

AN ABSTRACT OF THE THESIS OF

Gene E. Shippentower for the degree of Master of Science in Fisheries Science presented on October 5, 2007.

Title: Development of a Progeny Marker for Steelhead.

Abstract approved:

Carl Schreck

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This study was undertaken to determine if strontium chloride could be used to create a trans-generational otolith mark in steelhead (*Oncorhynchus mykiss*). I completed two strontium injection trials and a survey of juvenile steelhead from various steelhead hatcheries. The two trials measured Sr:Ca ratios in otoliths in response to injections and the survey measured the natural variation in Sr:Ca ratios in otoliths of juvenile hatchery steelhead in response to the natural variation. In 2003, adult female Willowa River, Oregon *O. mykiss*, were captured at the hatchery and evenly divided between a control group and two treatment groups. These females received an intraperitoneal injection of 1 cc/500 g of body weight of a physiologically isotonic solution (0.9% saline) containing concentrations of 0 (control), 1000, or 5000 parts per million (ppm) of strontium chloride hexahydrate ($\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$). Females were housed in a single outdoor tank until spawned artificially, and a distinct external tag identified each female within each treatment group. In 2004, female steelhead

were captured throughout the duration of the adult returns to the Umatilla River basin and injected with 0, 1000, 5000, or 20,000-ppm strontium. In both trials, progeny of fish treated with strontium had significantly higher Sr:Ca ratios in the primordial region of their otoliths as measured using an electron wavelength dispersive microprobe. There was no difference in fertilization rates of eggs and survival rates of fry among treatment groups. Progeny from treated mothers were on average larger than progeny of untreated mothers. The Sr:Ca ratios in otoliths collected from various populations of steelhead were greater than the control values measured in both injections studies. This study suggests that the marking technique works and the utility for such a technique could be used for empirical observations in determining the relative fitness of progeny of adult hatchery origin fish that spawn naturally. The variation in Sr:Ca ratios found among steelhead hatcheries suggests that care must be taken if the technique is employed where fish from more than one hatchery could potentially be involved.

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DEVELOPMENT OF A PROGENY
MARKER FOR STEELHEAD

by
Gene E. Shippentower

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Gene E. Shippentower, Author

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Introduction

Fish otoliths contain chemical patterns that represent records of the environment (Radtke 1989). Through the analysis of selected chemical elements, groups of fish can be distinguished (Campana et al. 2000). Recently, elemental analysis of calcified structures has been developed as a means to identify fish utilizing different environments (Elsdon and Gillanders 2002). Chemical marks occur because of changes in environmental chemical composition and differences in chemical utilization by the organism (Nielsen 1992). Different environments cause changes in elemental composition in otoliths in different parts of the otolith (Gallaher and Kingsford 1996; Wells 2000) because otoliths encapsulate growth patterns and key events in the life history of salmonids (Campana et al. 2000). The natural utilization of strontium into fish otoliths is of special interest because it potentially allows for the marking of small fish and determining occupancy of marine and freshwater environments (Kalish 1989).

Strontium occurs naturally in seawater (Horne 1969 as cited by Pollard et al. 1999), and freshwater (Riemen et al. 1994). However, the average concentration of strontium is greater in seawater (Kalish 1990). In a study to distinguish progeny of sympatric anadromous and non-anadromous salmonids, Kalish (1990) found progeny from sea-farmed brood stock had higher strontium: calcium (Sr:Ca) ratios than progeny of freshwater brood stock. Female fish that mature in the marine

environment absorb strontium and deposit the chemical into the developing ova. During early ontogeny, strontium becomes fixed into otoliths. Reiman et al. (1994) analyzed Sr:Ca ratios in two growth regions of otoliths sampled from juvenile sockeye salmon *Oncorhynchus nerka* for determining whether their parents were either a migratory (anadromous) or non-migratory life history type. The offspring of anadromous parents had elevated levels of strontium in the central primordia of the otolith, with reduced levels after the first annulus or the freshwater growth region. Offspring of freshwater parents showed no difference in strontium levels between the two growth regions. Zimmerman and Reeves (2000, 2002) had similar findings with otoliths sampled from juvenile rainbow trout *O. mykiss*. Juveniles from anadromous mothers had higher concentrations of strontium in the central primordium than juveniles from a non-anadromous mother. Strontium, as a natural tag, can therefore be used to identify an individual fish exposed to seawater across generations and can be detected in bony tissues (Schroeder et al 1995, 2001). The chemical and chronological properties of the otolith allow us to answer questions that are not possible using other calcified structures (Campana and Thorrold 2001). However, Guillou (1987) showed that opercula and vertebral bones from brook trout (*Salvelinus fontinalis*) reared in seawater had higher concentrations of strontium than bones from brook trout raised in freshwater. An assessment of otolith microchemistry has also been used for stock discrimination (Reiman et al. 1994) and determining movements between saltwater and freshwater environments (Elsdon and Gillander 2002).

Chemicals, including strontium, pass through three interfaces before being incorporated into the otolith; branchial uptake from the water, cellular transport within the circulatory system, and crystallization into a calcium carbonate matrix (Elsdon and Gillanders 2002). Otoliths may contain up to three forms of mineral carbonates: aragonite, vaterite, and calcite (Gauldie 1993) with aragonite allowing the incorporation of strontium (Howland et al. 2001). Vaterite is hypothesized as having a tighter crystal structure than aragonite, which may prevent the incorporation of elements such as strontium. Strontium is a divalent ion like calcium with similar ionic binding properties. Once incorporated into otoliths, strontium becomes metabolically inert (Yamada and Mulligan 1982), making it ideal for marking otoliths. Since the otolith is one of the first calcified structures to form, is present in salmon several weeks prior to hatch (Reiman et al. 1994), and exhibits daily growth, a change in habitat can cause a change in elemental composition (Stevenson and Campana 1992).

In addition to examining natural levels of strontium, researchers have conducted experiments to artificially elevate strontium levels in otoliths (Schroeder et al. 1995), statoliths (Hurley et al. 1985), scales (Ophel and Judd 1968; Snyder et al. 1992), vertebrae (Yamada and Mulligan 1982; Yamada et al. 1987, Schroeder 1995), and dorsal spines (Pollard 1999). Techniques used to elevate strontium levels include strontium-enriched diets (Yamada and Mulligan 1982; Snyder et al. 1992; and Pollard et al. 1999) and immersion baths (Yamada and Mulligan 1987; Schroeder et al. 1995).

Schroder et al. (2001) hypothesized that strontium in solution could be used to create a chemical mark if injected into females prior to spawning. A variety of marine fishes were tested, and otoliths from progeny of strontium-treated fish had greater concentration of strontium than progeny of non-treated fish. Injecting dissolved strontium into gravid females for marking otoliths of offspring suggests that a trans-generational mark is possible, although the current common techniques for applying a chemical mark in salmonids are still immersion baths and feeding strontium enriched diet.

Creating a strontium mark in bony tissue of salmonids by immersion baths or feeding is thought to be after the hatching stage of egg development and that any attempt to produce a strontium mark prior to hatching would be unsuccessful (Behrens-Yamada and Mulligan 1987). In this study, I evaluated whether injection of a strontium solution could artificially elevate the Sr:Ca ratios in the otoliths of *O. mykiss* progeny. In addition to investigating whether Sr:Ca ratios become elevated with treatment, I also assessed the effect of treatment on the survival and growth of progeny from treated females. Finally, I measured the natural Sr:Ca ratios in otoliths of juvenile *O. mykiss* collected from a number of Columbia basin and coastal hatchery populations to establish background information on Sr:Ca variation. This final step was used to determine if this technique can distinguish between the progeny of hatchery versus wild females that spawn naturally. Ultimately, such a mark would be useful for

assessing parentage of naturally-spawned juveniles for integrated hatchery programs where wild fish are collected for hatchery brood and hatchery fish are allowed to spawn in the wild. If a female hatchery fish received a strontium injection in order to create a trans-generational mark, and was subsequently released to spawn naturally, her offspring could be distinguished from the offspring of wild females by analyzing otolith microchemistry.

Methods

This investigation measured strontium and calcium in the sagittal otoliths, the largest of the three sets of otoliths found in salmonids. In 2003, 20 progeny were selected from each of 21 mothers. In 2004, 20 progeny were selected from each of 15 mothers. In addition to the progeny taken in the strontium injection trials, another 239 progeny were collected from various steelhead hatcheries for determining the natural variation of Sr:Ca ratios.

