toxicity and overt mortality, such doses are unlikely to be found in Stellar sea lion populations based on Arctic sampling (Macdonald and Bowers 1996). Effects from chronic exposure at sublethal concentrations are

possible, however, and may ultimately affect fitness of individuals, population growth, and sustainability. In this regard, the most direct effects of metals are those on the reproductive and endocrine systems (Sharara et al. 1998, McMaster 2001), which may reduce reproductive success and higher juvenile mortality. For example, Sydeman and Jarman (1998) found that elevated levels of copper were associated with premature parturition in some sea lion species. In addition, metals may also affect embryonic development, thus reducing survivability of offspring (Domingo 1995). Metals can also have effects on the excretory and immune systems (Bernier et al. 1995, Madden and Fowler 2000), leading to reduced health, which can have detrimental effects on the fitness of the organism. Effects of metals on the nervous system can be manifested as alterations in behavior, which in turn could affect survival and reproduction (Manzo et al. 1996). Finally, many metals can cause DNA damage, which could contribute to reproductive and immunological failures, and also can produce carcinogenesis (Snow 1992).

Metal contamination in the Arctic comes from five major sources: atmospheric transport, ocean currents, runoff from land, river inflow, and direct dumping into the ocean (Macdonald and Bowers 1996). Metal contaminants from anthropogenic sources are introduced mostly via atmospheric transport and local inputs from industrialized areas and mining operations (Macdonald and Bowers 1996). Within the Arctic ocean, surface currents direct the flow of contaminants. These metals then are transferred upward through trophic levels primarily via ingestion (Law 1996, Yu et al. 1993), although most metal contaminants do not biomagnify and occur at lower concentrations at higher trophic levels.

Some ingested metals are necessary for normal growth and survival. These essential metals are metabolically active in the body, and their uptake and retention is regulated in tissues because of their specific functions (Friberg et al. 1979). Most essential metals are either components of enzymes, or function as enzymatic co-factors. Other metals that are available in the environment do not perform specific metabolic functions, and their uptake and retention is determined by their specific physiology, environmental concentrations, and interactions with other elements (Suttle 1975). These non-essential metals are referred to as trace metals in this study.

Because ingestion is the major pathway for metal uptake, analyses of Steller sea lion prey and the contaminants that these fish contain should provide an indication of the contaminant loads in the sea lions themselves. Partially because it is non-invasive, scat analysis is the preferred technique for examining the diets of Steller sea lions (NMFS 2000), and hard parts of fish (otoliths or ear bones, opercles or gill covers, scales, eye lenses, and other bones) often pass through the sea lion digestive system relatively intact. Chemicals from the environment, including metals, are incorporated into fish hard parts as they form, and recent advances in elemental analysis have allowed the use of trace elements within boney structures, specifically the otolith, as indicators of water pollution (Dove and Kingsford 1998). For example, sand gobies (*Pomatoschistus minutus*) and sole (*Solea solea*) accumulate lead (Pb) and mercury (Hg) in their otoliths in proportion to their environmental concentrations (Geffen et al. 1998). Fossilized fish teeth have been shown to incorporate strontium (Sr) and the rare earth metal neodymium (Nd) at the time of growth, and the ratio of their incorporation was largely determined by their concentration in the environment (Elliott et al. 1998, Martin and Haley 2000).

One of the most powerful techniques to detect chemicals at trace amounts is inductively coupled plasma mass spectrometry (ICPMS). Laser-ablation ICPMS (LA-ICPMS) uses a high-powered laser beam focused on a small part of the sample material, vaporizing it and sweeping it into a plasma, which is then analyzed by a mass spectrometer. Geffen et al. (1998) used LA-ICPMS to measure mercury and lead concentrations in otoliths of plaice (*Pleuronectes platessa*), sand gobies, and sole for comparison to tissue concentrations. They found that the correlation between tissue and otolith concentrations was positive for mercury and negative for lead. A literature review over the past ten years yielded no studies comparing otolith and body tissue concentrations of metals other than lead and mercury.

This lack of knowledge about metals and their complex relationships warrants further study. The tools currently exist to investigate contaminant exposure from ingestion in Steller sea lions and to potentially trace sources of contamination using elemental analysis of fish otoliths obtained from scats. This study investigates the use of otoliths as an indicator of contaminant loads in Steller sea lion prey by analyzing the relationships between otolith concentrations of metals and concentrations in homogenized body tissues of three major prey species of the western sea lion stock: Pacific herring (*Clupea pallasii*), Atka mackerel (*Pleurogrammus monopterygius*), and Walleye pollock (*Theragra chalcogramma*). Metals tested in this study were chosen based on their potential for toxicity from chronic exposure, their regular incorporation into both otoliths and tissues, and their use by fisheries scientists to investigate various aspects of fish biology and fish life histories (Table 2.1). The techniques that I am employing also could be applied to other species that feed on marine fish.

Methods

Frozen Pacific herring, Atka mackerel, and Walleye pollock were supplied from ongoing captive Steller sea lion studies at the University of British Columbia (UBC) and shipped to Texas Tech University. These fish were caught by commercial fisherman in the Gulf of Alaska and donated to UBC and the Vancouver Aquarium; the exact location of capture is unknown. I removed the sagittal otoliths from each fish using standard procedures (Secor et al. 1991). Ceramic scalpels and plastic dissection trays were used to avoid metal contamination of the samples. Otoliths were rinsed with ultra-pure water, dried, and stored individually. The remainder of each fish was individually re-frozen and stored until tissues could be prepared and analyzed.

Otolith Preparation

Otoliths were embedded in Loctite, a non-reactive compound that hardens when exposed to ultraviolet light. After the Loctite had set, otoliths were sectioned transversely using a Buehler Isomet low-speed saw equipped with a diamond-embedded wafering blade (series 15HC). Otolith sections from each species were mounted on glass slides using Crystal Bond, a temperature-controlled, non-permanent adhesive, and polished using lapidary polishing cloths until the core of the otolith was visible.

Mounted otolith sections were shipped to Old Dominion University (ODU) in Norfolk, VA, for LA-ICPMS analysis. At ODU, a final cleaning polish was performed in a Class 100 clean room to avoid contamination. A Finnigan MAT Element 2 ICPMS was used to analyze samples that were introduced via a UV (266 nM) Nd:YAG laser ablation system (Merchantek LUV-266X) for solid material analysis. Eight metals were measured

as ratios of analyte to calcium (Ca) using a known standard for comparison. These included essential metals (Mg, Mn, Cu, and Zn) and trace metals (Rb, Sr, Ba, and Pb).

Two distinct sites on the otolith (the otolith core and the otolith edge just past the third annulus) were sampled for comparison to homogenized tissue samples because previous research has shown that the elemental concentrations in otolith cores are distinctly different from other regions of the same otolith (Ruttenberg et al. 2005).

Tissue Preparation

Tissue samples were analyzed for metals according to USEPA SW-846 protocols (Cobb et al. 2006, USEPA 1994). Fish tissues were thawed and homogenized using a Hamilton Beach blender, after which a 3 g (wet weight) sub-sample was taken from each fish for analysis. Prior to use, all glassware, Teflon, and other instruments were cleaned in a 1:1 HNO₃:H₂O solution and rinsed five times with milli-Q water. Tissue sub-samples had 3 ml of concentrated nitric acid added and were allowed to pre-digest overnight. Each sample was placed into a separate Teflon beaker, covered with a Teflon watch glass, and placed on a hot plate set at 90 °C. Samples were left on the hot plate, and an additional 3 ml of nitric acid was added every 30 minutes until the samples were clear and colorless. Once the samples were clear, they were removed from the hotplate for five minutes and diluted with 2 ml of milli-Q water and 3 ml of hydrogen peroxide (H₂O₂). Samples were returned to the hotplate until they no longer effervesced when H₂O₂ was added. Finally, samples were cooled to room temperature and diluted with 10 ml of hydrochloric acid (HCl) and enough milli-Q water to bring each sample to a volume of 50 ml. These samples were stored in plastic vials for one to sixteen days until they were analyzed using a Leeman Labs Inductively Coupled Plasma – Atomic

Emission Spectrometer (ICP-AES). All metals were analyzed using the ICP-AES except magnesium, which was analyzed using a Perkin-Elmer Atomic Absorption Spectrometer (AA).

Statistical Analyses

Correlation analyses were first conducted with data from all three fish species to look for relationships between otolith concentrations of an element and total body concentrations. Then each species was analyzed separately to look for correlations within species that might have been masked in the original analysis with the three species combined. One herring was removed from analysis due to the extremely high metal concentrations in the otolith samples ($> 3SD + \text{mean}$). Pearson product-moment correlation coefficients were estimated to assess linear relationships between otolith and total-body concentrations, and nonparametric Spearman rank-order correlation coefficients were estimated to assess monotonic (possibly non-linear) relationships between otolith and tissue samples. Because of the small sample sizes, levels of statistical significance (p-values) were estimated by random permutation, with 1000 iterations (Manly 2006). All analyses were done using MATLAB 6.5 (Mathworks). A sequential Bonferroni correction for experiment-wise error was estimated for each set of analyses within a single fish species.

Results

Mean concentrations of metals in fish tissues were generally less than one microgram per gram (Table 2.2). Lead is one of the metals of particular interest in this study due to its well-known toxicity, but the tissue concentrations were close to the

detection limits of the ICP-AES, resulting in several non-detected values. Therefore results for lead are not reported. Magnesium had the highest concentrations in homogenized tissues, but they were still well below chronic LC-50 concentrations (200 $\mu\text{g/g}$) for a six-month test in bluegill (*Lepomis macrochirus*) (EPA, ECOTOX database). Standard deviations were low compared to the means, indicating that metals concentrations in the tissues of individual fish from the same species were similar. This was not the case for otoliths, which had means ranging from approximately 86 ng/g (Rb in mackerel) to 2.5 mg/g (Zn in pollock) (Table 2.3). Standard deviations for otolith samples were often greater than mean concentrations, indicating a large amount of variance in the individual samples.

Otolith Core Samples and Body Tissues

Essential Metals

When data from all three fish species were combined, there were no significant relationships between samples taken from the otolith core and tissue samples (Tables 2.4, 2.5). When each fish species was analyzed independently, however, individually significant relationships were found in two species, despite the smaller sample sizes (and thus lower statistical power) in the intraspecific analyses. Herring had significant Pearson ($r^2 = 0.76$, $p = 0.01$) and Spearman correlations ($r^2 = 1$, $p = 0.01$) for zinc (Figure 2.1), but mackerel had no significant relationships for essential metals. Pollock had no significant Pearson correlations, but I found significant Spearman rank correlations for manganese ($r^2 = 0.78$, $p = 0.01$), copper ($r^2 = 0.54$, $p = 0.04$), and zinc ($r^2 = 0.66$, $p = 0.02$) (Figure 2.2). After the Bonferroni correction was applied, the Spearman rank

correlations for copper and zinc in pollock were no longer significant. However, the correlations for zinc in herring and for manganese in pollock remained significant.

Trace Metals

Only one trace metal showed an individually significant relationship when all three species were analyzed as a group (Tables 2.4, 2.5). Barium showed a significant correlation between core samples and tissue samples for both the Pearson test ($r^2 = 0.30$, $p = 0.01$) and the Spearman rank test ($r^2 = 0.38$, $p = 0.01$) (Figure 2.3). Herring and mackerel had no significant relationships when analyzed individually, but pollock had a negative Spearman rank correlation for strontium ($r^2 = 0.62$, $p = 0.01$) (Figure 2.4). All results showing a significant relationship individually were also significant after the Bonferroni correction.

Otolith Edge Samples and Body Tissues

Essential Metals

When all three species were tested as a group, one essential metal had an individually significant relationship between otolith edge samples and homogenized tissues (Tables 2.6, 2.7). Magnesium had significant Pearson ($r^2 = 0.15$, $p = 0.05$) and Spearman rank correlations ($r^2 = 0.12$, $p = 0.05$) (Figure 2.5). However, when each species was analyzed independently, only one species had a significant relationship. Pollock had a significant Pearson correlation for zinc ($r^2 = 0.53$, $p = 0.04$) (Figure 2.6) but no significant Spearman rank correlation. Herring and mackerel had no significant correlations for essential metals between otolith edge and tissue samples. After the Bonferroni correction was applied, there were no significant correlations for essential metals between otolith edge samples and fish tissues.

Trace Metals

Two trace metals had individually significant relationships between otolith edge and homogenized tissue samples when all three species were grouped together (Table 2.6, Table 2.7). Rubidium was significant for both the Pearson correlation ($r^2 = 0.37$, $p = 0.01$) and the Spearman rank correlation ($r^2 = 0.31$, $p = 0.02$), as was barium (Pearson: $r^2 = 0.43$, $p = 0.01$ and Spearman: $r^2 = 0.42$, $p = 0.01$) (Figure 2.7). When species were analyzed individually, there were no significant relationships for herring. Analyses for mackerel showed a negative Pearson correlation for strontium ($r^2 = 0.52$, $p = 0.04$) and a Spearman rank correlation for barium ($r^2 = 0.48$, $p = 0.05$) (Figure 2.8). Pollock analyses indicated a Pearson correlation for barium ($r^2 = 0.50$, $p = 0.05$) and a negative Spearman rank correlation for rubidium ($r^2 = 0.54$, $p = 0.05$) (Figure 2.9). The Bonferroni correction left no significant results for individual species, but the correlations for rubidium and barium across all three species remained significant.

Discussion

Otoliths and soft tissues have different mechanisms for the uptake and incorporation of metals. Otoliths incorporate metals largely by substitution for calcium in their CaCO_3 matrix, but metals can also be trapped as inclusions within the crystal lattice structure (Geffen et al. 1998). Once a metal has been incorporated into the otolith, there is no mechanism for removal (Campana and Neilson 1985). Soft tissues have evolved mechanisms to regulate the concentrations of metabolically active metals in specific ranges and may not regulate non-essential metals at all (Goyer 1996). During times of nutritional stress, tissue concentrations of essential metals can change

significantly as stores are depleted to offset the deficiency. The differences between otoliths and tissues in metal uptake, regulation, and resorption could prohibit any correlations between the two. Toxic metals in soft tissues are usually sequestered and retained in kidney, liver, or bone tissue, or they are excreted. However, Geffen et al. (1998) found significant relationships for lead and mercury in otoliths and fish organs, indicating that metal concentrations in hard structures can be correlated with at least some soft tissues. I found fewer correlations between otolith edge samples and tissues than was expected, and these correlations were with trace metals. Metal concentrations from otolith cores had as many correlations with tissues as otolith edge samples, but these correlations were mostly found with essential metals.

The study by Geffen et al. (1998) examined only correlations with different fish species grouped together; no relationships were tested within individual species. My results show that significant relationships found between otoliths and tissues when the three fish species were tested together disappeared from at least two of the species when they were tested individually. For example, I found a significant correlation for rubidium between otolith edge samples and homogenized tissues when all three species were combined. However, when species were tested individually, none of them showed significant results for rubidium. This indicates that comparisons across species may not be warranted, as different fish species can have different mechanisms for metabolizing and regulating metals. The point is also illustrated by the inverse situation, where individual species showed significant relationships, but there was no correlation when the three species were tested as a group. One potential reason is that individual species showed confounding relationships, positive or negative, between otoliths and tissues for

individual metals. When the three species were combined for analyses, the negative and positive relationships from the different species potentially cancelled each other out, creating a non-significant outcome. For example, both herring and pollock showed significant positive correlations for zinc between otolith core and tissue samples, whereas mackerel had a non-significant but negative relationship. When the three species were tested together, there was no significant relationship. Based on my results, individual species should be analyzed independently whenever possible.

The location on the otolith where the sample is taken from also has a distinct effect on whether or not there is a correlation with tissue samples. The core of the otolith represents material deposited during the first year of the fish's life, whereas the edge sample represents the recent time period. All fish in this study were at least three years old, based on the number of annuli present on otoliths. During the time between otolith core deposition and when tissue samples were harvested, several things could have affected elemental concentrations in tissues, while the otolith core remained unchanged. Shifts from larval and juvenile feeding habits and prey to adult habits and prey would affect metal ingestion, and movements from juvenile habitats to adult habitats could affect the environmental concentrations of metals. In addition, metabolic activities of adult fish often differ significantly from those of juvenile fish as energy usage is shifted from growth to reproduction. All of these changes suggest that metal concentrations in the core of an otolith are unlikely to be correlated with homogenized tissue concentrations from the adult fish. If correlations for metals between otolith concentrations and tissue concentrations are present, it is more likely they would be found for samples taken from the otolith edge because the edge layers were deposited

recently and were subject to the same conditions that the tissue had recently been exposed to. However, I found approximately the same number of significant correlations between otolith core samples and tissues as I did between otolith edge samples and tissues.

Three sources of experimental error that could have biased my results are the effects of transport on fish tissues, small sample sizes, and outlier effects. While being transported from Vancouver, BC to Lubbock, Texas, several fish completely or partially thawed and had to be re-frozen once they arrived in Lubbock. This caused water and blood loss from the fish tissues and could have affected tissue concentrations of metals. The thawing also caused some tissue degradation, which could have affected the results, as well. It is unlikely, however, that thawing and re-freezing affected metal concentrations in otoliths. Another potential source of bias is the small sample sizes in the study. Small sample size affects the power to detect correlations and the ability to distinguish between linear and non-linear relationships. The small sample sizes also increase the effects of outliers on the correlation results. Although extreme outliers (± 3 SD from the mean) were removed from the analysis, the small sample size left few data points to offset any smaller outlying values, and significant relationships could have been masked by these outliers (e.g. a Type II error). Variation in elemental concentrations measured in otoliths was much larger than the variation found in tissue samples. For example, in otolith core samples from pollock the standard deviation for zinc concentrations was one and a half times the mean value. The extreme variation in the otolith samples could be due to the alignment of calcium carbonate crystals at different locations on the otolith, which would affect elemental incorporation. This variation is likely to affect any statistical analysis performed. The effects of thawing and re-freezing

could not be corrected for, but random permutations in the statistical analyses were used to offset the small sample size and address the problem of outliers.

The majority of significant relationships between otolith core and tissue samples were essential metals (Mg, Mn, Cu, and Zn), whereas otolith edge samples showed the most correlations with tissues for trace metals. The simplest explanation for this could be a time lag in the regulation of essential metals in tissues, while trace metals simply fluctuate based on environmental factors (Suttle 1975). This would also explain the lack of correlations for essential metals between otolith edge samples and tissues. However, a time lag of several years for essential metal incorporation into tissue would be necessary to explain correlations between the otolith core and tissues, and such a long time-lag would defeat the purpose of metal regulation in body tissues. Further studies using known-age fish, controlled metal exposures, and fixed durations are needed to address whether there are time lags in tissue incorporation and regulation of essential metals with respect to otolith incorporation. Specific information on the regulation of various metals in fish tissues, the levels at which different metals are regulated, and how this regulation may differ between fish species would also be helpful in interpreting my results.

Essential metal concentrations at the otolith core showed one significant Pearson correlation and four significant Spearman rank correlations, all within individual species. Zinc showed significant Pearson and Spearman correlations in herring. The significant Spearman relationship and corresponding scatter plot of the herring data (Figure 2.1) indicate that the relationship between otolith core and tissue concentrations could be asymptotic. Most organisms biochemically regulate zinc concentrations in cells and tissues (Friberg et al. 1979), and if zinc concentrations in tissue reach their regulated

maximum, the tissue values will plateau even if otolith concentrations continue to increase. This would produce an asymptotic relationship that would be indicated by the Spearman rank correlation, but not necessarily by the Pearson correlation, which detects linear relationships. The other significant Spearman rank correlation tests for essential metals found significant correlations for manganese, copper, and zinc in pollock. The metabolism of copper is similar to that of zinc (Suttle 1975), and manganese is also metabolically regulated (Goyer 1996). The specific regulation of these metals, the lack of Pearson correlations, and the significant Spearman correlations could be indicative of asymptotic relationships for these three metals.

Trace metals (Rb, Sr, and Ba) showed more correlations between tissues and otolith edges than between tissues and otolith cores. These metals are not regulated for metabolic functions and do not perform specific functions within the body. Without specific pathways for regulation, these metals are more likely to fluctuate in body tissues with environmental levels (Goyer 1996). Some metals in tissues fluctuate at low concentrations without regulation, but once a specific threshold is reached where the metals begin to have adverse effects, excretory mechanisms are activated to remove the metal and return it to a non-toxic concentration. Trace metals also could be sequestered in specific parts of the body once high concentrations are reached, which would make the metal less available in the bloodstream for incorporation into the otolith. Either of these mechanisms would explain negative relationships between tissues and otolith concentrations of trace metals such as strontium in pollock (Figure 2.4) and mackerel (Figure 2.8), and rubidium in pollock (Figure 2.9). None of the essential metals showed significant negative correlations. Additionally, the incorporation of strontium and barium

in otoliths is influenced by temperature and salinity (Radtke et al. 1990, Thorrold et al. 1997), although it is unknown how these factors affect tissue concentrations. A study by Hanson and Zdanowicz (1999) found that tissue concentrations of trace metals were more correlated with environmental conditions than essential metals were. If environmental factors affect trace metal concentrations in otoliths and soft tissues in a similar fashion, it would explain the correlations I found between tissues and otolith edge samples. This would be consistent with my finding more correlations between trace metals and the otolith edge than between tissues and the otolith core. As with the essential metals, the significant Spearman rank correlations indicate potentially non-linear relationships between otolith concentrations and tissue concentrations of trace metals. Testing specific organs known to sequester metals, rather than homogenized tissues, may provide a better indication of relationships between metal concentrations in otoliths and total body burdens (Geffen et al. 1998).

The fish analyzed in this study were caught by commercial fisheries in the Gulf of Alaska, but the exact location of capture is unknown. These fish may or may not come from populations that are preyed on by Steller sea lions, but the low contaminant loads I found in fish tissues may indicate that metal contamination in sea lion prey is not a problem in at least some portions of the Gulf. Studies on marine birds and ringed seals in the Arctic have also shown low levels of metals (Mallory et al. 2004) in piscivorous predators.

Overall, my results indicate that otoliths display few significant correlations in concentration with metals in fish tissues and would not be accurate indicators of the total body burdens of these metals. However, several factors that affect the relationship

between tissue and otolith concentrations must be taken into account, including fish species, sampling site on the otolith, and the specific metal being analyzed. Fish species can differ in both metabolic functions and otolith formation characteristics (Geffen et al. 1998), and I found distinct differences in the relationships between tissues and otoliths among herring, mackerel, and pollock. I also found distinct differences between the otolith core and edge when compared to tissue concentrations, and different patterns of correlation between metabolically necessary metals and those that are not used by the body. In order to use otoliths to predict total body burdens in fish, some information on the factors listed above would be needed. Further studies are needed to determine the relationship between specific metals in otoliths and tissues for individual fish species under controlled conditions. Then otoliths collected from scat then could be used to estimate contaminant loads ingested by wild Steller sea lions without the need for invasive procedures such as blubber punches. My study simulated some of the characteristics of otoliths collected from scat, in that the location and environmental conditions where the fish were caught (consumed) were unknown. The effects of pinniped digestion were not addressed in this study and would need to be accounted for when using otoliths collected from scat to assess the effects of digestion on metal concentrations in otoliths. Other elemental signatures from these otoliths potentially can be related to the spatial distribution of prey items and help locate contamination sources.

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Table 2.1: Metals analyzed in fish tissues and otoliths, their metabolic function, and their toxic effects in humans (Goyer 1996; EPA, ECOTOX database). Some metals have no known metabolic function, indicated by --.

Element	Symbol	Essential or Trace	Metabolic Function	Toxic Effects From Chronic Exposure
Magnesium	Mg	Essential	enzyme co-factor	Lesion production, conjunctivitis, drops in blood pressure, fatigue, hallucinations, central nervous system depression, and skeletal muscle paralysis
Manganese	Mn	Essential	enzyme co-factor	Irritability, difficulty walking, speech disturbances, compulsive behavior, neuropsychiatric disorder with Parkinsonlike symptoms
Copper	Cu	Essential	enzyme component	Anemia, vomiting, hypotension, jaundice, coma, and Wilson's disease
Zinc	Zn	Essential	enzyme co-factor	Stomach cramps, nausea, vomiting, anemia, infertility (in rats)
Rubidium	Rb	Trace	--	Hyper-irritability, muscular spasms, and reproductive deficiencies
Strontium	Sr	Trace	--	Skeletal abnormalities, growth disruption, morphological abnormalities, carcinogenicity. Strongly affected by calcium nutrition
Barium	Ba	Trace	--	Decreased pulse rate, gastroenteritis, muscular paralysis, and ventricular fibrillation
Lead	Pb	Trace	--	Anemia, hypotension, sterility, neurotoxicity, nervous system disorders, neonatal deaths in males, coma, carcinogenicity.

Table 2.2: Mean concentrations and standard deviations for metals in the homogenized tissues of three species of fish. Metal concentrations found in Pacific herring (*Clupea pallasii*), Atka mackerel (*Pleurogrammus monopterygius*), and Walleye pollock (*Theragra chalcogramma*). *Lead (Pb) had > 30% non-detected values, so the results were not included in the statistical analyses.

	Mg ($\mu\text{g/g}$)	Mn ($\mu\text{g/g}$)	Cu ($\mu\text{g/g}$)	Zn ($\mu\text{g/g}$)	Rb ($\mu\text{g/g}$)	Sr ($\mu\text{g/g}$)	Ba ($\mu\text{g/g}$)	Pb ($\mu\text{g/g}$)*
<u>Pacific Herring (n = 5)</u>								
Mean	11.88	0.09	0.05	0.61	0.02	0.34	0.01	0.01
St. Dev.	1.58	0.05	0.01	0.10	0.00	0.15	0.00	0.01
<u>Atka Mackerel (n = 8)</u>								
Mean	15.85	0.04	0.07	0.39	0.03	1.11	0.07	0.01
St. Dev.	1.74	0.01	0.01	0.08	0.00	0.35	0.03	0.00
<u>Walleye Pollock (n = 8)</u>								
Mean	10.90	0.03	0.05	0.41	0.02	1.15	0.01	0.00
St. Dev.	1.97	0.01	0.01	0.08	0.00	0.64	0.00	0.00

Table 2.3: Mean concentrations and standard deviations for metals in fish otoliths. Values for core and edge samples on otoliths of Pacific herring (*Clupea pallasii*), Atka mackerel (*Pleurogrammus monopterygius*), and Walleye pollock (*Theragra chalcogramma*). * Samples from the edge of herring otoliths have an n = 4 due to the removal of an extreme outlier (> 3SD above the mean).

