IDENTIFICATION OF LIFE HISTORY VARIATION IN SALMONIDS USING OTOLITH MICROCHEMISTRY AND SCALE PATTERNS: IMPLICATIONS FOR ILLEGAL INTRODUCTIONS AND FOR WHIRLING DISEASE IN MISSOURI RIVER RAINBOW TROUT

by

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A dissertation submitted in partial fulfillment
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of

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ABSTRACT

Proper conservation and management of wild salmonid populations requires correct identification of the array of life histories present and their contribution to adult recruitment. I used otolith microchemistry to identify natal origin and scale patterns to determine outmigration age in Missouri River rainbow trout where whirling disease could potentially cause a population collapse. First, I reviewed the otolith microchemistry literature and concluded that there have been a limited number of freshwater studies, and there has been limited rigorous testing of the many instruments used. Second, I tested the efficacy of time-of-flight secondary ion mass spectrometry (ToF-SIMS) and found that Sr:Ca could be precisely measured in an otolith 'standard', there is a strong relation between qualitative ToF-SIMS results and concentrations measured with an electron microprobe, and the ambient water strongly influences otolith composition. Third, I analyzed the chemical composition of lake trout otoliths to identify the likely source of illegally transplanted lake trout in Yellowstone Lake. Changes in otolith microchemistry of suspected transplants accurately (>90%) determined the likely source of the introduction, and transect analyses indicated that lake trout have possibly been in Yellowstone Lake since as early as the mid-1980s. Finally, I used otolith microchemistry to identify natal origin and scale patterns to identify outmigration age in Missouri River rainbow trout. Otolith microchemistry was not an effective technique for this particular system because of similarities between the two major rainbow trout producing tributaries. However, I demonstrated a technique that uses the relation between otolith and water Sr:Ca to estimate sample sizes required to detect differences between streams. This can be used in future studies as a preliminary test to determine if otolith microchemistry is feasible. Scale patterns revealed that the yearling outmigration life history contributed most (88%) to adult recruitment in the mainstem fishery and the proportion of young-of-the-year to yearling outmigrants did not change post-whirling disease. Rainbow trout in the Missouri River have two obstacles to overcome: 1) severe whirling disease infection if the natal stream has *Myxobolus cerebralis*; and 2) low probability of surival if they manage to avoid severe infection but leave the stream too early.

CHAPTER 1

INTRODUCTION

Proper management and conservation of wild salmonids requires correct identification and knowledge of sources of recruitment, the array of life history strategies (e.g., age at migration) within a population, and contribution of these different sources and strategies to the fishery. However, identification and determination of the contribution of various sources and life history strategies to the adult population can be problematic. Direct methods such as trapping and tagging of juveniles and adults have been used to estimate the number of outmigrating juveniles, the timing of outmigration, and the contribution of specific life history types or stocks into the lake, river, or ocean (e.g., Kwain 1981; Seelbach 1993). Trapping and tagging, however, can be labor intensive and expensive, thus often limiting the number of fish that can be tagged or recaptured. For example, certain trap types can be difficult to operate in high flows and when there is a large amount of debris in the river (Todd 1994) and, depending on tag type, tagging typically needs to be done on a large scale in order to be economical (Kaill et al. 1990; Nielsen 1992). A successful tagging program requires that tags are retained for the length of the study, easily recognizable upon recovery, and do not increase mortality, influence growth, or alter fish behavior (Guy et al. 1996). However, these ideals are often difficult to attain when tagging very small fish for recovery years later, as adults. In addition, large numbers of fish need to be tagged, especially when the goal

is to estimate the contribution of stocks or life histories to adult recruitment, because of naturally high mortality rate of juvenile fish and therefore low recovery rates of tags.

Because of these limitations, indirect methods are increasingly used to identify the various life histories in fish populations. Natural marks, either morphologic or genetic, provide a useful tool for deciphering a great deal of information about individual or groups of fishes. Because the fish produce the marks themselves, using natural marks avoids the potential stress and injury of handling and tagging (Nielsen 1992; Guy et al. 1996). Use of indirect marks assumes that the marks are stable and that nearly all fish within a given group should be similarly affected and therefore carry the mark (Guy et al. 1996). Otolith microchemical analysis and scale growth pattern analysis are two important techniques that use natural marks to answer a variety of questions about the history of individual fish and the contribution of specific groups to mixed assemblages of adults.