Experimental Design

Trial 1- Twenty-one female summer steelhead returning to Big Canyon Acclimation Facility (Wallowa River, OR) were captured and divided into three groups-a control group and two treatment groups of seven fish each (Figure 1).

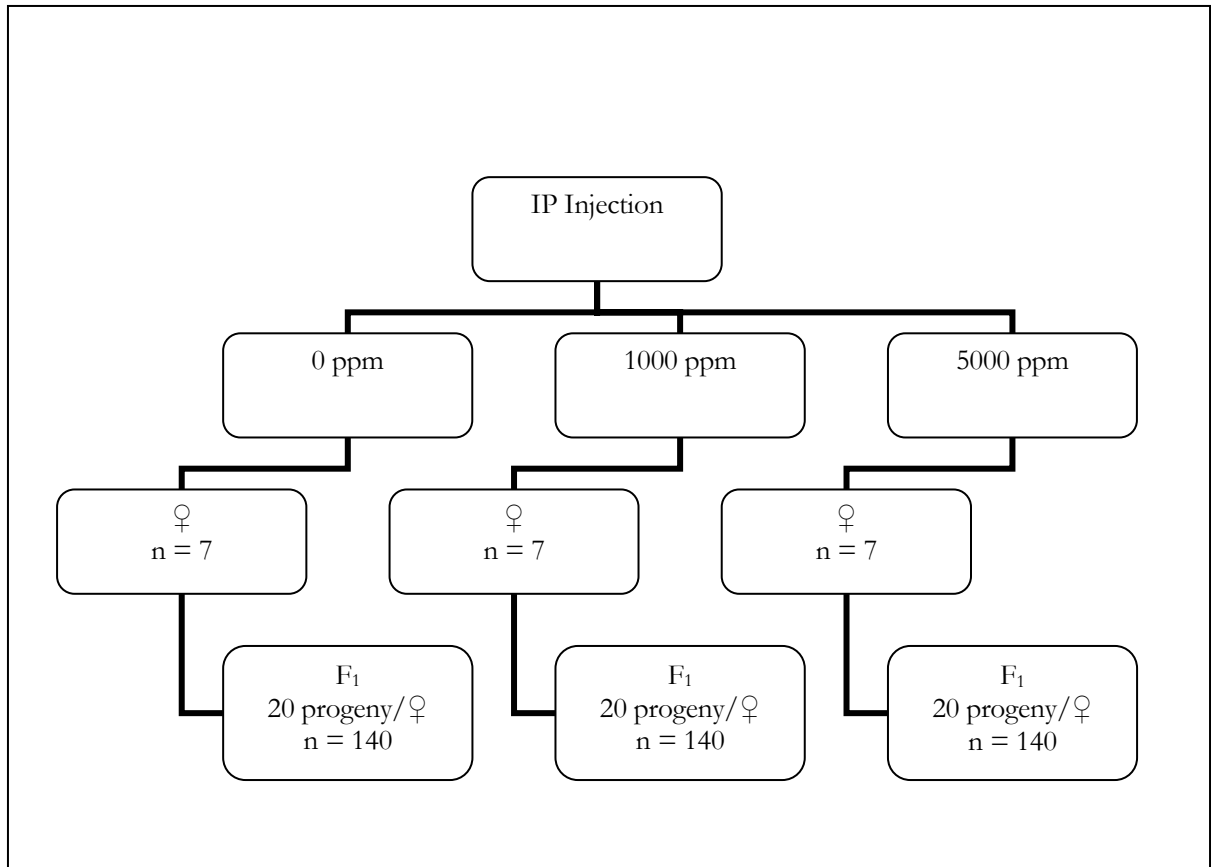


Figure 1. 2003 experimental design for testing the feasibility of creating a trans-generational chemical mark in steelhead. Female steelhead ($n = 21$) were divided evenly among three treatment groups. A sub-sample of twenty progeny from each female were killed for the collection of otoliths ($n = 420$). One otolith from each progeny was selected for five readings, data of which were pooled for calculating the mean Sr:Ca ratio. A fourth treatment of 20,000-ppm strontium was added during the second trial (2004).

Females ranged in weight from 1,670 g to 4,050 g. On March 27, 2003, females were anesthetized with tricaine methane sulfonate (MS-222 with a sodium bicarbonate

buffer), marked with an external anchor tag, and injected with 1cc/500 g of body weight of a physiologically isotonic solution (0.9% saline) containing concentrations of either 0 (control), 1000, or 5000 ppm of strontium chloride hexahydrate. Opercular punches served as secondary marks for identifying female fish to treatment group.

Spawning was not synchronous among the twenty-one mothers and thus female fish were examined once every seven days to determine whether they were ready for artificial spawning. Once a mother was determined to be ready, she was artificially spawned after being killed. Sperm from a minimum of three males was used to fertilize the eggs from each female, with some males used more than once. Eggs and milt collected from brood fish holding were transported to the Oregon Department of Fish and Wildlife's Willowa Hatchery in Enterprise, OR. Eggs were incubated in fiberglass Heath trays divided into thirds with vertical flow; this allowed eggs from three families to incubate simultaneously in a shared tray. Eggs separated according to treatment incubated for 31 days after fertilization.

I applied mechanical shocking for determining infertile eggs soon after they had reached the eyed stage, and 700 eggs per female were transported to Oregon State University's Fish Performance and Genetics Laboratory Corvallis OR for hatching (Table 1). Sixteen days after hatching, progeny from each mother were transferred from incubation trays to one of 21-0.65 meter diameter, 0.5-meter deep indoor

fiberglass circular tanks with continuous flow through water at 12 – 13 °C, with the offspring from each mother being assigned to an individual tank. Fingerlings were fed an Oregon[®] starter mash diet four to six times a day. Food particle size was increased as fish size increased. Mortality was recorded daily. After absorbance of the yolk sacs, a subsample of 20 swim-up fry from each mother was killed for the collection of otoliths, which occurred between August 29, 2003 and October 2, 2003.

Table 1. Steelhead spawning schedule and number of eggs collected from 21 females during the 2003 trial. Eggs were fertilized and then transported from Enterprise, Oregon to Corvallis, Oregon.

Egg Fertilization Date	Egg Transport Date	Number of females	Number of eggs
April 05	May 06	10	6,711
April 12	May 13	2	1,380
April 19	May 20	4	2,633
April 26	May 27	3	2,055
May 02	June 02	2	1,200

Trial 2- For the second trial I worked with steelhead returning to Three Mile Falls Dam located on the Umatilla River, OR. Forty-three hatchery steelhead (20 males and 23 females) were trapped and transported to Oregon State University's Fish Performance and Genetics Laboratory, Corvallis, OR. Females ranged from 1,460 g

to 2,180 g in weight. Females were grouped according to whether they were early (October 1- November 30, 2003) middle (December 1, 2003 – January 31, 2004) or late (February 1 – April 30, 2004) returning fish (Figure 2). Females were anesthetized with MS-222 as described above, marked with a passive integrated transponder tag, and injected at 1cc/500 g of body weight of a solution containing concentrations of either 0 (control), 1000, 5000, or 20,000 ppm of strontium chloride hexahydrate. Females were killed and artificially spawned with one female mated to one male.

