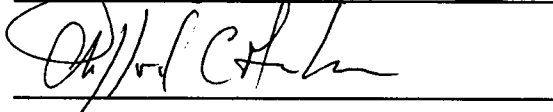


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I am submitting herewith a thesis written by Sandra L. Davis entitled "Laser Ablation Mass Spectrometry Analyses of Fish Scales for the Determination of *Morone Saxatilis* Movements Between Fresh Water and Saltwater Habitats." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Ecology.


C. C. Coutant, Major Professor

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LASER ABLATION MASS SPECTROMETRY ANALYSES OF FISH SCALES
FOR THE DETERMINATION OF *MORONE SAXATILIS* MOVEMENTS BETWEEN
FRESH WATER AND SALTWATER HABITATS

A Thesis

Presented for the

Master of Science

Degree

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Sandra L. Davis

August, 1994

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Abstract

Laser ablation combined with quadrupole mass spectrometry, a new technique being developed at Oak Ridge National Laboratory, was used to analyze Sr content in striped bass (*Morone saxatilis*) scales to investigate movements of these fish between fresh water and saltwater habitats. It is known that saltwater has a much higher concentration of Sr as compared to fresh water. Therefore, it was hypothesized that striped bass movements could be monitored through strontium levels in fish scales due to the fact that elements such as Sr are incorporated into fish hard parts as they grow.

The system was also used to test whether elements such as Rb fed in the diet would produce marking bands in coho salmon scales.

It was found that, due to fish scale morphology and perhaps the laser/mass spectrometer system used, Ca and Sr signals obtained were extremely variable. Strong and consistent Sr peaks were not observed. It was found that Rb fed in the diet of coho salmon, produced distinct, but inconsistent marking peaks in their scales. It was concluded that further analyses of fish scale morphogenesis would be necessary to determine how the scale incorporates

elements from the surrounding environment. It was also determined that more studies of the quadrupole and laser system were needed to test its suitability for the fish scale analyses.

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Chapter I

Introduction

A need for better management of fish populations has sparked an interest in determining fish movements between environments. This is extremely important for species which have high economic value. Striped bass (*Morone saxatilis*) are one such species of importance to aquatic specialists. Aquatic scientists believe that in order to obtain the best fishery yield possible, each individual breeding population, also known as a stock, should be managed separately. Therefore, it is very important to possess a means with which to distinguish between individual stocks of fish (Mulligan et al., 1987).

In past decades, striped bass numbers have been high, an encouraging fact for fishery managers who know the high demand for saltwater striped bass and their products (Mulligan et al., 1987). Striped bass have also been very important to fresh water sport fishing in the U.S. (Belanger et al., 1987). However, due to numerous factors, in the past few years, striped bass numbers have been declining, creating a need for better management practices (Mulligan et al., 1987). Supply of these fish cannot always keep up with the demand, causing market prices of fresh water and marine water striped bass to increase

(Goodyear et al., 1985). Illegal sales of fresh water striped bass labeled as marine fish have prompted authorities to search for a quick method of distinguishing the identical looking groups at the market place (Belanger et al., 1987).

Projects have been undertaken by fishery managers in the North Carolina and Virginia areas to restock declining estuarine populations with fresh water striped bass. These fishery managers need a technique which allows them to distinguish between fresh water hatchery-reared fish and naturally occurring estuarine fish after later capture. Distinguishing between the two types would allow managers to evaluate the status of natural reproduction in the stocks, as well as the status of the restocking program (Coutant and Chen, 1991).

It is believed that most striped bass reside in fresh water during early life, move to marine water for some time period and return to fresh water to spawn. It would be beneficial to have the capability to determine at what point in the fish's life the transition from fresh water to saltwater was made. Managers would also like to determine how long a fish stayed in each environment. Furthermore, there are some stocks of striped bass which may not be anadromous. These are among the questions which aquatic specialists would like to answer.

Chemicals laid down in fish hard parts, such as scales and bones, may offer clues to the environment occupied during fish growth. Coutant et al. (1992) stated that "fish scales are very much like a computer chip implanted at birth which records the location of the organism, temperature changes, growth rate, periods of disease or other stress, periods of reproduction, and the environment from which the mother came". Therefore, using fish scales as living records of fish life history is an interesting new prospect.

A new technique is currently being developed at Oak Ridge National Laboratory which may make the process of stock identification and fish movements much easier. The technique is called laser ablation mass spectrometry (LAMS) and it is used to chemically analyze fish scales (Coutant et al., 1992). This technique, which is funded through the Department of Energy, is being developed by the Environmental Sciences Division in association with the Health Sciences Division at ORNL.

Chapter II

Objectives and Hypotheses

Objectives

There were four main objectives for the thesis project:

1. To explore a new technique called laser ablation mass spectrometry for the analysis of fish scale microchemistry and determine if it has applications for fisheries scientists
2. To explore fish scale mounting techniques for laser analysis
3. To determine strontium and calcium levels at discrete locations in fish scales as indicators of fresh water/saltwater habitat and movements
4. To determine if exposing fish to an element, such as rubidium, through the diet produces a chemical "mark" in the fish scale

Hypotheses

H_O: Anadromous *Morone saxatilis* scales can be used to trace saltwater/fresh water movements through a strontium peak at the point in the fish's life of saltwater entry.

H_A: Anadromous *Morone saxatilis* scales cannot be used to trace saltwater/fresh water movements through a strontium peak at the point in the fish's life of saltwater entry.

H_O: Artificial chemical markers fed briefly to fish during hatchery rearing will be detectable as discrete peaks of concentration in fish scales.

H_A: Artificial chemical markers fed briefly to fish during hatchery rearing will not be detectable as discrete peaks of concentration in fish scales.

Chapter III

Description of Study Area

Striped bass scales were obtained from Pete Kornegay of the North Carolina Wildlife Resources Commission, Department of Inland Fisheries, Camden, N.C., Dr. Victor Vecchio of the New York State Department of Environmental Conservation, Stonybrook, N.Y., and from Michael Stangl of the Delaware Division of Fish and Wildlife. Tim Mulligan of the Pacific Biological Station, Nanaimo, B.C. provided coho salmon scales for comparison analyses.

The North Carolina scales were collected from fish in the lower Roanoke River/Albemarle Sound area during spring creel surveys. The scales were collected from mature females ranging in age from 6 to 8 years which were believed to be returning from marine waters to their spawning grounds in the Roanoke River.

The New York striped bass scales were collected from fish which originated in a New York hatchery on the Hudson River, migrated to marine waters and returned to the hatchery to spawn. These scales were collected from females ranging in age from 4 to 6 years. Because their habitat history is certain, these scales were used as a comparison or "control" for the North Carolina scales.

Striped bass scales were also obtained in the upper Delaware Bay from fish which were known to migrate between fresh water and saltwater. Delaware scales were obtained from males ranging in age from 4 to 5 years. These scales were used as another comparison for the Roanoke River striped bass scales.

Coho salmon scales were obtained after artificial marking feeding experiments in British Columbia. These fish were fed two separate specially enriched diets, one containing Rb and the other containing Sr. The age and sex of these fish were unknown. These fish were used as another comparison to the Roanoke River striped bass. They were used to determine if scales exposed to high supplemental concentrations over relatively short periods of time produced marks that were easily detected with our system (Figure 1¹).

All fish scales were removed from the fish by my collaborators using a spatula and forceps, dried and placed in manila sample envelopes (Table 1).

¹See appendix for table and figures.

Chapter IV

Background Information

Scale Structure and Growth

Fish scales begin to form soon after hatching. They are mostly composed of calcium phosphate in an organic matrix (Maekawa and Yamada, 1970). They also have as much as 3.8% fluorine, the major component of bones and teeth (Coote et al., 1991). As the scale grows outwardly, increments or rings are formed which represent daily and annual growth. The central or focus region of the scale represents the time near hatching. These rings have been compared to the annual growth rings in trees (Rhoads and Lutz, 1980) (Figure 2). Rings range in size from a few microns to 1mm and are spaced between 200 and 3000 μ m apart (Bigler, 1989). Each increment is composed of a calcium-rich, as well as a calcium-poor zone also known as the growth zone and discontinuous zones, respectively (Radtke et al., 1990). Figure 3 shows that the rings form a ridge-and-valley topography on the surface of the scale (Lagler et al. (eds.), 1962). Fryer and Schwartzberg, (1990) stated that narrowly-spaced rings on salmon scales represent slow or fresh water growth and widely-spaced rings represent fast or salt water growth (Figure 4). Fish scales grow by

laying down new material in the horizontal as well as vertical plane. There are three layers in the vertical plane: the upper osseous or calcified layer, the lower non-calcified fibrillary plate and a pre-osseous or osteoid zone between these two layers (Maekawa and Yamada, 1970) (Figure 5). In the vertical plane, growth first occurs in the osseous layer through deposition of new material composed of neutral heteropolysaccharides, acidic sulfated mucopolysaccharides and both mature and immature collagens. After the upper layer is laid down, the lower fibrillary plate, composed of collagen fibers, fills in underneath (Maekawa and Yamada, 1970). In addition to vertical growth, the scale grows concentrically outward through further deposition of calcium phosphate in an organic matrix, first forming an osteoid zone (Figure 6) and later becoming fully calcified (Waterman, 1970).

Because it has been shown that rings are laid down with annual or even daily markings, they have been used for many years to determine fish age (Coutant et al., 1992). The spacing patterns of rings have been used to determine growth histories of fish (Wood et al., 1988). In addition to concentrically-formed rings, scales are also marked with grooves or radii extending from the focus region outward (Waterman, 1970) (Figure 4).

Striped Bass Life Cycle

Striped bass (*Morone saxatilis*) range on the Atlantic coast from Florida to the Gulf of St. Lawrence as well as the Gulf of Mexico (Figure 1). When residing in marine waters, striped bass remain within a couple of miles from the coastline (Merriman, 1937). Landlocked populations found primarily in North and South Carolina (Ursin, 1977) have led fisheries managers to stock the species in many fresh water reservoirs throughout the continental United States. The degree to which the North Carolina (Albemarle Sound and Roanoke River) stock is migratory is uncertain.

After hatching in fresh or brackish waters of estuaries, young striped bass remain in or near their area of hatching for the first year. As the fish mature, they increase the distance they will move away from the natal area (Kohlenstein, 1981). Fish age 1 or 2 generally remain in the estuaries. In early spring, the majority of the adult population begins migrating to the ocean and moves northward along the coastline as far as maritime Canada (Chapoton and Sykes, 1961). The migrating population includes immature juveniles and mature adults. As fall approaches and water temperatures decrease, the population begins moving southward along the coast as far as the Carolinas. Mature adults (age 2 males or age 5-6 females) proceed to the natal hatching grounds in fresh or brackish

waters to spawn in April while immatures remain farther downstream (Kohlenstein, 1981). Kohlenstein (1981) also stated that mature females do not necessarily spawn every year, an interesting point which is very important to our analysis of fish movements.

Incorporation of Elements Into Scales

It has been shown by many researchers that hard parts, including vertebrae, otoliths, teeth, shells and scales, incorporate elements from the surrounding water as they grow. This has been demonstrated with whole-sample analyses (Lapi and Mulligan, 1981; Bagenal et al., 1973; Belanger et al., 1987). Lapi and Mulligan (1981) stated that, "the ideal tissue for chemical analysis is one containing a separable record of time spent in fresh water and saltwater". It is believed that since they are metabolically inert and will therefore retain the elemental concentration after exposure, fish scales are very good records of exposure history (Lapi and Mulligan, 1981). Bagenal et al. (1973) reported that as the scale matrix is laid down, it tends to incorporate materials such as local contaminants and trace metals, as well as isotopic ratios which are linked to changes in temperature and salinity. Belanger et al. (1987), used chemical analysis of whole

fish scales to successfully differentiate between striped bass from fresh water and saltwater.

Water temperature changes have been monitored using fish scales. Gauldie et al. (1986), determined that as water temperatures increase, uptake of Zn and Mn by fish scales increases. Whole scale analysis for these elements allow scales to be used as indicators of temperature fluctuations. Lee and McKenzie (unpublished) also performed analyses on whole fish scales. They demonstrated that it may be possible to monitor PCB exposure through chlorine concentrations in fish scales.

It has been demonstrated that all fish hard parts, including scales and bones, have a tendency to take up stable strontium from the surrounding water environment (Yamada et al., 1979). Strontium is very similar to calcium structurally; they have the same valence (+2) and similar ionic radii (Ca, 0.99Å; Sr, 1.13Å) (Amiel et al., 1973). Therefore, strontium easily replaces calcium in fish scales as they grow (Bagenal et al., 1973). Skoryna (1981) stated that the higher the concentration of strontium in the environment, the more substitution of strontium for calcium will occurs in hard parts. There is a considerably higher concentration of strontium in the ocean verses fresh water (0.09 mM/kg Sr:10.3 mM/kg Ca verses 0.00068 mM/kg Sr:0.35 mM/kg Ca) (Kalish, 1990).

Theoretically, this concentration change should be reflected in the fish scales.

Previously Used Methods to Monitor Fish

Several methods have been used previously to monitor fish population origin and movements between habitats. They included microanalysis of whole fish hard parts, chemical marking techniques and non-chemical marking techniques.

Coutant (1990), described three microanalytic methods which have been used to analyze fish hard parts. The first was electron microprobe spectroscopy. This method used a focused beam of electrons to bombard a biological sample, in this case, fish otoliths. This method was advantageous in that it did not require destruction of the sample and it was possible to obtain data from small, discrete locations on the otolith. However, electron microprobe spectroscopy was limited in that only elements with atomic number 11-92 could be detected. This range was adequate for Sr and Ca detection, but we wanted to expand our later analyses to detect elements such as Pb and Hg. Since the data was in the form of an energy spectrum, it became difficult to evaluate the concentration of a single element such as Sr or Ca. The electron microprobe has however been demonstrated as useful for evaluating Sr:Ca ratios in

otoliths linked to water temperature changes (Radtke et al., 1990; Townsend et al., 1989).

The second method described by Coutant (1990) was atomic absorption spectrometry. This method was undesirable in that each sample had to be ashed before analysis and only one element at a time could be detected.

Neutron activation analysis was the third method described by Coutant (1990). Disadvantages of this method, which required exposing the sample to a neutron source in a nuclear reactor, included the necessity of sample destruction for analysis and extremely high cost.