	Mg		Mn		Cu		Zn		Rb		Sr		Ba		Pb	
	<i>(uMol/Mol Ca)</i>		<i>(uMol/Mol Ca)</i>		<i>(nMol/Mol Ca)</i>		<i>(nMol/Mol Ca)</i>		<i>(nMol/Mol Ca)</i>		<i>(mMol/Mol Ca)</i>		<i>(uMol/Mol Ca)</i>		<i>(nMol/Mol Ca)</i>	
	<u>Core</u>	<u>Edge</u>														
<u>Pacific Herring (n = 5)*</u>																
Mean	293.8	122.2	9.8	1.4	1164.8	414.6	2488.7	1383.7	90.2	73.0	1.5	1.5	2.8	1.8	39.9	37.2
St. Dev.	128.82	48.79	13.08	1.26	1105.49	465.37	1559.03	1833.35	4.28	16.65	0.26	1.17	2.41	2.14	21.74	50.27
<u>Atka Mackerel (n = 8)</u>																
Mean	160.7	223.7	9.5	5.3	927.8	1499.3	2379.6	3330.9	86.7	139.3	1.8	2.2	5.0	14.5	38.4	59.5
St. Dev.	54.32	187.00	6.99	2.28	557.03	1413.50	3049.22	5678.46	36.86	40.79	0.32	0.86	2.10	12.59	38.52	57.22
<u>Walleye Pollock (n = 8)</u>																
Mean	135.2	92.1	14.9	2.8	856.1	1741.7	2672.7	2342.5	125.3	96.5	2.2	3.1	1.6	6.3	38.1	98.2
St. Dev.	56.92	63.32	29.27	1.65	420.28	1907.39	4169.31	2783.11	64.47	19.74	0.55	0.65	0.32	7.96	37.94	125.56

Table 2.4: Pearson product-moment correlations for otolith core samples and homogenized tissue samples. Shown are sample sizes (N), Pearson product-moment correlation coefficients (r) and significance levels (p). * indicates an individually significant correlation. ** indicates a significant relationship after sequential Bonferroni correction.

	N	Essential Metals								Trace Metals					
		Mg		Mn		Cu		Zn		Rb		Sr		Ba	
		r	p	r	p	r	p	r	p	r	p	r	p	r	p
Pacific Herring	5	0.37	0.52	-0.57	0.34	-0.06	0.86	0.87	0.01**	-0.05	0.96	0.18	0.66	-0.18	0.71
Atka Mackerel	8	0.15	0.71	-0.25	0.61	-0.03	0.94	-0.63	0.06	-0.15	0.71	-0.19	0.60	-0.43	0.94
Walleye Pollock	8	-0.39	0.35	0.34	0.53	0.49	0.18	0.42	0.23	-0.08	0.86	-0.67	0.07	0.40	0.34
All species	21	-0.04	0.80	-0.13	0.42	0.07	0.70	0.06	0.77	-0.17	0.48	-0.03	0.90	0.55	0.01**

Table 2.5: Spearman rank correlations for otolith core samples and homogenized tissue samples. Shown are sample sizes (N), Spearman rank correlation coefficients (r) and significance levels (p). * indicates an individually significant correlation. ** indicates a significant relationship after sequential Bonferroni correction.

	N	Essential Metals								Trace Metals					
		Mg		Mn		Cu		Zn		Rb		Sr		Ba	
		r	p	r	p	r	p	r	p	r	p	r	p	r	p
Pacific Herring	5	0.60	0.37	-0.40	0.46	0.10	0.97	1.00	0.01**	0	1	0.1	0.96	0.1	0.93
Atka Mackerel	8	0.31	0.38	0.14	0.72	0.02	0.94	-0.50	0.21	-0.06	0.91	0.17	0.61	-0.05	0.86
Walleye Pollock	8	-0.52	0.21	0.88	0.01**	0.74	0.04*	0.81	0.02*	-0.46	0.20	-0.79	0.01**	0.55	0.15
All species	21	-0.10	0.61	0.23	0.29	0.17	0.36	0.11	0.61	-0.36	0.08	0.11	0.58	0.61	0.01**

Table 2.6: Pearson product-moment correlations for otolith edge samples and homogenized tissue samples. Shown are sample sizes (N), Pearson product-moment correlation coefficients (r) and significance levels (p). * indicates an individually significant correlation. ** indicates a significant relationship after sequential Bonferroni correction.

	N	Essential Metals								Trace Metals					
		Mg		Mn		Cu		Zn		Rb		Sr		Ba	
		r	p	r	p	r	p	r	p	r	p	r	p	r	p
Pacific Herring	4	-0.54	0.5	-0.33	0.69	-0.86	0.22	0.76	0.13	-0.45	0.98	-0.57	0.29	-0.62	0.37
Atka Mackerel	8	0.08	0.81	0.04	0.91	-0.2	0.66	-0.47	0.15	0.16	0.76	-0.72	0.04*	0.66	0.08
Walleye Pollock	8	0.18	0.73	0.05	0.94	-0.3	0.43	0.73	0.04*	-0.39	0.35	0.08	0.79	0.71	0.05*
All species	20	0.39	0.05*	-0.3	0.16	-0.19	0.39	-0.09	0.64	0.61	0.01**	0.13	0.53	0.66	0.01**

Table 2.7: Spearman rank correlations for otolith edge samples and homogenized tissue samples. Shown are sample sizes (N), Spearman rank correlation coefficients (r) and significance levels (p). * indicates an individually significant correlation. ** indicates a significant relationship after sequential Bonferroni correction.

	N	Essential Metals								Trace Metals					
		Mg		Mn		Cu		Zn		Rb		Sr		Ba	
		r	p	r	p	r	p	r	p	r	p	r	p	r	p
Pacific Herring	4	-0.80	0.32	0.20	0.72	-0.20	0.80	0.80	0.30	0.00	0.98	-0.80	0.24	-0.80	0.31
Atka Mackerel	8	-0.07	0.85	0.29	0.58	-0.19	0.66	-0.60	0.09	-0.01	0.99	-0.47	0.25	0.69	0.05*
Walleye Pollock	8	0.29	0.50	0.12	0.80	0.02	0.99	0.55	0.12	-0.73	0.05*	0.36	0.37	0.24	0.60
All Species	20	0.35	0.05*	0.00	1.00	-0.07	0.70	-0.09	0.67	0.56	0.02**	0.09	0.63	0.65	0.01**

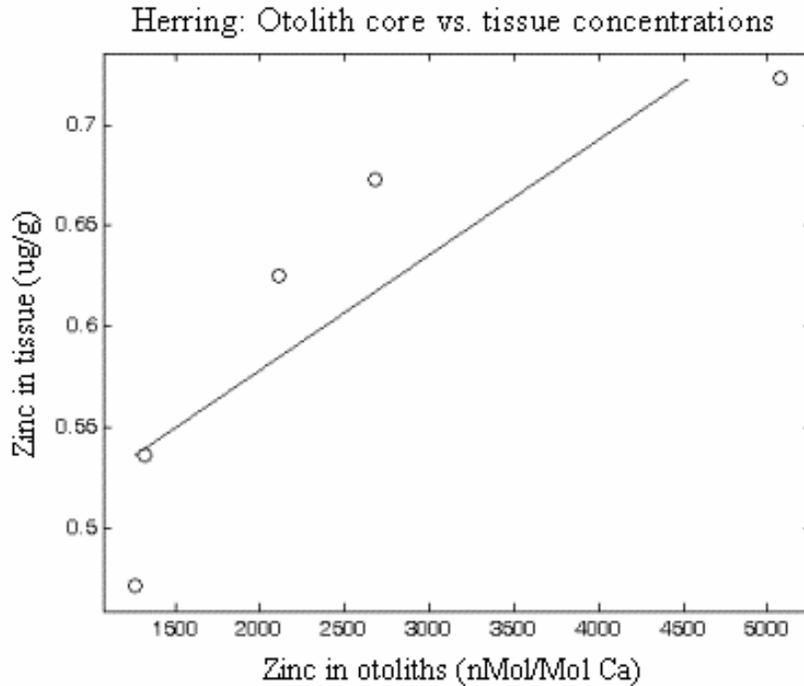


Figure 2.1: Essential metals with significant relationships between otolith core samples and homogenized tissues in Pacific herring (*Clupea pallasii*). Zinc had significant Pearson product-moment and Spearman rank correlations between the otolith core and tissue samples individually and after a sequential Bonferroni correction. The line represents the best-fit line indicated by the Pearson product-moment analysis.

Pollock: Otolith core vs. tissue concentrations

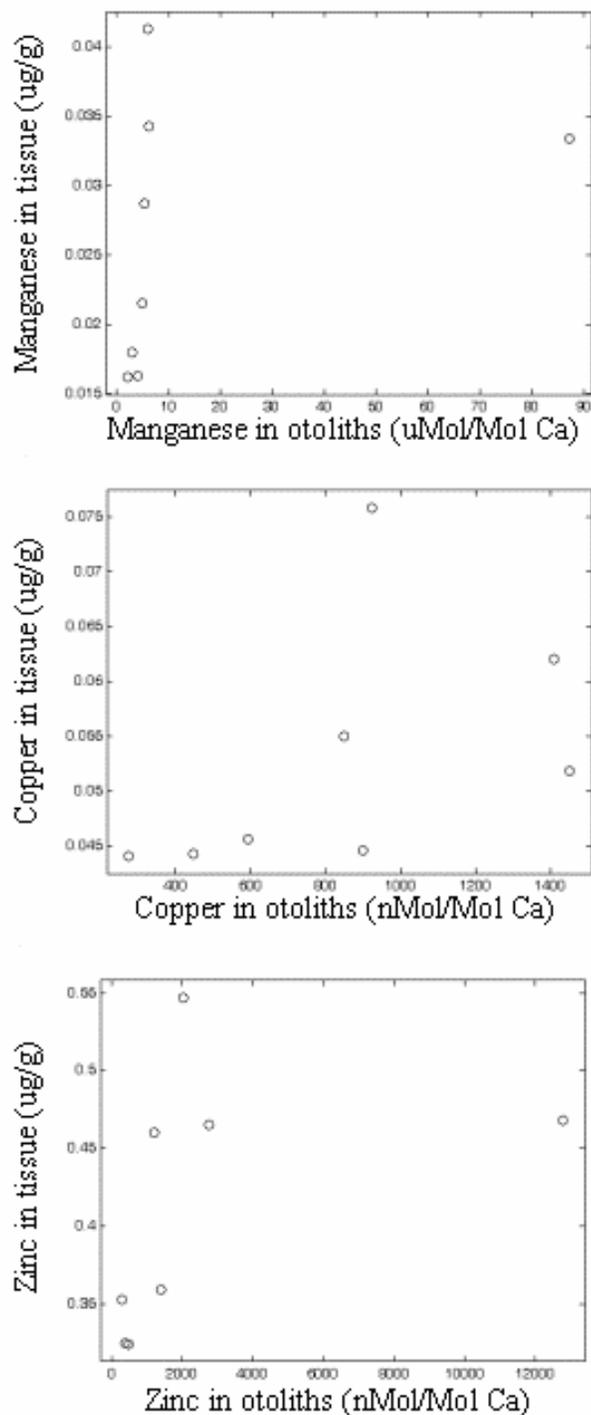


Figure 2.2 Essential metals with significant relationships between otolith core samples and homogenized tissues in Walleye pollock (*Theragra chalcogramma*). Manganese, copper, and zinc had individually significant Spearman rank correlations, but only Mn remained significant after a sequential Bonferroni correction.

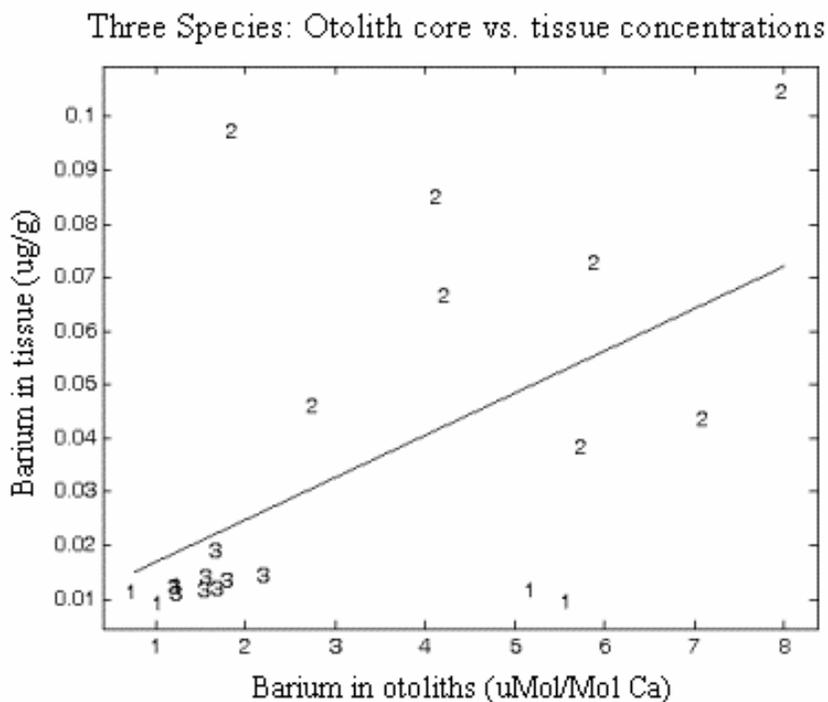


Figure 2.3: Trace metals with significant relationships between otolith core samples and homogenized tissues across three species: (1) Pacific herring (*Clupea pallasii*), (2) Atka mackerel (*Pleurogrammus monopterygius*), and (3) Walleye pollock (*Theragra chalcogramma*). Barium had significant Pearson product-moment and Spearman rank correlations both individually and after a sequential Bonferroni correction. The line represents the best-fit line indicated by the Pearson product-moment analysis.

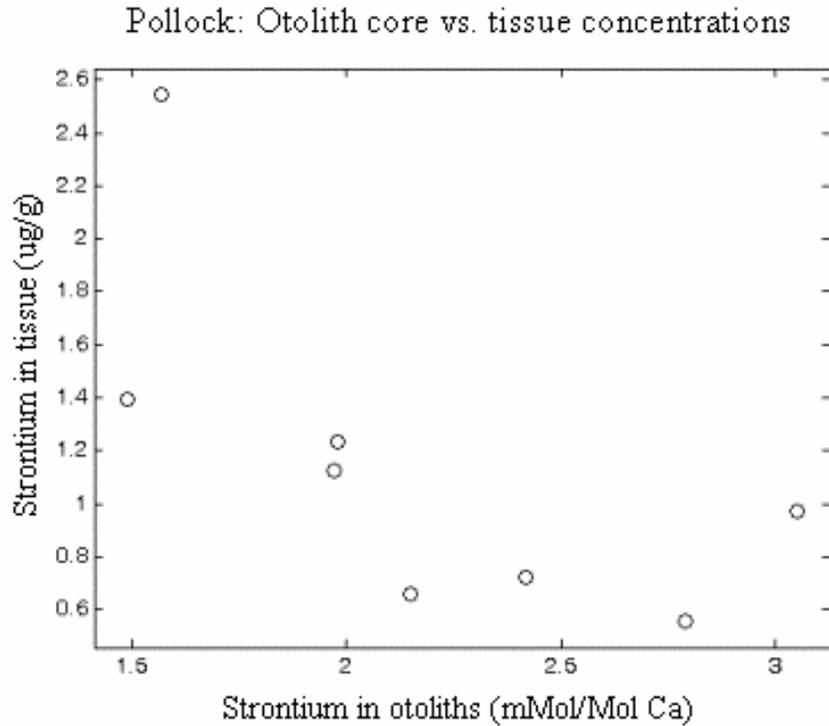


Figure 2.4: Trace metals with significant relationships between otolith core samples and homogenized tissues in Walleye pollock (*Theragra chalcogramma*). Strontium had a significant negative Spearman rank correlation individually and after a sequential Bonferroni correction.

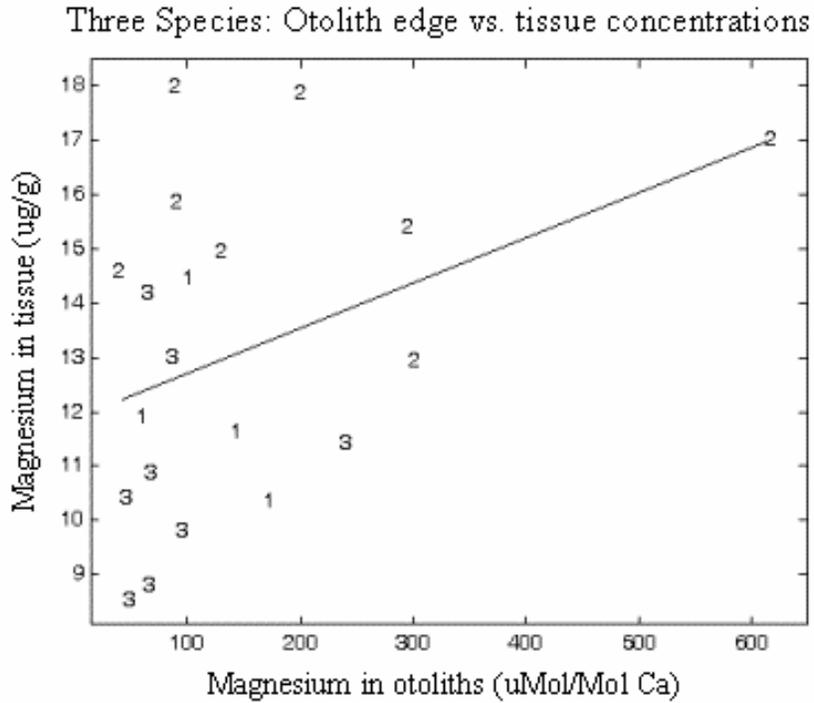


Figure 2.5: Essential metals with significant relationships between otolith edge samples and homogenized tissues across three species: (1) Pacific herring (*Clupea pallasii*), (2) Atka mackerel (*Pleurogrammus monopterygius*), and (3) Walleye pollock (*Theragra chalcogramma*). Magnesium had individually significant Pearson product-moment and Spearman rank correlations, but showed non-significant relationships after a sequential Bonferroni correction. The line represents the best-fit line indicated by the Pearson product-moment analysis.

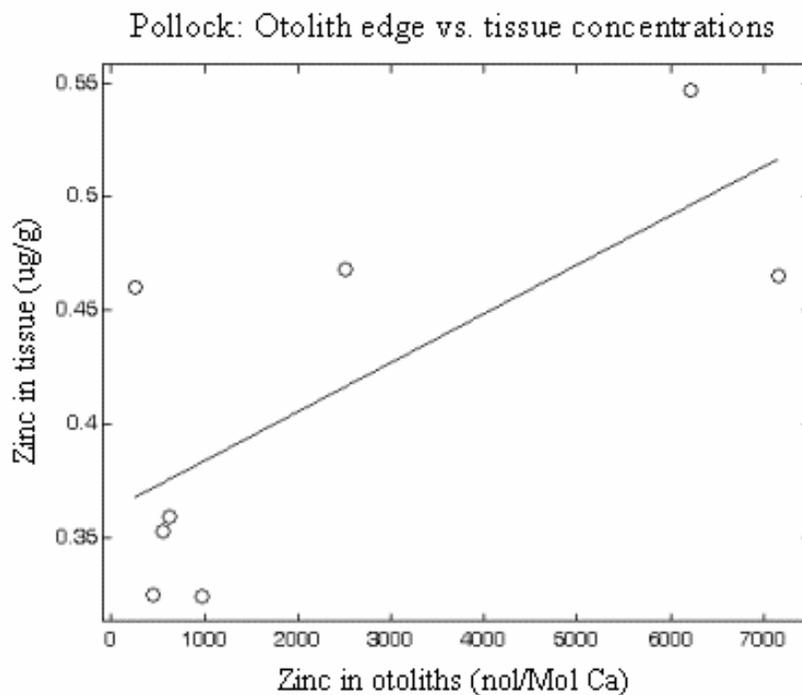


Figure 2.6: Essential metals with significant relationships between otolith edge samples and homogenized tissues in Walleye pollock (*Theragra chalcogramma*). Zinc had an individually significant Pearson product-moment correlation, but showed a non-significant relationship after a sequential Bonferroni correction. The line represents the best-fit line indicated by the Pearson product-moment analysis.

All Species: Otolith edge and tissue concentrations

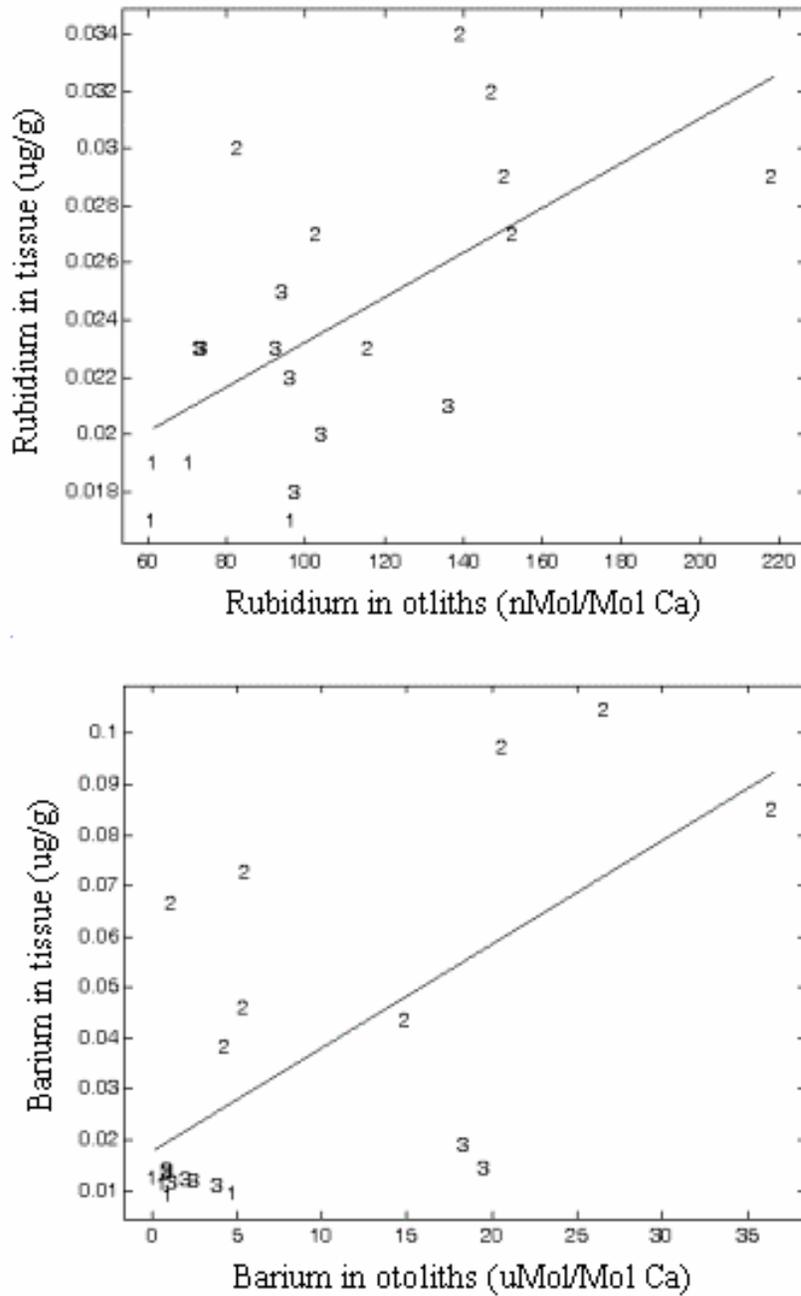


Figure 2.7: Trace metals with significant relationships between otolith edge samples and homogenized tissues across three species: (1) Pacific herring (*Clupea pallasii*), (2) Atka mackerel (*Pleurogrammus monopterygius*), and (3) Walleye pollock (*Theragra chalcogramma*). Rubidium and barium had significant Pearson product-moment and Spearman rank correlations both individually and after a sequential Bonferroni correction. The line represents the best-fit line indicated by the Pearson product-moment analysis.