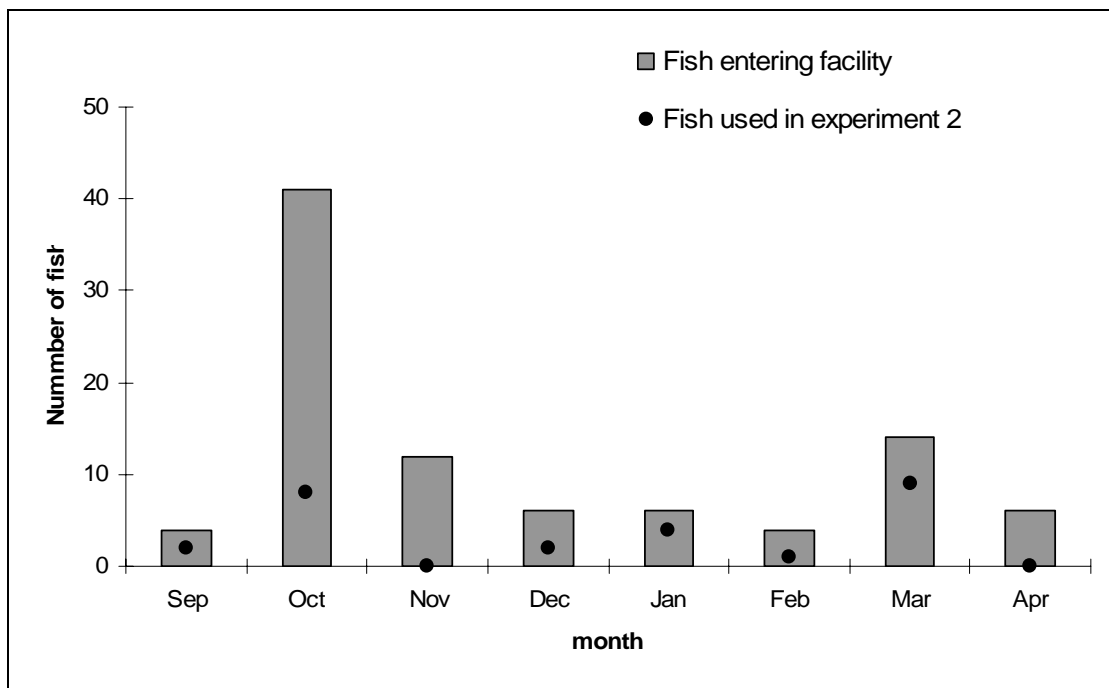


Figure 2. Number of hatchery steelhead returning to Three Mile Falls Dam Umatilla River, OR in late 2003 and early 2004. Columns indicate the total number of hatchery females entering the trap and dots indicate the number of steelhead used in the second trial.

The Umatilla stock used in this trial required transportation, and there was some mortality associated with hauling stress and copepod infestation. Thus, in the second trial I collected progeny from 15 families, which incubated in heath trays and were then transferred into 0.65 m diameter, 0.5-meter deep fiberglass circular tanks with continuous flow through water at 12 – 13 °C as in the first trial. Fish were fed Oregon[®] bio-moist pellets until satiated twice a day to preclude food from becoming a limiting factor for growth. For progeny of each mother, growth rates were measured periodically beginning when the fish were stocked into circular tanks (day 16 post hatch), size at culling to 400 fish (day 31 post hatch), and at 60, 74, and 88 d post hatch. Batch weights were measured at day 16 and 31 post hatch. The total number of surviving fish in each family was calculated on day 16-post hatch. For determining number of fry per female, I weighed three samples of progeny from each mother and counted the number of individuals per sample. Three averages were calculated and pooled for determining an overall average weight. For the day 31 measurement, I counted 400 progeny (or all fish present) from a circular rearing tank into a tare-weighted container. I batch weighed and calculated the average weight. The subsequent estimates of growth were based on pooled growth rates calculated by weighing 30 individual progeny from each family at day 60, 74, and 88. An absolute growth formula $(Y_2 - Y_1) / (t_2 - t_1)$ was used for calculating growth rate between each time interval (Busacker et al., 1990).

Hatchery sampling

In order to understand the natural variability of strontium in otoliths, I collected juvenile steelhead from steelhead hatcheries throughout Oregon and Idaho (Table 2).

Sagitta otoliths were extracted from these fish to measure Sr:Ca ratios.

Table 2. Steelhead hatcheries in Oregon and Idaho where 20-100 juvenile steelhead were collected from each hatchery in order to determine natural between-stock variation in otolith Sr:Ca ratios in 2004. Five different populations were sampled from Magic Valley hatchery of Idaho.

Hatchery	Region	Location	n	Date
Rock Creek	Southwest	Idleld Park, OR	40	July 12
Bandon	Southwest	Bandon, OR	20	July 13
Elk River	Southwest	Port Orford, OR	20	July 13
Cole Rivers	Southwest	Trail, OR	20	July 14
Round Butte	High Desert	Madras, OR	20	July 14
Oak Springs	High Desert	Maupin, OR	20	July 15
Magic Valley	Magic Valley	Twin Falls, ID	100	July 28

Sampling of progeny and analyses

At otolith sampling, fish were measured (total and fork length) and weighed. Pairs of sagittal otoliths were removed from each fish and stored dry (Stevenson and Campana 1992). One otolith from each fish was used for elemental analysis with the other kept in reserve. Otoliths were mounted with Crystal Bond® thermoplastic glue, sulcus side down, onto glass slides and ground in the sagittal plane to the primordium with sand paper, starting with 1200 grit and ending with 2000 grit until the central primordium and surface scratches appeared to be on the same focal plane when viewed using a compound microscope (Figure 3). Otoliths were then polished with 0.05 μm alumina paste and rinsed with distilled water. Multiple finished otoliths were mounted to glass slides and carbon coated prior to microprobe analysis.

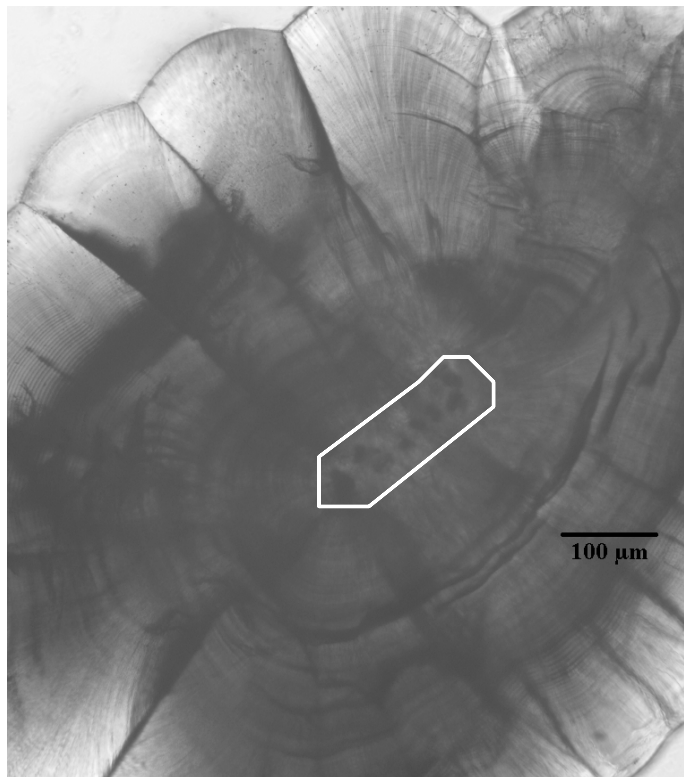


Figure 3. An example of a finished otolith ground and polished to the central primordium. Five sample sites measured within the white outlined primordial region were used to determine average Sr:Ca ratio for each otolith. The area marked is the initial site of calcium carbonate deposition.

Electron probe microanalysis is a quantitative analysis that enables identification of elements and their concentrations (Potts et al.1995). Electrons are emitted from an electron gun and are accelerated or energized onto a solid (Jones 1992). The electrons strike the solid and emit X-rays (Reed 1975; Mulvey 1983; Jones 1993) and electrons (Jones 1992). From X-ray emissions, elements present can be assessed qualitatively and quantitatively by characterizing wavelength and intensity of X-ray lines. X-rays are detected and characterized by wavelength dispersive spectrometry (Jones 1992).

Elemental analysis was conducted using a Cameca SX-100 wavelength dispersive microprobe (Cameca, Paris, France).

The Oregon State University Electron Microprobe Laboratory maintains a Cameca SX-100 Electron Microprobe (Cameca, Paris, France; Munich, Germany) equipped with 5 wavelength dispersive spectrometers (WDS) and one energy dispersive spectrometer (EDS) with a thin window for light element detection. The probe has wavelength dispersive crystals for high-sensitivity trace element analysis and a high precision sample stage with 0.1 μm stepping capability. The SX-100 is capable of quantitative analyses for elements with an atomic weight (Z) from 4 (Be) to 92 (U) uranium and has the capability for high-resolution elemental mapping and trace element analysis at low concentrations (generally to 100's of ppm for $Z > 6$). The electron probe has a high speed back scattered electron (BSE) imaging system, transmitted and reflected light imaging, and automated calibration and analysis.

Elemental analysis was conducted following the methods outlined by Toole and Nielsen (1992) and Zimmerman and Nielsen (2003) using strontiantite (SrCO_3 -USNM R10065), and calcite (CaCO_3 -USNM 136321) as Sr and Ca standards, respectively. A 15-kV, 50-nA, 10-40 μm diameter electron beam was used for the analysis of the central primordium. Sixty second counting times were used for each element with counts for all elements taken simultaneously. Strontium was measured using the

thallium acid phthalate diffracting crystal and calcium was measured with the pentaerythritol (PET) diffracting crystal within the central primordium region only. Large TAP and PET crystals were used for these analyses due to the higher signal generated by the greater crystal surface area, and resultant greater analytical sensitivity than regular crystals. Data output from the electron microprobe was considered acceptable if the total weight percents calculated for oxygen, carbon, calcium, and strontium totaled $100 \pm 4\%$. Any analyses outside these criteria were excluded from the analysis. It was assumed that otoliths are predominantly calcite and the percent of the otolith made up of carbon was set to a constant according to the atomic weight of carbon ($Z = 12.02$). The atomic weight of oxygen was calculated by applying stoichiometry, a mathematical treatment for determining quantitative relationship among the four elements that comprise an otolith.