X-ray fluorescence spectrometry is a method which has been used to analyze fish hard parts such as vertebrae (Mulligan et al., 1983; Yamada et al., 1979; Calaprice et al., 1975). The sample is exposed to a beam which causes it to produce X-rays characteristic of elements contained in the vertebrae. This method has been used to differentiate between different stocks of salmon through detection of elements characteristic of their aquatic habitat (Mulligan et al., 1983). Gauldie et al. (1986) described inductively coupled argon plasma optical emission spectrometry as a method to analyze whole otoliths for ion content which can be related to water temperature changes in the past.

Laser ablation mass spectrometry may be an excellent new method to detect artificial and natural chemical

markers in fish scales. This would allow quick and easy separation of fish stocks through chemical markers from natal streams and separation of hatchery verses wild species through detection of artificial chemical markers.

Previously used chemical marking methods have been labor intensive, required extensive handling of fish and often require sacrificing the fish. Strontium and tetracycline have been used to artificially mark hatchery fish vertebrae through the diet (Yamada et al., 1979; Chart and Bergersen, 1988). Unfortunately, the fish must be sacrificed to identify the mark. Chemical markers can also be applied through injection. Michibata and Hori (1981), injected samarium into fish abdominal cavities. Detection of this mark was expensive and required sacrificing the fish because the fish had to be exposed to a beam of thermal neutrons in a nuclear reactor.

Other markers have been used which are not chemical in nature. These methods are also labor intensive and many require sacrificing the fish. Encoded wire tags can be implanted into the snout. This method required much fish handling. Tag detection was with a magnetic field. Decoding the tags required sacrificing the individual to remove it from the snout (Chart and Bergersen, 1988). Passive integrated transponders, which emit a low frequency radio signal, can be implanted into the body cavity (Chart and Bergersen, 1988). Tags can be decoded within a live

individual but involve high cost and effort for implantation.

The presence of various types of parasites characteristic of certain regions have been used to identify stocks of fish, as well as learn about their movements between regions. This method had a high degree of accuracy but required extensive parasite surveys and can only be performed by workers with a firm knowledge of parasitology (Chart and Bergersen, 1988).

Laser Ablation Mass Spectrometry

Laser ablation mass spectrometry (LAMS) is a technique normally used in the chemical analyses of non-biological samples. In our case, we are using this technique for the chemical analysis of fish scales. The technique involves the use of a vacuum chamber containing the sample, a Neodymium:YAG laser and a quadrupole mass spectrometer. An IBM computer is used in conjunction with this equipment for the purposes of data acquisition and analyses (Figure 7).

The sample of interest is placed in the chamber, containing three ports (windows), which must be under vacuum for the analyses to proceed. The Nd:Yag laser is directed at the sample through one port. The laser ablates a small puff of material from the surface of the sample. Sample movement across the laser beam is controlled through

computer-driven motors and can be monitored using a video camera installed at another port in the chamber. The ablated ions enter the quadrupole mass spectrometer detector which has been tuned to the element of interest. Relative chemical signals are recorded by the computer and stored in a compressed format for later analyses.

Advantages of LAMS Over Other Methods

Laser ablation mass spectrometry is a new method being tested for the chemical analysis of fish scales. Using fish scales rather than other hard parts in the technique decreases sample preparation and increases the types of fish which can be analyzed. Fish scales need only be cleaned before analysis as opposed to vertebrae and otoliths which must be cut and finely ground. Due to the ease of scale removal, it is possible for fishery managers to quickly remove a few samples at a time from literally hundreds of fish. Many samples are available per fish, so chemical results are easily confirmed. Scales are a very desirable source of information because they can be obtained without sacrificing the fish. Therefore, it is possible to take multiple samples from rare stocks or brood stocks without depleting the population (Coutant, 1990).

Compared to the methods described earlier, laser ablation mass spectrometry has much more chemical

sensitivity. In fact, the laser ablation mass spectrometry technique has the capability of detecting all elements except helium and neon (Coutant, 1990). One of the most important features of the LAMS technique is it allows the time factor in the scales, which is represented as annuli, to remain intact. This is not possible with the methods described above. Using the annuli, one can relate chemical contaminants to years. One can follow the increases and decreases in contaminant levels over time (Coutant and Chen, 1991). The LAMS technique also allows vertical sampling at deeper levels in the scale (Coutant and Chen, 1991).

Additional Applications for LAMS

The future applications for the LAMS technique, if proven to be effective and economical, seem to be endless. It could be a valuable new tool for scientists to utilize in the study of fish and wildlife species and the environment they live in.

LAMS would be an innovative new technique for the detection of artificial chemical marks used to identify hatchery-reared species of fish verses their wild counterparts. Rare earth elements such as rubidium, terbium and europium have been used to artificially mark hatchery-raised salmon populations (Coutant, 1990).

Previously, mark detection has been accomplished through whole otolith, scale or vertebral analysis. LAMS provides the capability of quick and relatively easy mark detection from hatchery fish mixed with wild fish in marine water without destroying the individual to obtain the sample.

LAMS would also be an asset in the differentiation between wild fish stocks through elements contained in the scales which are characteristic of their natal streams. In order to help evaluate the status of certain rare stocks of Pacific salmon, it is necessary to differentiate between the stocks after they have become mixed in the ocean (Coutant et al., 1992). Analyzing scales for elements which have previously been found to be unique to their streams of origin would accomplish this goal. Using scales instead of internal bones would prevent depletion of already dwindling numbers of fish.

Scientists need better and more economical methods of using fish and wildlife as bioindicators. LAMS potentially provides such a method. Water contamination could be monitored through fish scales (Coutant, 1990). It will be possible to detect contaminants such as lead and mercury in streams, rivers and oceans. Because scales are left intact, water contamination can be linked to specific annuli, thus providing clues as to date and location of exposure. These clues can eventually lead to identification of the pollutant source. Sampling scales

over time could also provide information as to success or failure of water cleanup projects. Other aquatic species such as coral and clams also provide a record of water pollution and temperature histories through their annual rings (Dodge and Gilbert, 1984).

The LAMS technique can give useful information concerning movements of terrestrial mammals. Terrestrial mammals such as bears and coyote, which have teeth composed of annually deposited cementum layers (Linhart and Knowlton, 1967), may be tracked through naturally occurring elements present in soils. Artificial marking of the teeth could be accomplished through the diet, also allowing movements to be monitored. A fast, easy and cost effective method for monitoring movements of certain populations would help in the development of environmental impact statements by determining how often these populations reside in potential development sites (Coutant, 1990).

New insight into the migration patterns of whales can be gained through the LAMS technique. Marine mammals also deposit dentine and cementum layers in teeth as they age (Scheffer and Myrick, 1980). Analysis of tooth samples for elements characteristic of water quality or element ratios which can be related water temperatures may give use new information as to location of these mammals during all phases of migration.

Chapter V

Equipment

Former Systems

In adapting the laser ablation mass spectrometry technique for use with fish scales, we have explored numerous equipment and sample-holder designs. These components were in use in the Health Sciences Research Division at Oak Ridge National Laboratory. For our purposes, each of the former systems had design flaws which necessitated a new modified system. While the final fish scale results of this project are very important, we felt that the progression of testing several equipment setups also deserved mention.

The first system we used included a spherical vacuum chamber 13-inches in diameter in association with a quadrupole mass spectrometer. Pacific salmon scales were mounted individually on a stainless steel sample holder for laser analysis. The advantage of this system was the ease of sample viewing and changing samples provided by the large size of the vacuum chamber.

The minuses outweighed the pluses dramatically. They consisted of alignment problems, limited capability of the

mass spectrometer, chemical interference from the sample holder, and instability of the sample holder arm.

We encountered alignment problems between the laser beam, sample and quadrupole detector due to the fact that the chamber was not specifically designed for this project. Misalignment affected our chemical signal by lowering the amount of ablated material that the quadrupole detector was able to pick up. Small amounts of ablated material did not produce a strong signal on the quadrupole mass spectrometer, therefore making it difficult to detect Sr in the fish scales.

Using the quadrupole type of mass spectrometer, we were only able to detect one element at a time, thus greatly increasing the amount of time needed to analyze one scale. The high-Q head (Q describes the capacitance and inductance of a circuit) used with the mass spectrometer determines what range of elemental masses can be detected. The high-Q head available was only able to cover the mass range below 100 Daltons. This range was adequate for detection of Sr and Ca, but we were also interested in expanding the project to look at environmental pollutants such as Pb and Cd.

We had difficulty with the stainless steel sample holder for this system. This sample holder was found to adversely affect our data because the stainless steel contained too many contaminating elements which were picked

up along with the ablated scale material by the quadrupole detector. The contaminants confounded the element signals we were targeting.

A further problem with this system was instability of the sample holder. It was mounted within the vacuum chamber on an arm approximately 14-inches in length which moved the sample in the X and Y directions via a set of external X-Y Compumotors (Figure 8). The length of the arm amplified background building vibrations, making the sample holder very unstable. This instability caused the laser shot accuracy on the sample to be very low. This situation was untenable because the material tested with this system was the extremely small scales of Pacific salmon. Instability caused much difficulty in their analysis. It was necessary to decrease the laser beam diameter to the point that the beam would not focus consistently on a single spot and, therefore, produced highly variable and unreliable data (Figure 9).

The second system setup we used included a time-of-flight mass spectrometer and a new spherical vacuum chamber 5 inches in diameter. The advantage of this system was the ability to detect a whole spectrum of elements at one time. With the new vacuum chamber, we eliminated the alignment problem between the laser beam, quadrupole detector and sample. Instead of using a long, motorized arm to move the sample, the new chamber included a movable stage with

motors mounted directly beneath the sample. This orientation eliminated any vibration problems. Unfortunately, it was determined that for this system to work effectively, further design modifications would be necessary for the vacuum chamber. The chamber design was such that background signals would be very large and would subsequently 'swamp-out' the signal in the time-of-flight mass spectrometer which we were trying to detect. In order to correct this problem, it would be necessary to install pulse deflectors in the chamber which would have required a delay of 2-3 months.

The third system consisted of a modified 5-inch diameter vacuum chamber coupled with a quadrupole mass spectrometer. Again, this system only allowed detection of one mass at a time. After many attempts and failures to obtain useful chemical data using salmon scales in this system, it was decided to move to the fourth and final system.

Final System

The system which was finally determined to best suit our needs included the modified 5-inch diameter vacuum chamber, quadrupole mass spectrometer, and a new high-Q head which allowed for a higher mass cutoff for analyses. A pulsed Nd:Yag laser was used to produce a 355 nm

wavelength beam for ablation purposes. Switching from the stainless steel sample holder to one made of aluminum avoided the problem of sample contamination. It was also decided to begin our analyses with larger striped bass scales (10,000-15,000 μ m in diameter) instead of the extremely small Pacific salmon scales (1,800-4,000 μ m in diameter) (Figure 7). This system also utilizes the mechanical stage instead of the long arm to prevent sample vibration. Due to the small size of the chamber (5-inches in diameter), the transmission distance between sample and quadrupole detector was greatly shortened. A video camera was mounted at one of the ports in the chamber in order to view placement of the laser beam and monitor sample movement (Figure 7).

Chapter VI

Scale Sample Preparation

Cleaning

Scale sample preparation for the LAMS technique was relatively simple. When the scales were first removed from the fish, they were covered with a layer of mucous and dirt. The presence of these contaminants on the scale during laser analysis would cause specific scale-element (such as Ca and Sr) detection to be very difficult. This difficulty is due, in part, to the thickness of the mucous layer which must be cleaned away before elements within the scale structure can be detected. Because laser ablation requires a very flat specimen surface, it was determined that reliable and accurate data could not be obtained with the mucous layer present. Therefore, before the laser analysis could be performed, all mucous, dirt and other debris had to be removed from the scale. Removal of the unwanted layer was accomplished through a combination of an ultrasonic bath and stiff brush. Scales were removed from the sample envelopes and placed directly into a beaker containing distilled water. The beaker was placed in a warm ultrasonic bath for 45 minutes, removed and cleaned

with a stiff brush, then returned to the bath for another 45 minutes.

After bathing, the scales were only handled with stainless steel forceps to prevent future contamination from skin contact. The cleaned scales were placed between sheets of Kimwipe®, an absorbent and lint-free laboratory tissue paper. The scales were pressed flat with 5 pound weights and allowed to dry overnight. The pressing was necessary to insure that the scales were as flat as possible for mounting and laser ablation purposes. When dried without pressing, scales tended to curl, making the mounting procedure very difficult.

Mounting

Scales were mounted individually on 3 cm diameter aluminum disks. Initially, 5-minute epoxy was used to attach the scales, but it was found that the combined heat from the laser light source and the vacuum of the chamber caused the scales to pop off the aluminum disk during laser ablation. A mounting compound called Permabond® Industrial Grade metal bonding adhesive was determined to work much better. Due to the fact that the scales had to be as flat as possible for laser analysis, they were glued onto the aluminum disk and placed in a C-clamp to dry for approximately two hours. After the Permabond® adhesive

had dried, reference lines were drawn on all sides of the scales to define the edges during viewing on the video monitor (Figure 10). This gridding was necessary because the experimenter used the image on the video monitor to guide the movement of the laser across the scale.

Chapter VII

Data Acquisition and Analysis

Three sets of responses were taken for each fish scale. Each set consisted of two closely parallel rows of laser shots; one row to analyze for Ca and the other row to analyze for Sr. We analyzed for both elements in order to use the Sr:Ca ratios in our data analyses. Each set of two laser rows was a transect from the focus region of the scale to the outer edge (Figure 10). The laser beam was focused to approximately 50 μ m in diameter. Each laser row consisted of discrete ablated spots 150 μ m apart. At each spot, the laser ablated with 200-450 pulses depending on factors such as thickness of the scale and number of pulses necessary to penetrate the scale. These factors were determined prior to analysis of each scale.

A Microsoft TrueBasic computer program, developed by Ron Phillips of the Health Sciences Division, controlled the data acquisition process. The program allowed specification of the number of shots to use per spot, as well as the distance between spots for each scale. It automatically collected all relative signal readings from the quadrupole and stored them in compressed file format. The compressed file format was necessitated by the large size of the data files. A second TrueBasic program, also

developed by Ron Phillips, was used to convert compressed data into a decompressed format readable by the PSiPlot plotting program, filter data and average all pulses for each spot. The averages were imported into PSiPlot for graphing.