Mackerel: Otolith edge and tissue concentrations

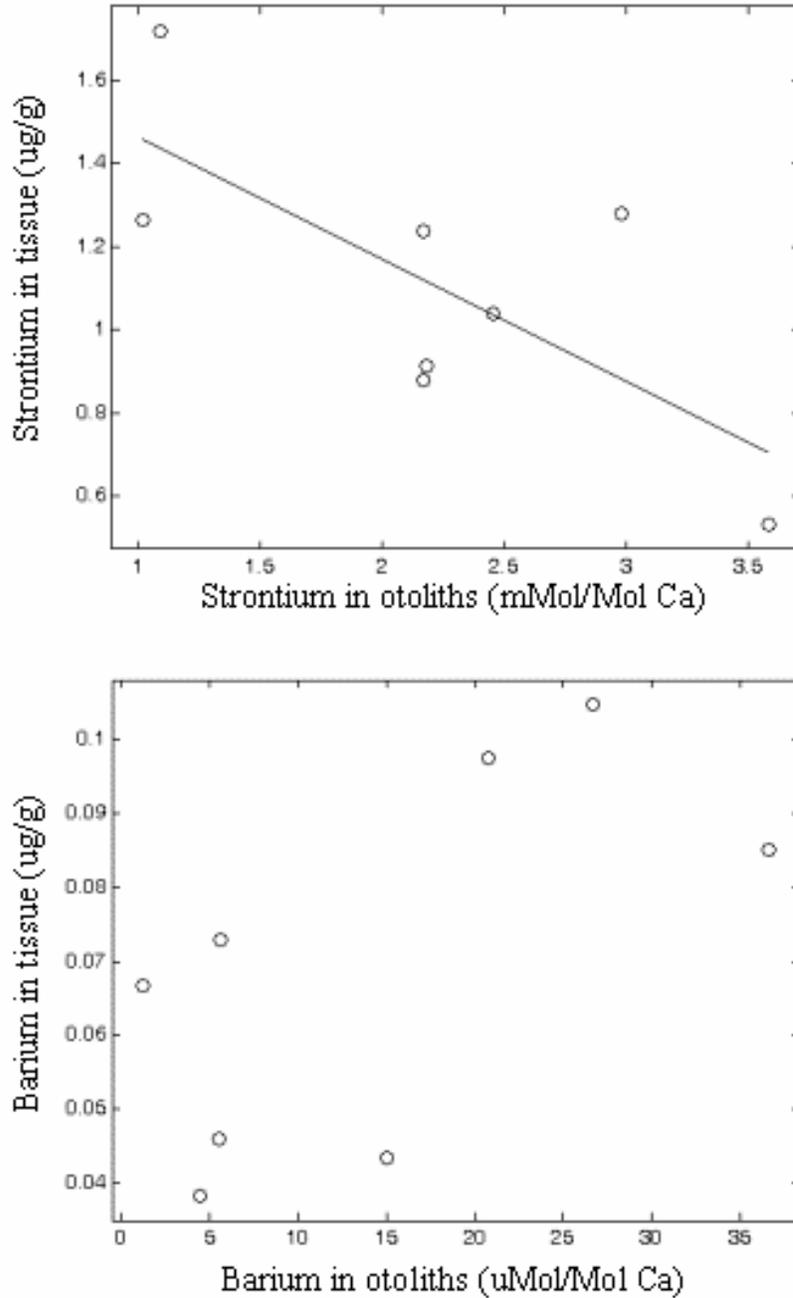


Figure 2.8: Trace metals with significant relationships between otolith edge samples and homogenized tissues in Atka mackerel (*Pleurogrammus monopterygius*). Strontium had an individually significant negative Pearson correlation, and barium had a individually significant Spearman rank correlation. Neither relationship was significant after a sequential Bonferroni correction. The line represents the best-fit line indicated by the Pearson product-moment analysis.

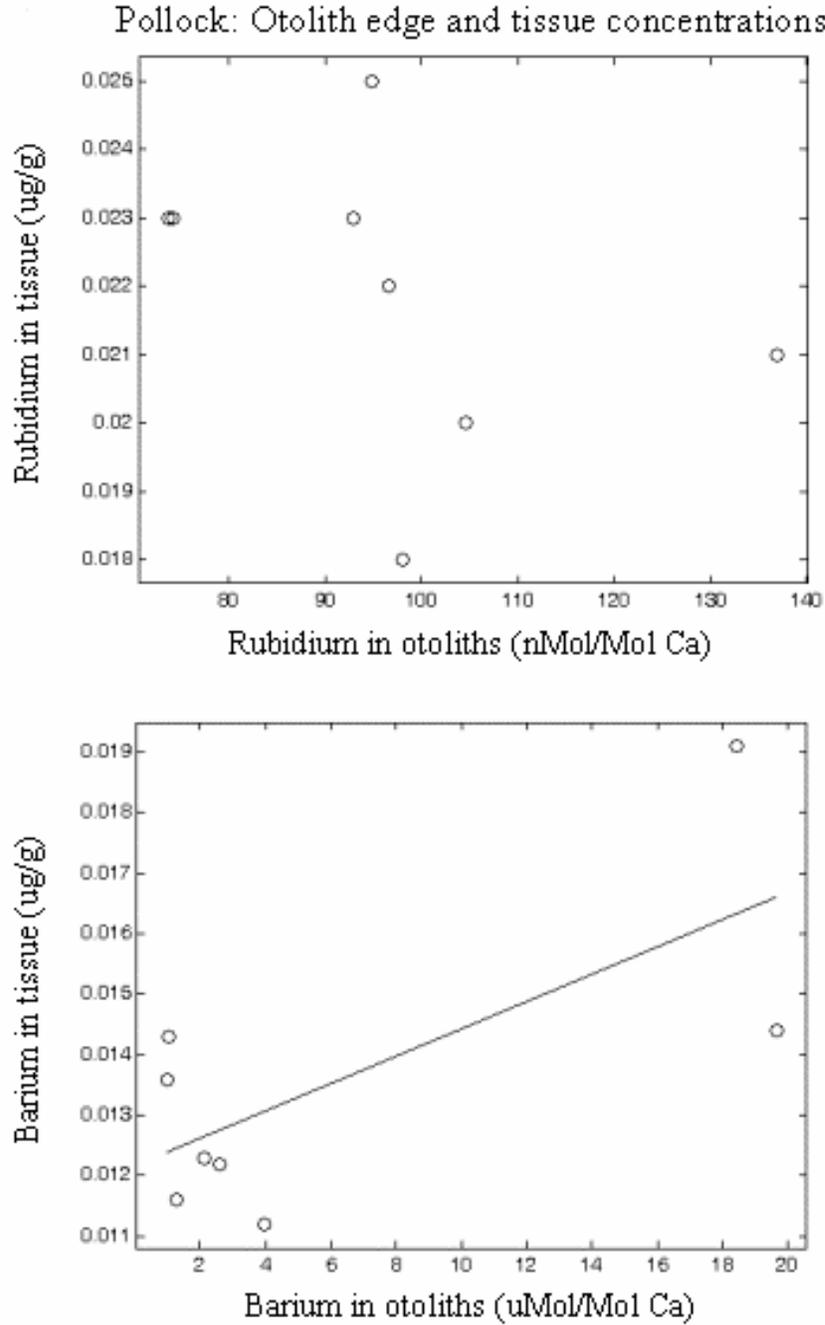


Figure 2.9: Trace metals with significant relationships between otolith edge samples and homogenized tissues in Walleye pollock (*Theragra chalcogramma*). Rubidium had a significant negative Spearman rank correlation, and barium had a significant Pearson correlation. Neither relationship was significant after a sequential Bonferroni correction. The line represents the best-fit line indicated by the Pearson product-moment analysis.

CHAPTER III

PREY DISCRIMINATION IN STELLER SEA LIONS (*EUMETOPIAS JUBATUS*) USING ELEMENTAL ANALYSIS OF OTOLITHS RECOVERED FROM SCAT

Introduction

The Steller sea lion (*Eumetopias jubatus*) population has been in decline since the 1960s and has been listed as threatened or endangered on the U. S. Endangered Species list since 1990. Research has indicated that nutritional stress is a potential factor in the decline, but the direct cause of this stress has not been determined. One hypothesis is that a change in the prey base, such as changes in diversity/quality or availability, may be responsible for the Steller sea lion decline in the western Gulf of Alaska (Merrick et al. 1997, Rosen and Trites 1999). The changes in Steller sea lion diet could be a result of shifts in oceanic conditions, competition with commercial fisheries, or any other factor that affects the distributions and abundances of fish species.

Scat analysis is widely used for examining the diets of pinnipeds in the wild (Sinclair and Zeppelin 2002); however, only physical examinations of fish bones, including otoliths, and other fish remnants are employed at present. Several studies on different pinniped species have examined the inherent biases in, or unreliability of, diet analysis using physical examination of otoliths recovered from scat. For example, Dellinger and Trillmich (1988) reported underestimates of the number of sprat (*Sprattus sprattus*) in the diets of California sea lions (*Zalophus californianus*) and South American fur seals (*Arctocephalus australis*) based on counts of otoliths recovered from scat, and da Silva and Neilson (1985) and Tollit et al. (1997) reported similar findings for other

small fish in the diets of harbor seals (*Phoca vitulina*). Captive feeding studies have shown that prey size, meal size, and the size of the pinniped affect the percentage recovery and condition of otoliths recovered from scat (Tollit et al. 2003). Two methods have been used to offset errors associated with physical analysis: numerical correction factors that attempt to account for completely digested otoliths (Bowen 2000), and the all-structure technique, where diet analysis is undertaken using all of the hard prey remains present in the scat (Tollit et al. 2003). Although these methods reduce the error of diet reconstruction, both studies highlighted certain limitations. Numerical correction factors are not always available for key species and do not account for double-counting of a single fish based on the remains found in scat. Both methods are partially limited by the inability to assign extremely digested otoliths and bones to a known prey species. This occurs most often for gadoids, flatfish, salmonids, and rockfish, which are all sea lion prey fish (Sinclair and Zeppelin 2002).

Otoliths act as data storage units for fish, acquiring incremental rings of calcium carbonate (CaCO_3) and organic material as a fish ages, and unlike other bones and scales, otoliths are not subject to resorption during nutritional stress (Campana and Neilson 1985). The suspension of the otolith in endolymphatic fluid and its acellular nature may account for this exemption from resorption (Fowler et al. 1995). Thus, the otolith retains a complete picture of the growth history of the fish, and otolith isolation from metabolic reworking also makes it a storehouse of environmental information (Campana and Thorrold 2001).

Chemicals from the environment are incorporated into otoliths based on chemical structure, environmental factors such as salinity and temperature, and metabolic factors.

For example, strontium (Sr) has the same valence as calcium (Ca) and substitutes for it in fish hard parts, but Sr uptake can be dependent on temperature and salinity (Radtke et al. 1990, Secor and Rooker 2000). Barium (Ba) incorporation has been linked to salinity as well and shows a negative correlation with Sr incorporation (Thorrold et al. 1997). The incorporation of other elements, such as magnesium (Mg), is affected by otolith precipitation rates and somatic growth (Martin and Thorrold 2005). These relationships between elemental concentrations in otoliths and external conditions mean that otoliths contain a record of an individual fish's movements and location throughout its life (Campana 1999), and each otolith has a unique microchemistry (i.e., its elemental fingerprint).

Elemental analysis of otoliths has become a standard method for fisheries scientists to discriminate among fish stocks (Gillanders 2001), examine geographic/spatial locations (Elsdon and Gillanders 2003), describe the environmental conditions experienced by a fish at different times during its life (Martin et al. 2004), trace migration pathways (Martin and Thorrold 2005), and use as a metabolic indicator (Schwarz et al. 1998). Several techniques have been employed for elemental analysis of otoliths and other fish hard parts, and one of the most powerful is inductively coupled plasma mass spectrometry (ICPMS). Laser-ablation ICPMS (LA-ICPMS) uses a high-powered laser beam focused on a small part of a solid sample, vaporizing it and sweeping it into a plasma stream of an inert gas, which is then analyzed by a mass spectrometer. The technique is rapid and combines the advantages of ICPMS with *in situ* micro-sampling. With the precision of laser sampling, different stages of a single fish's life can

be analyzed from the growth rings of one otolith, and the minute area needed for LA-ICPMS sampling would allow for broken or highly eroded otoliths to be analyzed.

Otoliths recovered from pinniped scat are often fragmented or highly eroded by the digestive process, and elemental analysis of otoliths recovered from pinniped scat can potentially answer a number of important questions about fish that have been consumed. For example, partially digested otoliths could be used to determine percentage species composition more accurately than physical examinations alone and could potentially provide useful information on the location of Steller sea lion foraging grounds, which fish stocks are being consumed, and thus whether competition is occurring with commercial fisheries. However, marine mammalogists to date have not utilized elemental analysis techniques developed by fisheries scientists.

The current study was conducted to examine if otoliths recovered from Steller sea lion scat retain enough chemical microstructure to be useful for elemental analyses; the potential problem being the effect of digestion on the chemical structure of otoliths. Changes in the elemental fingerprints of otoliths due to differential digestion would affect how much information is available for species discrimination, fish stock separation, determination of spatial locations, and life history analyses. Although the focus of this study is on Steller sea lions due to their endangered status and possible impacts from commercial fisheries, the techniques that I am employing can be applied to otoliths recovered from other pinniped and marine mammal species for which hard remains are collected for dietary analysis.

Methods

Otolith preparation

Walleye pollock (*Theragra chalcogramma*), Atka mackerel (*Pleurogrammus monopterygius*), and Pacific herring (*Clupea pallasii*) are key prey of wild Steller sea lions in the Gulf of Alaska (Sinclair and Zeppelin 2002), and their otoliths can be differentiated morphologically by external examination. Thirty whole, frozen fish from each species were supplied from ongoing studies at the University of British Columbia and shipped to Texas Tech University. I removed the sagittal otoliths from each fish using standard procedures (Secor et al. 1991). Ceramic scalpels and plastic dissection trays were used to avoid trace metal contamination of samples. Fish were measured to the nearest ± 1 mm and otoliths to ± 0.1 mm. Otoliths were rinsed with milli-Q water, dried, and stored individually prior to the feeding study and analysis.

One randomly selected otolith from each fish was fed to one of two juvenile female captive sea lions (SSL1 #F97HA, SSL2 #F97SI) held at the Vancouver Aquarium Marine Science Center, and the other otolith was prepared for LA-ICPMS analysis without being digested (i.e., pristine). Prior to Steller sea lion feeding, otoliths were placed in a double layer of gelatin capsules and inserted into the body cavities of decapitated herring (aiming to mimic the protection of the skull bones and head tissue during digestion). Scat was collected from both sea lions for the following seven days, and otoliths and otolith fragments that survived digestion were recovered from scat via elutriation (Bigg and Olesiuk 1990).

Digested otoliths were assigned to a species based on morphological features such as size and shape (Harvey et al. 2000), and pristine and digested otoliths from each

species were measured and prepared for LA-ICPMS analysis. Otoliths were embedded in Loctite, a compound that hardens when exposed to ultraviolet light. After the Loctite had set, otoliths were sectioned using a Buehler isomet, low-speed saw equipped with a diamond embedded wafering blade (series 15HC). Otolith sections from each species were mounted on glass slides using Crystal Bond, a temperature controlled, non-permanent adhesive, and polished using lapidary polishing cloths until the core of the otolith was visible. The diameter of each otolith was measured across the transverse axis of the otolith section using a micrometer and a dissecting microscope.

Previous studies have shown that the elemental fingerprints of otolith cores are distinctly different from other regions of the same otolith (Ruttenberg et al. 2005), so two microsamples were taken from each otolith: one from the core of the otolith, which represents the larval and juvenile life stages, and the other from the edge, which represents the post-juvenile life stage. All core samples were taken interior to the first annual ring on each otolith. Because the edge of a digested otolith does not correspond to the edge of a pristine otolith, all edge samples were taken just exterior to the third annual ring, which was the most exterior section available on all otoliths. Mounted otolith sections were shipped to Old Dominion University (ODU) in Norfolk, VA for LA-ICPMS analysis. At ODU, a final cleaning polish was performed in a Class 100 clean room to avoid contamination. A Finnigan MAT Element 2 ICP-MS was used to analyze samples that were introduced via UV (266 nm) Nd:YAG laser ablation system (Merchantek LUV-266X) for solid material analysis. Nine elements (^{12}Mg , ^{25}Mn , ^{29}Cu , ^{30}Zn , ^{37}Rb , ^{38}Sr , ^{39}Y , ^{56}Ba , and ^{82}Pb) were measured as ratios of analyte to calcium using

a known standard for comparison; these elements were chosen based on their widespread use in fisheries studies (Campana 1999).

Statistical analyses

All statistical analyses were carried out using MATLAB version 6.5 (Mathworks), and the functions for discriminant analysis (*discrim*), stepwise discriminant analysis (*stepdisc*), and multivariate analysis of variance (*manova*) are available at: www.biol.ttu.edu/Strauss/Matlab/matlab.htm.

Changes in mean diameter due to digestion were calculated for each species, and the percent decrease in diameter was calculated by dividing the change in mean diameter by the mean diameter of pristine otoliths for that species. These changes were then analyzed for significance using left-tailed t-tests for each fish species. A sequential Bonferroni correction was used to account for experiment-wise error.

Data sets were log-transformed for normalization, and values that were outside of the mean \pm 3 standard deviations for each element measured were removed as outliers. My statistical analysis required intact data sets, so outlying values were imputed using the expectation-maximization method described by Strauss et al. (2003). Data were then transformed back to their original units to facilitate interpretation. Data comparing the elemental fingerprints of pristine and digested otoliths were analyzed using a discriminant analysis in conjunction with a MANOVA to determine if digestion had a significant effect on the elemental fingerprints that would preclude their use in assessing the life histories of prey fish. Data for species discrimination were bootstrapped in conjunction with a stepwise discriminant analysis to determine the smallest subset of elements that would consistently distinguish among species. The results of the stepwise

discriminant analysis were then used in a MANOVA with randomized permutations to determine whether the differences in elemental concentrations among species were significant ($p < 0.05$). Post-hoc power estimates were calculated using noncentrality interval estimation procedures (Steiger and Fouladi 1997).

Results

Digestion effects

Otoliths from different species were affected to different degrees by the process of digestion, both physically (Table 3.1) and chemically (Figure 3.1). Digestion had a significant effect on otolith diameter for all three species (Table 3.2). The smallest otoliths (herring), showed significant changes in the elemental fingerprint for core and edge samples, whereas the larger otoliths (mackerel and pollock) each had one sampling location that showed a significant difference between elemental fingerprints and one that showed a non-significant difference ($p > 0.05$).

Pacific herring were the smallest fish used in the study, and their otoliths had the smallest diameter of the three species tested (Table 3.1). However, the proportional extent of physical digestion on herring otoliths fell between those of mackerel and pollock. Samples taken from the edge of herring otoliths showed a significant change in elemental concentrations ($p = 0.009$), as a result of digestion with a post-hoc estimated power of 0.79 for the MANOVA (Figure 3.1a). Core samples from these otoliths indicated a significant effect ($p = 0.001$) as well, with a post-hoc estimated power of 0.96 (Figure 3.1b).

Atka mackerel otoliths were larger, on average, than herring otoliths, and their diameters were less affected by digestion (Table 3.1). Samples taken along the edge of the otolith showed significant differences in their elemental fingerprints before and after digestion ($p = 0.002$) with a post-hoc power estimate of 0.93 (Figure 3.1c). The core sample data were not significantly different ($p = 0.08$), but the post-hoc estimated power for this MANOVA was only 0.39 (Figure 3.1d). Although digestion should have less of an effect on core samples because they are protected by the exterior (edge) layers of CaCO_3 , the low power level must be considered when interpreting the results.

Walleye pollock had the largest otoliths prior to digestion and showed the greatest change in diameter during the digestion process (Table 3.1). In pollock, the edge samples were non-significant ($p = 0.07$), but this test also had a low post-hoc power estimate of 0.35 (Figure 3.1e). The lack of significance was unexpected, given the extent of physical degradation to the otoliths, but the low power may simply indicate an inability to detect an effect. The lack of significance in the edge samples is further confounded by the highly significant effect of digestion on the elemental concentrations in the core samples ($p = 0.001$), which had an post-hoc estimated power of 0.86 (Figure 3.1f).

Species discrimination

Discriminant analysis of samples taken along otolith edges indicated that species discrimination was highly significant using only Sr concentrations for both pristine ($p = 0.002$) and digested ($p = 0.001$) samples (Figure 3.2). Post-hoc estimated power for these analyses was 0.91 and 0.95, respectively. When pristine ($n = 29$) and digested ($n = 24$) otolith data were pooled, strontium concentrations significantly discriminated between species ($p = 0.001$), with an post-hoc estimated power of 0.91. These results indicate that

elemental concentrations in samples taken along the edge can distinguish between species when otoliths show varying degrees of digestion and physical erosion.

Analyses of samples taken at the otolith core showed distinct differences in five elemental concentrations among species (Figure 3.3). The MANOVA included data for Ba, Rb, Sr, Y, and Mg concentrations, and both pristine and digested otoliths showed significant differences among species ($p = 0.001$ for both analyses). The post-hoc estimated power was 0.77 for pristine samples and 0.93 for digested samples. When pristine and digested otolith data were pooled for core samples, the results showed significant differences in elemental concentrations ($p = 0.001$) with an post-hoc estimated power of 0.92. Thus, samples from otolith cores also distinguish among species despite varying effects of digestion on different otoliths.

Discussion

Digestion effects

Analyzing the microchemistry of otoliths recovered from scat can provide additional information on Steller sea lion prey for dietary analysis. My results indicate that digestion does affect the elemental fingerprint of an otolith, but digestion of otoliths did not diminish my ability to distinguish among fish species using elemental analysis.

The effects of digestion on the microchemistry of otoliths differed among species and between the core and edge sampling locations. These differences are likely related to otolith size and otolith morphology. Pacific herring were the smallest fish in this study and had the smallest otoliths. Herring otoliths showed significant differences in elemental concentrations after digestion at both edge and core locations (Figure 3.1a and

3.1b), and they showed a larger proportional change in diameter than did the mackerel otoliths (Table 3.1), which had a slightly larger initial diameter. The largest otoliths, from pollock, showed the greatest change in diameter (Table 3.1), and both mackerel and pollock had one sampling location (core or edge) that showed a highly significant difference in elemental signatures due to Steller sea lion digestion.

Small otoliths have a lower recovery rate from scat than larger otoliths, but those that are recovered tend to pass through the gastrointestinal (GI) tract more quickly than do larger otoliths (Tollit et al. 1997). Because the stomach functions as an acid bath, dissolving ingested material, otoliths that pass through more rapidly have less exposure to the effects of digestion. Larger otoliths apparently are retained by the pyloric sphincter until they are reduced enough to pass through it (Tollit et al. 1997). However, smaller otoliths have a larger surface area to volume ratio, which increases the area of the otolith that is exposed to digestive acid. So, it is possible that an otolith of an intermediate size would be the least affected by digestion because it would pass through the digestive tract more quickly than would large otoliths, but the surface area to volume ratio would be less than with small otoliths. The amount of surface area exposed to digestive acid and the length of time an otolith is retained in the stomach can also be affected by otolith morphology. More spherical otoliths expose less surface area and are perhaps less likely to become wedged into crevices or folds in the stomach or intestines. Thus, round otoliths would pass through the GI tract more quickly than would angular or oddly shaped otoliths and would present less surface area to digestive acids, mitigating the effects of digestion. My data on the physical effects of digestion support the idea of intermediately sized otoliths being less affected, but my microchemistry data are

equivocal. Herring and mackerel elemental fingerprints are consistent with the physical changes in the otolith, with the core of the mid-sized otolith, from mackerel, failing to show a significant change in the elemental fingerprint. If digestion has a minimum effect on mid-sized otoliths, the otolith would pass through the digestive tract quickly enough that the core would be protected from digestion by the surrounding CaCO_3 material, and the elemental signature of the core would remain fairly intact. My lack of significant results from mackerel core data are consistent with this scenario, but the low power estimate for the test raises some question as to my ability to detect a difference if one is indeed present.

The low power estimates for my mackerel core and pollock edge analyses present problems in interpreting my results. The ability to detect an effect from digestion in these two tests was approximately half of the levels for the other tests, which all showed highly significant effects. My small sample size could be the reason for the low power in these two tests, but the sample size is consistent across all six of my tests, so differences in power are likely to come from another source. Low power levels could be caused by greater variability in elemental concentrations in my mackerel core and pollock edge samples, which could be an artifact of the sample itself. Increased variation also could be caused by how and where elements are incorporated into the otolith in pollock and mackerel. Differential digestion of elements incorporated into otoliths could depend on whether an element is embedded in the CaCO_3 or the organic matrix of the otolith layers. In order to address the effects of digestion on otolith microchemistry, it would be helpful to have more information on the exact mechanisms for elemental incorporation into otoliths and how this differs between species. Further studies with larger sample sizes

also would be helpful in addressing the low power issue. No other research on the effects of digestion on otolith microchemistry has been found for comparison to help with interpreting my results.

Species distinction

Although the data demonstrate that digestion has a significant effect on the microchemistry of an otolith, I was still able to distinguish among fish species using elemental analysis. The ability to identify species based on broken or heavily degraded otoliths in scat will improve species resolution and hence the accuracy of dietary analyses from scat collection. My data suggest that elemental fingerprints can distinguish between species in pristine and digested otoliths, even when the degree of digestion varies.

For samples along the edge of the otoliths, Sr was the only element that was needed to distinguish species with a reliability of > 90%, indicating a potential difference in salinity and temperature among the species' habitats. Because herring are pelagic fish and Atka mackerel and pollock are both groundfish, the water masses that herring inhabit should have different salinities and temperatures, which would be reflected in Sr concentrations. The variation in Sr concentrations between pollock and Atka mackerel could be affected by many factors. For example, pollock live close to the bottom with little vertical migration (Eschmeyer and Herald 1983), whereas Atka mackerel are thought to show extensive diurnal, vertical movements (Nichol and Somerton 2002). Vertical migration would affect the water salinity and temperature as the fish move through the halocline and thermocline, and these changes could affect Sr incorporation. Previous studies have indicated that Walleye pollock and Atka mackerel in the Gulf of Alaska show different temperature preferences (Swartzman et al. 1994), which also could

affect Sr uptake, and the difference in Sr incorporation in otoliths may also be species-specific even in identical water conditions.

Core samples, representing the larval and potentially juvenile life stages, required more elements than the post juvenile (edge) samples to discriminate among species. All three fish species used in this study have planktonic larvae, whose movements are directed by the prevailing currents (Nichol and Somerton 2002, Schumacher and Kendall 1995, Lassuy 1989). This would lead to a greater similarity in larval habitats than in post-juvenile habitats and may explain why more elements were needed to differentiate between species at the larval/juvenile sampling location (i.e., the core). My analysis on core samples incorporated elements whose uptake is affected by external and internal factors. Sr and Ba concentrations are indicative of salinity and temperature, whereas Rb and Y incorporation are affected by environmental concentrations (Chittaro et al. 2005), and Mg uptake is largely determined by somatic growth rates and otolith precipitation rates, rather than by environmental conditions (Martin and Thorrold 2005). Based on my results, species living under similar environmental conditions or with similar life histories retain unique elemental fingerprints that distinguish the species even after Steller sea lion digestion.