Statistical analysis

To test for a treatment effect of strontium injection on the Sr:Ca ratio in the progeny, the experimental design was analyzed using one-way analysis of variance (ANOVA) with sub sampling. The maternal parent received the treatment with observations measured as Sr:Ca ratios in nucleus of otoliths collected from the progeny. ANOVA was used to test for differences between group means (Keuhl, 2000; Ramsey and Schafer, 2002). If significance was detected Tukey's multiple comparisons procedure

was used for investigating which treatment groups differed from others. Diagnostic plots, (Insightful S-Plus, 2005) were used for determining outliers and normal distribution of the data.

Results

Trial 1- There was no difference in average weight of adults between treatment groups ($F_{2,18} = 0.09$, $P = 0.92$). There were no adult mortalities in the time between injections and spawning. Approximately 50 % of the females spawned within 9 d after injection (Table 3).

Fertilization rates to the eyed stage of development ranged from 53% to 100% with no difference in mean survival between treatment groups ($F_{2,18} = 0.11$, $P = 0.90$).

However, there was one family in each of the 0, 1000, and 5000 ppm treatment groups where dead eggs outnumbered the live eggs and required mechanical removal prior to sampling. In addition, within the 5000 ppm treatment group one female produced only a small number of eggs because she was not sufficiently ripe. The removal of these families from the dataset changed the survival rate to the eyed stage and ranged from 85% to 100% with no difference in mean survival among treatment groups ($F_{2,14} = 1.15$, $P = 0.35$). Daily mortality of fingerlings was sporadic with very few fish dying

among treatment groups. Otoliths were collected from fish of various sizes for each treatment group (Figure 4).

Table 3. Spawning schedule and number of female Wallowa steelhead spawned after injection treatments with concentrations of 0, 1000, and 5000-ppm strontium.

Egg Fertilization	Days after injection	Treatment		
		0 ppm	1000 ppm	5000 ppm
April 05	9	4	2	4
April 12	16	1	0	1
April 19	23	1	2	1
April 26	30	1	2	0
May 02	36	0	1	1
Total		7	7	7

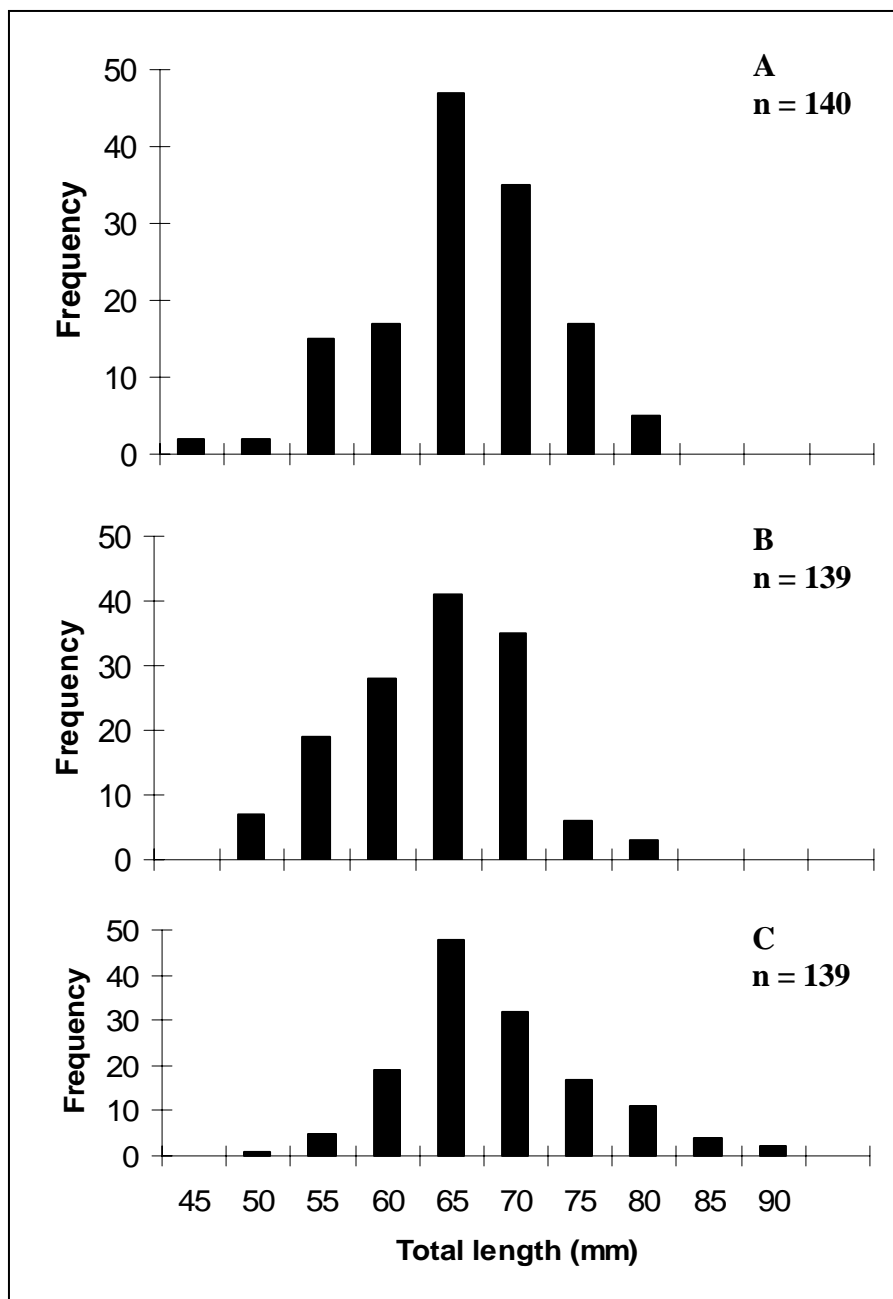


Figure 4. Length frequencies for *Wallowa* progeny in the three treatment groups from the 2003 trial A) control B) 1000 ppm strontium, and C) 5000 ppm strontium. Length values are grouped into 5 mm intervals.

Microchemistry

Trial 1-I found a difference in mean Sr:Ca ratios among the three groups ($F_{2,415} = 98.4$, $P = < 0.0001$, Figure 5). The average Sr:Ca ratio increased as the injected strontium concentration increased from 0 ppm to 1000 ppm and again from 1000 ppm to 5000 ppm. The average change in Sr:Ca ratio between the 0 ppm and 5000 ppm treatment groups was an increase of 4.04×10^{-4} , with a 95% confidence interval from 3.33×10^{-4} to 4.75×10^{-4} (Tukey's HSD). This represents a 30% increase in values between the 0 and 5000 ppm treatment groups.

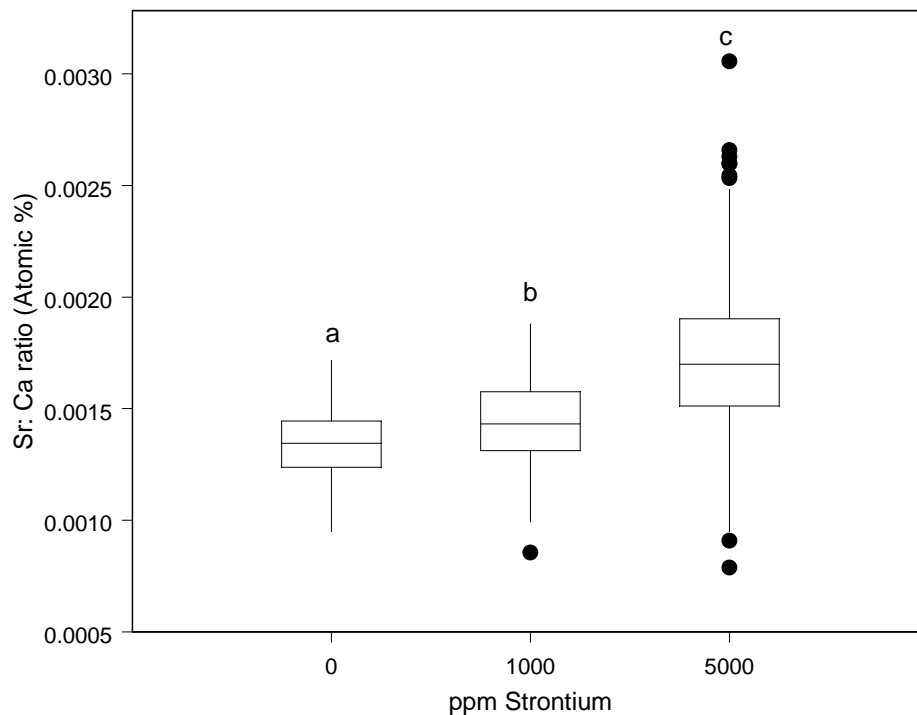


Figure 5. Sr:Ca ratios in progeny of Wallowa steelhead among three treatment groups (N = 418) in 2003. Different letters indicate a significant difference in means.