In order to determine the fish's age during peaks and lows of Sr, it was necessary to relate laser shots with annuli. Therefore, it was necessary to take photographs of the scales before and after mounting them on the sample holder. Using a 35mm camera mounted on a microscope, pictures were taken before mounting because the adhesive used to mount the scales made it very difficult to distinguish the annuli. These pictures were blown up to 8-1/2 x 11 to view the annuli. Pictures were taken again after laser ablation in order to match laser shots on the scale with annuli. Using the scale photographs taken before and after laser analysis, we matched up any data points showing Sr or Rb peaks with annuli, thus giving some indication of the chronology of peaks.

Experiments Performed

Four types of studies were performed on the fish scales. We tested for Ca and Sr data variability within a scale, repeatability of Ca and Sr data within a scale and

between scales from the same fish, as well as the detection of the artificially fed chemical marker Rb.

Data variability studies were performed on all four types of fish scales including 5 Roanoke River striped bass, 5 New York hatchery striped bass, 3 Delaware Bay striped bass and 6 coho salmon (Table 1). These studies were performed to determine if the chemical signals detected by the quadrupole mass spectrometer were relatively consistent throughout the fish scales.

Repeatability of data studies were performed to determine if the chemical signal peaks were repeatable in different radii of the same fish scale and between multiple scales from the same fish. These analyses were performed on the same numbers and types of fish as the data variability analyses.

Further studies were performed to determine if artificial feeding of an element (Rb) would produce a Rb marker peak within a fish scale. These studies would also help to determine if high concentrations of element exposure over a relatively short period of time produced a more distinct peak in the fish scale. Rb detection studies were performed on 6 coho salmon scales.

Chapter VIII

Results and Discussion

LAMS Technique

After attempting the fish scale analyses with numerous equipment and sample-holder designs, it was determined that the most appropriate system for our needs included an aluminum sample-holder, modified 5-inch vacuum chamber, quadrupole mass spectrometer and a new high-Q head. This equipment was coupled with a pulsed Nd:YAG laser. The aluminum sample-holder eliminated contamination of the chemical signal through elements contained in the metal. The modified 5-inch chamber eliminated alignment, sample vibration problems and material transmission problems between the fish scale and quadrupole detector. Using the new high-Q head allowed higher mass elements to be detected.

Fish Scale Mounting Technique

It was determined scale mounting required that the scale must first be thoroughly cleaned and flattened. When 5-minute epoxy did not securely hold the fish scales in the

vacuum, it was discovered that a stronger compound, Permabond® Industrial Grade metal bonding adhesive, would be necessary. Lack of a clearly defined scale boundary visible on the video monitor necessitated the addition of reference lines drawn on each side of the scale before placement in the vacuum chamber.

Data Variability Along Radii

Data profiles for Ca, Sr, and Rb taken across the fish scale showed a high degree of variation. Element variation across the scales was evident in all four fish groups tested. The results we obtained showed that Ca, as well as Sr and Rb levels were very inconsistent across the scale. Figures 11 through 14 show the data variability across the scales for each of the fish groups tested.

Figure 11 shows relative Ca signal variation from 250 volts to 1500 volts in coho salmon scales. In between the high and low signals were numerous irregular ups and downs. The pattern of irregular ups and downs continued in each of the four groups of fish scales tested. Some peaks of Ca appear to occur at the annuli, but these did not occur at every annulus. Relative Ca signal varied anywhere from 310 volts to 1500 volts in a single scale radius of New York hatchery striped bass (Figure 12). Figure 12 shows that Ca concentration seemed to peak at some, but not all annuli.

In Roanoke River striped bass, relative Ca signals varied between 750 volts and 2400 volts (Figure 13), with more peaks occurring at the annuli. Delaware striped bass scales had relative Ca signals that varied from 400 volts to 1000 volts, peaks of signal at annuli as well as irregular ups and downs between the highest and lowest signals (Figure 14).

Data variability also occurred for relative Sr and Rb signals. Figure 15 shows relative Rb signals varied between 200 volts and 1100 volts. Rb peaks appeared at two annuli, but not at all annuli. Again, there was an irregular up and down pattern between the highest and lowest signals. In New York hatchery striped bass scales, we saw relative Sr signal variation between 250 volts and 2000 volts. Between the highest and lowest signal, we again saw the irregular up and down pattern. Sr peaks appeared at annuli 2 and 3, but not at annuli 1 (Figure 12). The pattern of variation, irregular ups and downs between highest and lowest signals, as well as, peaks of relative Sr signal was seen in Roanoke River striped bass scales (Figure 13) and Delaware Bay striped bass scales (Figure 14).

A general trend was observed for relative Ca signals. In most scale radii, it was observed that Ca concentration was higher at the focus region and decreased towards the outer edge of the scale. This trend was observed in coho

salmon, New York hatchery striped bass, Roanoke River striped bass and Delaware Bay striped bass (Figures 11-14). This trend was not, however, consistent for all radii in each type of scale.

Repeatability of Sr Data Among Radii and Scales

Data collected on the different scale radii may have each shown a peak of Sr, but this peak was not repeated in different radii of the same scale or in different scales from the same fish. This was true for all striped bass groups tested. Therefore, the peaks we saw cannot be assumed to be the actual mark we had hoped to see. Figure 16 shows Sr:Ca ratio profiles across multiple radii in New York hatchery striped bass. On radius 'B' a Sr peak was seen at annuli 2 and 4 but in radius 'C', there was a peak before annulus 1 and after annulus 6. This non-repeatability pattern was also shown in Figure 17. This figure shows Sr:Ca profiles across Roanoke River striped bass scales. Again, a definite peak was seen at position 15 for radius 'B' but no definite peak was seen in radius 'C'. We also observed a lack of repeatable data in Delaware striped bass scales. Figure 18 shows radius 'B' had a Sr peak at annulus 3 but radius 'C' had a Sr peak at annulus 1.5.

The lack of repeatable data was also observed between scales from the same fish. Figure 19 shows Sr:Ca profiles respectively for two scales from the same salmon specimen. Sr peaks were observed at position 4 in scale 3 and at positions 5 and 7 in scale 4. The non-repeatability factor was demonstrated in Roanoke River striped bass (Figure 20), New York hatchery striped bass (Figure 21), as well as Delaware striped bass (Figure 22).

Rb Marking Studies

Coho salmon artificially marked with Rb through the diet showed some instances of mark occurrence in their scales. Figure 23 shows a distinct peak of Rb at positions 3 and 5 in the first scale S-1. Figure 23 also shows Rb data for scale S-3. There was a definite Rb peak at position 4. Another double Rb peak was seen in salmon scale S-6 at positions 3 and 5 (Figure 23).

Discussion

The laser ablation mass spectrometry technique did not work as well as we had hoped for analysis of fish scales. We were not able to determine movements of striped bass between fresh water and saltwater habitats through a Sr peak in the scale at the point in the fish's life of

saltwater entry. In order to explain this, we looked to the equipment setup and the morphogenesis of the fish scales themselves.

We can state that the laser/quadrupole mass spectrometer system was fully operational and probably was not responsible for the lack of valid, repeatable peaks in Sr. Extensive tests using known metals, such as Al, produced strong and consistent quadrupole signals. However, it is possible that organic ions contained in the scale matrix and having masses similar to Ca and Sr may have been ablated and detected along with the elements of interest, thus confounding our results (Chen, 1994). Using the present equipment system, it was not possible to separate such organics from the elements we were interested in detecting.

It is possible that we do not fully understand the physio-chemical development of the fish scale and how it affects element uptake and storage. Relative Ca signals showed a great deal of variability across scale radii. This was not the expected result. We expected to see a relatively constant Ca signal across the scale given that this is the major scale component. The relatively constant signal would allow us to use Ca as a baseline in the Sr:Ca ratio and the Rb:Ca ratio. Figures 11 through 14 show the data variability across the scales for each of the fish groups tested. It is not yet completely understood why the

horizontal profiles appeared this way. One possible explanation is the ridge-and-valley topography of the scale. It is known that ridges have a higher degree of calcification and valleys are less calcified (Yamada and Watabe, 1979). Therefore, if the laser beam samples a spot on the ridge, we would see a peak in Ca as compared to the next spot sampling in the valley area. It follows that ridge areas with higher Ca concentrations would also show higher concentrations of Sr and Rb.

It is clear from data profiles taken across the scale (Figures 12-22), as well as vertical depth profiles (Figure 24), that Sr is found throughout the entire fish scale. It is not evident that Sr occurs only in the top calcified layer as previously believed, but actually occurs throughout the depth of the fish scale. Sr is found in varying amounts along the horizontal axis of the scale as well. We observed Sr peaks in multiple positions along a scale radius. This leads to the conclusion that elements in the water in which the fish resides are incorporated into the scale, but they may not remain in one discrete location as previously thought.

Elements in the water environment, such as Sr, may somehow be diffused into the scale matrix by processes that are more complex than originally believed. Description of the processes of scale deposition and calcification may help to explain this idea. The fish scale begins as a

collagenous osteoid plate (Everhart and Youngs, 1981). The plate becomes calcified through increased growth of hydroxyapatite crystal patches (Yamada and Watabe, 1979) (Figure 5). Maekawa and Yamada (1972) observed that flattening cells in the transitional zone between the pre-calcified and osseous layers help the calcification process in the pre-calcified zone (Figure 6). Following the deposition of the osteoid plate, deposition of the bottom scale layer, or fibrillary plate begins to occur (Everhart and Youngs, 1981). The fibrillary plate thickens and hardens through the addition of connective tissue as the scale grows (Yamada and Watabe, 1979) (Figure 6).

New growth of the fish scale first occurs at the margin through deposition of osteoid material which extends beyond the fibrillary plate (Everhart and Youngs, 1981). The calcification process begins at the oldest part of the scale and moves outward to the margin (Maekawa and Yamada, 1972). Because calcification is occurring continually within the scale, causing the focus region to become thicker and thicker, elements such as Sr may be diffused throughout the osseous and fibrillary plate matrices. Constant changes in the scale structure described above may prohibit concentration peaks of Sr only in discrete locations. Instead, the entire scale is "contaminated". This fact would make distinguishing fish age at the point

of saltwater entry by means of a rapid increase in Sr content very difficult.

It is possible that scale growth periods inhibit the formation of elemental marking bands on the scale. Van Oosten (1957) stated that "scales experience a pronounced retardation of growth once per year followed by a period of complete rest". These retardation and rest periods may inhibit adequate Sr uptake for elemental marking band formation during periods of the year that would coincide with saltwater habitation in striped bass. Perhaps the growth retardation period allows the scale time to diffuse Sr which has been incorporated and allow the mark to be "diluted".

Temperature changes may confound Sr concentrations in our scales. Otoliths, which are composed of materials similar to that of scales, have been used to follow fish life history patterns such as temperature and salinity exposures. It has been shown that the amount of Sr substitution for Ca is inversely related to temperature (Townsend et al., 1989). Townsend et al. (1989) also stated that coral aragonite, which is structurally similar to otoliths and scales, has a Sr to Ca ratio which reflects the Sr to Ca ratio of the sea water in which it resides. However, they determined that there is a possible temperature factor which can effect this ratio. These researchers found evidence that increasing water

temperatures produced a discrimination affect against the uptake and incorporation of strontium. Townsend et al. further suggested that because fish have much more complex physiology than coral, they would experience a much higher temperature-induced discrimination against Sr incorporation and therefore would have Sr to Ca ratios in their otoliths much lower than the surrounding higher water temperatures environment. It follows that fish scales would also experience a discrimination against Sr incorporation which could explain the lack of distinct Sr peaks following saltwater habitation.

Otoliths may be a better source of information concerning Sr exposure. Past studies have found that otoliths can be used to monitor temperature exposure histories of fish. Temperature histories are recorded in the otolith through Sr:Ca ratios (Radtke, 1984; Radtke, 1989; Gauldie et al., 1986, Radtke et al., 1988). Radtke (1984) stated that, in otoliths, Sr replaces Ca in an amount which is inversely related to water temperature. Otoliths may be better Sr:Ca record keepers because they are structurally much more simple than scales. Scales are composed of three layers of ever-changing calcium carbonate, whereas, otoliths are composed of one inorganically precipitated aragonite layer (Kalish, 1989).

Using artificially marked salmon scales, we tested the belief that high element (Rb) concentration and short

exposure time determined if a clear marking band would be evident in the scale. These scales gave some proof of this hypothesis. As discussed in the previous section, some distinct, although inconsistent Rb peaks were detected (Figure 23). Rb peaks were not found at consistent positions along the scale radii, but they were very distinct in each of the four salmon scales tested. Furthermore, the double Rb peaks seen in Figure 23 support the idea of chemical diffusion within the scale matrix.

The Next Step

Because LAMS has so many possible applications for the analysis of fish scales and other hard parts, further work should be done to refine the technique and correct its short-comings. One possible avenue for future research would be to analyze fish otoliths which are definitely known to produce and maintain bands of elements which have been incorporated from the surrounding water. Performing the LAMS analysis on these materials would allow problems in the equipment and technique to be pinpointed and corrected for later fish scale analyses.

Future work should include further analyses of the laser and quadrupole mass spectrometer to determine if this system must be altered to better detect the elements of interest. It was previously stated that organic ions

contained in the scale material, with masses close to that of Sr, may be released with the ablated material and thus confound the Sr signal. A new laser setup would be necessary to suppress these ions. A second laser, called a tunable wavelength dye laser, would be needed in conjunction with the existing Yag laser to accomplish the task. Another Yag laser would be needed to pump the dye laser. All in all, this process would involve much more complex instrumentation and monetary commitments. It would also require a much higher degree of technician training to perform the analyses. However, considering the great potential for the LAMS technique, perhaps it would be worthwhile to pursue this course of action.