The elements that were included for each sampling location were the same for pristine and digested otoliths, which indicates that the same suite of elements can be used to identify species regardless of the extent of digestion of an otolith. This would allow heavily digested otoliths that have been in the sea lion digestive tract for several days to be compared with otoliths that had passed through the tract in a shorter timespan to determine if they belong to the same species. Since LA-ICPMS analysis does not require

a large amount of sample material, the elemental fingerprints of broken or highly degraded otoliths that are incompatible with current identification techniques can be used in dietary analyses. There is also the potential for combining ICPMS analysis with the all-structure technique to further improve dietary analysis. All fish bones lay down calcified layers, and a cross-section of a bone would have rings similar to those of otoliths. If other fish bones contain elemental fingerprints that are similar to otoliths, ICPMS analysis could be employed on several structures to identify species and give a more accurate estimate of how many fish were consumed.

In summary, my results indicate that sea lion digestion has significant effects on otolith microchemistry, but these effects do not prohibit the use of digested otoliths in species determination for dietary analysis. The degree of digestion does not affect the ability to discriminate between species, and there is the potential for combining LA-ICPMS analysis with the all-structure technique to further improve the accuracy of dietary analyses performed on pinniped scats. Further study on the elemental fingerprints of other fish bones would have to be undertaken to determine if they are suitable for this type of analysis and what effects digestion has on their microchemistry. I may be able to correct for the effects of digestion on otolith microchemistry, making it possible to discriminate between fish stocks, determine spatial locations, and describe environmental conditions based on digested otoliths. This would allow critical fish stocks of Steller sea lion prey to be defined more accurately, affording them better protection. The clear next step is an *in vitro* study on the rates at which various elements digest out of otoliths to develop a regression between the time spent in the GI tract and the loss of individual elements from the otolith. My study is the first step toward using elemental analysis on

fish bones recovered from marine mammal scat, and the amount of information that this technique could provide, without invasive measures, requires that I further investigate its potential.

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Table 3.1: Sample sizes, mean values for physical measurements, and average proportional decrease in otolith diameter due to digestion for Pacific Herring (*Clupea pallasii*), Atka Mackerel (*Pleurogrammus monopterygius*), and Walleye Pollock (*Theragra chalcogramma*). Percent decrease in diameter represents proportional digestion of otoliths for each species.

Species	N	Mean fish length (cm) ± 1 SD	Mean otolith diameter (mm) ± 1 SD	Percent decrease in diameter
Pacific herring				
pristine	10	20.7 \pm 2.9	1.8 \pm 0.2	
digested	7		1.4 \pm 0.2	17.9
Atka mackerel				
pristine	10	36.2 \pm 1.9	2.3 \pm 0.3	
digested	9		1.8 \pm 0.2	13.2
Walleye pollock				
pristine	9	27.4 \pm 3.3	5.4 \pm 0.4	
digested	8		2.9 \pm 0.2	44.0

Table 3.2: Left-tailed Student's t-test results for changes in otolith diameter caused by sea lion digestion. The change in diameter was significant in all three species, even after correction for experiment-wise error.

	<u>N</u>	<u>t-stat</u>	<u>Calculated p-value</u>	<u>Bonferroni critical p</u>
Pacific herring	17	3.77	0.001	0.03
Atka mackerel	19	3.44	0.002	0.05
Walleye pollock	17	15.01	6.83E-10	0.02

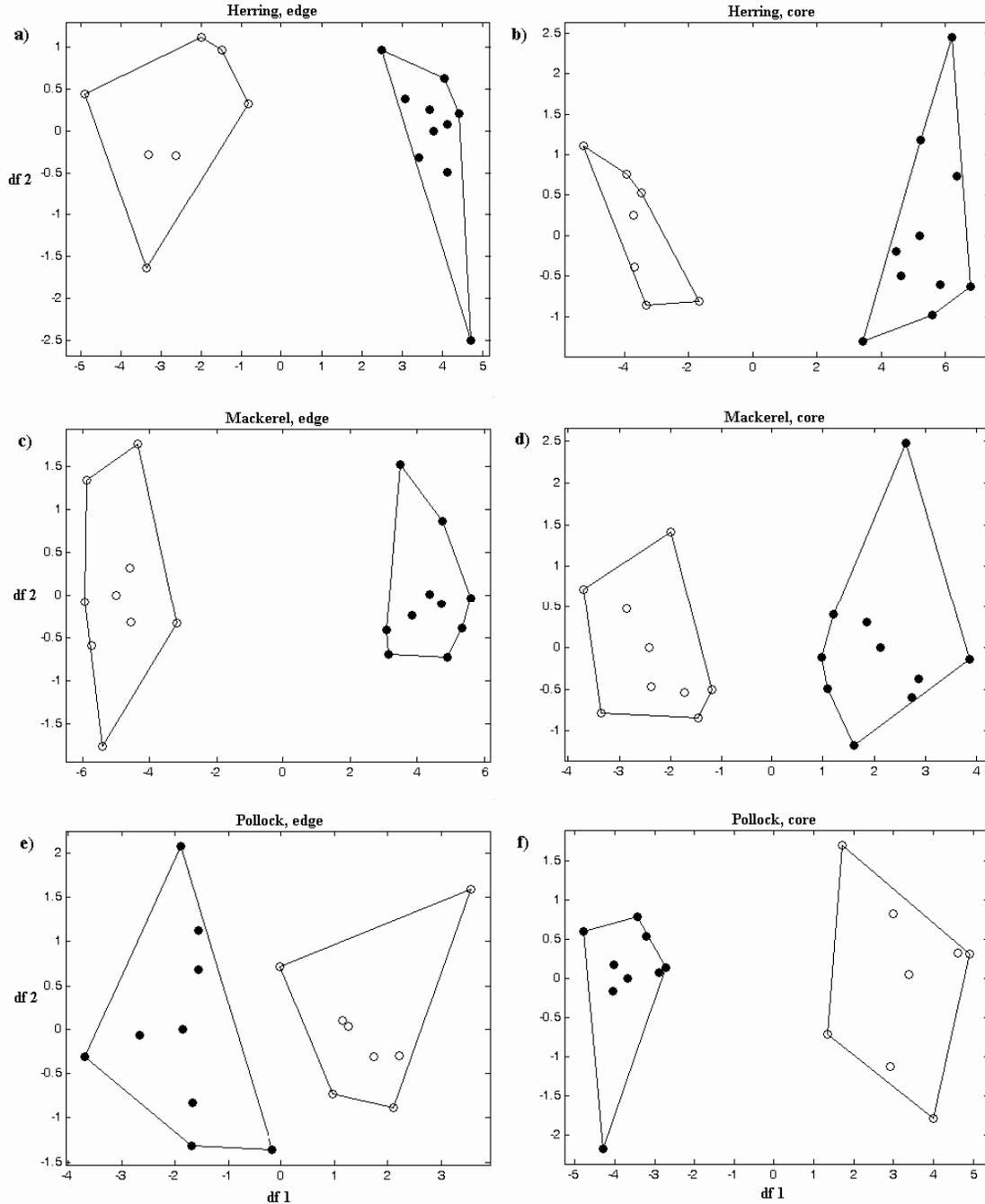


Figure 3.1: Discriminant function analysis plots for the effect of digestion on otolith microchemistry. Pristine otoliths (filled circles) compared to digested otoliths (open circles) for the same species. a) Edge samples - Pacific herring, b) Core samples - Pacific herring, c) Edge samples - Atka mackerel, d) Core samples - Atka mackerel, e) Edge samples - Walleye pollock, f) Core samples - Walleye pollock. Discriminant function 1 (df 1) represents the changes in elemental fingerprints of otoliths due to digestion and accounts for 100% of the discrimination. Discriminant function 2 (df 2) is an artificial function for the purpose of creating the scatterplot.

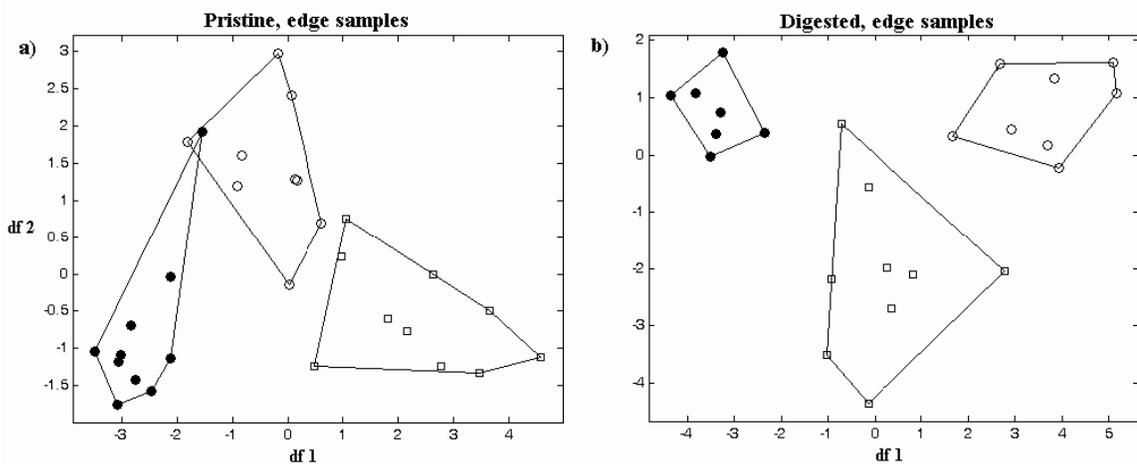


Figure 3.2: Discriminant function analysis plots for species discrimination at the otolith edge (post-juvenile life stage). ^{38}Sr was the only element required to distinguish between species, however, Pacific herring (filled circles), Atka mackerel (open squares), and Walleye pollock (open circles) were also differentiated when ^{12}Mg , ^{37}Rb , ^{38}Sr , ^{39}Y , and ^{56}Ba were all used in a MANOVA. a) In edge samples from pristine otoliths df 1 accounted for 80% of the discrimination and df 2 accounted for 20%. b) In edge samples from digested otoliths df 1 accounted for 87.5% of the discrimination and df 2 accounted for 12.5%.

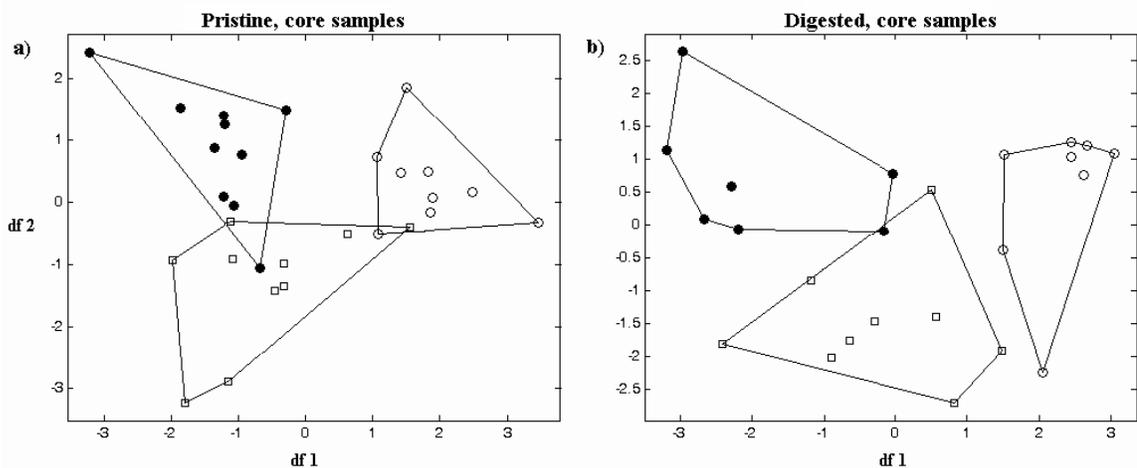


Figure 3.3: Discriminant function analysis plots for species discrimination at the otolith core (juvenile life stage). Concentrations of ^{12}Mg , ^{37}Rb , ^{38}Sr , ^{39}Y , and ^{56}Ba distinguished between Pacific herring (filled circles), Atka mackerel (open squares), and Walleye pollock (open circles). a) In core samples from pristine otoliths df 1 accounted for 67.1% of the discrimination and df 2 accounted for 32.9%. b) In core samples from digested otoliths df 1 accounted for 78.6% of the discrimination and df 2 accounted for 21.4%.

CHAPTER IV

A PRELIMINARY EXAMINATION OF THE USE OF EYE LENSES IN DIETARY ANALYSES OF STELLER SEA LIONS (*EUMETOPIAS JUBATUS*)

Introduction

Steller sea lions (*Eumetopias jubatus*) have been listed under the Endangered Species Act since 1990, and the western sub-population, or stock, has declined from over 300,000 animals to less than 30,000 over the past thirty-five years (Fritz and Stinchcomb 2005). While there are several factors that could contribute to this decline, evidence has indicated that nutritional stress is likely a major part of the problem (Rosen and Trites 1999). Any change in prey diversity, quality or availability could lead to nutritional stress and contribute to the Steller sea lion decline (Merrick et al. 1997, Rosen and Trites 1999). These changes could result from competition with commercial fisheries, shifts in oceanic conditions, or other factors that affect the distributions and abundances of prey fish species.

The physical examination of fish remnants, predominantly otoliths and other bones, recovered from scat is the most widely used technique for examining the diets of pinnipeds (Sinclair and Zeppelin 2002, Tollit et al. 2003). However, recent studies on different pinniped species have indicated there are inherent biases in this methodology. Studies on California sea lions (*Zalophus californianus*), South American fur seals (*Arctocephalus australis*), and harbor seals (*Phoca vitulina*) all report that small fish, such as sprat (*Sprattus sprattus*) and herring (*Clupea spp.*), are underrepresented in

pinniped diets based on counts of otoliths recovered from scat (da Silva and Neilson 1985, Dellinger and Trillmich 1988, Tollit et al. 1997). Gales and Cheal (1992) also found that dietary analysis for Australian sea lions (*Neophoca cinerea*) based on otoliths and squid beaks recovered from scat was completely unreliable due to the complete digestion of some of these structures. Although methods have been developed to minimize the errors associated with physical analysis of fish remnants, these methods do not account for the inability to assign extremely digested otoliths and bones to a known prey species and do not correct for double-counting of a single fish (Bowen 2000, Tollit et al. 2003). Additionally, the identification of fish hard parts recovered from scat is limited to a specific set of known species and structures, and an extended period of time must be taken to learn to identify digested structures (Cottrell and Trites 2002).

Eye lenses have the highest recovery rates of fish remains from pinniped scat and give the least-biased estimates of the number of prey items consumed. Cottrell and Trites (2002) recovered an average of 67% of eye lenses fed to captive Steller sea lions compared to an average of 26% of otoliths. In captive feeding trials with Antarctic fur seals (*Arctocephalus gazella*), 51% of eye lenses were recovered from scat, whereas 27% of otoliths were recovered (Staniland 2002). Studies on Australian sea lions found that eye lenses were present in over 50% of scats from captives and 95% of scats from wild sea lions, while otoliths were found in only 9% and 35% of scats, respectively (Gales and Cheal 1992). As with all physical counts, however, there are problems in using eye lenses for dietary analyses. Eye lenses can be eroded in the digestive tract, just as bones are (Gales and Cheal 1992), and there is also no way to differentiate among most fish species from physical examinations of eye lenses (Cottrell and Trites 2002). So, although

the physical analysis of eye lenses gives the least-biased estimate of the number of prey items consumed, there is currently no way to determine dietary proportions of individual fish species using this technique.

Fisheries scientists have recently begun to use elemental analysis to examine trace element incorporation in fish eye lenses. This technique is routinely used on fish otoliths to discriminate among fish stocks (Gillanders 2001), examine geographic/spatial locations (Elsdon and Gillanders 2003), describe the environmental conditions experienced by a fish at different times during its life (Martin et al. 2004), and trace migration pathways (Martin and Thorrold 2005). However, the use of elemental analysis on eye lenses is in the initial stages. At present, studies using elemental analysis of undigested eye lenses have differentiated fish populations and spatial locations (Dove and Kingsford 1998) but were unable to determine stock structure within a population (Gillanders 2001). Gillanders' study, however, involved small sample sizes ($n=5$) and was focused on correlations between otoliths and other structures (eye lenses, scales, and spines) for stock discriminations. Kingsford and Gillanders (2000) found that the concentration of rubidium (Rb) in eye lenses varied with depth of residence and that the distinct chemical composition, or elemental fingerprint, of an eye lens varied with depth. These results indicate that there is potential for the elemental analysis of eye lenses to provide more information on fish biology and life histories.

The favored technique for elemental analysis of fish otoliths and eye lenses is inductively coupled plasma mass spectrometry (ICPMS). ICPMS analysis has two main forms of sample introduction into the mass spectrometer: a solution-based approach and a laser ablation method. In solution-based ICPMS (SO-ICPMS), samples are placed in

solution by acid digestion. The solution is sprayed into flowing argon and passed into a torch, which is inductively heated. The resulting plasma is analyzed by the mass spectrometer. Laser ablation ICPMS (LA-ICPMS) uses a high-powered laser beam focused on a small part of solid sample material, vaporizing it and sweeping it into a plasma, which is then analyzed by a mass spectrometer. Both methods of sample introduction have benefits and drawbacks. SO-ICPMS requires more sample preparation, returns a mean value for the entire sample, and has a higher potential for sample contamination (Ludsin et al. 2006), but it has lower detection limits for most elements (Becker 2002). LA-ICPMS requires less sample preparation and less sample material, and it has a lower risk of contamination. However, the detection limits for LA-ICPMS are often higher than those for SO-ICPMS, and LA-ICPMS detection shows interference for some elements from polyatomic chemicals (Becker 2002). Previous studies on the elemental composition of fish eye lenses have all used SO-ICPMS to obtain the average concentration of an element for the entire eye lens.

LA-ICPMS has become the preferred technique for otolith analysis because of the small amount of sample needed, and its ability to spot-sample from several distinct locations on a single otolith. Otoliths lay down concentric layers as the fish ages, even in the absence of somatic growth, and they are not subject to resorption by the fish during nutritional stress. These rings incorporate trace elements from the environment based on the environmental concentrations and environmental conditions such as temperature and salinity. Increments start to form immediately after hatching in most species and have been shown to give accurate measures of fish age (Kalish 1989, Eka and Blay 2000).

Thus, otoliths contain a record of an individual fish's movements and location throughout its life (Campana 1999).

The eye lens possesses several of the characteristics that make otoliths a preferred structure for elemental analysis, and lenses may provide the same types of information on fish movements and life histories when analyzed via LA-ICPMS. Eye lenses form concentric layers comparable to growth rings, made up of fibrous cells that run from a point near the optic nerve longitudinally to the apex on the opposite side of the lens (Nicol 1989) (Figure 4.1). These cells are largely made up of structural proteins, with a crystalline matrix and an insoluble albuminoid component that increases in proportion as the lens ages (Nicol 1989). As the lens grows, epithelial cells at the outer surface differentiate into new fibrous cells, making the innermost portion of the lens (the core) the oldest (Dove and Kingsford 1998). Only the newly formed fibers and the epithelial layer contain organelles, which are absent in the mature fiber cells found in the inner portion of the lens (Horwitz 1993). The proteins in mature fiber cells remain intact for the lifetime of the fish, with no turnover after the cell has differentiated (Horwitz 1993). This, combined with the avascular nature of mature lens cells, leaves the eye lens with no mechanisms for removing trace elements from the tissue once they have been incorporated (Gillanders 2001, Horwitz 1993). Eye lens isolation from metabolic reworking potentially makes it a storehouse of information on fish ecology.

This study was conducted to further examine the use of eye lenses for dietary analysis in pinnipeds, specifically Steller sea lions. The morphology of eye lenses was examined for Walleye pollock (*Theragra chalcogramma*), Atka mackerel (*Pleurogrammus monopterygius*), and Pacific herring (*Clupea pallasii*), which are all

major prey species for Steller sea lions. First, I assessed the use of eye lenses to age fish based on the growth layers found in the lens. These layers may be analogous to the otolith annuli that are used to age both larval and adult fish (Campana and Thorrold 2001). Eye lenses recovered from scat could provide minimum ages for consumed fish, an age demographic for pinniped prey, and an estimate of prey size based on age-size conversions. Secondly, the effects of digestion on the dry mass of eye lenses was assessed to determine if a significant amount of material is lost during digestion, which could lead to changes in the elemental composition as well. Additionally, two eye lens samples were analyzed via LA-ICPMS to investigate elemental distributions across the eye lens, symmetry of elemental incorporation around the center of the lens, and the potential effects of sea lion digestion on the elemental composition of eye lenses. Elemental analysis of fish eye lenses via LA-ICPMS has the potential to not only distinguish among fish species based on the elemental fingerprint of lenses, but also to provide additional information on fish life histories, such as movements of prey fish prior to consumption and the location of sea lion foraging grounds.

Methods

Eye Lens Preparation

Whole, frozen fish from each of three species (Walleye pollock, Atka mackerel, and Pacific herring) were supplied from ongoing dietary studies at the University of British Columbia and shipped to Texas Tech University. Thirty fish from each species had both otoliths and eye lenses removed using ceramic scalpels and plastic dissection trays to avoid trace metal contamination. Otoliths were removed using standard

procedures (Secor et al. 1991). To remove eye lenses, the cornea of each eye was punctured and the optic nerve, suspensory ligaments, and lens muscle were severed. Eye lenses were cleaned with milli-Q water, air dried, and weighed prior to being stored individually. Otoliths were sectioned, mounted on glass slides, and polished using lapidary polishing cloths until the core of the otolith and annual rings were visible. Annual rings in each otolith were counted by one reader and validated by a second, and these annuli counts were used for comparison to growth layers found in sectioned eye lenses.

One randomly selected eye lens from each fish was fed to one of two juvenile female captive sea lions (SSL1 #F97HA, SSL2 #F97SI) held at the Vancouver Aquarium Marine Science Center, and the other eye lens was kept without being digested (i.e., pristine). Prior to Steller sea lion feeding, eye lenses were placed in two layers of gelatin capsules and inserted into the body cavities of decapitated herring (aiming to mimic the protection of the skull bones and head tissue during digestion). Scat was collected from both sea lions for the following seven days, and eye lenses that survived digestion were recovered from scat via elutriation (Bigg and Olesiuk 1990). Mackerel and pollock lenses were fed in the first of two feeding sessions, and mackerel and herring lenses were fed in the second session. The two sessions were separated by a span of several months.

Digested eye lenses were assigned to a species based on size, coloration, and feeding session. Five dry eye lenses, both pristine and digested, from each species were weighed, re-hydrated using milli-Q water, and split in half using sterilized scalpels. Lens halves for each fish were allowed to air dry, and two individual readers counted growth layers on five pristine eye lenses for mackerel and pollock and four lenses for herring.

LA-ICPMS Analysis

One half of each lens was shipped to the ICPMS lab at the Research School of Earth Sciences at the Australian National University (ANU). At ANU, lenses were embedded in an epoxy resin and polished prior to elemental analysis. A Hewlett Packard Agilent 7500 ICPMS was used to analyze samples that were ablated by an EXCIMER UV laser and introduced into the mass spectrometer. An initial laser raster was performed across the surface of each eye lens parallel to the axis of fiber elongation to remove surface contamination, and then a second raster was performed across the track of the first for actual data collection (longitudinal, or 'long', raster). The lens was rotated 90 degrees, and two more burns were performed perpendicular to the first two (latitudinal, or lat, raster) (Figure 4.2). Fourteen isotopes of twelve elements (^{25}Mg , ^{31}P , ^{43}Ca , ^{55}Mn , ^{57}Fe , ^{63}Cu , ^{65}Cu , ^{66}Zn , ^{68}Zn , ^{85}Rb , ^{88}Sr , ^{138}Ba , ^{139}La and ^{208}Pb) were measured. These elements were selected based on their widespread use in fisheries studies (Campana 1999), and their presence in the eye lens based on a Scanning Electron Microscope preliminary backscatter scan. Two isotopes of copper and zinc were analyzed to allow testing for interference in elemental detection from polyatomic chemicals. Preliminary results are presented here for two mackerel lenses (one pristine and one digested).

Data Analysis

Growth Layers and Age

A Pearson product-moment correlation analysis was used to investigate the relationship between the number of rings found in eye lenses and otoliths from the same fish. Otolith annuli are regularly used to determine fish age, and correlations between the two types of rings would indicate that eye lenses could be used to age fish as well. The

slope of the least-squares regression line from the data was then tested to determine if it was significantly different from one.

Eye Lens Weight

The effect of digestion on the mass of eye lenses was analyzed using a separate t-test for each fish species, with a sequential Bonferroni correction to account for experiment-wise error. A significant decrease in mass due to digestion could be indicative of a change in elemental concentrations.

Elemental Concentrations

Counts from the mass spectrometer were adjusted for background, corrected for machine drift, and converted to micrograms/gram ($\mu\text{g/g}$) using a known National Institute of Standards and Technology (NIST) 612 standard for trace elements. Preliminary results from the LA-ICPMS analysis on the two eye lens samples were analyzed for mean concentrations of each element, for elemental distribution symmetry around the center of the eye lens, and for significant effects from digestion. Mean concentrations for each raster, long and lat, on each eye lens were calculated after data sets had been truncated to account for mass spectrometer ramp-up and any elemental spikes caused by the laser running off the edge of the eye lens. The small sample size precluded statistical analysis, so visual analyses was used to examine the symmetry of elemental distributions and any large-scale effects on the distribution pattern from digestion. Symmetry was analyzed around the center of the lens for a single raster, and by comparing long and lat rasters from the same lens. Distributions were considered symmetrical when there were similar overall trends and patterns of dips and peaks, although the magnitude of dips and peaks did not have to be the same. The distributions of all four rasters, long and lat on each

lens, were examined to determine symmetry around the center of the lens. Similarity between perpendicular rasters was examined by comparing long and lat from the same lens.