Otolith microchemistry is a rapidly growing and evolving field of research. As otoliths grow, they incorporate ions from the surrounding endolymph on their surface (Campana and Neilson 1985). Because changes in the aquatic environment influence the composition of the endolymph, changes in environmental conditions such as salinity, temperature, and water chemistry are thereby reflected by changes in the otolith microchemistry (Kalish 1991). Therefore, the environmental conditions that the fish experience determine the elemental composition of the otoliths (Secor 1992).

Analysis of the chemical composition in fish otoliths has been used to discriminate among stocks and populations (Campana et al. 1994; Thresher et al. 1994;

Edmonds et al. 1995; Campana et al. 2000; Gillanders 2001); validate ages (Fenton et al. 1991; Kastelle et al. 2000; Weidman and Millner 2000); determine environmental and migration histories (Secor 1992; Secor et al. 1995; Howland et al. 2001; Limburg et al. 2001); and detect pollution (Papadopoulou et al. 1980; Hanson and Zdanowicz 1999; Saquet et al. 2002). Identification of natal origin is another common application (e.g., Thorrold et al. 1998b; Ingram and Weber 1999; Wells et al. 2003). For example, Thorrold et al. (1998a) were able to classify adult American shad *Alosa sapidissima* into their natal tributary with over 80% accuracy based on the analysis of the juvenile portion of their otoliths. Kennedy et al. (1997) found that strontium isotope ratios in the vertebrae and otoliths of Atlantic salmon *Salmo salar* was related to the ratio in the ambient water and therefore useful for identifying their rearing stream.

Scale growth pattern analysis has had many different applications in fisheries work. The most common use of growth pattern analysis has been in the ageing of fish. Over 100 years ago, it was recognized that annual patterns in the spacing of the scale circuli (ridges on the scale) could be used to estimate the age of fish. These seasonal patterns are a result of changes in growth of the fish and thus the scale; therefore, fast growth during the summer results in generally wider spaced circuli than slower growth during the winter. However, other patterns and marks, not associated with seasonal changes in growth, are also observed in scales. These non-seasonal patterns in the spacing and physical characteristics of the circuli are generally also caused by changes in the growth of the fish due to migration, spawning, or stocking. Analysis of these scale growth patterns has commonly been used to separate hatchery and wild fish. Separation

of the two groups is based on the fact that hatchery fish tend to grow faster and more uniformly than wild fish and thus have wider and more uniform circuli spacing (Schwartzberg and Fryer 1993).

Changes in the growth pattern, as reflected in the scales, can therefore be used to determine when fish migrate to a new environment with a different level of productivity, for example, when salmon migrate from their natal stream to the ocean (Tsiger et al. 1994; Bernard and Myers 1996). Similar changes in growth have been observed in potamodromous (migration within freshwater) populations of trout that migrate from spawning streams to a lake. Rosenau (1991) was able to identify two outmigration patterns in adult rainbow trout *Oncorhynchus mykiss* from Lake Taupo, New Zealand, by counting the number of circuli and measuring their spacing. Once growth patterns are identified, life histories, origin (wild or hatchery), and the contribution of each life history type can then be estimated from samples from a mixed population (e.g., Rosenau 1991) and used to examine long-term trends in distribution and relative abundance (Bernard and Myers 1996).

The wild rainbow trout population inhabiting the blue-ribbon section of the Missouri River near Helena, Montana, is an area where such techniques might prove useful in identifying the contribution of various spawning tributaries and outmigration strategies to the mainstem population in light of the presence of whirling disease. Whirling disease is caused by the non-indigenous parasite *Myxobolus cerebralis* and was first detected in rainbow trout in Little Prickly Pear Creek, a major spawning tributary in

May 1996. Since then, the parasite has become widespread in the drainage (Leathe et al. 2002).