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APPENDIX

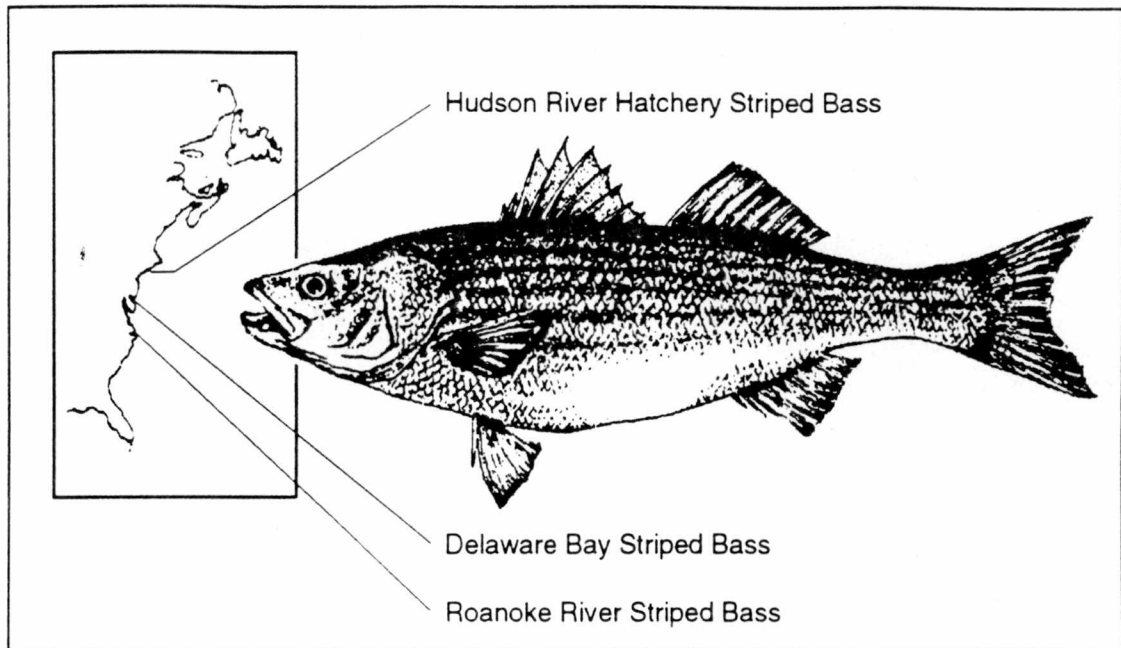


Figure 1: Geographic range of *Morone saxatilis* on the east coast of the United States. Arrows indicate where each of the three groups of striped bass were obtained.

Adapted From: Ursin, M.J. 1977. pp. 140-143. In: A guide to fishes of the temperate Atlantic coast. E.P. Dutton, New York.

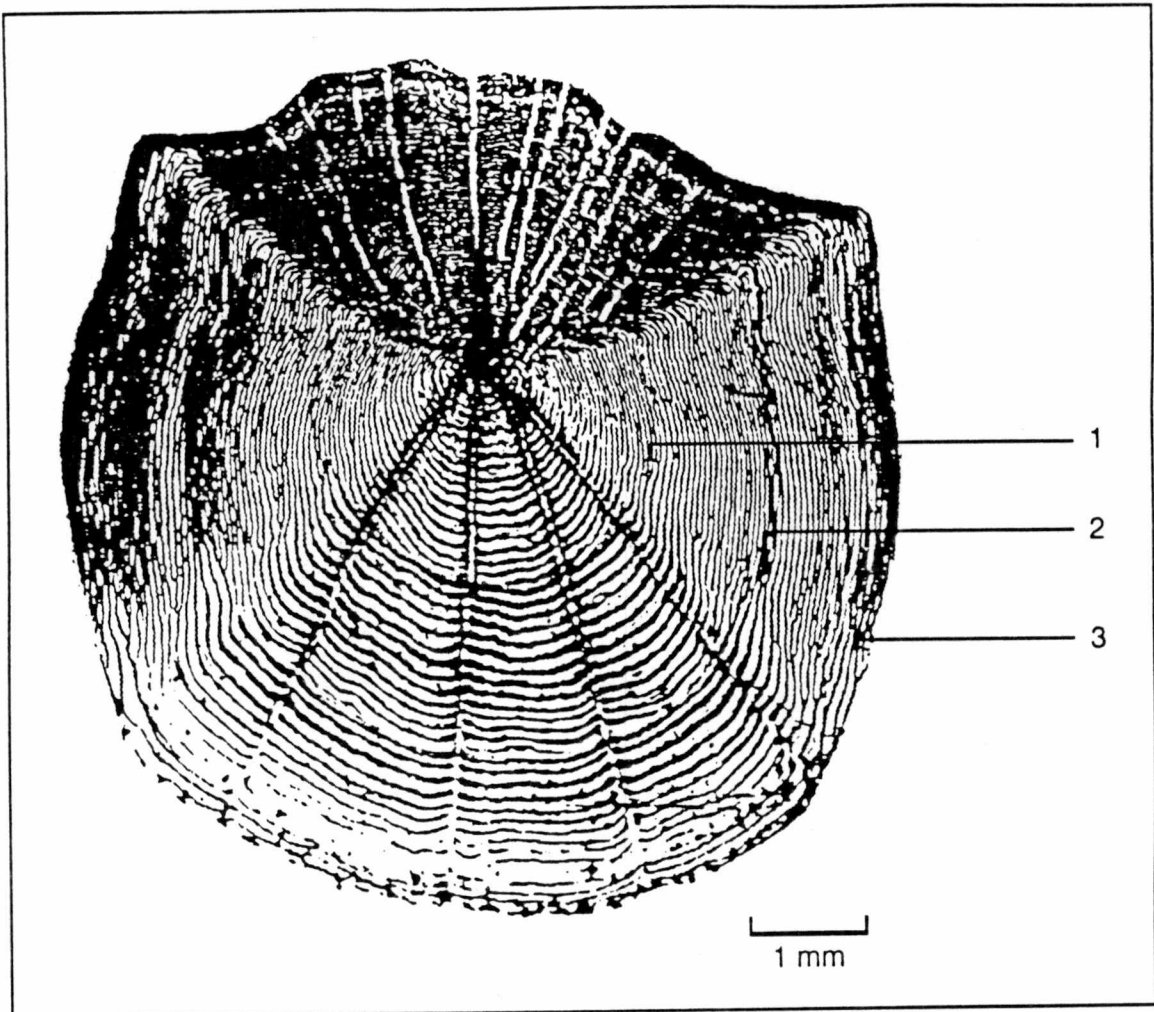


Figure 2: Fish scale showing growth rings and three annuli.

Source: Coutant, C.C., C.H. Chen, and J. Stewart, Jr. 1992. Salmon stock identification using laser microprobe chemical analysis. Seed Money Proposal.

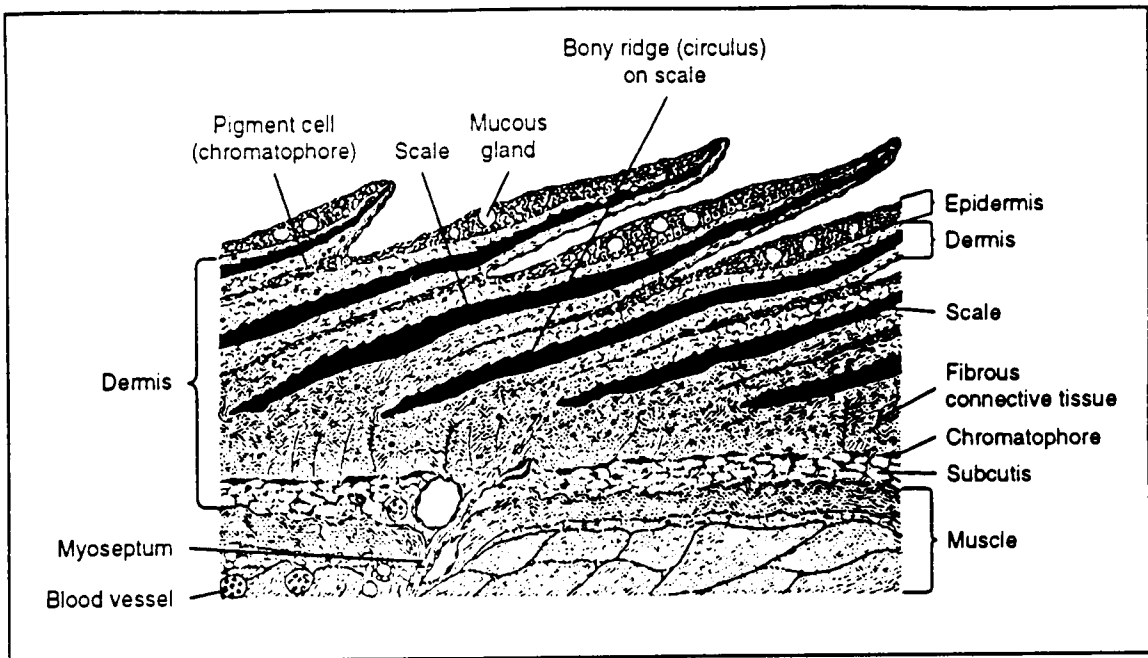


Figure 3: Fish scale implanted in the skin showing ridge and valley structure.

Source: Lagler, K.F., J.E. Bardach, R.R. Miller, and D.R.M. Passino (eds.). 1962. Skin, pp. 104-128. In: Ichthyology. John Wiley and Sons, New York.

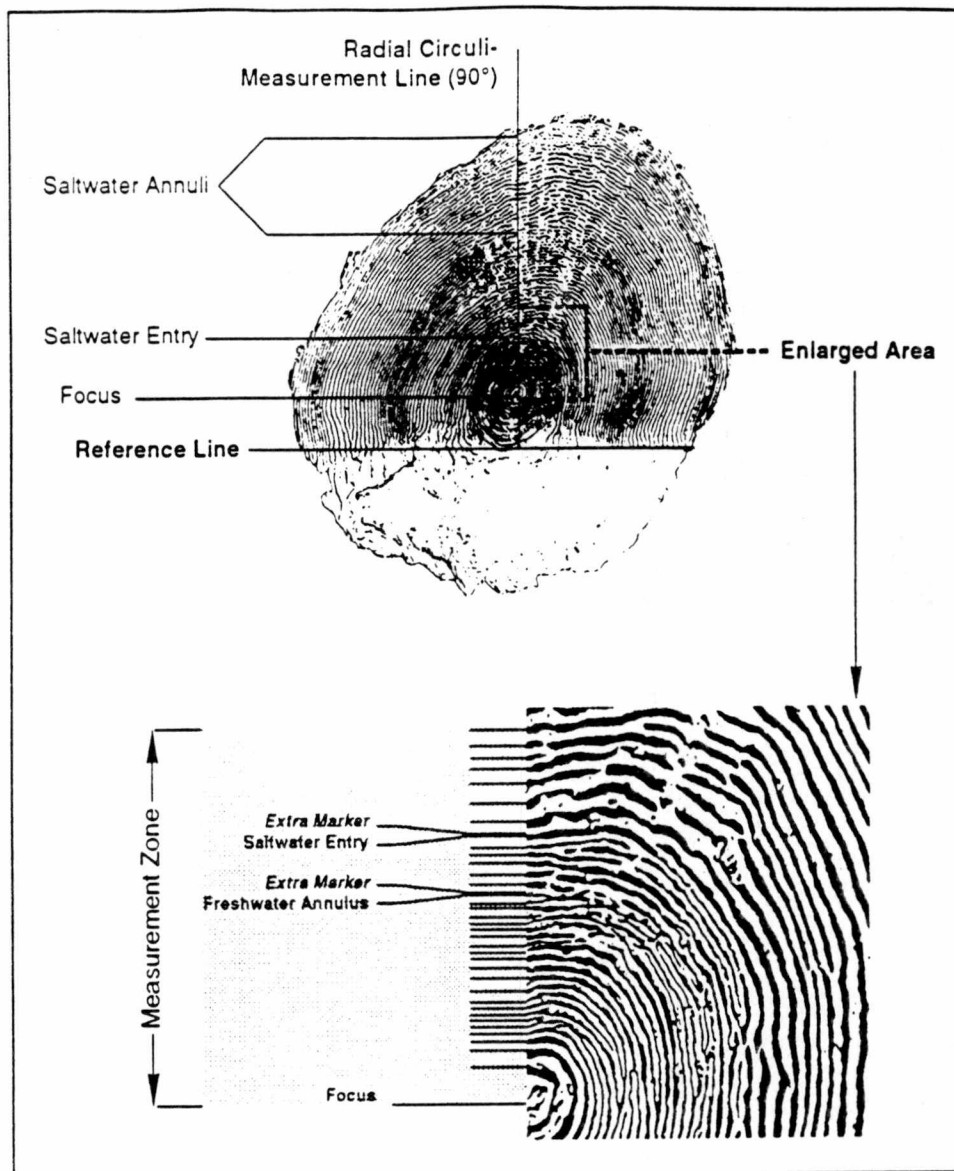


Figure 4: Two types of growth reflected in fish scales. Fresh water growth is represented by narrowly-spaced rings and saltwater growth is represented by widely-spaced rings.

Source: Fryer, J. and M. Schwartzberg. 1990. Experiments in identifying hatchery and naturally spawning stocks of Columbia Basin spring chinook salmon using scale pattern analysis. Tech. Rpt. 90-3. Columbia River Intertribal Fish Commission. 31pp.