Results

Growth Layers and Age

Microscopic observations of eye lens cross-sections revealed the highly fibrous nature of the lens (Figure 4.3). These fibers form layers within the lens, but the layers are not as distinct as the annuli in otoliths. This made it difficult to isolate individual growth layers and determine the number of layers in a single lens. For my analysis, growth layers were distinguished by structural breaks that could be traced around the entire lens and by shifts in translucence and color shading (Figure 4.4). To address the problem with layer distinction, layers were counted on two separate occasions, with two weeks between counting sessions. A second reader counted growth layers independently, and these counts were used to resolve any discrepancies when they occurred. Otolith data were not available while eye lenses were analyzed to avoid biasing the counts. Reader agreement between the second reader and my first counts for eye lens growth layers was 57%, but between the second reader and my second counts the agreement was 86%. In two eye lenses out of the fourteen, the second reader did not agree with either of my counts, although my two counts were the same. In each of the two eye lenses where there was no agreement, the second reader was one layer off from my count, above it for one lens and below it for the other (Table 4.1). In 64% of the fish examined (n=14), the growth layers in eye lenses corresponded to the number of annuli in fish otoliths plus one, and in 21%

of fish the number of growth layers in the lens was equal to the number of annuli in otoliths. In the remaining two fish, both of which were mackerel, the number of growth layers in the eye lens was equal to the number of annuli in the otolith plus two. The results of a Pearson product-moment correlation analysis indicated a significant relationship between the number of growth layers in an eye lens and the number of annuli in the corresponding otolith ($r = 0.45$, $p = 0.05$) (Figure 4.5). However, the slope of the least-squares regression line (0.45) was shown to be significantly different from one ($t = -3.12$, $df = 13$) at the 0.01 significance level.

Eye Lens Weight

The morphology of eye lenses showed little obvious change from sea lion digestion. The exterior surface of digested eye lenses had less surface debris and appeared polished compared to pristine eye lenses, but the overall shape and segmentation of the lens appeared to remain intact (Figure 4.6). Cross-sections of pristine and digested otoliths had similar appearances (Figure 4.7), which suggests that the interior of the eye lens may be somewhat protected from erosion in the digestive tract. Although the exterior surface of eye lenses appeared smoother, the quantitative effects of sea lion digestion on eye lens dry mass were insignificant. A randomized, nested ANOVA incorporating five pristine and five digested eye lenses from each fish species ($n = 30$) showed no significant difference in eye lens weights from sea lion digestion ($p = 0.77$), although there was a significant weight difference among fish species ($p = 0.001$) (Table 4.2).

Elemental Concentrations

For copper and zinc, where two isotopes of the element were measured to test for interference, the distributions of the isotopes across the eye lens paralleled each other almost perfectly. Spikes and dips were located in the same location, although their magnitudes often differed. Linear regressions between the concentrations of the two isotopes for each element had slopes close to one, as indicated by r , and were significantly different from zero, as indicated by p ($0.96 < r < 0.99$, $p < 0.001$) (Figure 4.8). Given this indication of little or no interference from polyatomic chemicals, only the more biologically active isotope was further analyzed.

For the long and lat rasters on each eye lens, the mean, standard deviation, coefficient of variation, minimum, and maximum concentrations were determined for each element (Table 4.3). The range of values (maximum minus minimum) for some elements was large compared to the mean due to spikes in element concentrations, which increased the variation. The mean concentrations of calcium for all four rasters, long and lat on both lenses, were within one standard deviation of each other, and this was also true for zinc. The mean concentrations for iron, copper, rubidium, and strontium were within one standard deviation of each other for long and lat rasters on the same eye lens, but showed greater differences when compared across the two lenses. Magnesium and manganese had mean concentrations for long and lat that were within one standard deviation for the digested lens only, whereas mean concentrations for each raster on the pristine lens were just outside of one standard deviation. Conversely, barium and lanthanum had mean values for long and lat rasters that were within one standard deviation for the pristine lens but showed greater differences on the digested lens.

If the layers in the eye lens represent concentric growth rings, elemental distributions should appear symmetric when moving outward from the center of the lens. When graphs of elemental data were overlaid onto photos of the respective lens cross-section to obtain an image of the changes in elemental concentrations across the lens, some elements displayed symmetry around the lens core and some did not (Figure 4.9). Magnesium (Figure 4.10), phosphorous (Figure 4.11), copper (Figure 4.12), zinc (Figure 4.13), and strontium (Figure 4.14) showed symmetrical distributions around the center of the eye lens in at least three of the four rasters, long and lat on the pristine lens and long and lat on the digested lens. Iron (Figure 4.15) and lead (Figure 4.16) were asymmetrically distributed around the lens core for at least two of the four rasters. Barium (Figure 4.17) had symmetrical distributions around the lens center for long and lat on the digested eye lens, but the rasters on the pristine lens had a lot of noise in the signal, which hindered any analysis of symmetry. Manganese (Figure 4.18), rubidium (Figure 4.19), lanthanum (Figure 4.20), and calcium (Figure 4.21) had too much noise in their elemental signals to visually assess symmetry at all.

When long and lat rasters were compared for each eye lens, some elements showed similar trends in distribution and others did not. Magnesium, phosphorous, zinc, and strontium all displayed similar trends when the two rasters from one eye lens were compared. Copper, iron, and lead did not show similarity between the long and lat rasters, and barium distributions did not appear to be similar but were affected by signal noise. The similarity between long and lat rasters could not be compared for manganese, rubidium, lanthanum, and calcium due to the noise in their elemental signals.

Periodic spikes and/or dips in elemental concentrations occurred for most of the elements analyzed. These abrupt changes in elemental concentrations were generally located symmetrically around a central point, but the magnitude of the changes was not always equal. Copper, lanthanum, and barium showed more of tendency to spike than did other elements whose elemental distributions were generally smooth, with a fairly constant amount of noise and few spikes.

Discussion

My results indicate that the analysis of fish eye lenses can provide more information about fish life histories and pinniped diets than is currently being obtained. Although a few studies have used elemental analysis of the whole lens to distinguish between fish populations and locations of residence, the use of LA-ICPMS on the incremental layers found in the eye lens increases the amount of information potentially available.

The correlation analysis found a significant relationship between the number of growth layers in eye lenses and the number of annuli in otoliths, but the relationship was not one-to-one, which was what I was looking for. Previous studies using fish eye lenses as an indicator of age relied on the weight of the eye lens (Burkett and Jackson 1971, Carlton and Jackson 1968). These studies found that the mean lens weight and age were significantly correlated, but the distributions for adjacent age classes overlapped, which prevented the accurate aging of some individuals. I found that 79% of eye lenses had more growth layers than the number of annuli on the corresponding otolith. Research has shown that rapid growth rates can mask the ring structure of an otolith, making some

annuli difficult to distinguish (Burkett and Jackson 1971), and that age determination from otoliths is confounded by incomplete years of growth in some fish where the annuli have yet to be formed (Carlton and Jackson 1968). Masking of annuli and the absence of an annual marker in fish during an incomplete year of growth could lead to other structures, such as the eye lens, indicating a greater age than the otolith of the same fish.

However, otolith annuli counts have been validated for numerous fish species, whereas the growth layers found in eye lenses have not been examined in known-age fish. It is possible that the eye lens has an additional layer that forms during the larval stage or that growth layers are not laid down in the lens precisely annually. My data indicate that it is likely eye lens growth layers are not laid down annually, but still have a correlation to fish age. Controlled studies with known-age fish are needed to validate eye lens growth layers as indicators of fish age. My study was limited by the small age range of fish included, and eye lenses from a larger age sample need to be analyzed, as well.

The fibrous nature of the lens can make growth layers difficult to distinguish (Figure 4.3). Contaminants and other impurities can become trapped among the fibers, causing cataracts and other disruptions of lens layers (Hargis and Zwerner 1988). Other factors, such as nutritional imbalances and genetic aberrances, can also create lens abnormalities that affect the layered structure (Hargis and Zwerner 1989). These aberrations in lens formation can lead to indistinct growth layers and random breaks within growth layers that hinder accurate counts. My method for splitting eye lenses in half was too coarse for the structure of the lens, and growth layers were sometimes damaged and compressed when the eye lenses were bisected (Figure 4.23). A precise

tool, such as a microtome or laser, should be employed when taking cross-sections to minimize damage to growth layers.

However, even with the potential counting discrepancies there was a significant correlation between the number of growth layers in lenses and the number of annuli in otoliths. These results indicate that eye lenses could be a useful tool for aging fish. Cross-sections of digested eye lenses recovered from sea lion scat showed growth layers as distinct as those in pristine lenses, suggesting that lenses recovered from scat can be used to age prey fish. My results indicate that the mass of the eye lens is not significantly affected by digestion, which introduces the possibility of aging prey fish using both eye lens weight and growth layer counts to get an accurate measure of fish age. Given that eye lenses are recovered more frequently from scat than otoliths and appear to show fewer effects from digestion, eye lenses recovered from scat may be more accurate for aging prey fish than their otolith counterparts.

The high recovery rate for eye lenses suggests that some characteristic of the lens makes it more resistant to digestion by acid than other fish structures, and the effect of sea lion digestion on eye lens weight was not significant for herring, mackerel, or pollock. The spherical shape of the eye lens may allow for relatively rapid passage through the gastrointestinal (GI) tract, and the crystalline/albuminoid structure of the eye lens has been shown to withstand acid digestion. In studies where damselfish (*Parma microlepis*) eye lenses were dissolved in acid (Dove and Kingsford 1998, Gillanders 2001), heat had to be employed in order to completely dissolve the lens sample. The eye lenses of Pacific herring, Walleye pollock, and Atka mackerel may be particularly resistant to acid digestion, as I was unable to completely dissolve lenses using either a

microwave digestion or hot plate methodology. As eye lenses age, the relative concentration of insoluble albuminoid increases (Nicol 1989), and high concentrations of insoluble material could account for the lack of digestion of eye lenses in the pinniped GI tract.

Preliminary elemental analyses on two mackerel eye lenses indicated that elemental concentrations display distinct distributions across the lens. Because eye lenses grow by laying down concentric rings of fibers, I expected elements to display radial symmetry outward from the center of the eye lens. This symmetry would be evident when looking at the two halves of a single raster and when comparing the two rasters, long and lat, from the same eye lens. The expectation of symmetry in elemental distributions across the lens was not supported for all of the elements analyzed, however, and there are several factors that could influence the symmetry of an elemental distribution. In general, an individual element tended to either show symmetry around the core and similarity between the two rasters, or the element displayed asymmetrical distributions for both comparisons. Copper was the only element that was symmetrical around the core, but did not have similar distributions for the two rasters within a lens, and the noisy signals for some other elements could have been masking any symmetry.

Although some elements displayed symmetrical trends overall, the fibrous nature of the lens may preclude precise point-to-point symmetry. Individual fiber cells elongate across the lens independently of each other, allowing for a somewhat random pattern of placement of cells elongating at the same time within the growth layer. Growth layers within the eye lens may have mean elemental values that are symmetric around the center, but the random placement of fibers would suggest that symmetry within growth

rings is not likely. My method of cross-sectioning the lens, which compressed the lens in some places and stretched it in others, could also have affected the symmetry of an elemental distribution across the lens, stretching or compressing a portion the distribution when compared to other parts of the lens. Cataracts and other inclusions could also disrupt a symmetrical elemental distribution. Additional studies using more refined methods and larger sample sizes will help to clarify my preliminary results.

Concentration spikes or dips occurred for most elements at some point along the elemental distributions, although some elements visibly had more spikes than others. Some of these spikes are artifacts of the LA-ICPMS technique. As the laser moves across the surface of the lens, particles are kicked up into the plasma causing an artificially high count. Spikes or dips can also be artificially caused when there is epoxy resin left on the surface of the lens, and the laser runs across the epoxy rather than the sample itself. I am working with collaborators at ANU to distinguish between spikes and dips that are artifacts and those that represent real changes in elemental concentrations.

Peaks and dips that represent real changes in elemental concentrations could have several causes. In addition to cataracts and other inclusions, abrupt changes in elemental concentrations could be caused by changes in the orientation of the fibers in the eye lens at a specific point. The fibers of the eye lens are not symmetrical themselves (Figure 4.24), and a change in their alignment could affect whether or not atoms of specific elements adhere to the lens and become incorporated. Spikes or dips in elemental concentrations also could indicate a natural break between eye lens growth layers or a shift between the crystalline structure and the albuminoid in the lens. The specific causes

of elemental spikes and dips within the distribution, and what these abrupt changes indicate, were outside of the scope of this study.

The changes in trends and patterns within an elemental distribution are what distinguish one eye lens from others. Although my sample size precludes any statistical tests, the calculated mean concentrations of each element for each laser sample were almost all within one standard deviation of the other mean values for that element. Based on my results, sea lion digestion did not have a large effect on elemental concentrations, if there was any distinguishable effect at all. Elemental mean concentrations and distribution patterns were generally similar between pristine and digested eye lenses, and there was not a significant change in the mean eye lens weight due to digestion. This bodes well for using digested eye lenses to examine the life histories of prey fish.

Further studies are required to validate the preliminary results from the elemental analysis. An ICPMS study including more than one fish species and a larger sample size is necessary to determine if species can be distinguished by elemental analysis of eye lenses. A controlled laboratory study would be ideal, where eye lenses from each fish could be marked so that the pristine and digested eye lenses from the same fish could be directly compared to each other. This would remove some of the within lens variation from the analysis. Controlled laboratory studies have been used to examine contaminant incorporation into fish tissues at varying environmental contaminant levels (Geffen et al. 1998), but none has examined the relationship between contaminant loads in eye lenses and those in other fish tissues and organs. Eye lenses do incorporate contaminants from the environment (Hargis and Zwerner 1989), and lenses may be useful as indicators of

contaminant loads in other fish tissues and thus as indicators of contaminant ingestion by pinnipeds.

The incorporation of contaminants and other trace elements into the eye lens is likely to be affected by other environmental characteristics in addition to environmental concentrations of an element. Numerous studies have been done to analyze the effects of temperature, salinity, and environmental concentrations on trace elements incorporated into otoliths, and similar studies should be done for eye lenses. The elemental distributions across eye lenses appear to be analogous to the unique elemental fingerprints of otolith, and may provide information useful for discriminating among fish stocks, examining geographic/spatial locations, and describing the environmental conditions experienced by a fish at different stages of its life.

This study has positive implications for the use of eye lenses recovered from pinniped scat in dietary analyses. Eye lenses possess several of the characteristics that make otoliths a useful tool for studying fish populations, such as growth increments that appear to correspond with age and distinct elemental distributions across the lens. In addition, eye lenses appear to be more resistant to pinniped digestion than otoliths. Different elements show distinct patterns and obvious trends across the eye lens, and these may be indicative of the environmental conditions during the corresponding stage of the fish's life. If elemental concentrations in eye lenses, like otoliths, are indicative of the environmental characteristics of a specific location or water mass, they may be useful in locating sea lion foraging grounds or changes in foraging locations over time. Identifying fish species from eye lenses using elemental analysis would allow for more precise determinations of the dietary proportions of different species based on eye lenses

recovered from scat. The levels of contaminants found in eye lenses may also provide an estimate of the contaminant levels ingested by sea lions from their prey. Based on my results, analyses of eye lenses have the potential to significantly increase the amount of information available from scat analysis, and further studies will determine exactly how much information can be obtained from the analysis of digested eye lenses.

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Table 4.1: Reader agreement for eye lens growth layers. Two independent readers counted eye lens growth layers on fourteen eye lenses. Reader one counted growth layers on two separate locations, while reader two counted layers once. Reader two's counts were used to resolve discrepancies when reader one's counts did not agree.

<u>Growth Layer Counts</u>			
<u>Fish ID</u>	<u>Reader 1</u>		<u>Reader 2</u>
	<i>a</i>	<i>b</i>	<i>c</i>
Pol 5	4	4	5
Pol 19	3	4	4
Pol 24	3	3	3
Pol 32	3	4	4
Pol 33	4	3	3
Mac 6	4	4	4
Mac 43	3	4	4
Mac 46	3	3	3
Mac 49	4	4	4
Mac 57	4	4	4
Her 17	3	4	4
Her 19	4	4	4
Her 2	4	4	3
Her 10	4	4	4

Table 4.2: Mean values and standard deviations for dry lens weight in Walleye pollock (*Theragra chalcogramma*), Atka mackerel (*Pleurogrammus monopterygius*), and Pacific herring (*Clupea pallasii*). A randomized, nested ANOVA was used to analyze changes in eye lens dry masses caused by sea lion digestion (n = 30).

	Pristine		Digested	
	<u>MEAN</u>	<u>(ST.DEV.)</u>	<u>MEAN</u>	<u>(ST.DEV.)</u>
Pacific Herring	6.7	(1.00)	5.5	(2.47)
Atka Mackerel	19.2	(7.76)	16.2	(4.54)
Walleye Pollock	21.1	(9.02)	19.6	(4.78)

<u>SOURCE</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>p</u>
Digestion treatment	1	26.50	26.51	0.092	0.769
Among species within digestion treatment	4	1155.30	288.82	9.016	0.001
Within species	24	768.80	32.03		
TOTAL	29	1950.6			

Table 4.3: Elemental data summary from LA-ICPMS analysis of two Atka mackerel (*Pleurogrammus monopterygius*) eye lenses, one pristine and one digested. Mean concentrations, standard deviations, coefficients of variation (CV), and minimum and maximum values are reported for each element for each raster. Several elements have CVs that are greater than one, indicating the standard deviation was greater than the mean (i.e., there was a lot of variance in the data), which is partially due to false peaks and spikes.

	<u>Mg25</u> ($\mu\text{g/g}$)	<u>P31</u> ($\mu\text{g/g}$)	<u>Ca43</u> ($\mu\text{g/g}$)	<u>Mn55</u> ($\mu\text{g/g}$)	<u>Fe57</u> ($\mu\text{g/g}$)	<u>Cu65</u> ($\mu\text{g/g}$)	<u>Zn66</u> ($\mu\text{g/g}$)	<u>Rb85</u> ($\mu\text{g/g}$)	<u>Sr88</u> ($\mu\text{g/g}$)	<u>Ba138</u> ($\mu\text{g/g}$)	<u>La139</u> ($\mu\text{g/g}$)	<u>Pb208</u> ($\mu\text{g/g}$)
<u>Pristine pollock, parallel to axis of fiber elongation (long raster)</u>												
MEAN	17.53	742.23	72.83	0.19	11.78	0.49	3.73	0.02	0.16	0.39	0.18	0.44
ST. DEV.	24.95	311.93	47.84	0.64	25.60	0.66	1.32	0.04	0.11	0.93	0.62	2.68
CV	1.4	0.4	0.7	3.3	2.2	1.4	0.4	2.1	0.7	2.4	3.4	6.1
MIN	0.48	11.05	5.59	0.00	0.00	0.02	0.09	0.00	0.00	0.00	0.00	0.00
MAX	164.44	1144.42	583.13	6.80	348.75	5.39	9.22	0.38	0.69	7.11	5.19	26.39
<u>Pristine pollock, perpendicular to axis of fiber elongation (lat raster)</u>												
MEAN	10.19	1041.89	69.70	0.09	7.06	0.79	4.77	0.02	0.15	0.24	0.12	0.08
ST. DEV.	7.03	246.78	144.57	0.09	8.46	0.42	1.75	0.02	0.10	0.28	0.28	0.11
CV	0.7	0.2	2.1	0.9	1.2	0.5	0.4	0.9	0.7	1.2	2.3	1.3
MIN	3.27	174.22	27.05	0.00	0.00	0.07	1.80	0.00	0.03	0.01	0.00	0.00
MAX	105.95	1634.35	4460.47	0.83	121.34	4.97	25.89	0.25	0.77	3.03	3.12	2.99
<u>Digested pollock, parallel to axis of fiber elongation (long raster)</u>												
MEAN	13.63	465.39	116.13	0.08	2.75	0.92	4.25	0.01	0.44	0.48	0.16	0.05
ST. DEV.	9.69	197.84	165.61	0.07	5.42	1.20	1.25	0.01	0.62	0.39	0.27	0.03
CV	0.7	0.4	1.4	0.9	2.0	1.3	0.3	1.9	1.4	0.8	1.7	0.6
MIN	3.75	117.91	18.15	0.00	0.00	0.20	2.05	0.00	0.11	0.02	0.00	0.00
MAX	165.61	996.74	1359.13	0.84	1.84	2.84	3.84	4.84	4.82	3.33	4.02	0.30
<u>Digested pollock, perpendicular to axis of fiber elongation (lat raster)</u>												
MEAN	13.21	794.12	69.57	0.03	0.78	0.31	3.98	0.00	0.28	0.17	0.04	0.02
ST. DEV.	7.82	244.12	56.24	0.04	1.90	0.16	1.15	0.00	0.24	0.27	0.07	0.01
CV	0.6	0.3	0.8	1.6	2.4	0.5	0.3	1.2	0.9	1.6	1.8	0.5
MIN	2.28	385.19	32.62	0.00	0.00	0.07	1.79	0.00	0.08	0.00	0.00	0.00
MAX	34.64	1361.84	609.57	0.56	49.32	1.85	8.31	0.02	2.32	2.72	0.63	0.08

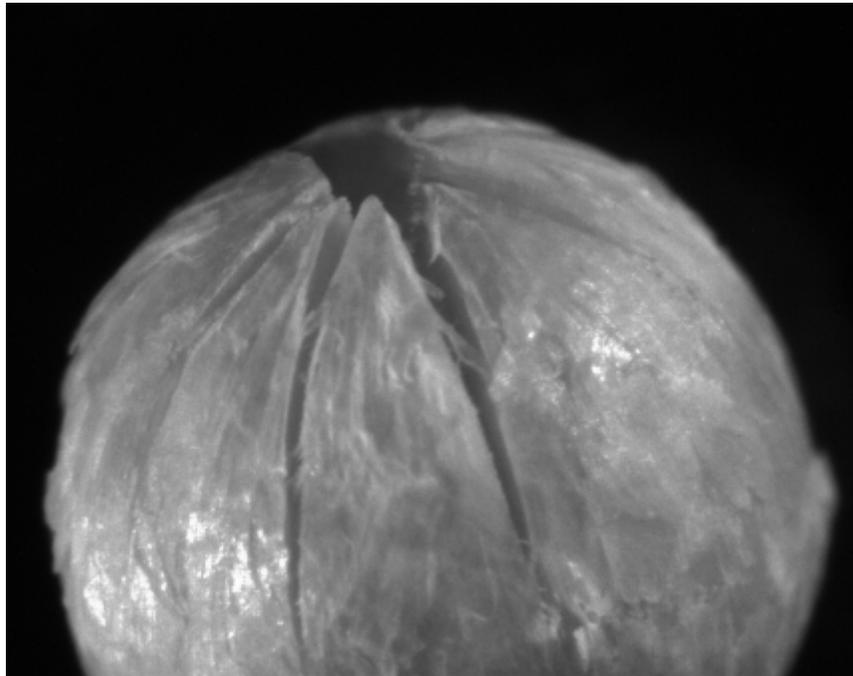


Figure 4.1: A dissecting microscope photo of an Atka mackerel (*Pleurogrammus monopterygius*) eye lens. This image shows the sutures where lens fibers terminate at the apex opposite the optic nerve.

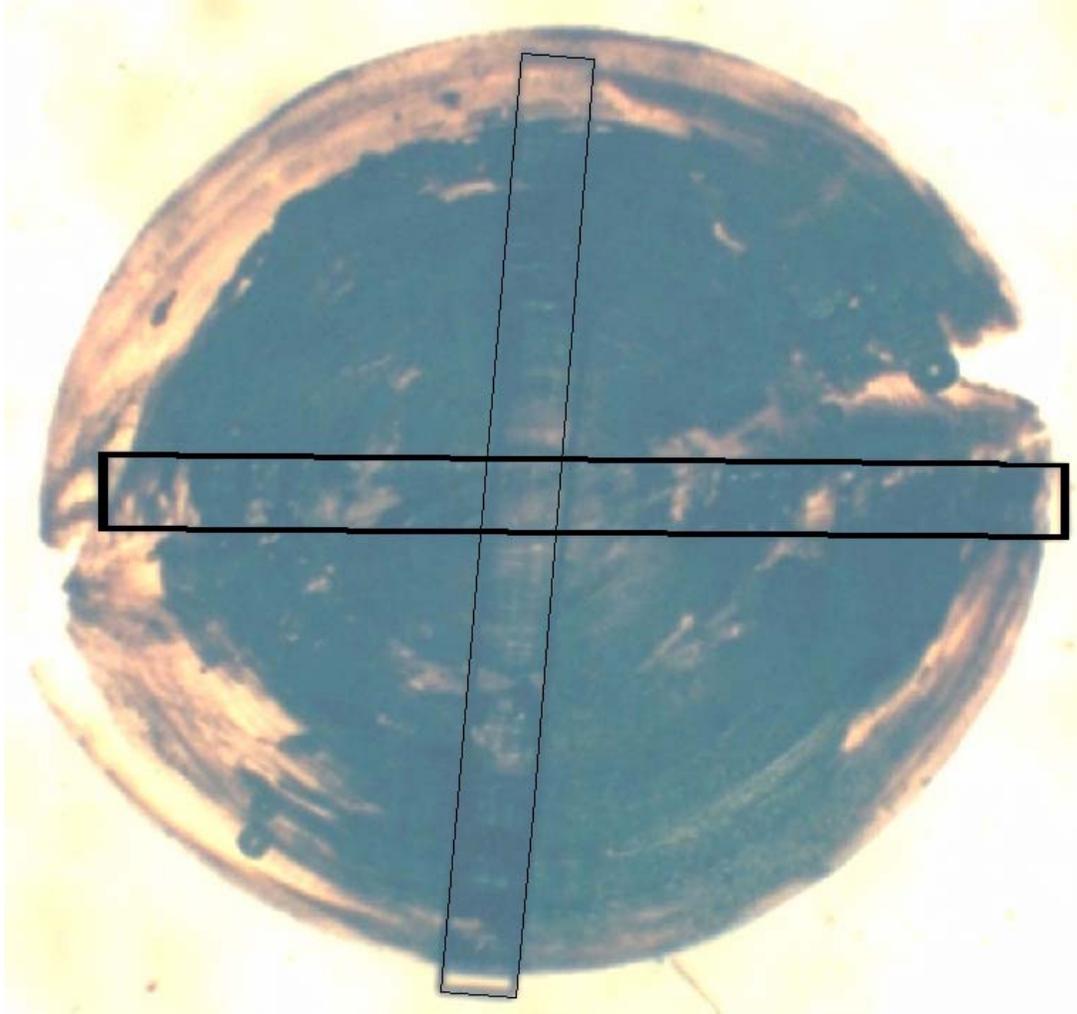


Figure 4.2: An Atka mackerel (*Pleurogrammus monopterygius*) eye lens half showing tracks from the Excimer laser. Each track represents two laser rasters across the lens: one cleaning sweep and a second raster used to collect data on elemental concentrations. The horizontal track (long), boldly outlined, runs roughly parallel to the axis of fiber elongation. The vertical track (lat) runs roughly perpendicular to the axis of fiber elongation. Ideally, both rasters run across the core of the eye lens.