Given the high rate of spread and severity of infection, a collapse in rainbow trout recruitment similar to the Madison and Colorado rivers (Nehring and Walker 1996; Vincent 1996) is anticipated in this section of the Missouri River. An outbreak of whirling disease would be detrimental to this wild trout fishery because rainbow trout comprise 75% of the Missouri River trout assemblage. The severe declines (up to 90%) in some prized wild trout fisheries have been attributed to *M. cerebralis* (Nehring and Walker 1996; Vincent 1996). The severe population declines are a result of poor recruitment of fish less than 2 years old because of high mortality, especially in young-of-year fish (Vincent 1996). However, large population declines from whirling disease have not been observed in all infected systems. Severe population declines have not been reported in California or Oregon despite *M. cerebralis* having been present for several decades (Modin 1998; Sandell et al. 2001). Currently in Montana, 87 waters in 13 of the 22 major river drainages are whirling disease positive (Bartholomew and Reno 2002). However, to date, severe whirling disease outbreaks and salmonid population declines in Montana have been reported in only a few sites (E. R. Vincent, Montana Fish Wildlife and Parks, unpublished data).

Where and when fish spawn and rear in relation to the disease may play an important role in determining the risk of infection (Downing et al. 2002). Therefore, it is important to understand the relation of fish life history to whirling disease susceptibility because of the complex two-stage life cycle of the disease (see Wolf and Markiw 1984).

Rainbow trout can be infected by *M. cerebralis* at any stage in their life from free-swimming larva to adulthood (Markiw 1991, 1992), but are most susceptible to whirling disease infection within a relatively narrow time window of 9 weeks post-hatch (756 degree days of development, Ryce 2003). This window of high susceptibility occurs because *M. cerebralis* attacks the cartilage of the skeleton and cranium, which is most prevalent at this stage (Hedrick et al. 1998)

A possible management strategy to maintain rainbow trout populations in whirling disease infected systems is to identify, protect, and enhance specific rainbow trout life history types with characteristics that limit overlap with *M. cerebralis*-infected *Tubifex tubifex* during the vulnerable juvenile stage and the release of the infective triactinomyxon stages (Downing et al. 2002). Sentinel cage studies suggest that whirling disease infection can vary markedly even within highly infected systems (Hiner and Moffitt 2001; Sandell et al. 2001). Tubificid worm surveys also indicate that *T. tubifex* is present in nearly every stream, but its abundance varies greatly and it may be absent in some sections, even in infected river systems (B. Kerans, Montana State University, unpublished data). In addition, temperature may play a role in whirling disease infection risk. Sentinel cage studies have suggested that there are periods in the early spring and fall when the water is too cold, and in mid summer when the water is too warm, for infection to be severe (MacConnell and Vincent 2002). Therefore, trout that are spawned and reared in areas of low infection risk could potentially bypass the vulnerable period to *M. cerebralis* infection. In addition, trout with life histories that emerge during times of low triactinomyxon stage production also could potentially minimize serious

infection. Thus, matching rainbow trout life history and risk to *M. cerebralis* infection within a watershed has the potential to offset whirling disease impacts to a wild trout fishery, and may explain why population declines are more severe in some areas than others.

Missouri River rainbow trout appear to exhibit several life history types. Rainbow trout in this section of the river primarily use three tributaries for spawning (Little Prickly Pear Creek, Sheep Creek, and the Dearborn River), each with varying degrees of parasite prevalence. Data from fry outmigrant trapping suggest that the length of fry rearing in tributaries varies from 1 month to 1 year (Montana Fish, Wildlife and Parks, unpublished data). Adult rainbow trout in the mainstem are assumed to be the progeny of tributary spawners, but information on the relative contribution of each tributary and each potential life history recruited to the fishery is lacking.