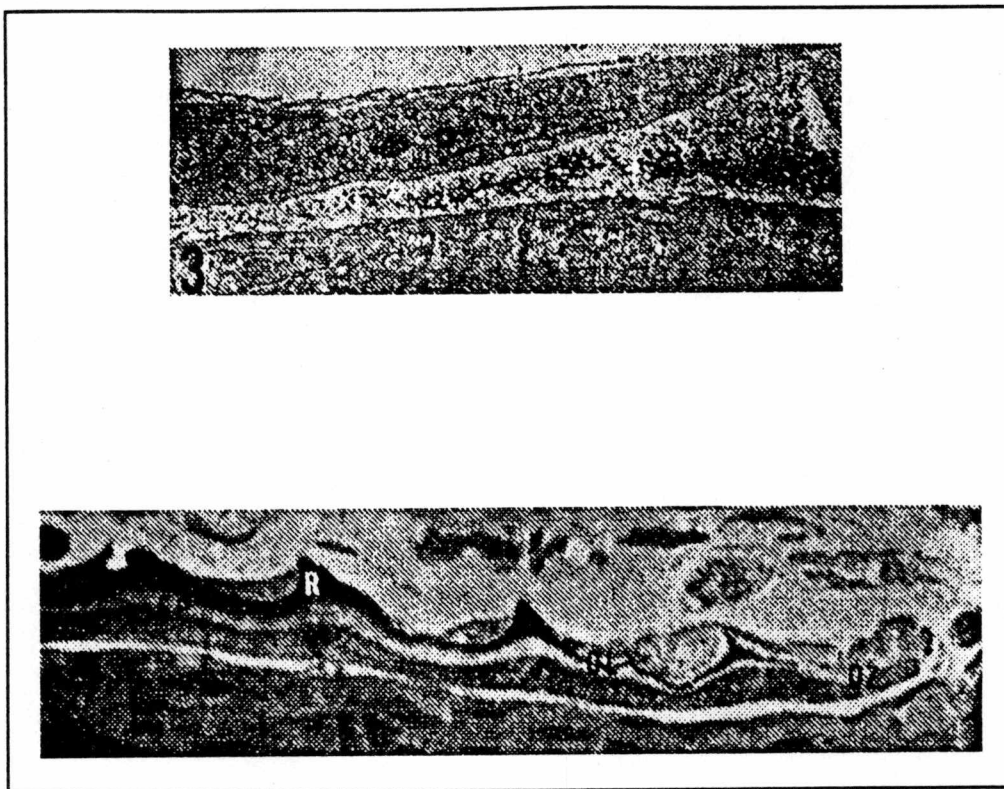


Figure 5: Three layers of fish scales. **R** represents a calcified ridge; **OL** represents pre-osseous zone; **OZ** represents fibrillary plate.

Source: Maekawa, K. and S.B. Yamada. 1972. Morphological identification and characterization of cells involved in the growth of the goldfish scale. Japanese J. Ichth. 19:1-10.

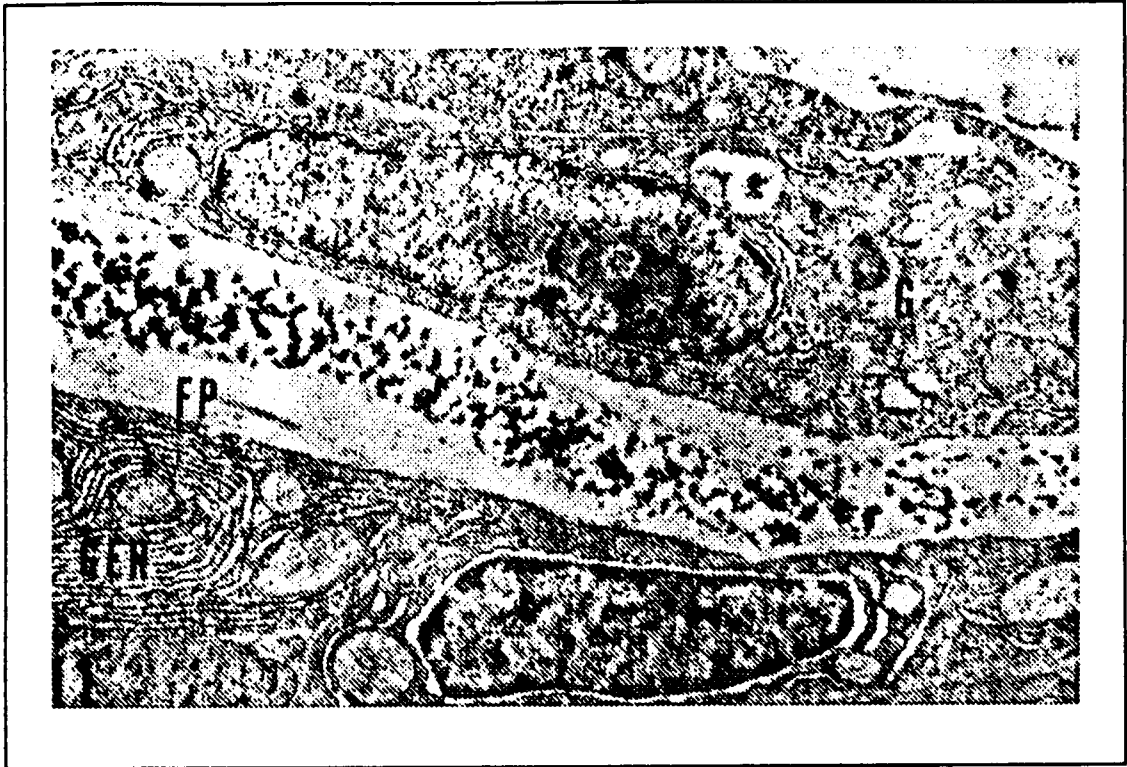


Figure 6: Calcification of the fish scale. **FP** represents non-calcified fibrillary plate. Above **FP** is the pre-osseous zone (speckled) and the calcified zone (white).

Source: Maekawa, K. and S.B. Yamada. 1972. Morphological identification and characterization of cells involved in the growth of the goldfish scale. Japanese J. Ichth. 19:1-10.

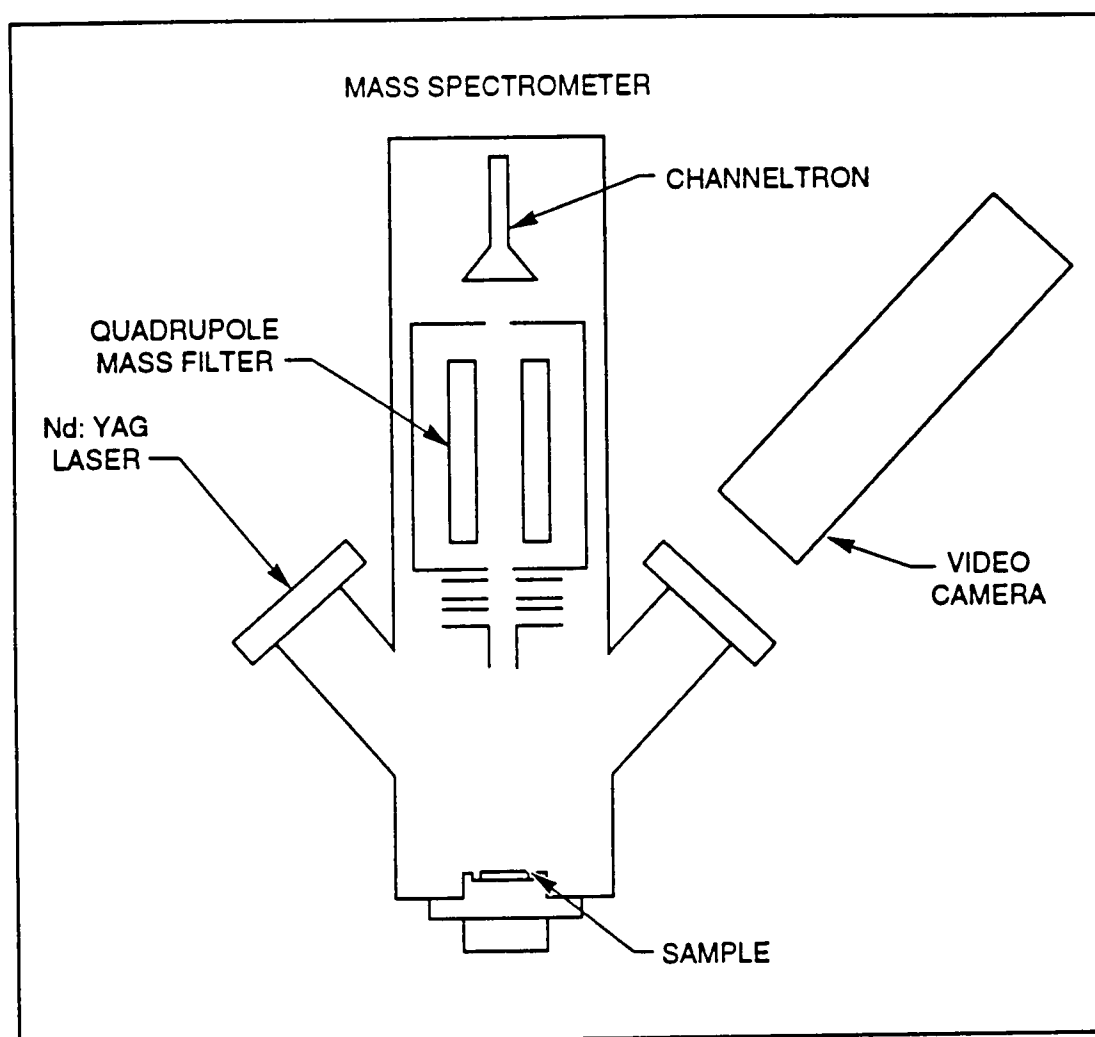


Figure 7: Nd:YAG laser, modified vacuum chamber and quadrupole mass spectrometer.

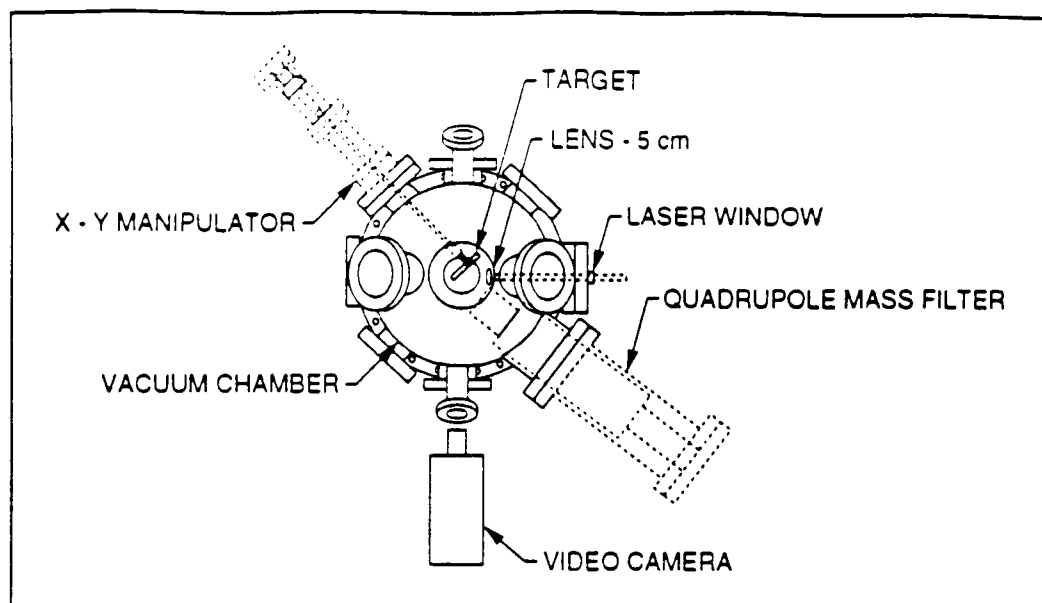


Figure 8: 13-inch diameter vacuum chamber, quadrupole mass spectrometer and 14-inch sample arm.

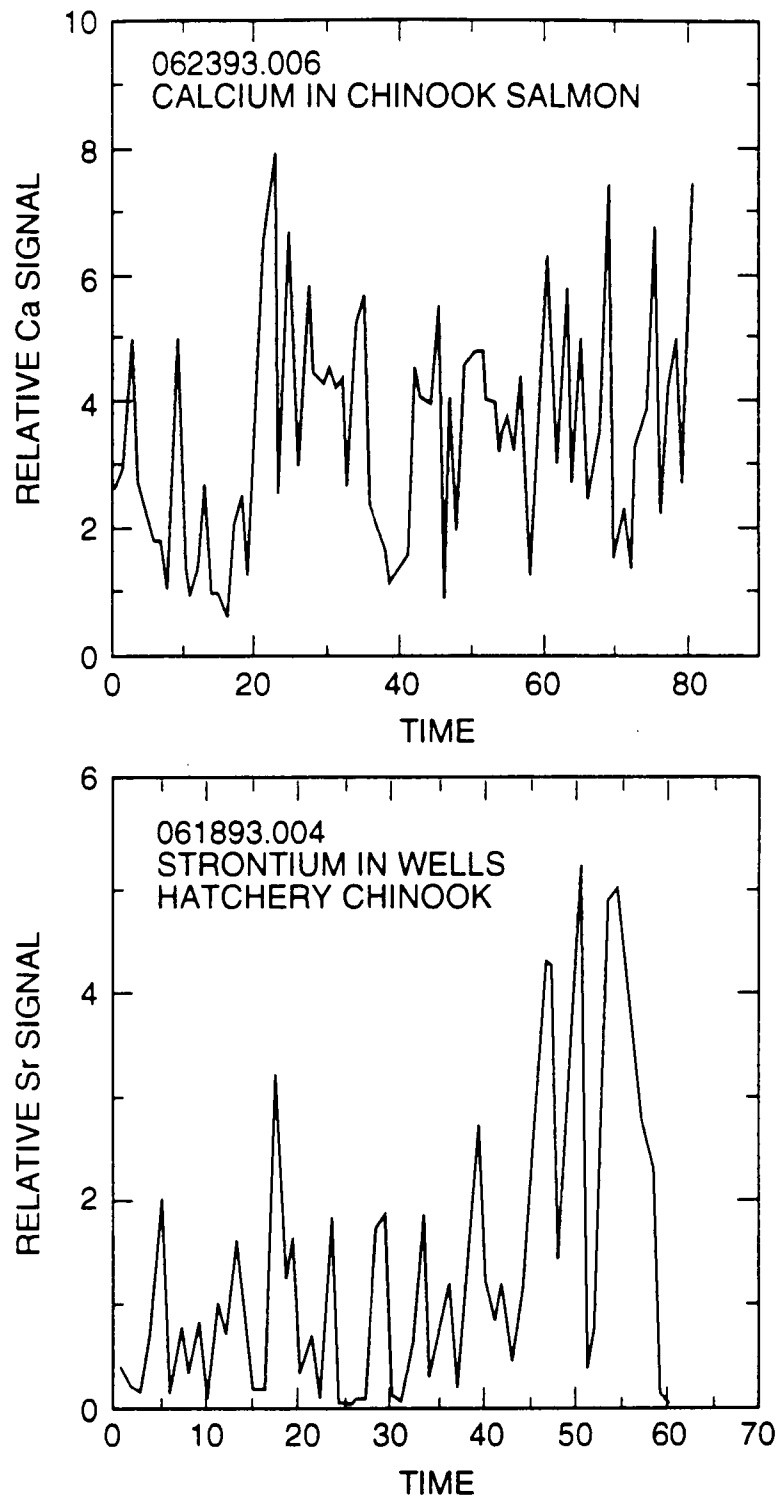


Figure 9: Highly variable Ca and Sr signals obtained with 13-inch diameter vacuum chamber, quadrupole mass spectrometer and 14-inch sample arm.