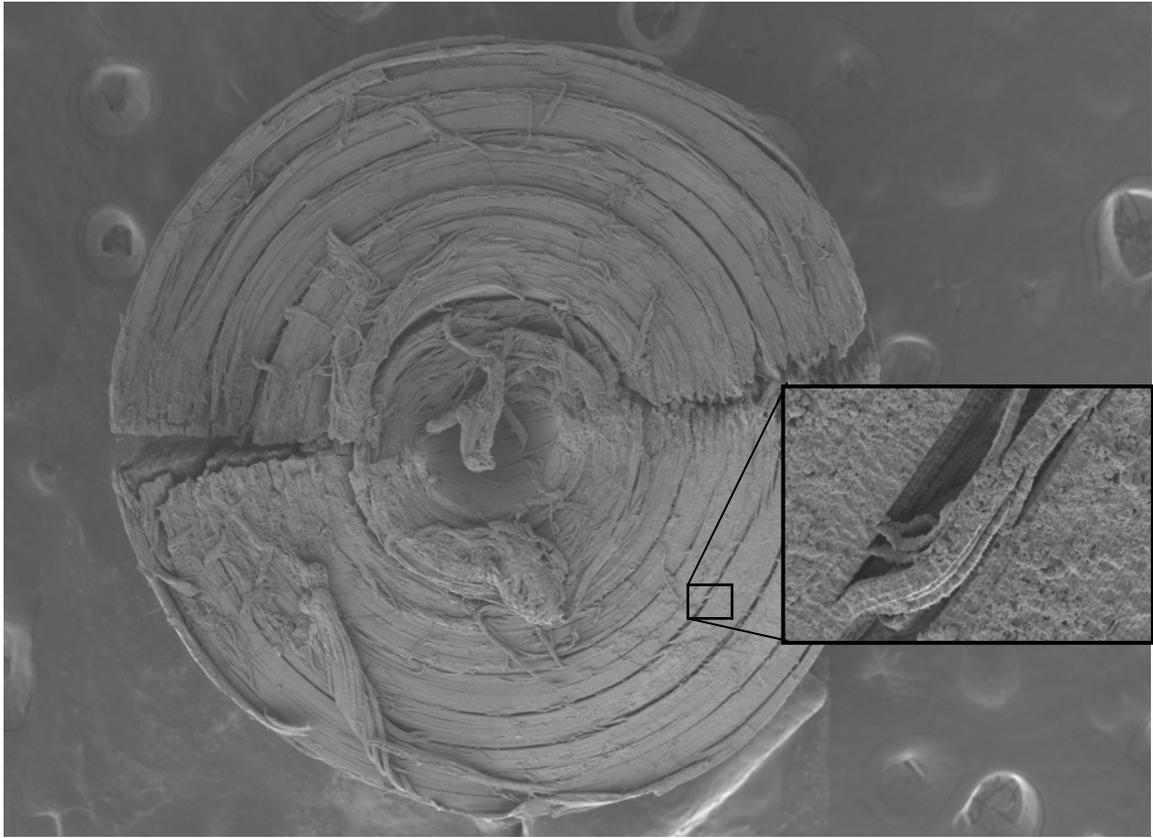


Figure 4.3: A scanning electron microscope image of a Pacific Herring (*Clupea pallasii*) eye lens after spatter coating. The SEM allows for individual fibers to be distinguished in the inset, but the spatter coating obscures the growth layers used for aging.



Figure 4.4: Growth layers in a Walleye pollock (*Theragra chalcogramma*) eye lens. Layers were distinguished by color, translucence, and structural breaks that ran entirely around the lens. Three growth layers were counted in this lens, and the nodule at the core of the lens is missing.

Comparing otolith annuli and eye lens growth layers

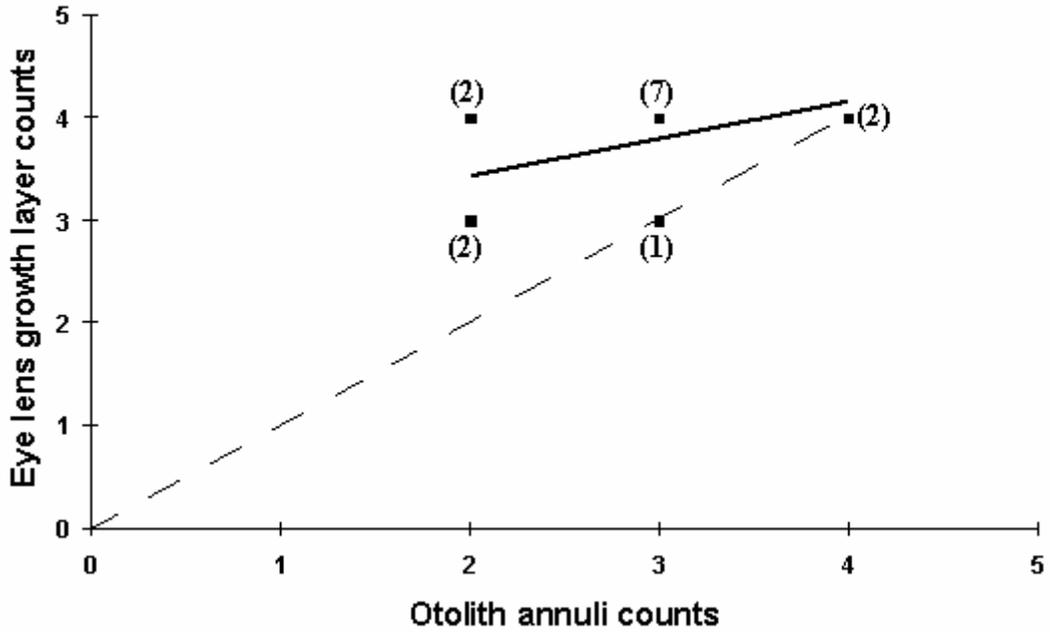


Figure 4.5: A comparison of otolith annuli and eye lens growth layers. Numerals in parentheses represent the number of fish that had that specific combination of otolith annuli and eye lens growth layer counts ($n = 14$). The dashed line represents a one-to-one regression line, while the bold line is the least-squares regression line calculated from the data with a slope of 0.45. A Pearson product-moment correlation between annuli and growth layers showed a significant correlation, however a test of $\beta_1 = 0$ indicated that the slope is significantly different from one ($t = -3.12$, $df = 13$) at the 0.01 significance level.

a.



b.

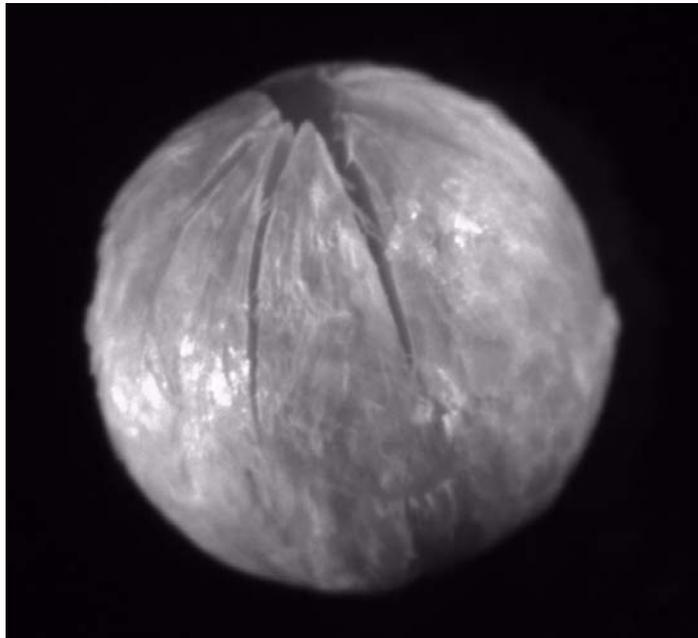
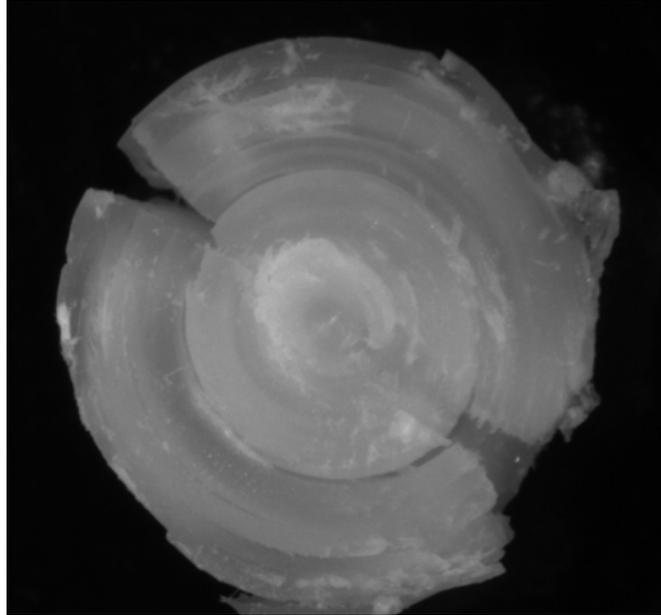


Figure 4.6: Pristine (a) and digested (b) Atka mackerel (*Pleurogrammus monopterygius*) eye lenses. These dissection microscope images show the external appearance of the eye lens.

a.



b.

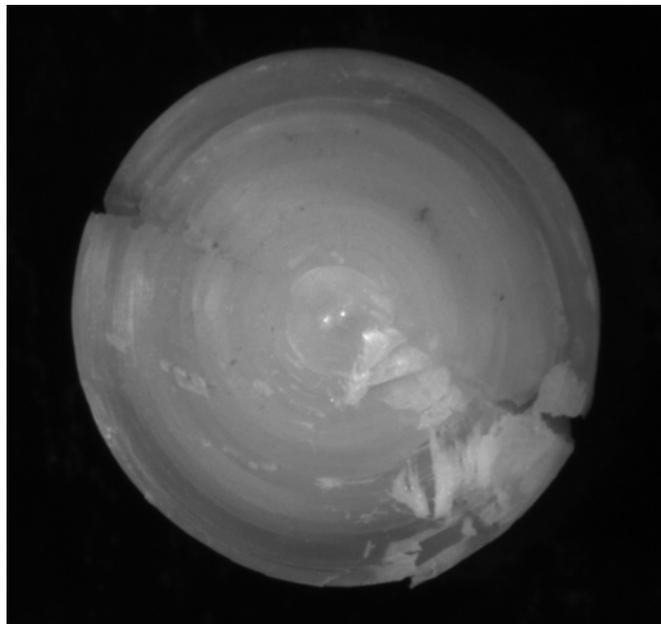


Figure 4.7: Cross sections of two different Walleye pollock (*Theragra chalcogramma*) eye lenses. a) An undigested lens and b) a digested lens both show growth layers laid down over time in the lens, although glare from the light source on the digested lens inhibits lens distinction.

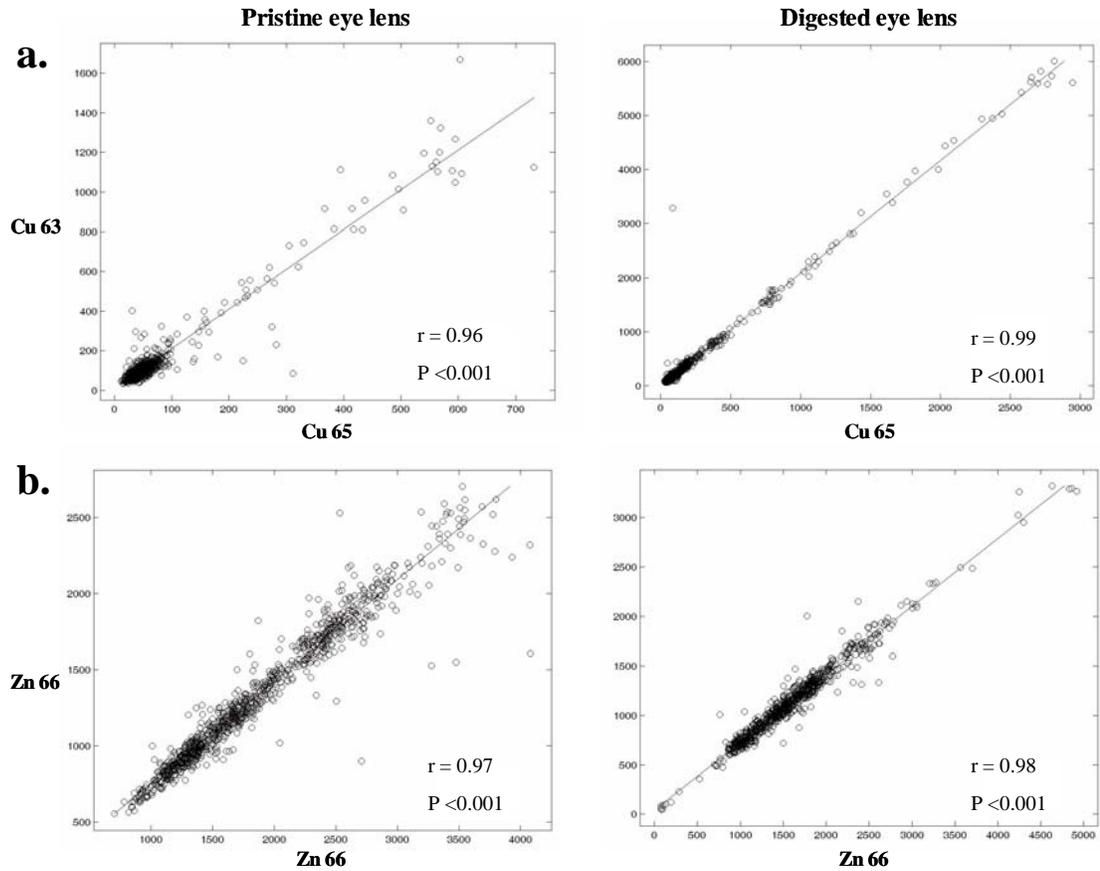
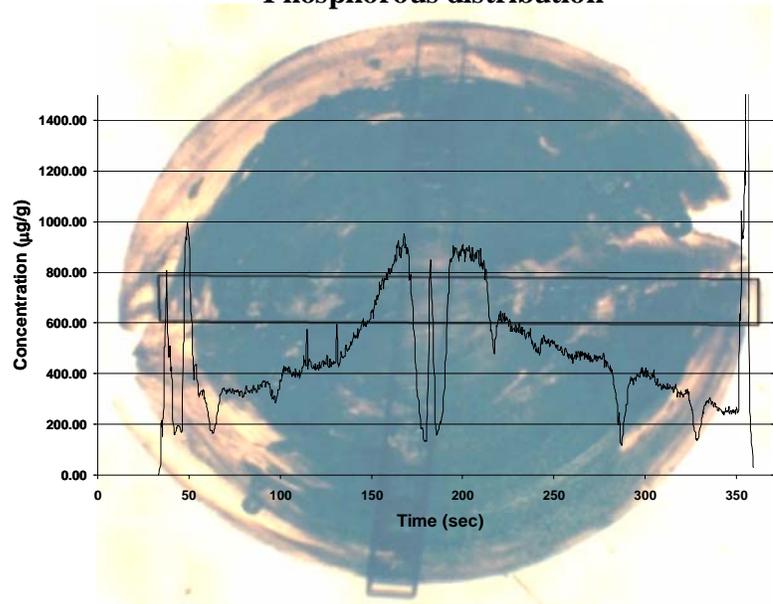


Figure 4.8: Testing for polyatomic interference. Linear regressions between the two isotopes of a) copper and b) zinc from each laser raster had slopes close to one ($0.96 < r < 0.99$), indicating little or no interference.

a.

Phosphorous distribution



b.

Lanthanum distribution

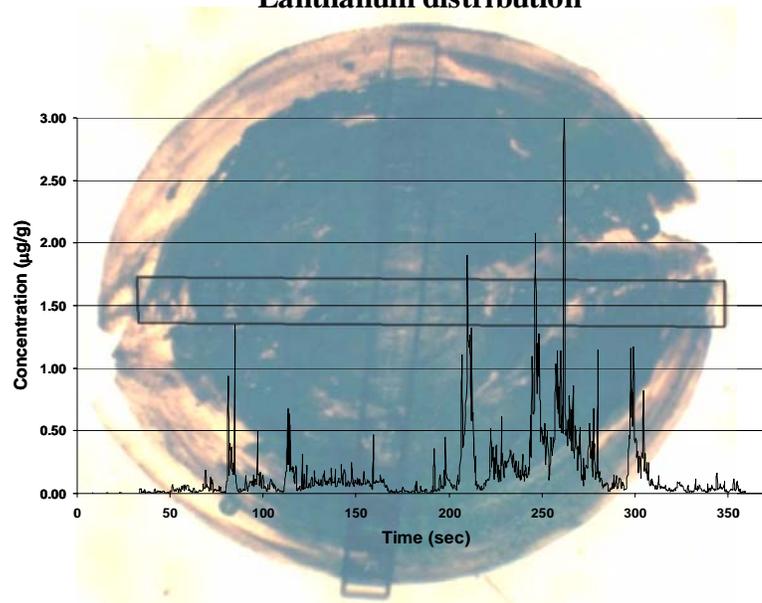


Figure 4.9: Symmetry of phosphorous and lanthanum around the center of an eye lens. Elemental distributions for a) phosphorous and b) lanthanum along the long raster of the same Walleye pollock (*Theragra chalcogramma*) eye lens. While phosphorous displays a symmetrical distribution, lanthanum does not.

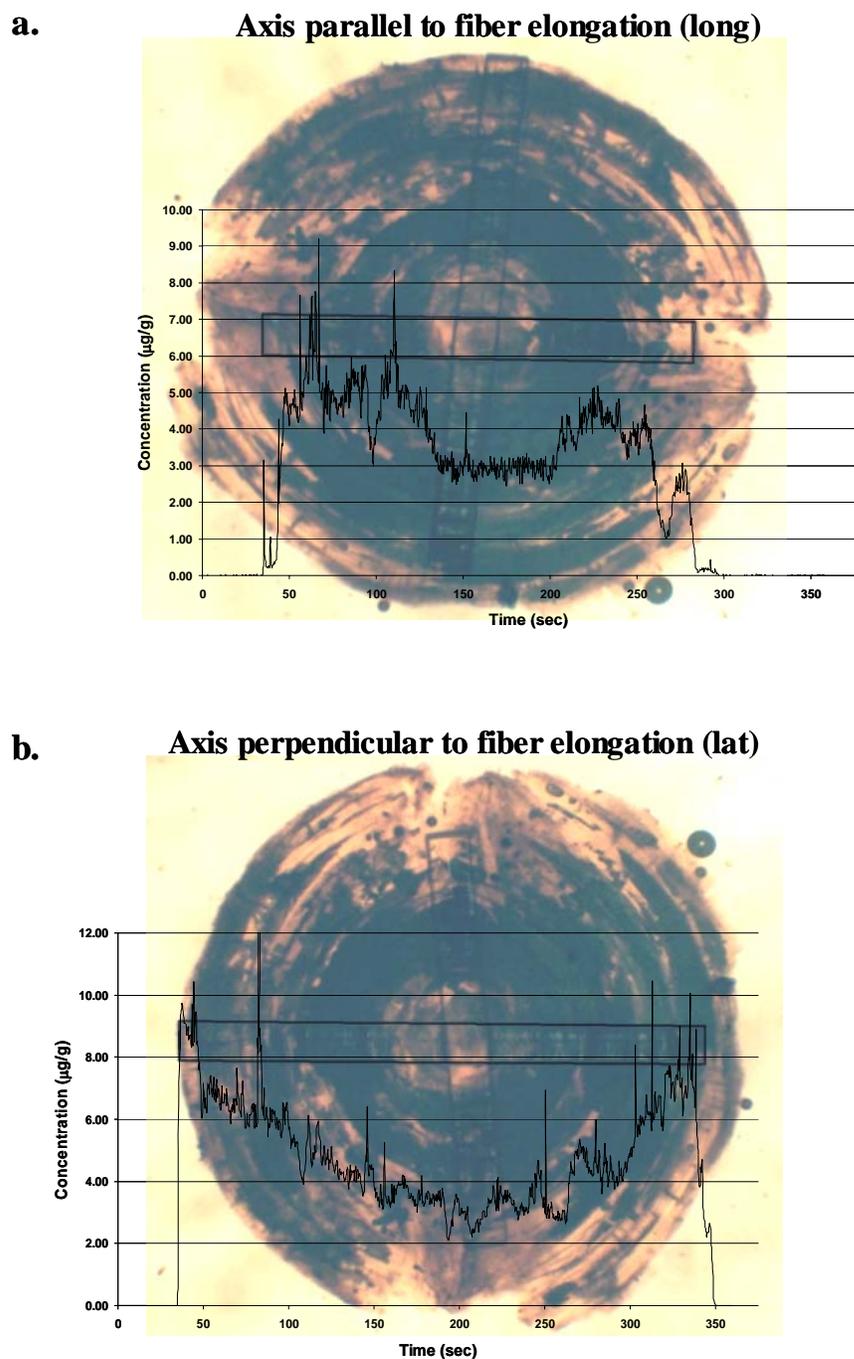


Figure 4.10: Elemental distributions for zinc in long (a) and lat (b) rasters on the same Walleye pollock (*Theragra chalcogramma*) eye lens. Zinc concentrations show a similar trend of increase outward from the center, although values at the outer edges of long (a).

Magnesium distributions

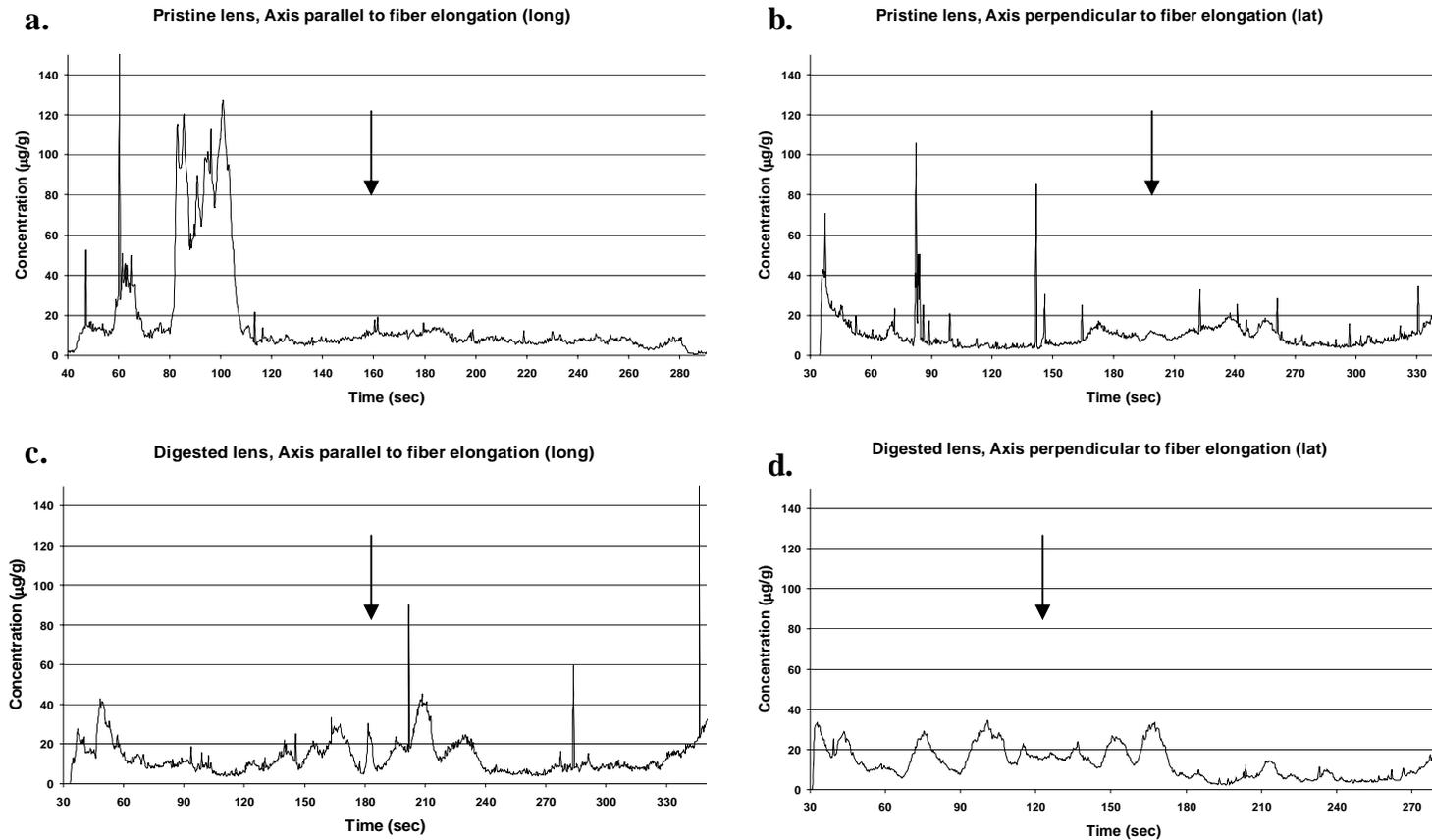


Figure 4.11: Magnesium distributions. a) The pristine lens parallel to fiber elongation. b) The pristine lens perpendicular to fiber elongation. c) The digested lens parallel to fiber elongation. d) The digested lens perpendicular to fiber elongation. Arrows indicate the approximate center of each lens for that distribution, and b), c), and d) show symmetry around that point. Disregarding the magnitude of peaks, trends between the long and lat rasters on each lens were considered to be similar.