The data in terms of atomic weight percent show evidence that mean Sr:Ca ratios in three treatment groups are different. I found a difference in mean Sr:Ca ratios at 9 ($F_{2, 197} = 43.46$, $P < 0.0001$), 16 ($F_{1, 38} = 27.49$, $P < 0.0001$), 23 ($F_{2, 76} = 24.78$, $P < 0.0001$), 30 ($F_{1, 58} = 29.96$, $P < 0.0001$), and 36 ($F_{1, 37} = 53.34$, $P < 0.0001$) days after injection, although not all three groups were available for comparison on all spawning days. Progeny of strontium treated mothers showed consistent differences in

otolith Sr:Ca ratios compared to non-treated females irrespective of the duration between injection and spawning (Figure 7).

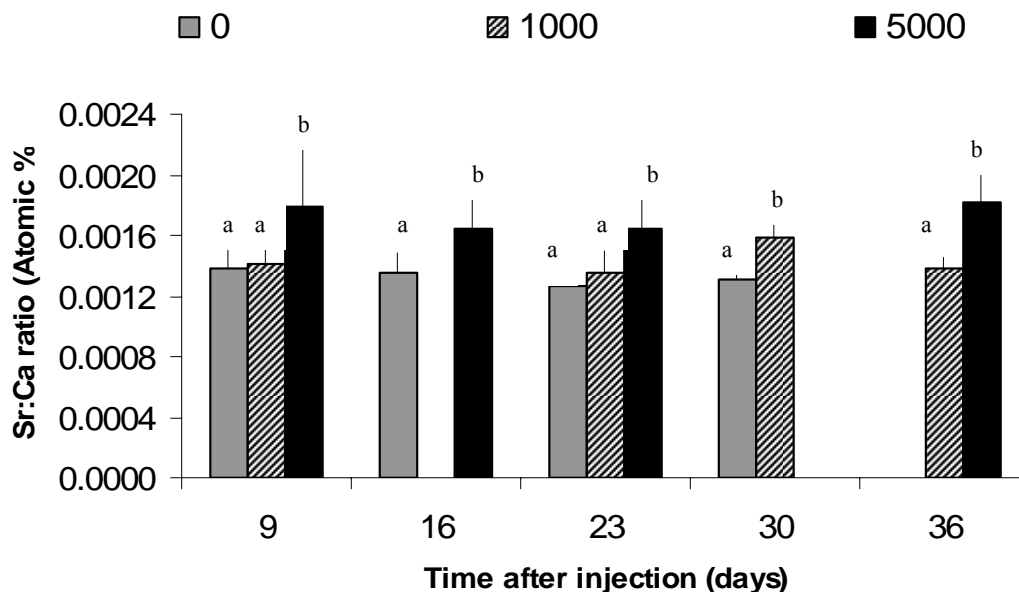


Figure 6. Sr:Ca ratios in the otoliths of progeny from artificially-spawned mothers sampled at different time intervals following injection. Different letters indicate a significant difference among treatments within a given time period.

Trial 2- There was no difference in average weight in adults between treatment groups ($F_{3,11} = 0.39$, $P = 0.76$). There was a 40% mortality rate among the Umatilla stock steelhead with 10 adult mortalities in the time between injection and spawning.

Eggs from each family hatched 25 days after fertilization. The number of fry per female ranged from 293 to 7,967 with no difference in average fry counts among the four treatment groups ($F_{3,11} = 2.10$, $P = 0.16$). However, there was one family in the

each of the 1000 and 5000-ppm treatment groups where there was low survival to the fry stage, perhaps due to mishandling of eggs or fry. The removal of these families changed the fry count data set and ranged from 1,485 to 7,967 with no difference in mean fry numbers between treatment groups ($F_{3,9} = 1.38$, $P = 0.31$). Daily mortality of fingerlings was sporadic, with very few fish dying among treatment groups. The survival of progeny was high in all treatment groups.

Mean Sr:Ca ratios differed among the four groups ($F_{3,263} = 19.81$, $P = < 0.0001$, Figure 7). The greatest difference in Sr:Ca ratio was observed between the 0 ppm and 20,000 ppm treatment groups, with a mean ratio increase of 5.01×10^{-4} in the 20,000-ppm treatment group relative to the 0 ppm group, with a 95% confidence interval from 3.31×10^{-4} to 6.72×10^{-4} (Tukey HSD). This is a 38% increase in values between the 0 and 20,000 ppm treatment groups.

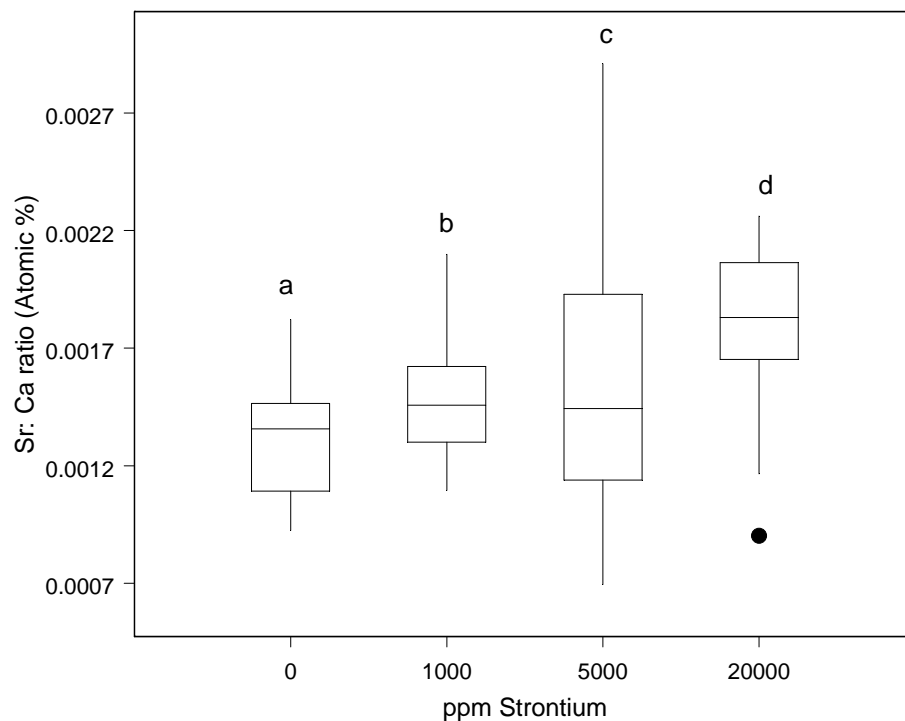


Figure 7. Sr:Ca ratios measured in 267 otoliths among four treatment groups in the second trial. Different letters indicate a significant difference in means.

Average growth rates were statistically different among the four groups at 60 ($F_{3,416} = 22.05$, $P < 0.0001$, Figure 8), 74 ($F_{3,416} = 3.15$, $P = 0.02$, Figure 8) and 88 ($F_{3,416} = 20.14$, $P < 0.0001$, Figure 8) days post hatch. However, there was no discernable pattern in growth rate difference among treatments, and the maximum observed difference in rate was 0.02 g/day greater growth rate for the 0-ppm (control) group relative to the 1000-ppm. This difference would result in a 29 g (1-oz) difference in

weight over a four-year period, which is probably biologically insignificant.

Furthermore, there was no discernable pattern to the difference in growth rate, indicating that the difference can be attributable to natural variability.