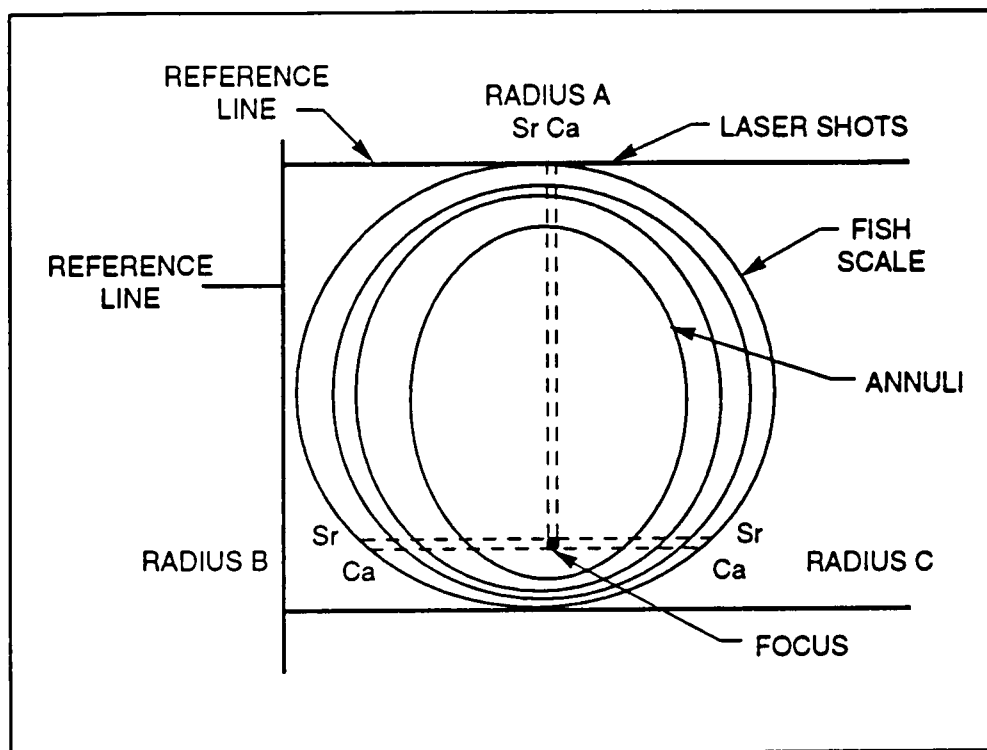


Figure 10: Diagram of laser paths across the fish scale radii. Diagram shows three sets of Sr and Ca data taken for each scale. Reference lines used with the video camera are also shown here.

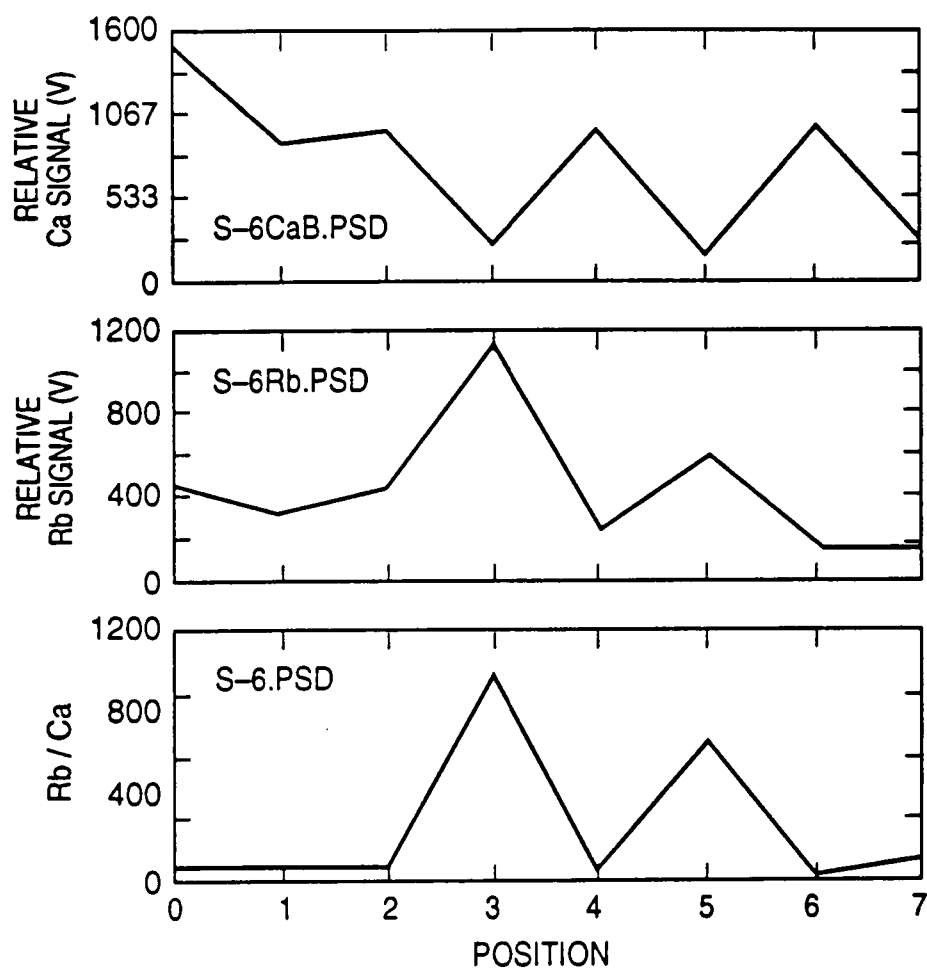


Figure 11: Ca, Rb, and Rb:Ca data variability shown in Pacific coho salmon scales.

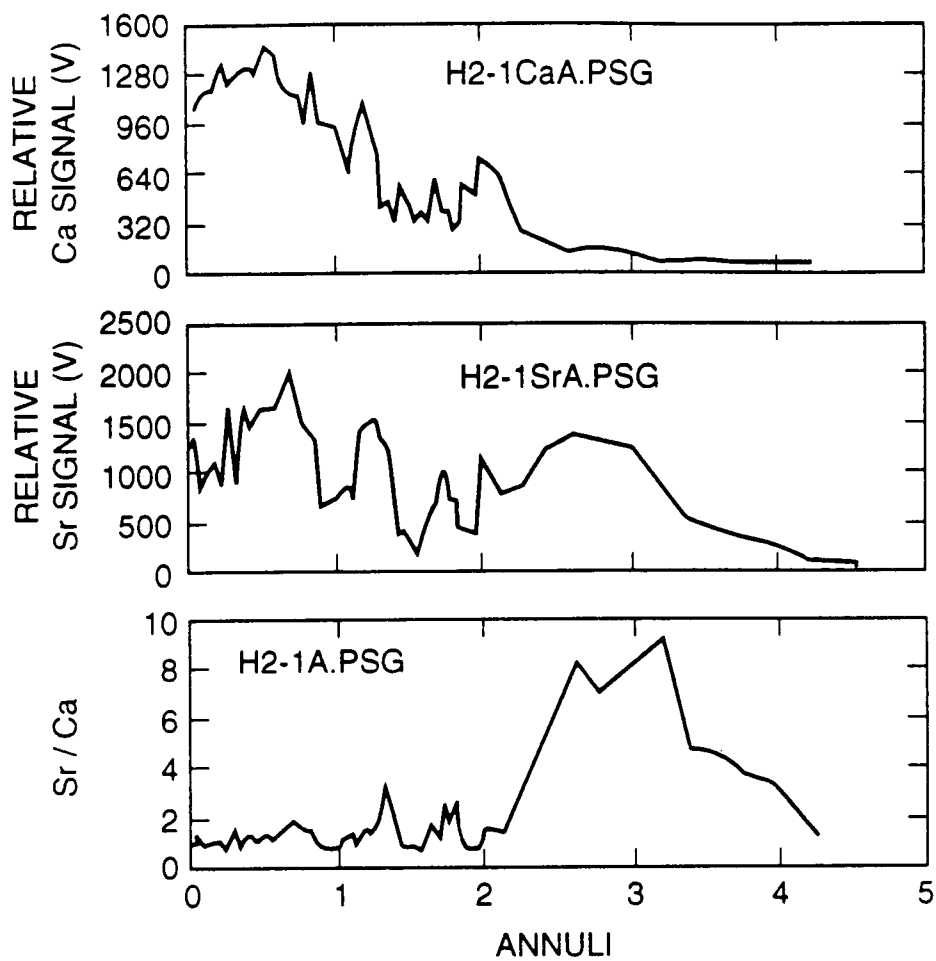


Figure 12: Ca, Sr, and Sr:Ca data variability shown in New York hatchery raised striped bass scales.

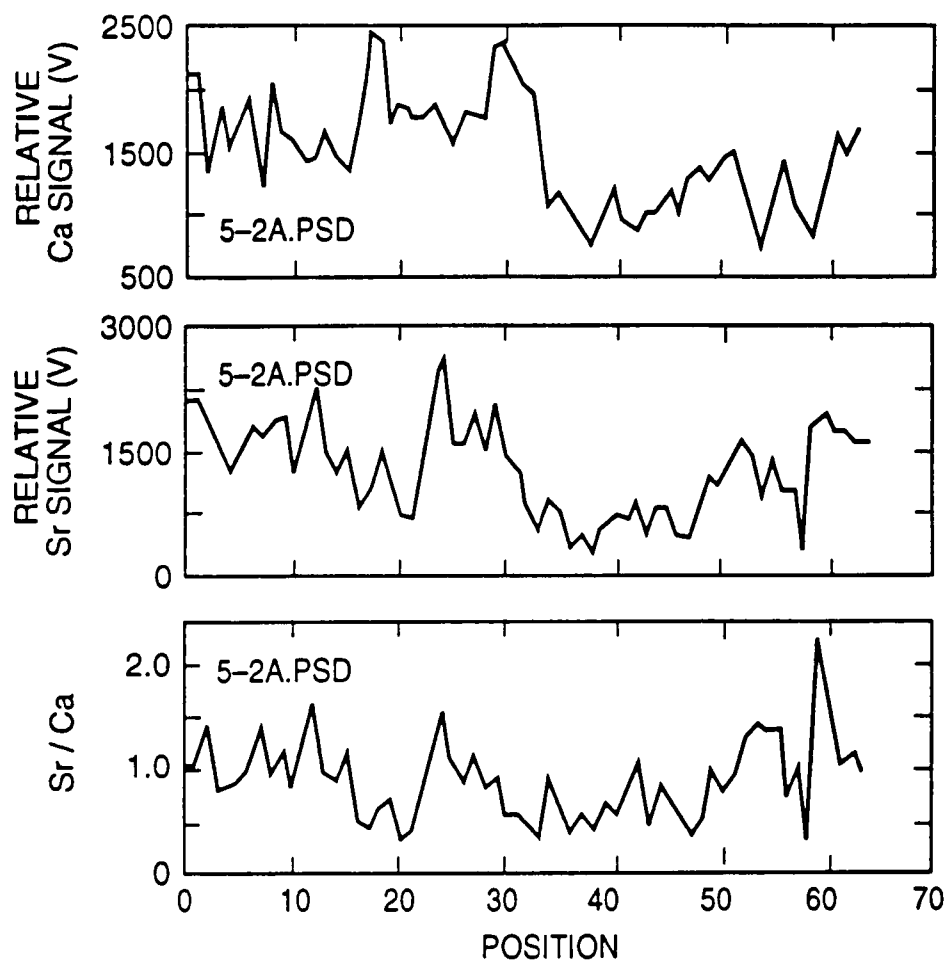


Figure 13: Ca, Sr, and Sr:Ca data variability shown in Roanoke River striped bass scales.

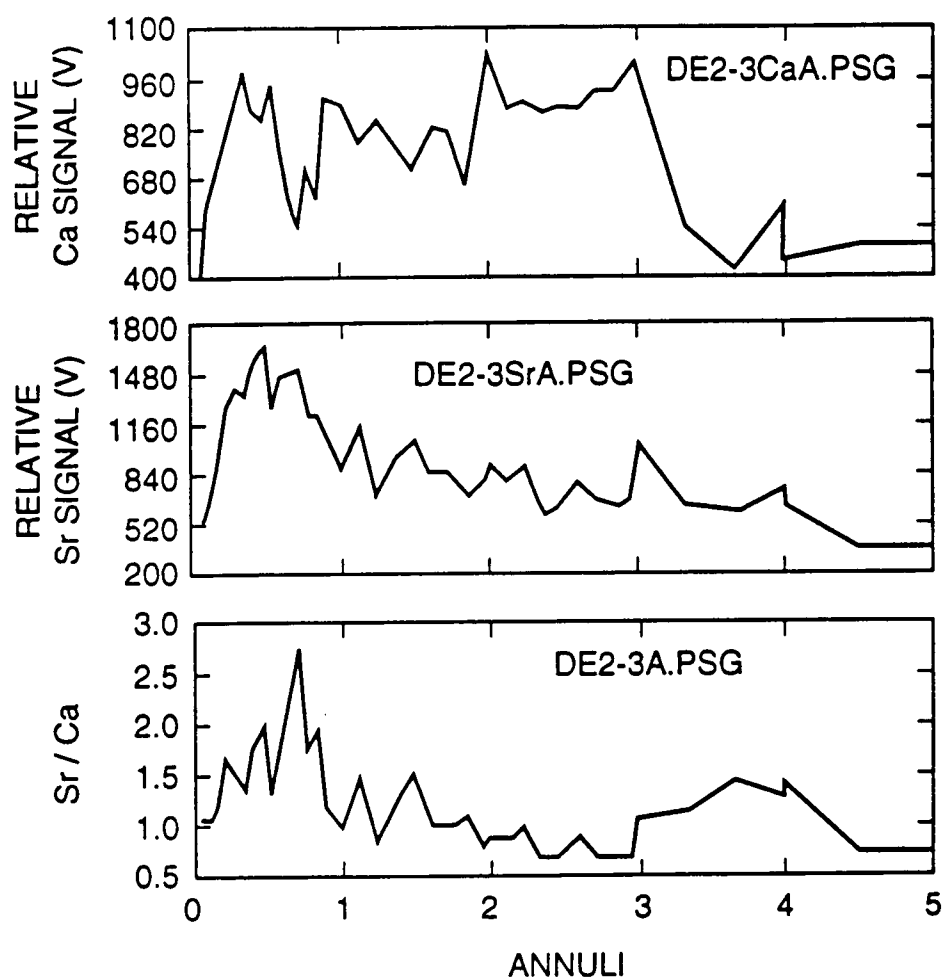


Figure 14: Ca, Sr, and Sr:Ca data variability shown in Delaware Bay striped bass scales.