Phosphorous distributions

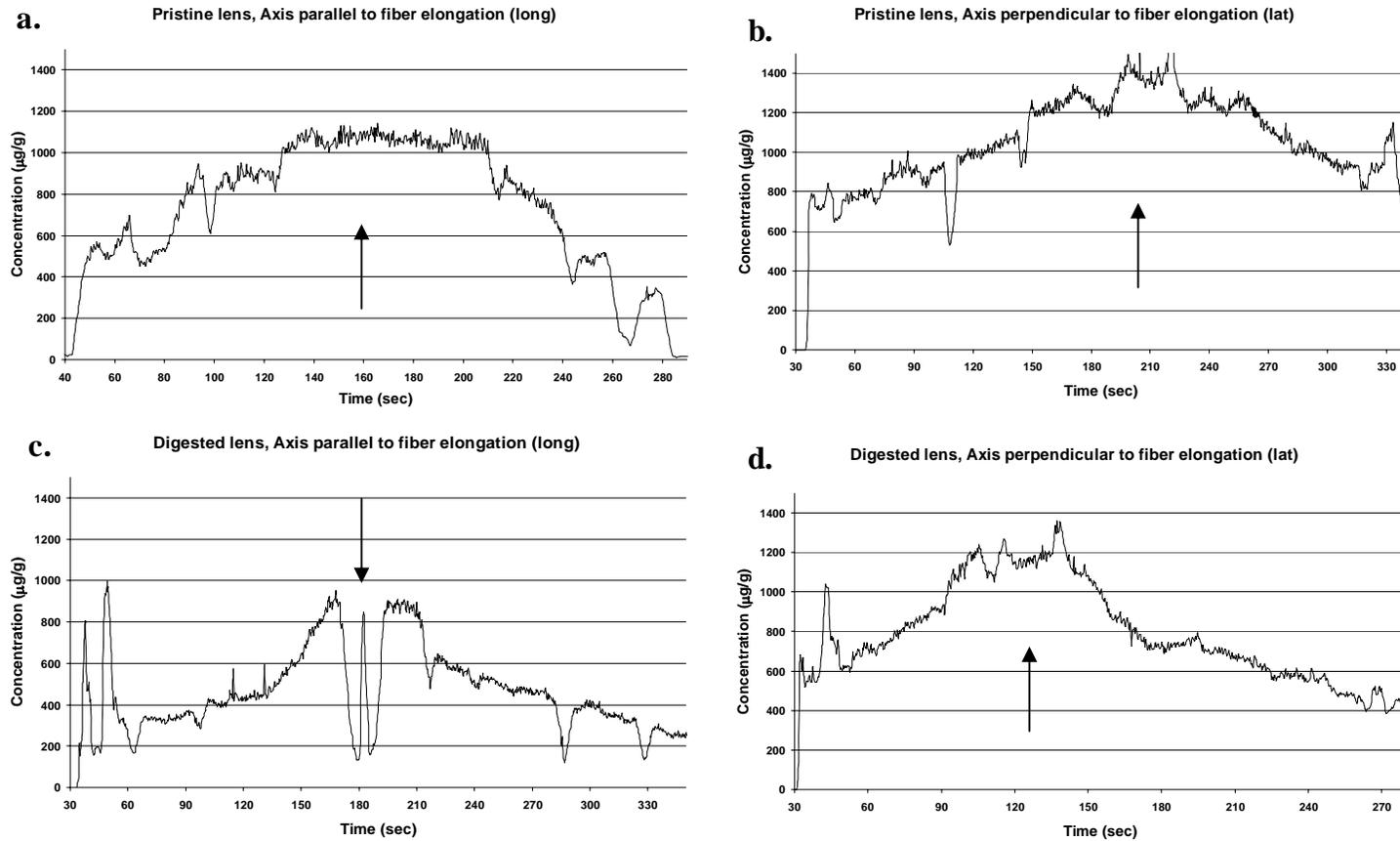


Figure 4.12: Phosphorous distributions. a) The pristine lens parallel to fiber elongation. b) The pristine lens perpendicular to fiber elongation. c) The digested lens parallel to fiber elongation. d) The digested lens perpendicular to fiber elongation. Arrows indicate the approximate center of each lens for that distribution, and all four rasters show symmetry around that point. Disregarding the magnitude of peaks, trends between the long and lat rasters on each lens were considered to be similar.

Copper distributions

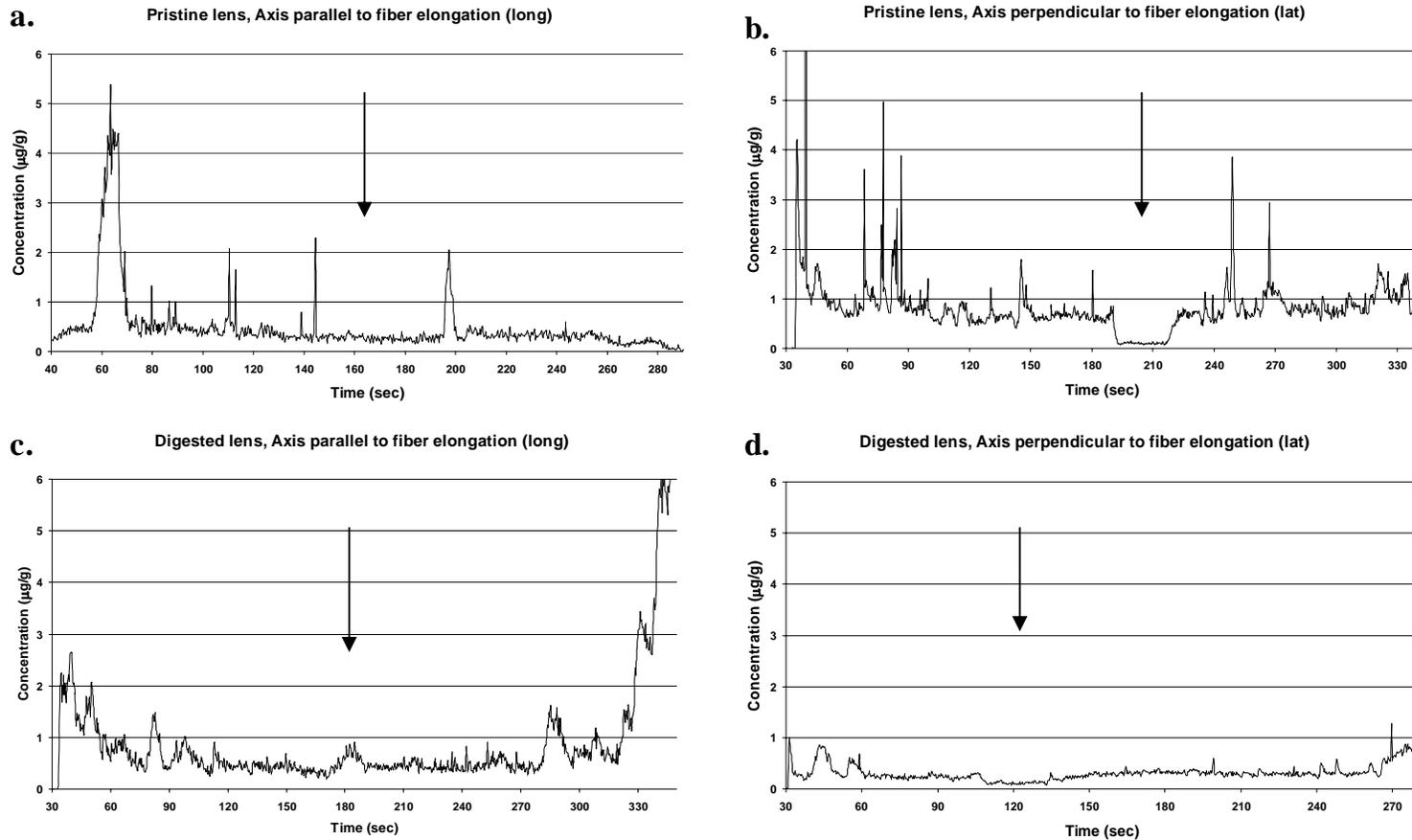


Figure 4.13: Copper distributions. a) The pristine lens parallel to fiber elongation. b) The pristine lens perpendicular to fiber elongation. c) The digested lens parallel to fiber elongation. d) The digested lens perpendicular to fiber elongation. Arrows indicate the approximate center of each lens for that distribution, and a), b), and c) show symmetry around that point. Trends between the long and lat rasters on each lens did not appear to be similar, but the signals for a) and d) had a lot of noise.

Zinc distributions

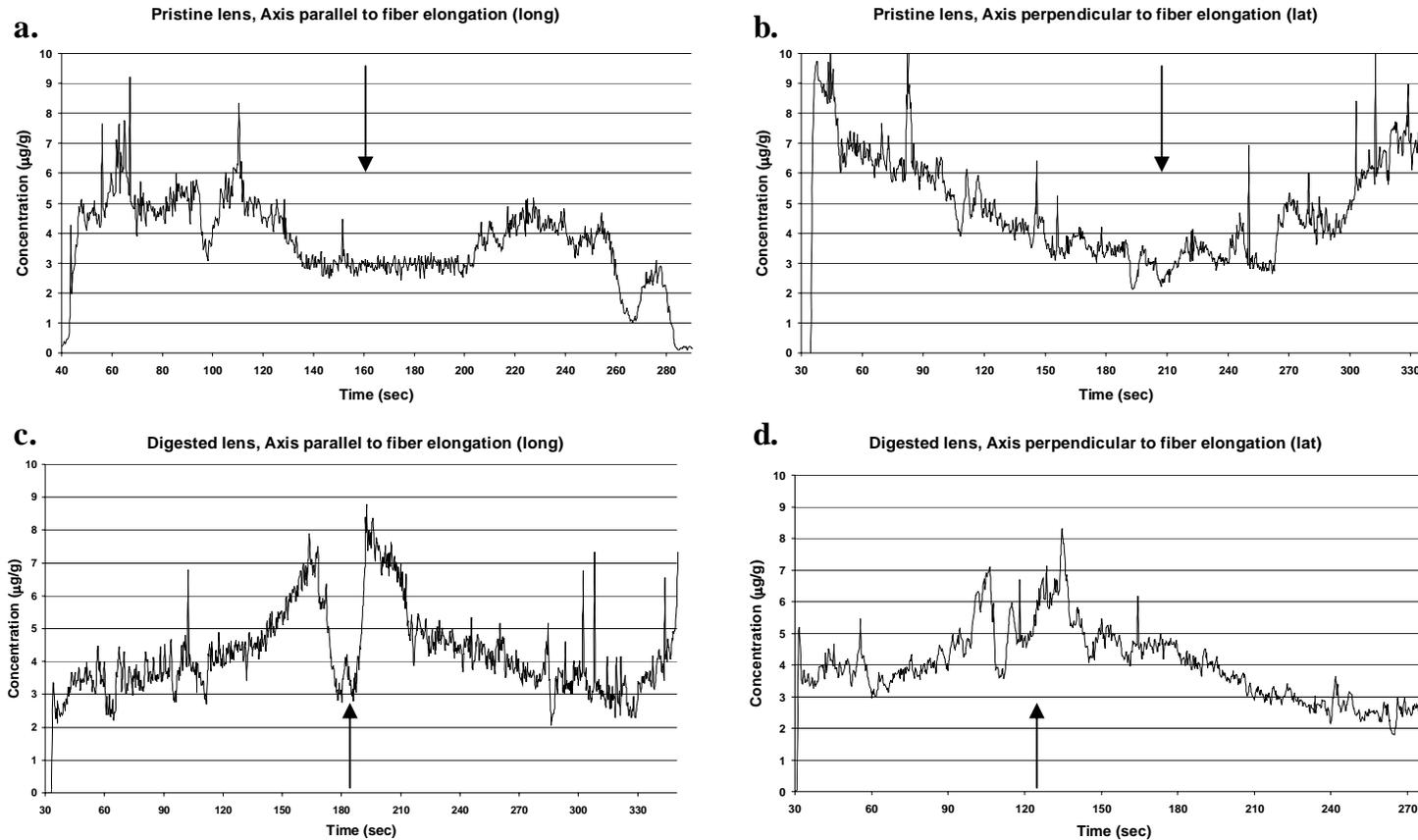


Figure 4.14: Zinc distributions. a) The pristine lens parallel to fiber elongation. b) The pristine lens perpendicular to fiber elongation. c) The digested lens parallel to fiber elongation. d) The digested lens perpendicular to fiber elongation. Arrows indicate the approximate center of each lens for that distribution, and all four rasters show symmetry around that point. Disregarding the magnitude of peaks, trends between the long and lat rasters on each lens were considered to be similar.

Strontium distributions

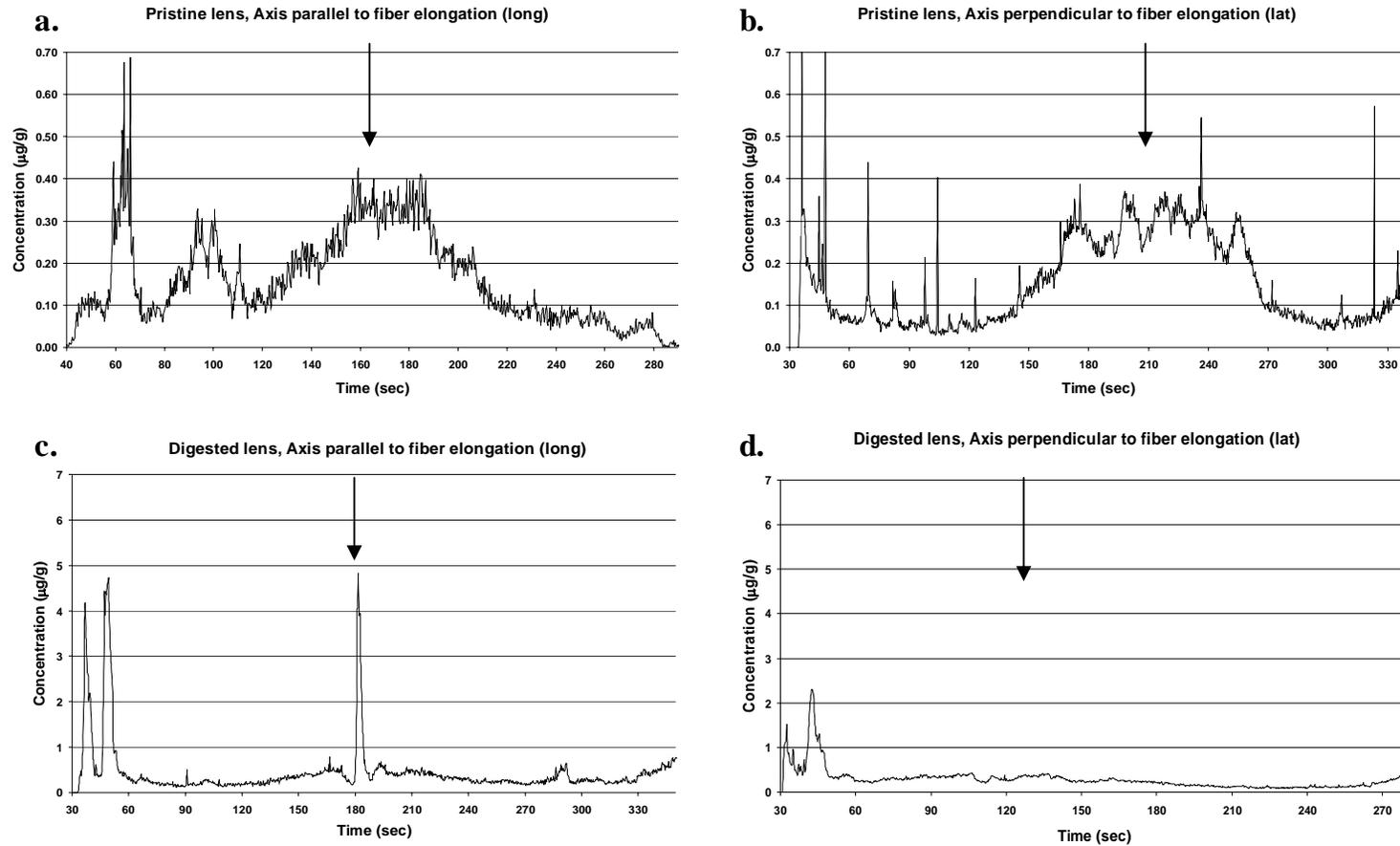


Figure 4.15: Strontium distributions. a) The pristine lens parallel to fiber elongation. b) The pristine lens perpendicular to fiber elongation. c) The digested lens parallel to fiber elongation. d) The digested lens perpendicular to fiber elongation. Arrows indicate the approximate center of each lens for that distribution, and a), b), and c) show symmetry around that point. Disregarding the magnitude of peaks, trends between the long and lat rasters on each lens were considered to be similar.

Iron distributions

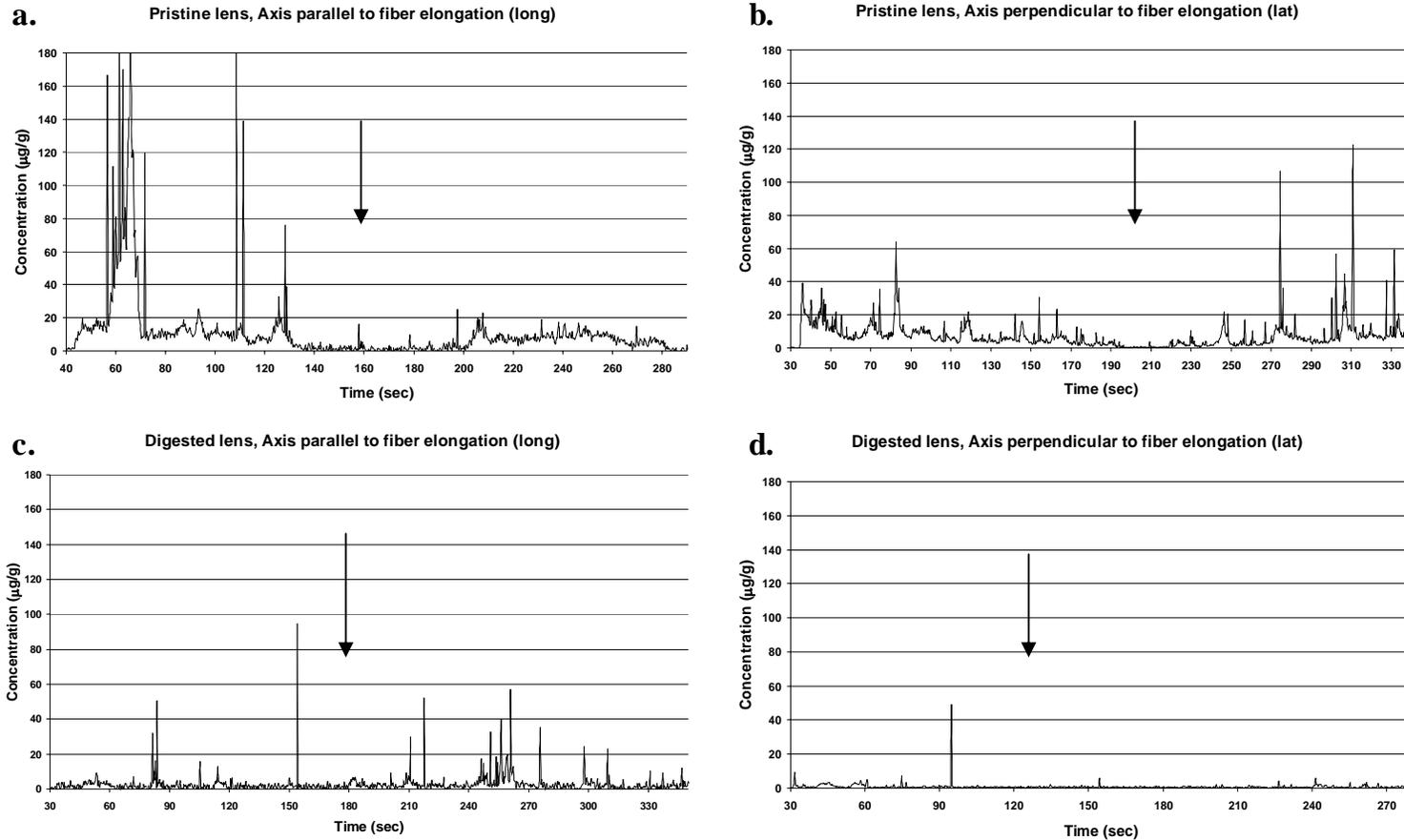


Figure 4.16: Iron distributions. a) The pristine lens parallel to fiber elongation. b) The pristine lens perpendicular to fiber elongation. c) The digested lens parallel to fiber elongation. d) The digested lens perpendicular to fiber elongation. Arrows indicate the approximate center of each lens for that distribution. While a) and b) appear symmetrical, c) and d) do not. Trends between the long and lat rasters on each lens were not considered similar but were affected in some rasters by signal noise.

Lead distributions

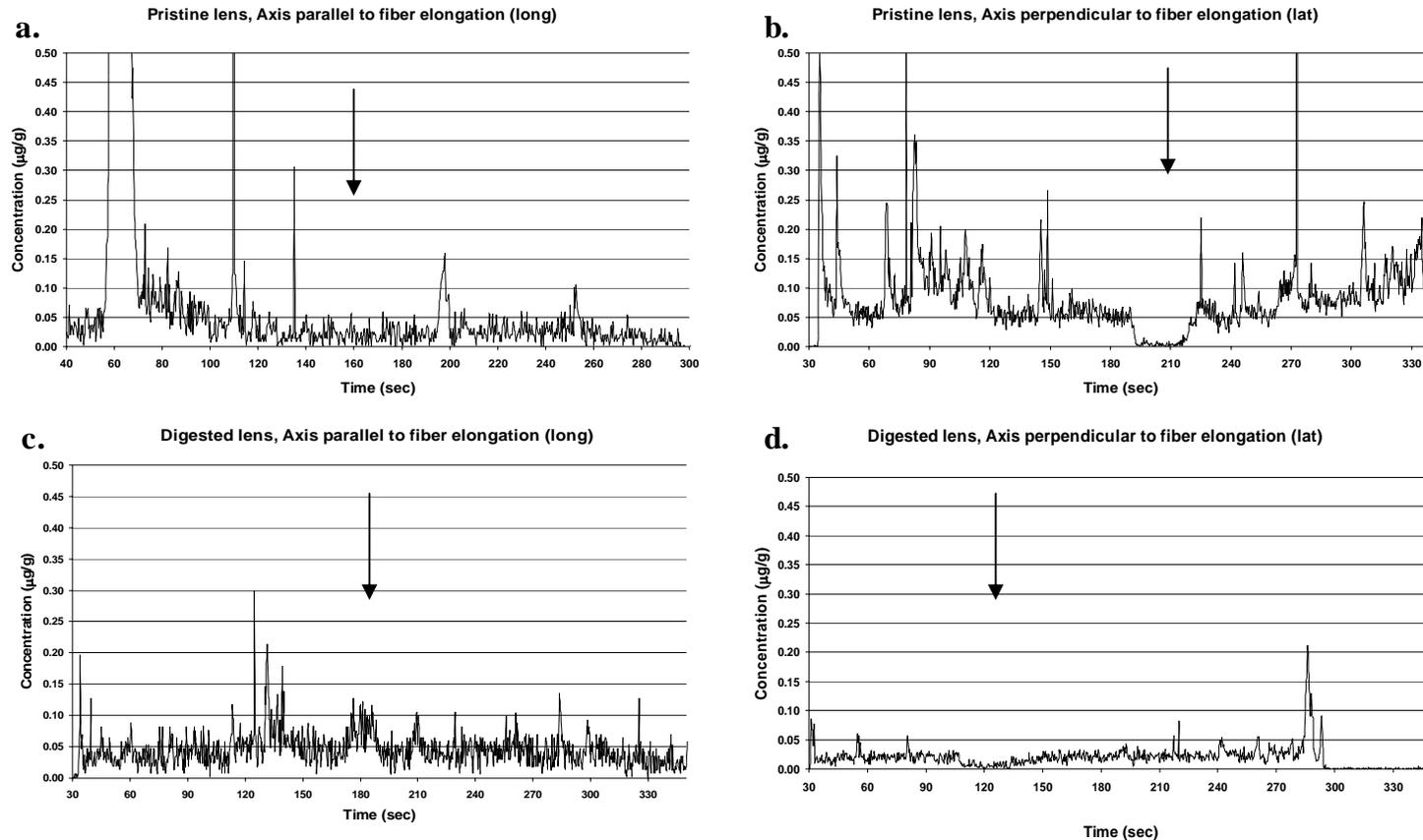


Figure 4.17: Lead distributions. a) The pristine lens parallel to fiber elongation. b) The pristine lens perpendicular to fiber elongation. c) The digested lens parallel to fiber elongation. d) The digested lens perpendicular to fiber elongation. Arrows indicate the approximate center of each lens for that distribution, and b) and c) display symmetry around that point. a) and d) do not appear symmetric, but show a lot of signal noise. Trends between the long and lat rasters on each lens do not appear to be similar.