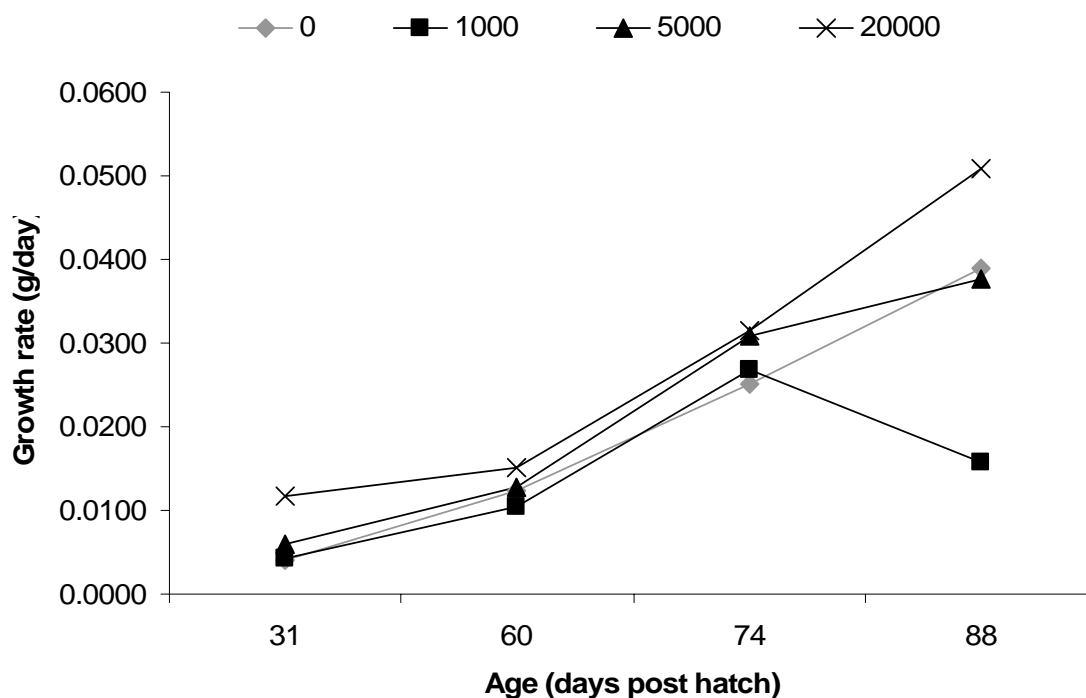


Figure 8. Growth rate for progeny of strontium treated and non-treated Umatilla steelhead females (2004 trial). Sample sizes for each treatment were: 0 (n = 120), 1000 (n = 90), 5000 (n = 150), and 20,000 (n = 60) ppm for each respective Sr treatment group. Growth rate at day 31 was calculated from comparing batch weights at day 16 and 31. Growth rates on subsequent dates were calculated from weights of individual fish.

Natural Sr: Ca ratios observed in hatchery steelhead

Strontium to calcium ratios among otoliths sampled from various hatchery stocks in Oregon and Idaho varied from 0.0018 to 0.0030 (Table 4). These values were equal to or greater than the values measured in the control and treated fish in both strontium injections trials.

Table 4. Otolith Sr:Ca ratios, in atomic percent, measured in young of the year steelhead populations in Oregon and Idaho. The numbers in parenthesis are one standard deviation from the mean.

Stock	Sample size	Average Wt. (g)	Average TL (mm)	Sr:Ca ratio
Umatilla (OR)*	67	1.6	54	0.0013 (0.0002)
Wallowa (OR)*	140	2.3	64	0.0013 (0.0002)
Rock Creek (OR)	20	0.9	47	0.0018 (0.0002)
Oak Springs (OR)	20	7.8	93	0.0019 (0.0002)
Round Butte (OR)	19	6.3	89	0.0020 (0.0002)
Saw Tooth (ID)	20	1.7	56	0.0020 (0.0002)
Dworshak (ID)	20	2.8	68	0.0021 (0.0009)
Cole M. Rivers (OR)	20	2.2	64	0.0021 (0.0002)
Pahsemeroi (ID)	20	0.9	47	0.0022 (0.0002)
Elk River (OR)	20	2.9	66	0.0023 (0.0004)
B-run (ID)	20	0.7	43	0.0023 (0.0003)
East Fork (ID)	20	1.3	52	0.0023 (0.0003)
Rock Creek (OR)**	20	0.5	39	0.0023 (0.0003)
Bandon (OR)	20	3.3	70	0.0030 (0.0003)

* = control data from trials

** = winter run steelhead, the remainders were summer run steelhead.

Discussion

This study tested the utility of using intra-peritoneal strontium injection to create a trans-generational tag, using adult hatchery origin fish from two populations of steelhead. There was a positive relationship between Sr injection dose and the Sr:Ca ratio in otoliths. The data suggest that progeny of strontium-treated steelhead mothers have on average, small, but elevated levels of strontium relative to that of progeny from untreated mothers. This work represents to my knowledge, the first project in which intraperitoneal cavity injections of strontium chloride were used to chemically mark otoliths of progeny from anadromous rainbow trout prior to spawning. On average, intraperitoneal cavity injections of strontium chloride can produce a detectable mark in otoliths collected from the progeny of mother steelhead.

The concentrations and exposure of strontium used in this study had no adverse effects on the survival of adult females, fertilization rates of eggs, or the growth and survival of parr. The slower growth rates for the 1000 and 5000-ppm treatments in trial 2, groups at 88 days post hatch (Figure 8) may be explained by poor fish health or differences in females in which the progeny originated (i.e. sample size, genetics, size, age). There were two families that experienced high mortality rates due to cold-water disease as verified by Lou Holtz-fish pathologist, Oregon Department of Fish and

Wildlife. Although these families were removed from the growth study a sub-sample of those killed were used for measuring Sr:Ca ratios.

In the 2003 trial, the sub-samples of progeny from each female were treated as an independent replicate in determining a treatment affect. A nested analysis of variance showed a difference in mean Sr:Ca ratios among the three treatment groups ($F_{2, 18} = 183.93$, $P = < 0.00001$, Figure 9) and among females within treatment groups ($F_{18, 21} = 20.03$, $P = < 0.00001$, Figure 9).

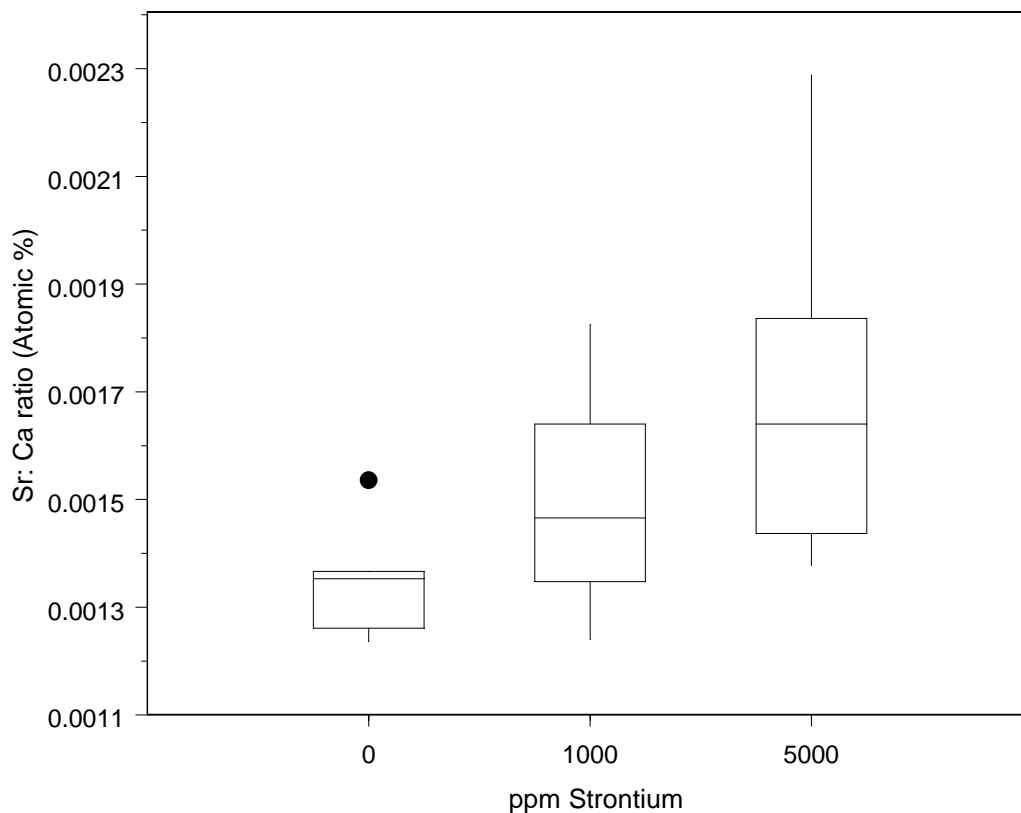


Figure 9. Mean Sr:Ca ratios calculated by pooling sub-samples for 21 females divided evenly among three treatment groups containing 0, 1000, and 5000-ppm strontium in trial 1.