In general, there is little published research on life history variation of potamodromous stocks of wild salmonids (Behnke 1992). However, there are well known differences in migratory life histories. Rainbow trout exhibit all forms of potamodromy (Northcote 1997). Much of the research on the timing and age at outmigration of potamodromous rainbow trout populations has been on lacustrine-adfluvial populations. Typically, juveniles remain in the tributaries one to three years before migrating to the lakes (Van Velson 1974; Biette et al. 1981). In some populations, many of the juveniles will migrate before they are 1 year old (Rosenau 1991; Hayes 1995) or even as recently emerged fry (Hayes 1988). For example, Rosenau (1991) identified two predominant juvenile life history types in adult spawners

from Lake Taupo, New Zealand: juveniles either migrated from their natal stream as young-of-year, or over-wintered and left their natal stream as yearlings. The proportion of returning spawners among the two life history types differed markedly, however. Back-calculation of fish length at time of migration indicated that the mean length at migration was similar among the tributaries (140 mm) and that the minimum length at migration was 94 mm. In contrast, Stephens (1989) found that many fish smaller than 94 mm migrated to the lake. The data therefore suggested that juveniles that migrated downstream to the lake at sizes less than 94 mm were strongly selected against.

Hayes (1995) also found multiple migratory life histories in rainbow trout from Lake Alexandrina, New Zealand. Based on otolith growth patterns, he estimated that 55% of adults sampled from the lake had migrated from inlet streams a few days after emergence (mean length = 25.8 mm), 28% had spent some time rearing in the inlet streams, and 17% had reared in the outlet stream. In a study of Scotts Creek (one of Lake Alexandrina's inlet streams), 96% of the rainbow trout migrated to the lake as recently emerged fish (Hayes 1988). These two studies confirm that there are a number of different juvenile rainbow trout life histories and illustrate that not all life histories are equally successful in contributing to adult recruitment.

The purpose of this research was to develop methods for identifying life history variation and contribution of different sources to a fishery, specifically to determine if there are rainbow trout life histories in the Missouri River that limit exposure to whirling disease. I also wanted to estimate the contribution of the spawning tributaries and life

history types to the mainstem fishery and the changes in composition as whirling disease spreads through the system.

To identify natal origin of the rainbow trout in the Missouri River I used otolith microchemistry and scale growth pattern analysis to identify age at migration. However, there has been a limited number of studies that have applied otolith microchemistry techniques to freshwater systems (see Chapter 2), so in this document there is a emphasis on the development of otolith microchemistry techniques and assessing the efficacy of otolith microchemistry in freshwater fisheries applications.

The dissertation is organized into four self-contained chapters. In Chapter 2, I review the current state of knowledge in the field of otolith microchemistry with an emphasis on freshwater otolith microchemistry studies and necessary considerations when using otolith microchemistry to assess life history variation and stock contribution. In Chapter 3, I investigate the efficacy of time-of-flight secondary ion mass spectrometry (ToF-SIMS) for the chemical analysis of fish otoliths. Because ToF-SIMS has never been used to analyze otoliths, I first test the precision of the instrumentation using an otolith 'standard'. I then compare ToF-SIMS results with an established and accepted analytic technique. Finally, I compare otolith and water composition in order to evaluate if differences observed in the otoliths are directly related to the chemistry of ambient water. In Chapter 4, I demonstrate the potential power of freshwater otolith chemical analysis. In this chapter I use otolith microchemical analyses to determine the origin and timing of the illegal introduction of lake trout *Salvelinus namaycush* in Yellowstone Lake, Yellowstone National Park, Wyoming. The introduction of lake trout into the lake

afforded an opportunity to assess the predictive ability of otolith microchemistry to identify origin of individual fish inhabiting geographically close water bodies and to demonstrate a new and unique application of otolith microchemistry as a forensics tool for investigating illegal fish introductions. In Chapter 5, I apply otolith microchemistry techniques in an attempt to identify natal tributary in wild rainbow trout from the Missouri River. I assess the contribution of the various spawning tributaries to the mainstem river adult assemblage in the face of spreading whirling disease infection in the system. In addition, the feasibility of otolith microchemical analyses using water chemistry as a preliminary test is explored. I also integrate into the chapter investigations into the use of scale patterns to identify the age at outmigration and contribution of the strategies compared to outmigrant trapping done by Montana Fish, Wildlife and Parks. I conclude the dissertation by relating these findings to how life history variation and source of recruitment may influence the effects of whirling disease as it continues to spread and impact the highly valued rainbow trout fishery in the Missouri River.