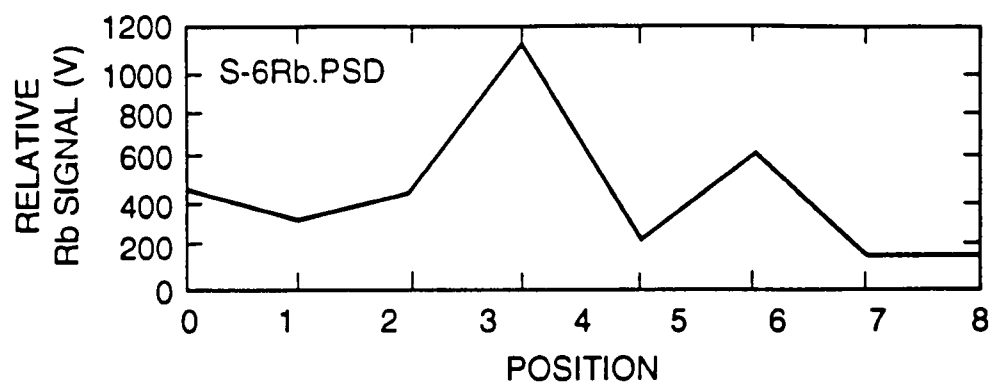


Figure 15: Rb data variability shown in Pacific coho salmon scales.

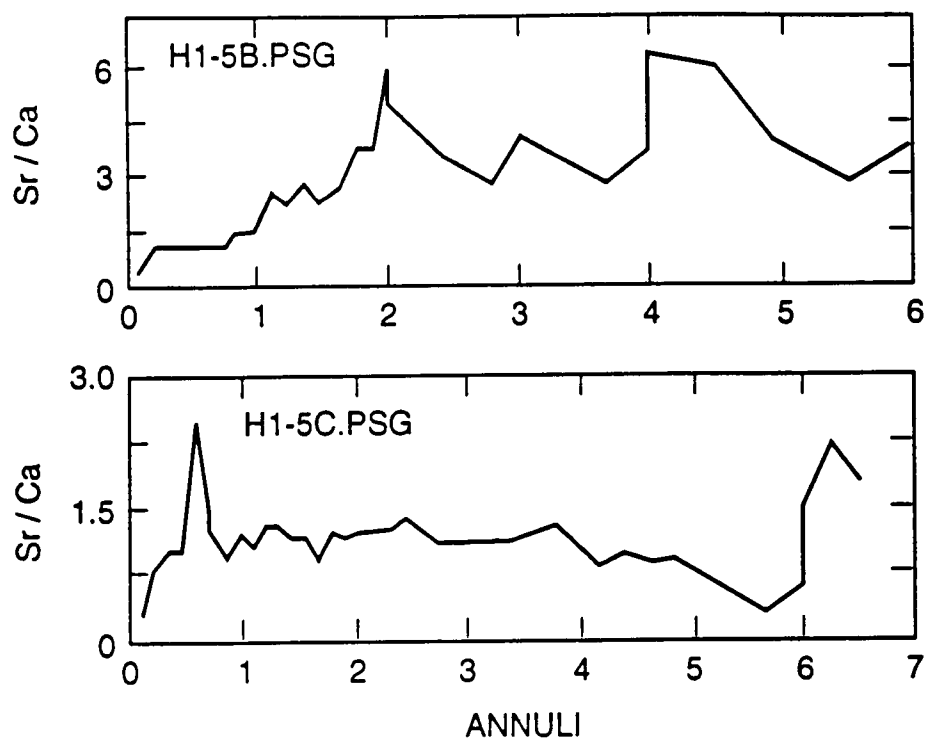


Figure 16: Non-repeatability of data shown in two radii from a New York hatchery raised striped bass scale.

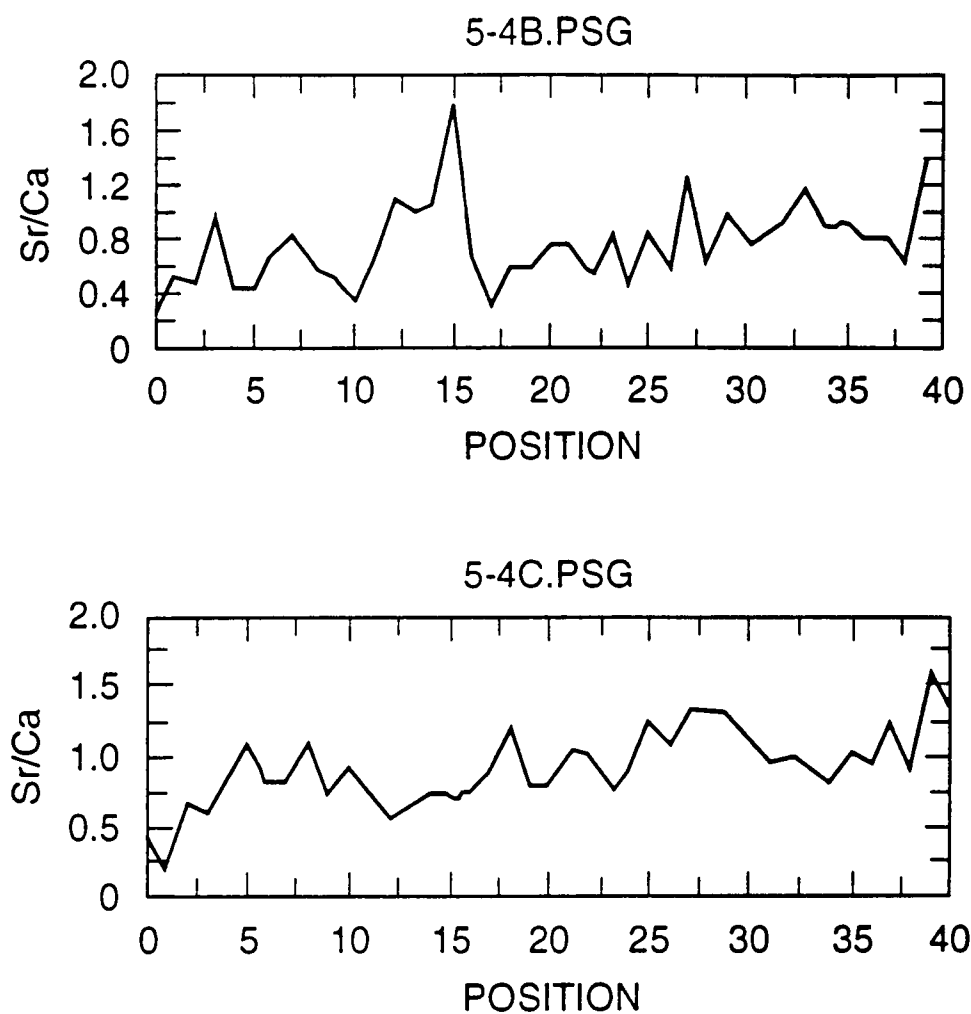


Figure 17: Non-repeatability of data shown in two radii from a Roanoke River striped bass scale.

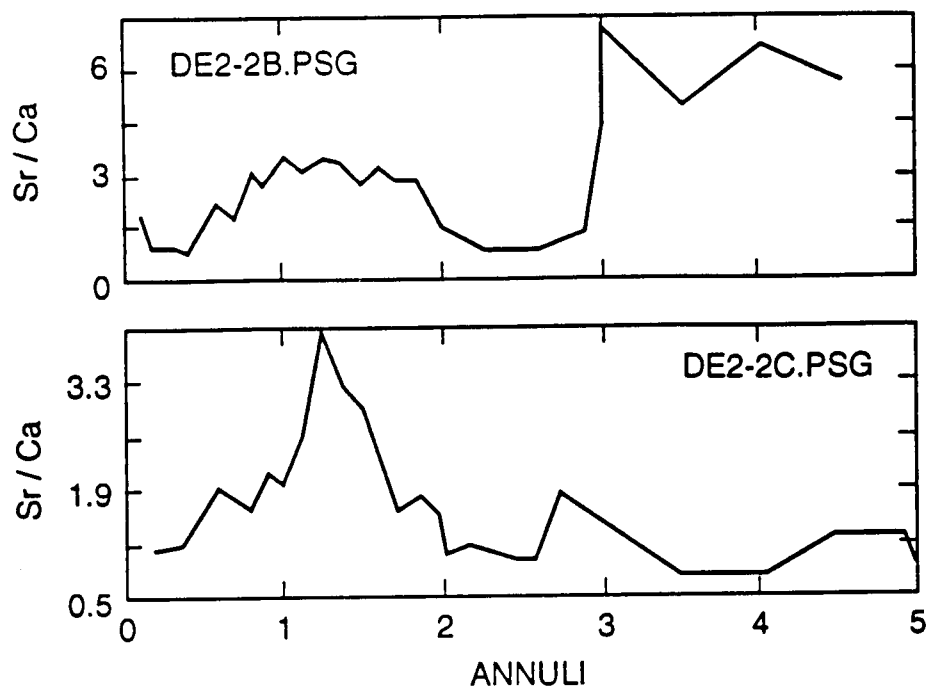


Figure 18: Non-repeatability of data shown in two radii from a Delaware Bay striped bass scale.

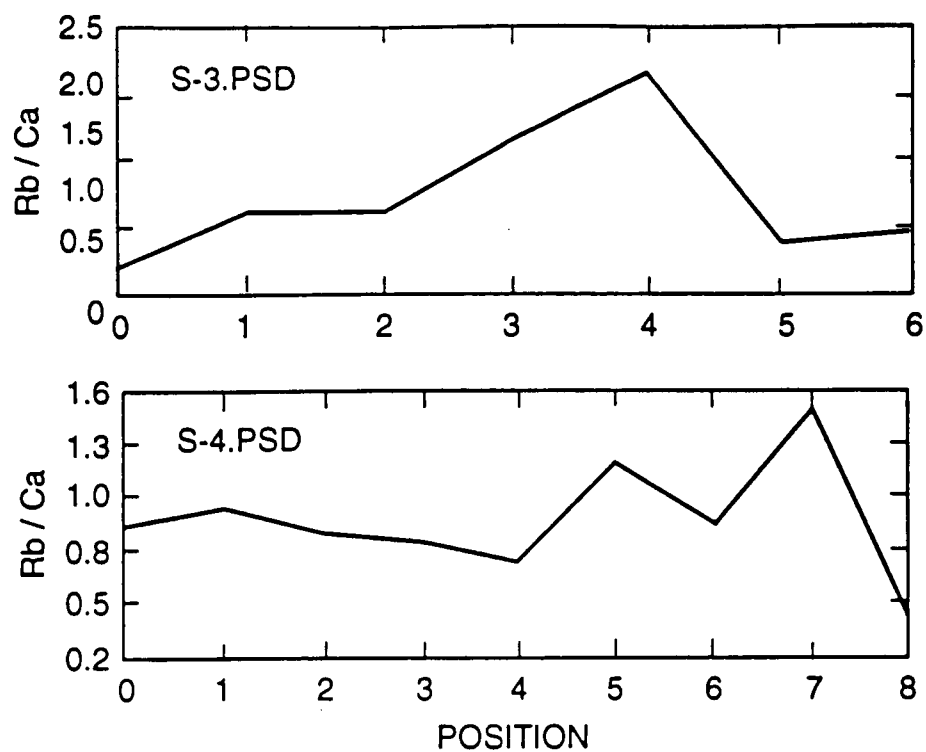


Figure 19: Non-repeatability of Sr data between two scales from the same Pacific coho salmon.

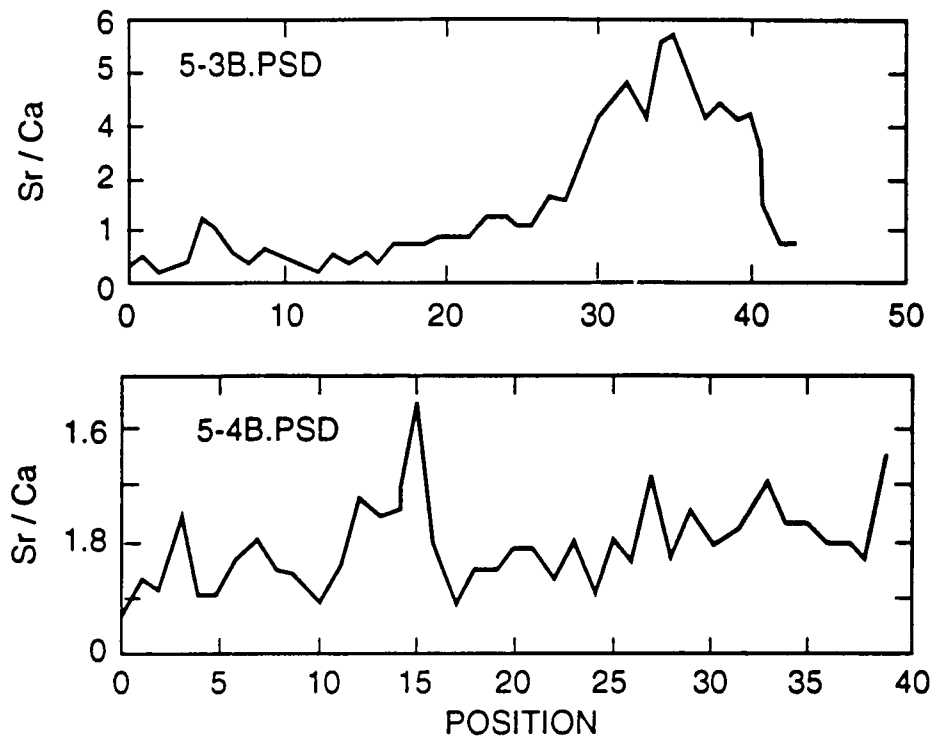


Figure 20: Non-repeatability of Sr data between two scales from the same Roanoke River striped bass.