The variability for the trial using Wallowa River brood showed approximately 61% of variation occurs at the treatment-to-treatment level; 14% of variation occurs at the female within treatment level; and 25% of variation occurs at the otolith within female

level. The Sr:Ca ratios vary strongly from treated and untreated females but do not vary as much among females within treatments.

Results from the second trial were similar to first trial in that there is a difference in mean Sr:Ca ratios among four treatment groups ($F_{3, 11} = 34.07$, $P = < 0.00001$, Figure 10) and among females within treatment groups ($F_{11, 15} = 18.87$, $P = < 0.00001$, Figure 10).

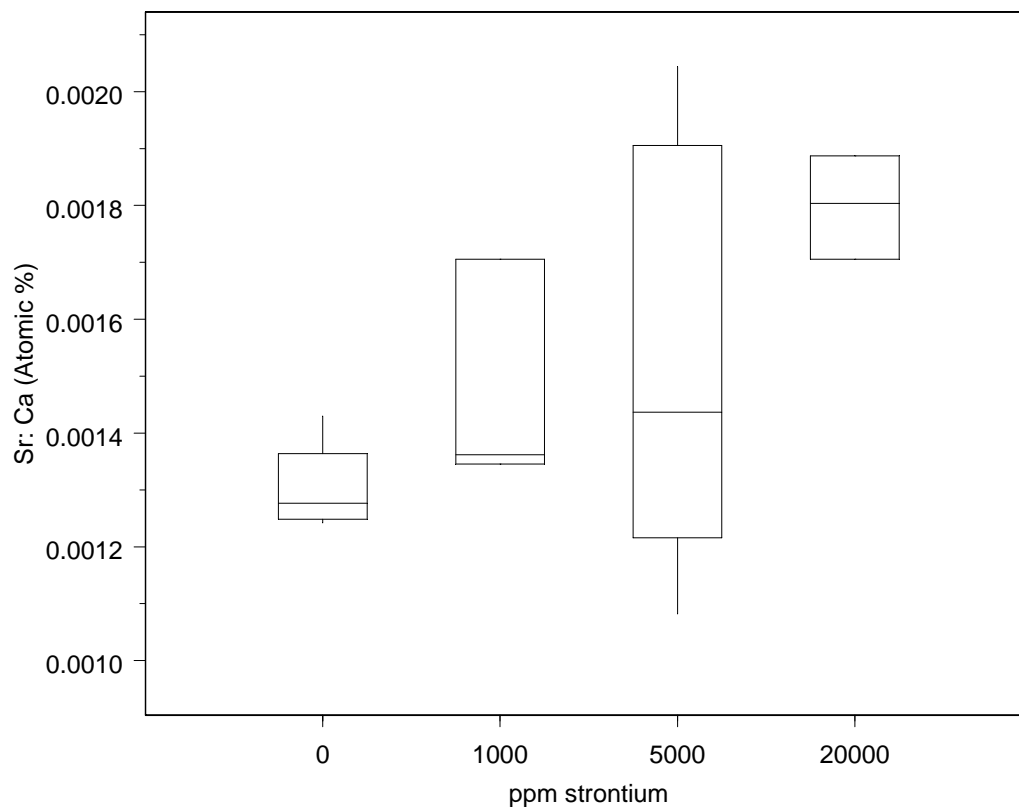


Figure 10. Mean Sr:Ca ratios in Umatilla steelhead calculated by pooling subsamples for 15 females among four treatment groups containing 0 (n = 4), 1000 (n = 3), 5000 (n = 5), and 20,000 (n = 3) ppm strontium.

The variability for the trial using Umatilla River brood showed that approximately 17% of variation occurs at the treatment-to-treatment level; 63% of variation occurs at the female within treatment level; and 19% of variation occurs at the otolith within female level. Sr:Ca ratios vary strongly between females within treatments but do not

vary as much in treated and untreated females. The coefficient of variation (CV) for the Sr:Ca ratio using Wallowa River brood was 34%. The CV for Umatilla River brood was 29%.

Treatment with 5,000 and 20,000-ppm strontium in solution artificially elevated Sr:Ca ratios in the otoliths of *O. mykiss* progeny. However, there is an overlap in the data between the different treatment groups. In the first trial, using Wallowa River steelhead, the treatment with the greatest difference compared to controls was the 5000-ppm treatment group. The data show 52 otoliths contributing 100% of the values ≥ 0.0018 ; 33 otoliths contributing 87% of the values $> 0.0016 < 0.0018$ and 41 otoliths at 48% of the values $> 0.0014 < 0.0016$. The otoliths in the control group show 44 otoliths contributing 52% of the values $> 0.0014 < 0.0016$ and 70 otoliths at 91% of the values $> 0.0012 < 0.0014$ (Figure 11).

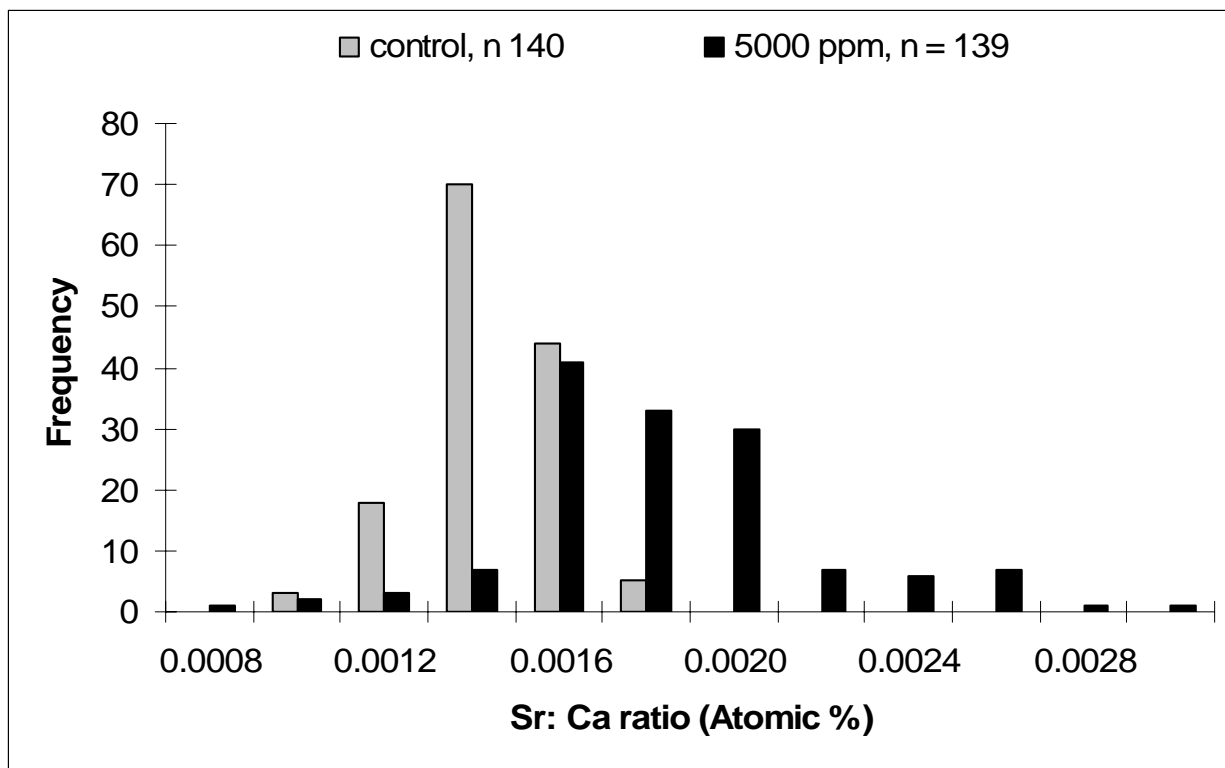


Figure 11. Frequency of relative Sr:Ca ratios for comparing the 0 and 5000-ppm strontium groups using Wallowa river steelhead in trial 1.

In the second trial, using Umatilla River steelhead, the greatest difference compared to controls was the 20,000-ppm treatment group. The data show 15 otoliths contributing 100% of the values ≥ 0.0002 ; 13 otoliths contributing 93% of the values $> 0.0018 < 0.002$, and 12 otoliths contributing 75% of the values $> 0.0016 < 0.0018$ (Figure 12).