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CHAPTER 2

REVIEW OF CURRENT APPLICATIONS AND APPROACHES FOR CHEMICALLY ANALYZING THE CALCIFIED STRUCTURES OF FISH

Introduction

The information storage capabilities of the calcified structures of fish have long been recognized. Scales have been used to age fish for over a century (Carlander 1987) and otoliths for almost as long (Jones 1992). Apart from the observation of daily growth rings by Pannella in 1971 and subsequent development of the daily increment technique (Jones 1992), the most promising advance in unlocking the information stored in these structures is in the field of otolith microchemistry. This is not a new field (Thresher 1995), but in the last decade the number of otolith microchemistry related studies in the literature has increased dramatically. Campana (1999) reported that prior to 1980 only 6 papers discussed otolith microchemistry, but by 1998 157 papers had been published; this number has now increased to at least 219 papers as of 2003. The study of the chemical composition of otoliths and other calcified structures date back to the 1950s. In a study on the biogeochemical deposition of Sr, Odum (1957) found that the ratio of Sr:Ca in freshwater fish otoliths was lower than the concentration in saltwater fish otoliths. It was readily recognized that the information that could potentially be gathered from these structures would have many applications to fisheries science and management. For example, the Fisheries Agency of Japan (1967) realized that analysis

of these elements could provide information on stock structure and movements of Pacific salmon.

Since the initial studies, there has been a proliferation of research in the area of the chemical composition of the calcified structures of fish, particularly otoliths (Campana and Thorrold 2001). Currently, otolith microchemistry is regarded as a tool capable of answering questions that are not easily answered by conventional fisheries techniques (Campana and Tzeng 2000). For example, otolith microchemistry can be used to determine the environmental and physiological history of individual fish – information that is difficult or impossible to obtain using traditional tagging techniques or genetic analysis.

Early research was hampered in part by the lack of sensitivity of the instruments available (Thresher 1995). Since the groundbreaking studies (e.g., Calaprice 1971; Gauldie and Nathan 1977; Lapi and Mulligan 1981), there have been vast improvements in analytical techniques and new, more accurate, and sensitive instruments have been developed. However, each analytical technique has its own level of accuracy and precision and sets of advantages and disadvantages. Thresher (1995) noted that over a dozen different methods are available to analyze otolith chemistry. In researching the literature, I found references to the use of at least 17 different instruments. The growth and evolution of the field of otolith microchemistry research fits nicely into a research and development cycle paradigm outlined by Jones (1995) (Figure 2.1) where, for some instruments, it has been determined that their capabilities are not adequate for otolith microchemistry studies, despite their wide-spread use early on (e.g., energy dispersive

electron microprobes, ED-EM), while others, such as laser ablation inductively coupled plasma mass spectrometry (LA-ICPMS), are quickly becoming regarded as one of the 'instruments of choice'. However, with the increased number of otolith microchemistry papers and the suite of instruments used to answer a multitude of fisheries questions, there have been a limited number of comprehensive studies testing the efficacy of these instruments in analyzing otolith composition (notable exceptions being Gunn et al. 1992; Arslan and Paulson 2003; Zimmerman and Nielsen 2003). In addition, there have only been a few papers that have rigorously compared the performance of the instruments used or the results from various labs using the same analytic techniques (e.g., Campana et al. 1997; Secor et al. 2002).

Furthermore, until recently, there have been relatively few papers dealing with the application of otolith microchemistry techniques to freshwater species. For example, during the 1980s, at least 38 microchemistry papers were published. Marine and estuarine species were the subject of 23 of the studies and anadromous species for 13 papers. Freshwater species were only studied in two of the papers. The reason for this is unclear and difficult to pinpoint. One explanation is that in the early studies, the elements that were in concentrations high enough to be measured by the analytical techniques at the time (> 100 ppm) tended to be the elements that are found in lower concentrations in freshwater than in seawater (e.g., Ca, Na, K, Sr, P). Some of these elements, namely Sr, were found to be much lower in otoliths of freshwater fish than marine species, and in the freshwater growth zone versus the marine growth zone of anadromous fish otoliths. It was thought that the concentration and variation (among

locations) of these elements would be too low in freshwater otoliths to detect differences, given the sensitivity of the instruments at that time (B. Rieman, USFS, pers. comm.). Subsequently, otolith microchemistry investigators have realized that it is the ratio of element concentration to Ca concentration in the otolith, rather than the absolute concentration, that is often the more appropriate measure (Campana 1999). In addition, with the development of more sensitive analytical techniques more elements can now be accurately measured, including elements such as the rare earth elements (REE) that tend to naturally occur in higher concentrations in freshwater than seawater (Campana 1999; Arslan and Paulson 2003).