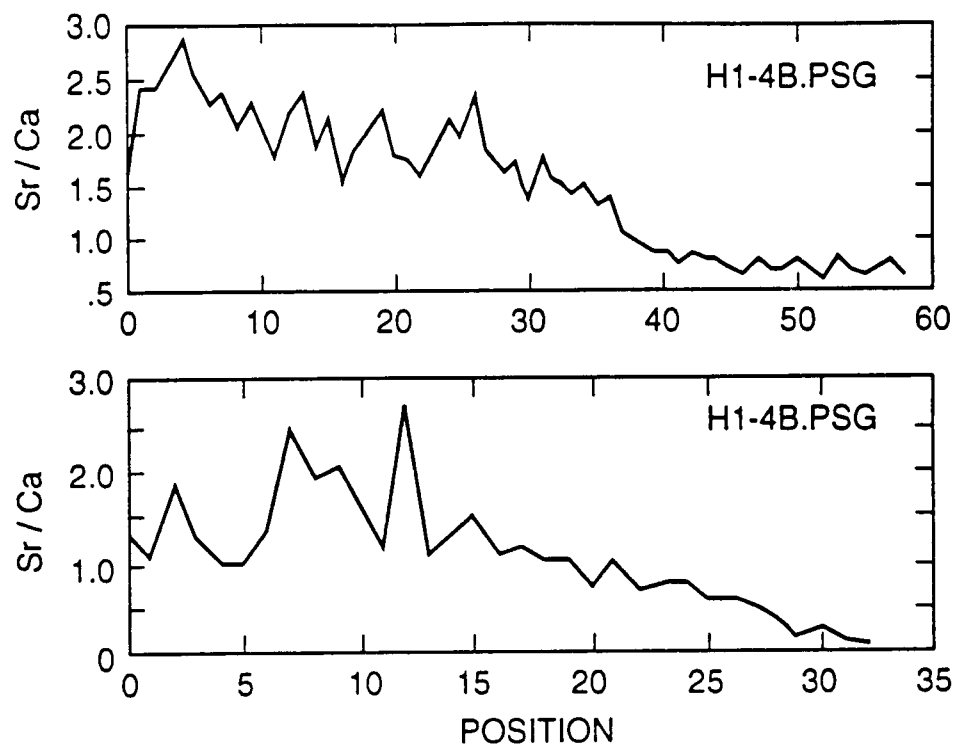


Figure 21: Non-repeatability of Sr data between two scales from the same New York hatchery raised striped bass.

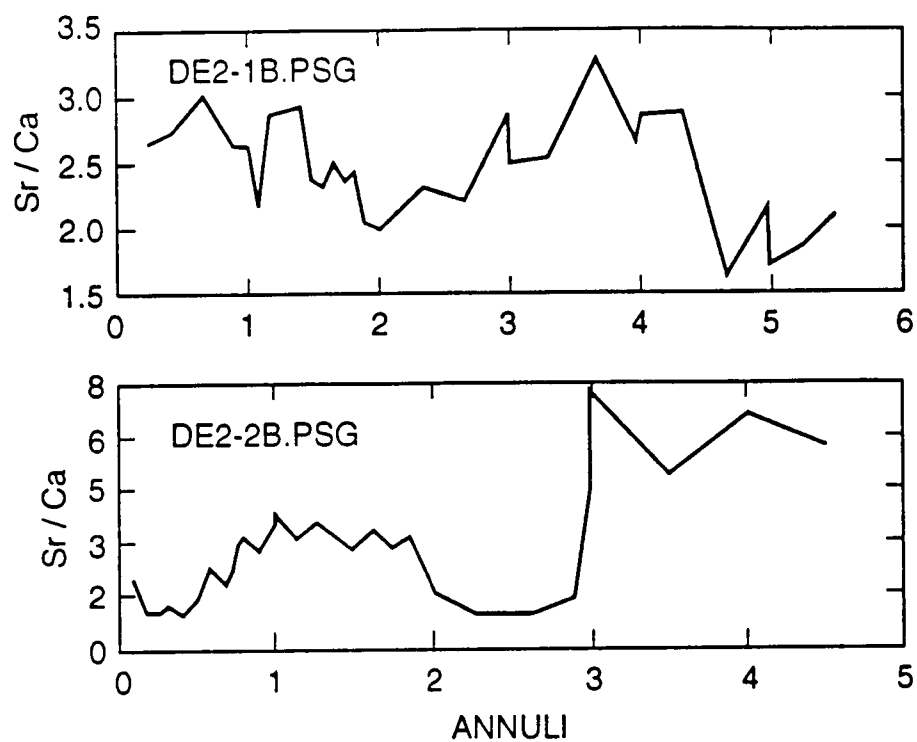


Figure 22: Non-repeatability of Sr data between two scales from the same Delaware Bay striped bass.

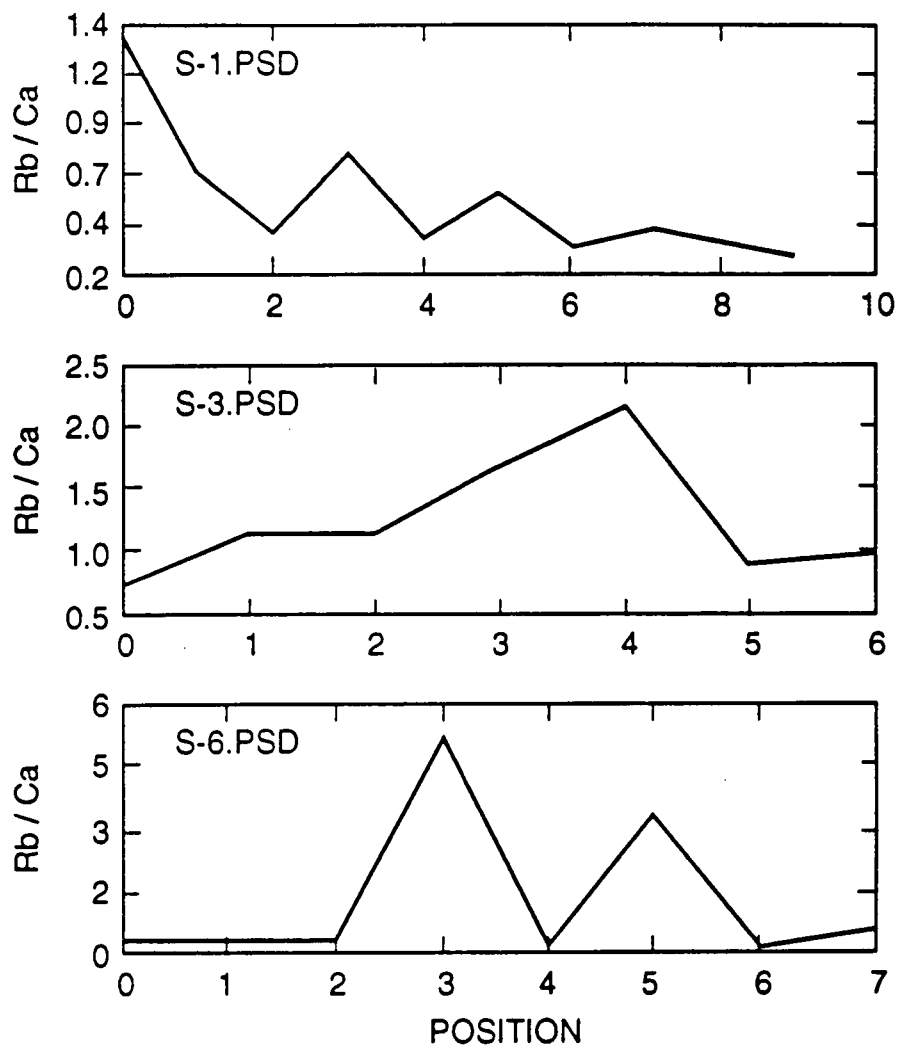


Figure 23: Rb peaks seen in Pacific coho salmon scales.

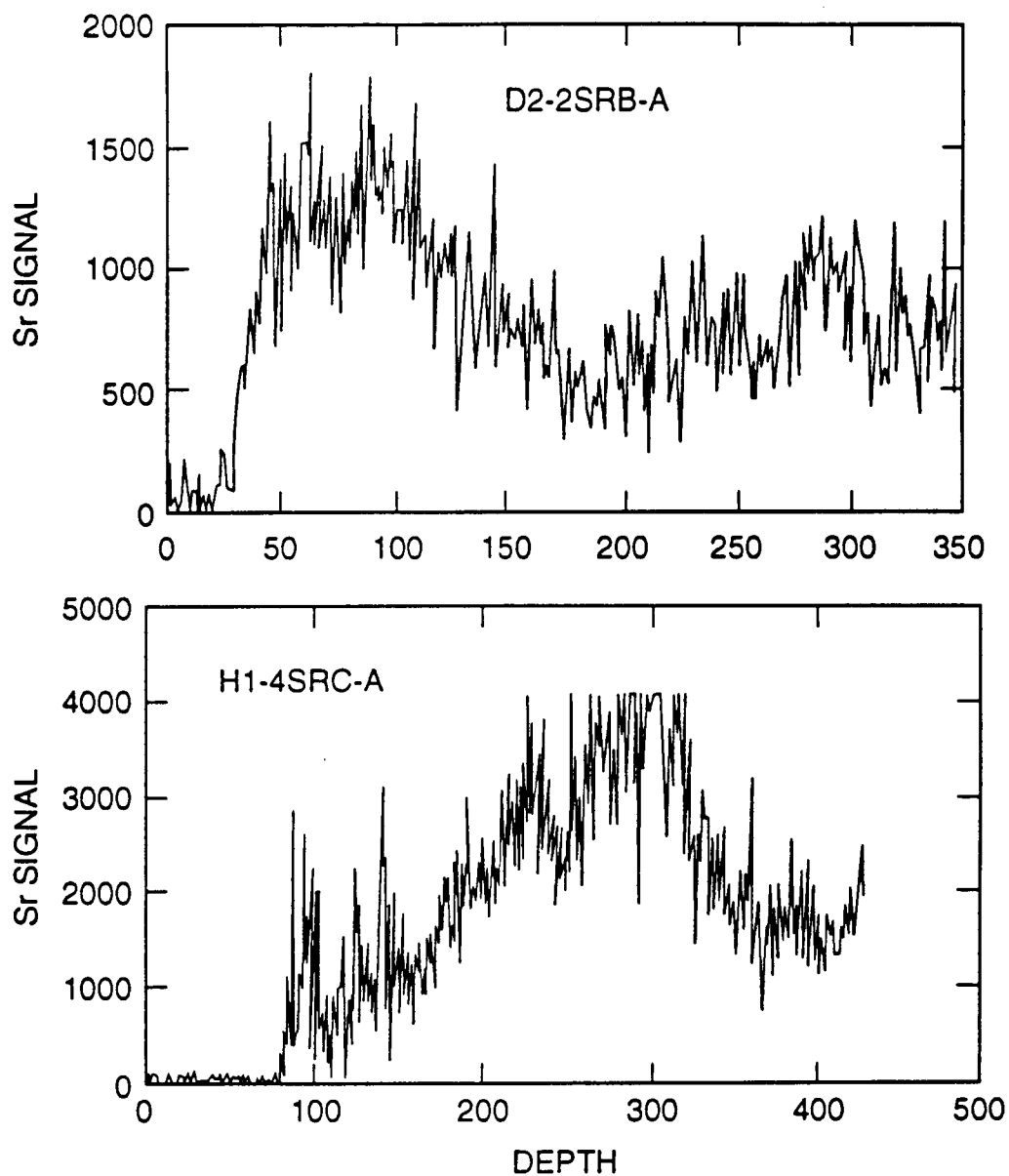


Figure 24: Sr data variability in vertical depth profiles from Delaware Bay striped bass and New York hatchery striped bass.

Table 1: Fish scale specimens used in laser ablation study.

Sample #	Species	Age	Sex	# Scales	Location
061893	Sockeye salmon			1	Pacific North West
062393	Sockeye salmon			1	Pacific North West
062493	Sockeye salmon			1	Pacific North West
4-2	Striped Bass	8	F	1	Roanoke River
5-1	Striped Bass	6	F	4	Roanoke River
5-2	Striped Bass	6	F		Roanoke River
5-3	Striped Bass	6	F		Roanoke River
5-4	Striped Bass	6	F		Roanoke River
H1-2	Striped Bass	6	F	4	New York Hatchery
H1-3	Striped Bass	6	F		New York Hatchery
H1-4	Striped Bass	6	F		New York Hatchery
H1-5	Striped Bass	6	F		New York Hatchery
H2-1	Striped Bass	4	F	1	New York Hatchery
DE2-1	Striped Bass	4	M	3	Delaware Bay
DE2-2	Striped Bass	4	M		Delaware Bay
DE2-3	Striped Bass	4	M		Delaware Bay
S-1	Coho Salmon			6	Pacific North West
S-2	Coho Salmon				Pacific North West
S-3	Coho Salmon				Pacific North West
S-4	Coho Salmon				Pacific North West
S-5	Coho Salmon				Pacific North West
S-6	Coho Salmon				Pacific North West

Vita

Sandra Lynn Davis was born in Hartford, Connecticut on December 18, 1966. She attended elementary schools in Columbus, Ohio and Oak Ridge, Tennessee. She graduated from Oak Ridge High School in June, 1985. Following high school, she attended undergraduate school at Auburn University, Alabama studying zoology and University of Tennessee, Knoxville studying animal science. She received a Bachelor of Science degree in Animal Science in December, 1989. She began graduate study in 1991, pursuing a Master of Science in Ecology through a research assistantship with the University of Tennessee and Oak Ridge National Laboratory. She graduated from University of Tennessee, Knoxville in August, 1994 with a Master of Science in Ecology.