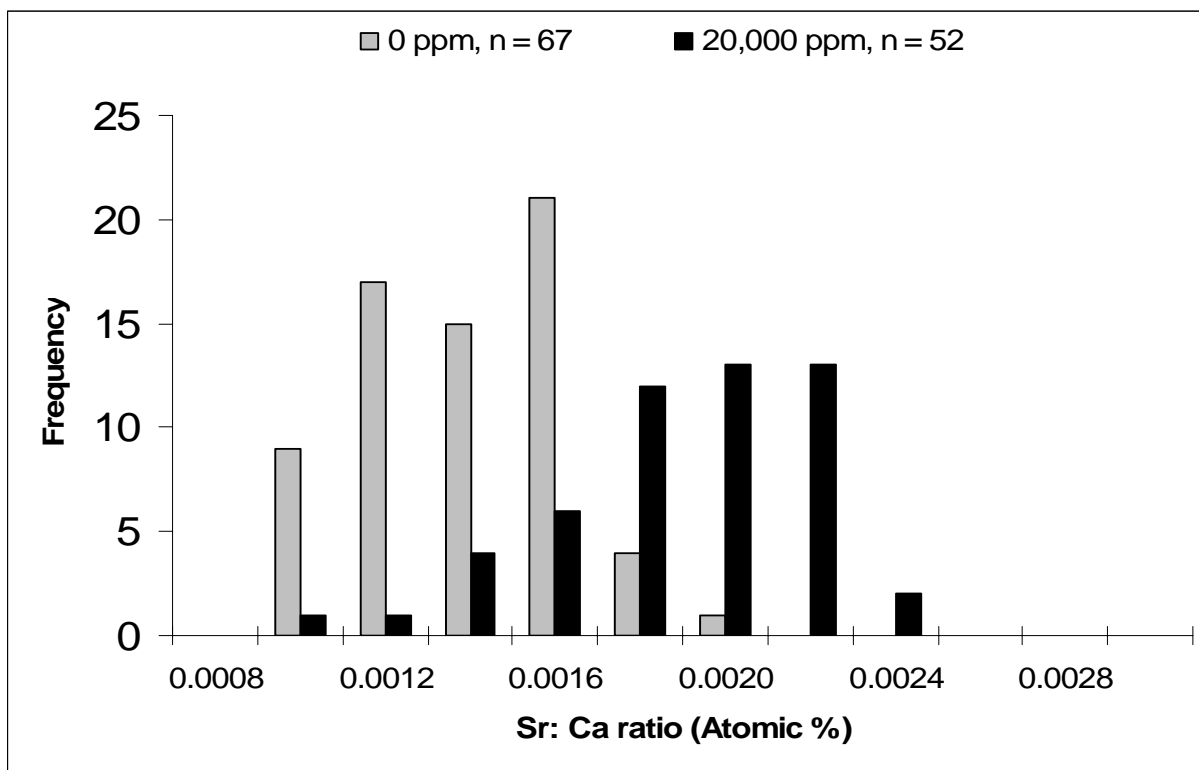


Figure 12. Frequency of relative Sr:Ca ratios for comparing the 0 and 20,000-ppm strontium groups using Uamtila river steelhead in trial 2.

These data indicate that precision could be increased by reducing the treatment-to-treatment variation and females within treatments variation for obtaining estimates that are more precise. Increasing the number of progeny for analysis would increase the precision of each female. However, it would be more effective to focus on reducing the variation that occurs at the female within treatment level by increasing the number females (Gottelli and Ellison 2004).

Adults returning to the Wallowa River were collected at the same time and location, confining the results to twenty-one hatchery steelhead from that particular river. However, sampling of the adult returns to the Umatilla River occurred at the early, middle, and late part of the run. Therefore, the results can be generalized to the population of hatchery steelhead. Sub-sampling females for the second trial could potentially become one of the greatest sources of variability due to fish size, ova development, and time between injection and spawning. Survival rates for female adults differed between the two injection trials. There was no mortality among the Wallowa stock but there was about a 40% mortality rate among the Umatilla stock used in the second trial.

The optimum timing for creating a strontium mark using the immersion bath technique or through feeding is thought to be after hatching and that any attempt to produce a strontium mark prior to hatching would be unsuccessful (Behrens-Yamada and Mulligan 1987). Because of the ontogeny of embryos and yolk absorption of sac fry, feeding a strontium-enriched diet for marking bony tissue at these life stages is futile. However, the manipulation of strontium in water can produce a detectable mark in scales and otoliths. For example, in a study to mark non-feeding embryos from sockeye salmon (*Onchorhynchus nerka*), eggs were fertilized and subsequently treated with strontium throughout their incubation period (Behrens Yamada and Mulligan 1987). In a similar study, *O. keta* and *O. nerka* fry were immersed in a

strontium chloride solution for a 24-h period (Schroder et al. 1995). These authors artificially elevated strontium levels in bony tissue of salmon prior to hatching and conversion from endogenous to exogenous feeding. Schroder et al. (1994) suggest that in order to evaluate the survival of salmon produced artificially, it is necessary to mark the fry of hatchery spawning events. The consequences for marking millions of fry rapidly are labor intensive and sometimes impractical due to their size. Immersion baths provide a technique for marking millions of fish without individual handling by delivering a constant flow containing strontium to their rearing water. Results from this study show an elevation in strontium by injecting mothers prior to egg fertilization. Although the differences between treated and untreated fish are not as discrete as the studies using immersion baths, the injection technique provides an opportunity for marking millions of eggs without individual handling. In this study, individual fish cannot be marked uniquely and measuring elemental parameters such as strontium produce statistical data to assign an individual to a group rather than absolute identification.

The presence of strontium in otoliths from the control groups shows a maternal influence in the strontium signal passed from mother to developing eggs (Zimmerman and Reeves 2000, 2002). Strontium-Calcium ratios varied substantially in juvenile steelhead among populations located in the states of Oregon and Idaho (Table 7). The

average Sr:Ca ratios for these hatchery-reared fish are greater than those observed in the controls and treated fish in this trial.

The natural absorption of strontium into fish otoliths is of special interest because of the utility for marking small fish and determining life history strategies (Kalish 1989). Otoliths of fish contain ecological data that may be accessible if the proper analytical techniques are employed and by doing so otoliths may serve as a data storage unit (Radtke 1989). However, the complexities about the factors that influence the way in which elements are incorporated into otoliths are not fully understood (Howland et. al. 2001). The amount of variability observed in otoliths may not be consistent over space and time because of physical and environmental factors such as geology, precipitation, and evaporation of water.

The unique chemical and chronological properties of otoliths allow researchers to ask questions about the life history of an individual fish (Campana and Thorrold 2001). For anadromous and catadromous species, the Sr^{2+} content contained in otoliths or statoliths provide a valuable tool for understanding their life history and habitat use of both adults and their offspring. In order to provide information on the population structure and movements of individual fish, two assumptions must be met: 1) calcified structures of fish are not susceptible to dissolution or absorption and 2) growth is continuous throughout life (Tzeng and Tsai 1994). The different chemical

compositions of sea and freshwater suggest that discrimination between marine and freshwater stocks could be determined by analyzing skeletal composition (Thresher 1999) using other available techniques (Howland 2001).

Further work for determining a progeny mark for steelhead may include multiple injections instead of a one-time injection. The injections could be administered into the abdomen, into the muscle, or a combination of both. In addition to strontium, the injections could contain magnesium and barium. Intramuscular injection may increase the strontium concentrations in the circulatory system. The mechanism for the uptake of strontium is relatively unknown. Since the injections employed in my study were into the abdomen, the eggs may have been receptive to an ionic exchange of strontium ions across the membrane of the egg (Alderdice 1988). Analysis of scales (Behrens Yamada and Mulligan 1982; Snyder et al. 1992) sampled from the progeny of treated mothers could be tested to investigate a non-lethal method for detecting a chemical mark.

Conclusion

In this work I artificially elevated strontium levels in progeny otoliths following injection of strontium solution into gravid female steelhead. The concentrations of strontium were non-toxic as evidenced by having no negative effect on the growth or

survival of steelhead progeny. The methods used in this trial provide a foundation for testing strontium as a trans-generational mark in salmonids. The variation in Sr:Ca ratios found among steelhead from different hatcheries suggests that care must be taken if the technique is employed where fish from more than one hatchery could potentially be involved.

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