I was interested in applying otolith microchemical techniques to answer questions about life history variation in wild rainbow trout *Oncorhynchus mykiss* in the Missouri River near Helena, Montana, where whirling disease had been detected in 1996 (see Chapter 1). At the inception of this project there were a limited number of freshwater otolith microchemistry studies and there was no one instrument that was clearly the preferred choice for otolith microchemistry studies. Therefore, the initial question of this study was to determine which analytical technique currently in use would be suitable, or if another new but untested technique might be appropriate.

When embarking on an otolith microchemistry study to answer a fisheries research question there are three basic considerations that need to be addressed. First, what is the question that you are trying to answer? Is it, for example, a question of stock identification, or determination of life histories present, or migration strategy? Second, what elements or isotopes could be potentially the most useful for helping to answer the

question? Third, which analytical technique is most appropriate to use given considerations 1 and 2?

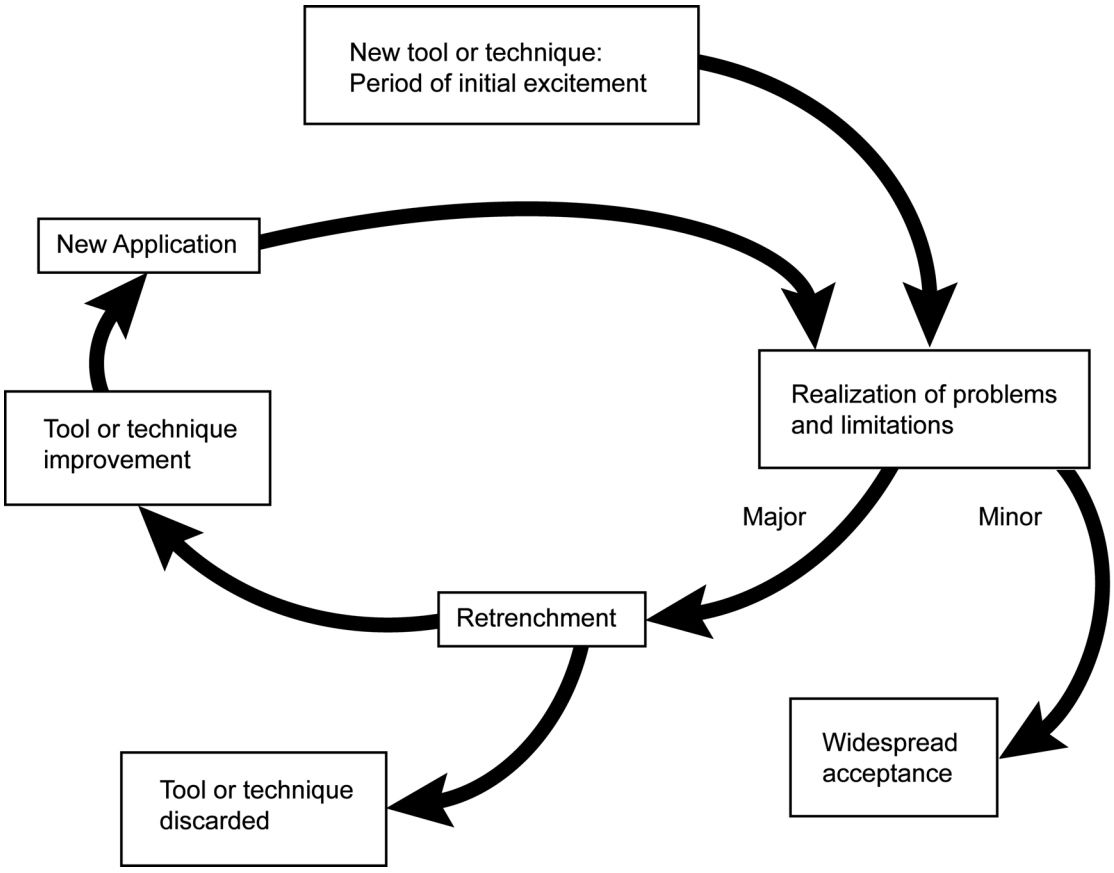


Figure 2.1. Research tool paradigm, illustrating the steps involved in the development and acceptance of new research tools and techniques. Diagram from Jones (1995).