Barium distributions

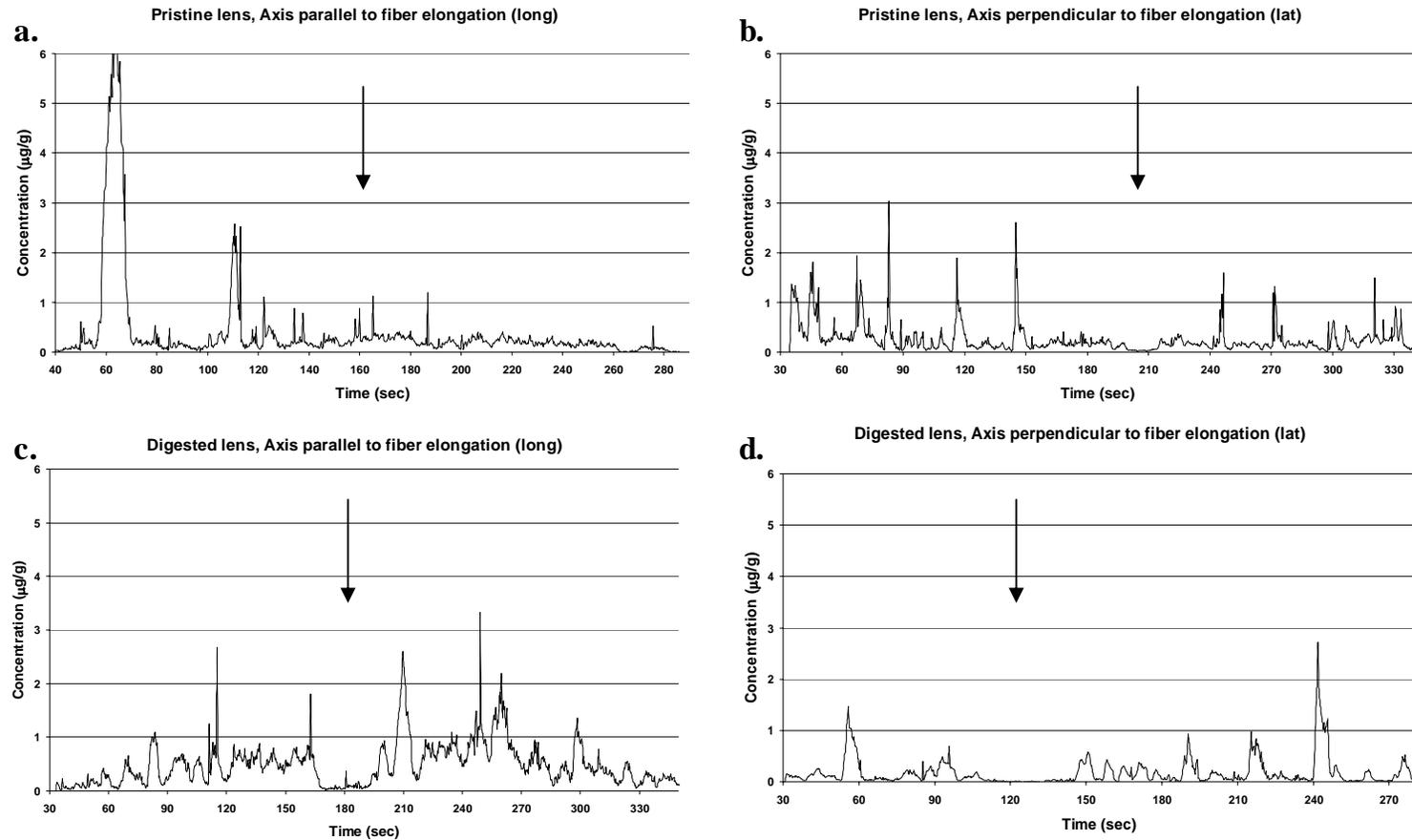


Figure 4.18: Barium distributions. a) The pristine lens parallel to fiber elongation. b) The pristine lens perpendicular to fiber elongation. c) The digested lens parallel to fiber elongation. d) The digested lens perpendicular to fiber elongation. Arrows indicate the approximate center of each lens for that distribution. While c) and d) appear symmetric, a) and b) are questionable. Trends between the long and lat rasters on each lens were not considered similar but were affected by signal noise.

Manganese distributions

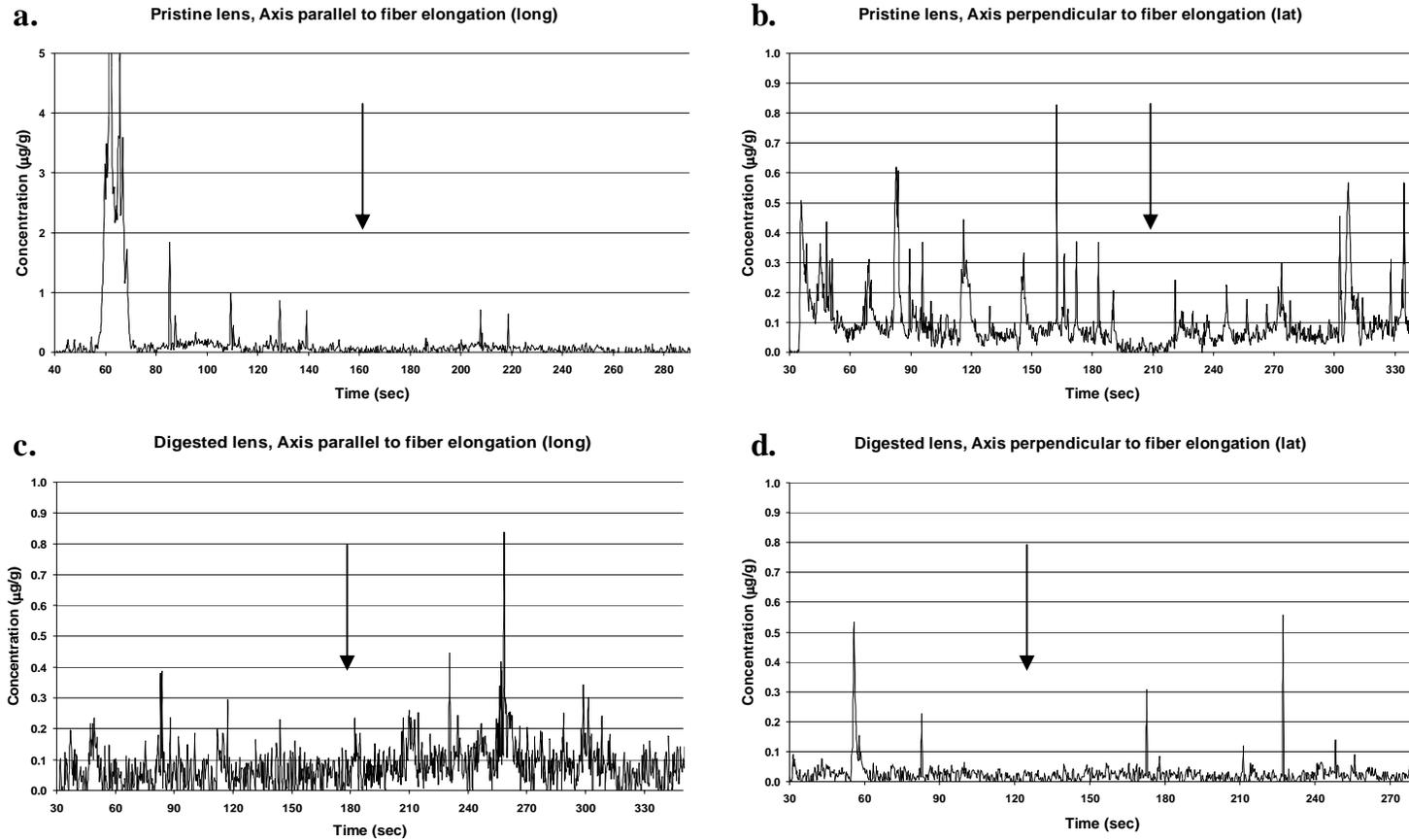


Figure 4.19: Manganese distributions. a) The pristine lens parallel to fiber elongation. b) The pristine lens perpendicular to fiber elongation. c) The digested lens parallel to fiber elongation. d) The digested lens perpendicular to fiber elongation. Arrows indicate the approximate center of each lens for that distribution. While b) appears symmetrical, the rest of the distributions have too much signal noise to analyze visually.

Rubidium distributions

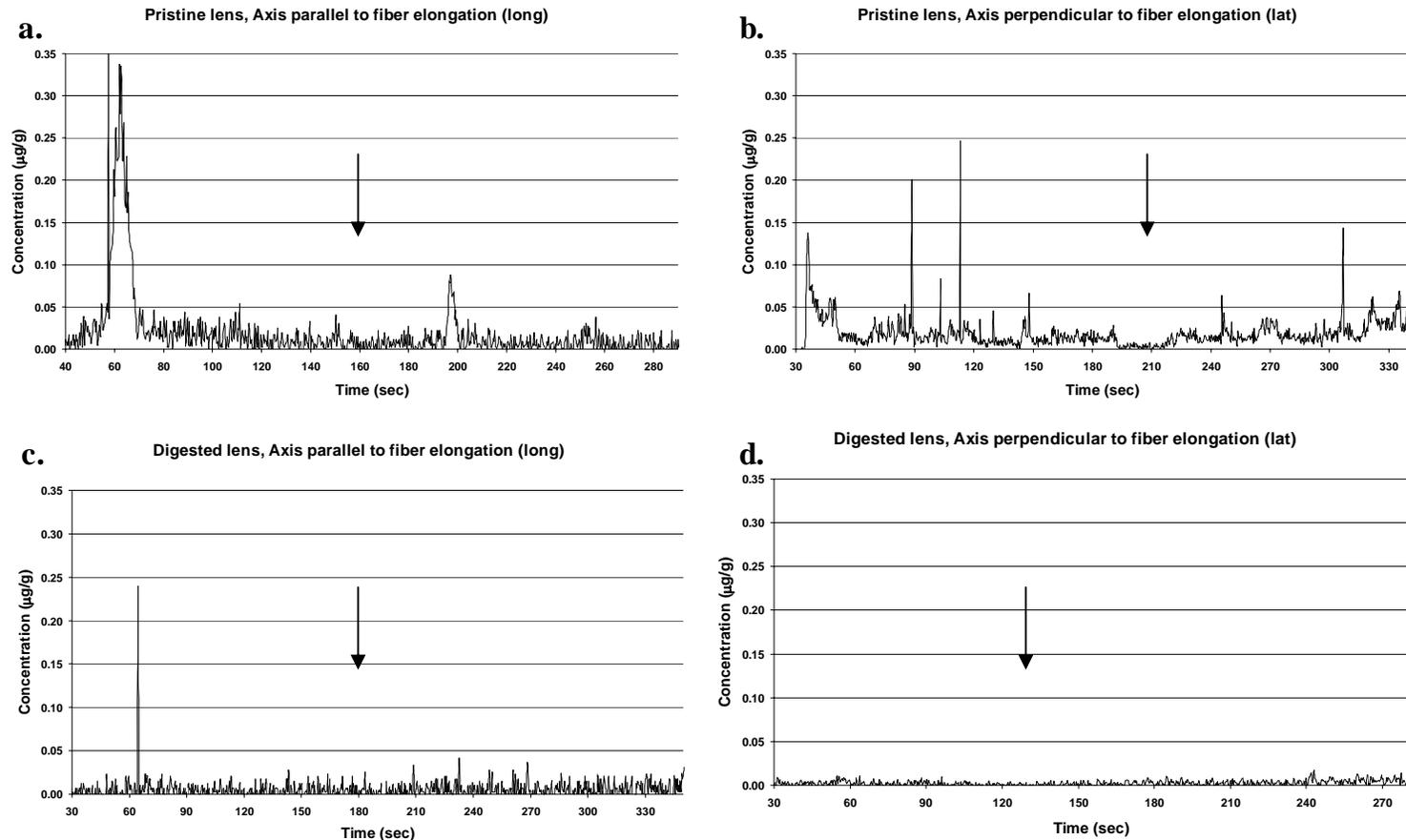


Figure 4.20: Rubidium distributions. a) The pristine lens parallel to fiber elongation. b) The pristine lens perpendicular to fiber elongation. c) The digested lens parallel to fiber elongation. d) The digested lens perpendicular to fiber elongation. Arrows indicate the approximate center of each lens for that distribution. While b) appears symmetrical, the rest of the distributions have too much signal noise to analyze visually.

Lanthanum distributions

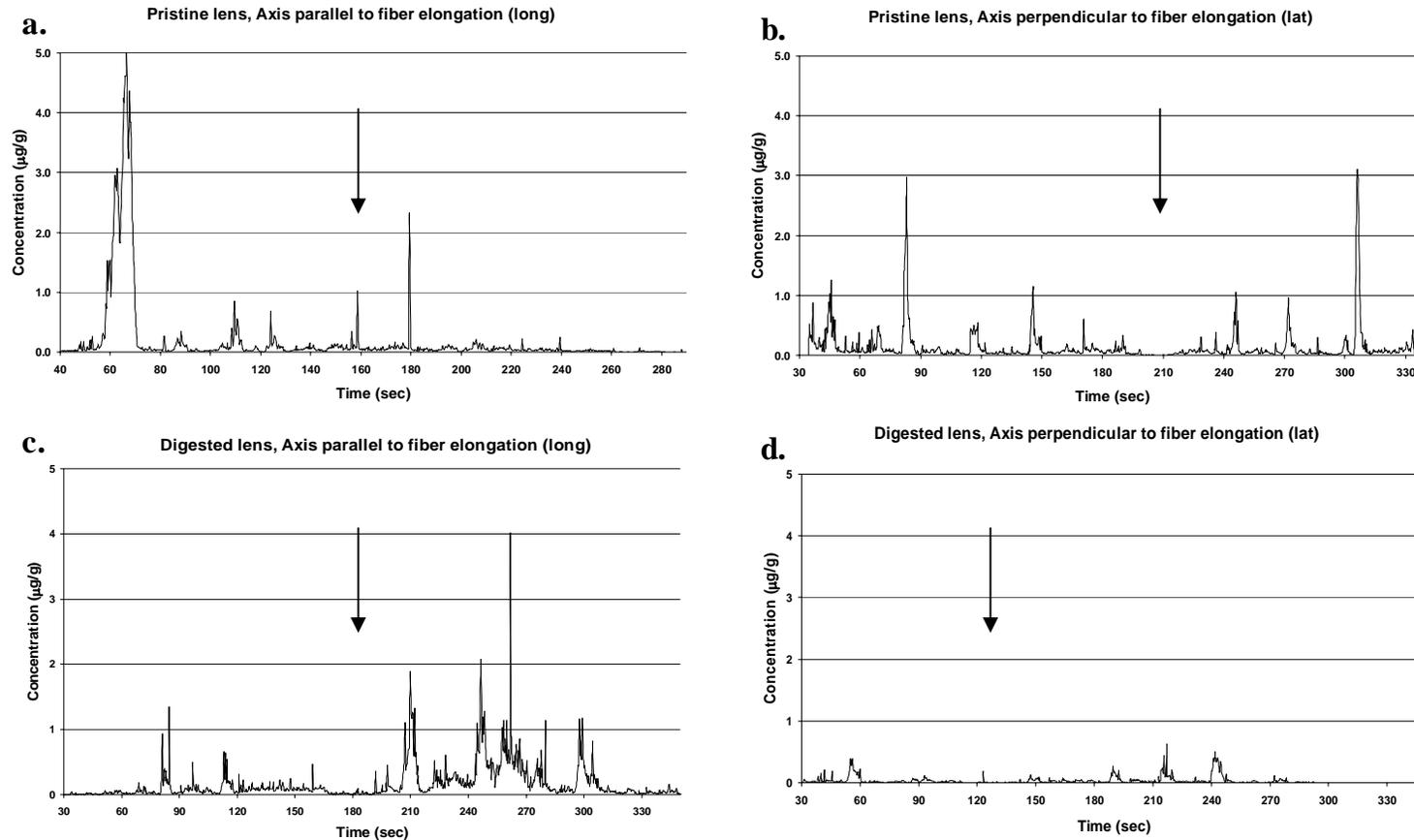


Figure 4.21: Lanthanum distributions. a) The pristine lens parallel to fiber elongation. b) The pristine lens perpendicular to fiber elongation. c) The digested lens parallel to fiber elongation. d) The digested lens perpendicular to fiber elongation. Arrows indicate the approximate center of each lens for that distribution. While b) appears symmetrical, c) does not and the other two distributions have too much signal noise to analyze visually.

Calcium distributions

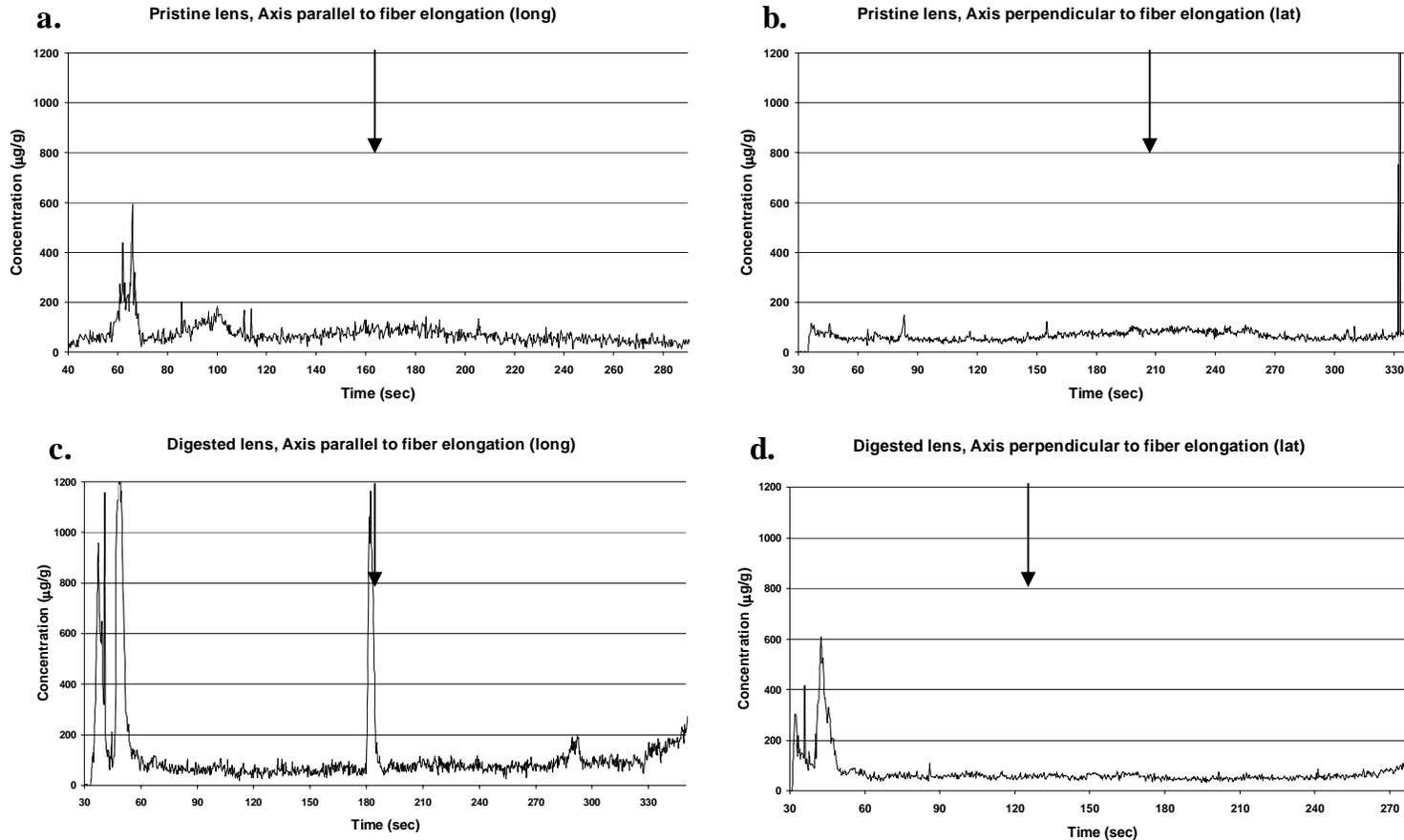


Figure 4.22: Calcium distributions. a) The pristine lens parallel to fiber elongation. b) The pristine lens perpendicular to fiber elongation. c) The digested lens parallel to fiber elongation. d) The digested lens perpendicular to fiber elongation. Arrows indicate the approximate center of each lens for that distribution. While c) appears symmetrical, the rest of the distributions have too much signal noise or no trends to analyze visually.



Figure 4.23: Damaged growth layers in a Walleye pollock (*Theragra chalcogramma*) eye lens. This cross section shows where the central growth layers have been compressed and damaged during splitting by a scalpel blade.

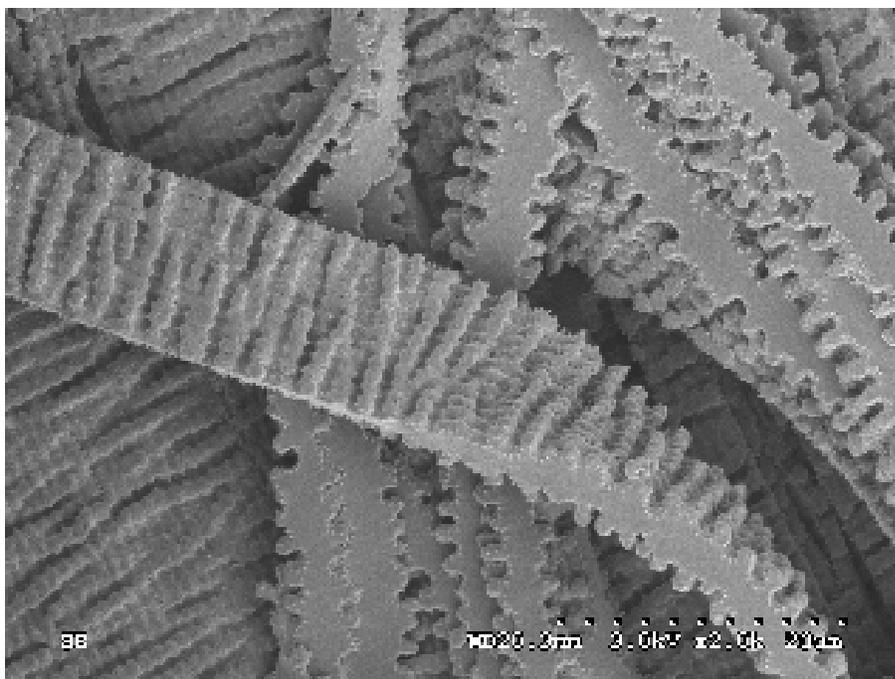


Figure 4.24: A Scanning Electron Microscope image of fiber asymmetry. This SEM photo shows the structure of the fibers that make up a Walleye pollock (*Theragra chalcogramma*) eye lens. Fiber orientation could effect elemental incorporation into the eye lens.

CHAPTER V

CONCLUSION

Dietary analyses for marine mammals are problematic in general because direct observation of foraging is difficult, if not impossible. The endangered status of the Steller sea lion and the current moratorium on research prohibit any direct interactions with wild sea lions, and so dietary information available from scat analysis has become increasingly important. Although marine mammalogists use physical analyses of fish remnants recovered from scat to estimate the total number of prey items consumed and dietary proportions for different prey species, research using current methods of physical analysis is reaching the limit of extractable information.

By employing the elemental analysis techniques that scientists studying fish populations have come to rely on, the amount of information available from fish remnants in scat can be increased. Otoliths and eye lenses are frequently recovered from scat, and they are the preferred structures for elemental analysis because of their incremental growth patterns and independence from resorption and metabolic re-working. Although elemental analysis on fish eye lenses is a relatively new application of the technique, its use on otoliths has been well validated and has become a standard methodology.

Concentrations of metal contaminants in undigested otoliths generally showed such poor correlations with metal levels in fish tissues prior to passage through a sea lion digestive tract that digested otoliths were not compared to tissue samples. The calcium carbonate structure of otoliths appears to have a different affinity for metals than do soft

tissues, and metal incorporation into otoliths is not regulated by the same mechanisms that control soft-tissue concentrations. Given the lack of a discernible relationship, otoliths are not likely to be useful to determine contaminant ingestion by pinnipeds. However, the proteinaceous structure of the eye lens may make it a more suitable predictor of contaminant levels in soft fish tissues.

The effects of digestion on otolith morphology vary with the time spent in the gastrointestinal tract (Tollit et al. 1997). Digested otoliths can be graded based on physical degradation (Tollit et al. 1997), and otoliths that show the least physical erosion are the most likely to be useful for elemental analysis. Even if it is not currently possible to use highly digested otoliths to determine information about prey life histories, as my data suggest, the elemental fingerprints of digested otoliths can still be used to distinguish among fish species. Otolith fragments that cannot be assigned to a species based on physical analysis can be identified using elemental analysis. This would improve accuracy in determining the dietary proportions of individual species in sea lion diets because otolith fragments that cannot be identified physically can be assigned to the appropriate species using elemental analysis.

Fish eye lenses, which currently play a minor role in dietary studies, show fewer effects from digestion than do otoliths and have the potential to be accurate indicators of fish age, although further testing with a greater age-range of fish is necessary to validate my findings. Age-size regressions that are used to estimate fish lengths can be coupled with counts of lens growth layers to obtain estimates of prey size, which is not available via current methods. The elemental distributions of eye lenses display distinct patterns for some elements but appear to be random for others, which creates a unique elemental

fingerprint similar to those found in otoliths. The symmetrical patterns found in the lens for some elements suggest that the incorporation of several trace metals is somehow being regulated or affected by external conditions. Because of the lens' resistance to physical effects from sea lion digestion, I would postulate that the elemental distributions are also resistant to changes from digestion and that digested eye lenses will not differ significantly from undigested lenses in their elemental distributions. This aspect of fish eye lenses has not been studied previously and was beyond the scope of this study, but it has the potential to be useful to fisheries scientists and marine mammalogists.

The elemental analysis of otoliths and eye lenses recovered from Steller sea lion scat clearly can provide additional information to that currently available from physical analysis. As researchers look for new ways to investigate the diets of marine mammals, they should look across disciplines and incorporate techniques from other branches of science that allow for increased information without increased disturbance.

Literature cited

Tollit, D.J., M.J. Steward, P.M. Thompson, G.J. Pierce, M.B. Santos, and S. Hughes.
1997. Species and size differences in the digestion of otoliths and beaks:
Implications for estimates of pinniped diet composition. *Can. J. Fish. Aquat. Sci.*
54:105-119.