The purpose of this chapter is to provide an overview of the state of knowledge of the field of otolith microchemistry with respect to the basic considerations outlined above. I focus on four areas: 1) the capabilities of otolith microchemistry analysis for answering fisheries science questions, with particular attention paid to freshwater applications; 2) the composition of otoliths and the relative usefulness of the various elements and isotopes for otolith microchemistry studies; 3) the variety of analytical

techniques available to analyze the composition of otoliths and their relative advantages and limitations; and 4) some of the issues that potentially affect the quality of the data, such as storage, handling, and use of standards.

Applications of Chemical Analysis

The analysis of the chemical composition of calcified structures is useful for answering a number of different fisheries science questions. These include the identification of stock or population structure; determination of environmental and migration histories; age validation; pollution detection; and detection of induced chemical marks, to name a few of the more common applications. Most studies have addressed questions relating to marine, estuarine, and anadromous species, but many of the same questions are important to the understanding of the biology and ecology of freshwater species. There have been few otolith microchemistry studies involving freshwater species, and these will be addressed at the end of this section.

The identification of stock or population structure is one of the main applications of otolith microchemistry. These types of analyses are based on the premise that the chemistry of the water a fish inhabits imparts a natural tag or 'elemental signature' in the otolith or skeletal structure that can then be used to discriminate among groups of fish from different spawning and rearing locations (Campana 1999). This elemental signature, which is typically based on analyzing concentrations of a number of elements, has been widely used to identify unique stocks in the marine environment with the goal of better managing the fisheries (e.g., Edmonds et al. 1991; Campana et al. 1994; Campana and Gagné 1995; Patterson et al. 1999; Campana et al. 2000; Gillanders 2001).

This approach has also been used to identify specific nursery habitats and determine the level of population connectivity (Gillanders and Kingsford 1996; Thorrold et al. 1997b; Thorrold et al. 1998b) or used to investigate source of recruitment of larval fish (Swearer et al. 1999; Gillanders 2002; Thorrold et al. 2002). For example, Swearer et al. (1999) found evidence that local retention and recruitment of larval bluehead wrasse *Thalassoma bifasciatum* on their natal reef occurs more often than previously thought.

Elemental signatures are particularly robust when there are predefined groups of fish that are characterized before they mix and when there are sufficient differences in the otolith compositions among the groups; in short, group membership is defined based on the chemical composition of otoliths from groups of known origin (Campana 1999; Thorrold et al. 2002). Understanding the reason for the observed differences in the chemical composition among groups is not critical for elemental signatures to work; rather, the fact that the differences exist and that that they are a powerful distinguishing characteristic of a group or stock for a given point in time is sufficient (Campana et al. 2000).

Occasionally, though, differences among the groups of fish of interest may be insufficient for accurately distinguishing group membership. In a study of two-banded bream *Diplodus vulgaris*, Gillanders et al. (2001) detected few differences among fish located > 100 km apart, likely due to homogeneity of the coastal waters in that area of the Mediterranean Sea. Several studies have also suggested that temporal variation in water chemistry might, in some cases, confound the spatial signature. For example, Gillanders et al. (2002) found that estuarine waters and chemical composition of juvenile

snapper *Pagrus auratus* was not always consistent from year to year. Similarly, Campana et al. (2000) found that elemental fingerprints in Atlantic cod *Gadus morhua* from the Gulf of Saint Lawrence were robust over the short term (< 2 years), but less so over the long term (4-13 years). Therefore, it is important that a researcher be aware of the potential for temporal variation in water chemistry and to collect a library of elemental fingerprints from each site and for each year over the time frame of interest (Gillanders 2002).

Another major application of otolith microchemistry is in the reconstruction of the environmental history of fish; i.e., the relation of otolith composition to changes in the temperature, salinity, and water chemistry the fish experience throughout their lives (e.g., Gauldie et al. 1986; Northcote et al. 1992; Radtke and Kinzie 1996). Stable oxygen isotope ratios ($\delta^{18}O$) (Kalish 1991b; Thorrold et al. 1997a), and Sr:Ca ratios (Townsend et al. 1989; Radtke et al. 1990; Townsend et al. 1992; Townsend et al. 1995) have both been used as proxies for temperature. However, reconstruction of individual temperature histories has had limited success with some cold water species (Campana 1999). Retrospective analyses of paleoclimate using stable oxygen isotopes in fossilized otoliths have used similar principles as the reconstruction of environmental history in modern otoliths (e.g., Devereux 1967; Patterson et al. 1993; Smith and Patterson 1994). However, the relation between Sr:Ca ratios and temperature, can be confounded by salinity (Campana 1999). Salinity histories, like temperature histories, have also been determined using Sr:Ca ratios (e.g., Limburg 1995; Tzeng et al. 1997; Radtke et al. 1998) and stable oxygen isotopes (e.g., Meyer-Rochow et al. 1992; Northcote et al.

1992) as proxies. To date, examination of salinity histories using Sr:Ca ratios are generally restricted to detection of anadromous and catadromous migrations (Campana 1999).

More commonly, changes in otolith composition resulting from movement from one environment to another (e.g., freshwater to marine), have been used to discriminate between hatchery and wild fish (e.g., Schwartzberg et al. 1995; Elfman et al. 1999) and anadromous from non-anadromous (resident) fish (e.g., Kalish 1990; Rieman et al. 1994; Elfman et al. 1999; Howland et al. 2001; Babaluk et al. 2002; Zimmerman and Nielsen 2003). In this application, series of measurements along the otolith growth axis, or two-dimensional mapping of an entire otolith, have been used to estimate age at migration and determine type of migration history (e.g., Secor 1992; Limburg 1995; Thorrold et al. 1997b; Arai and Miyazaki 2001; Limburg et al. 2001).

Annual or seasonal changes in the elemental or isotopic composition of otoliths can be used as an objective method to validate the age estimates from more traditional (and subjective) ageing methods. For example, variations in Sr concentrations or Sr:Ca have been correlated to the visible banding patterns in otoliths (Radtke and Targett 1984; Seyama et al. 1991; Halden et al. 1995). Stable oxygen isotope ratios, which cycle seasonally in otoliths, have been used to validate ages in fish (Weidman and Millner 2000). Age estimates have also been validated using radiochemical dating. This approach to ageing fish is based on decay rate of naturally occurring isotopes such as ^{210}Pb and ^{226}Ra (Bennett et al. 1982; Campana et al. 1990; Fenton et al. 1990; Fenton et

al. 1991; Kastelle et al. 2000), or measuring the radiocarbon from nuclear testing fallout (Kalish et al. 1996; Campana 1997; Kalish et al. 1997; Campana and Jones 1998)

Intentionally-induced chemical marks in the calcified structures of fish have also been used to validate age estimates or as batch marks to identify hatchery fish (Campana 1999). Typically, calcium-binding fluorescent chemicals such as oxytetracycline, alizarin red, and calcein have been used to mark fish otoliths (Campana and Thorrold 2001). But otoliths have also been chemically marked by elevating Sr in the water or the diet (e.g., Yamada et al. 1979; Yamada and Mulligan 1982; 1987; Brown and Harris 1995; Schroder et al. 1995). Ennevor and Beames (1993) and Ennevor (1994) experimented with using elements from the lanthanide group (La, Sm, and Ce) to mass mark hatchery coho salmon *O. kisutch* with good success. However, the quality of the marks is dependent on selectivity of the otoliths to the elements used (Campana 1999).

Microchemical analysis of otoliths and other calcified structures has also been used to detect the presence of or elevated levels of elements associated with pollution (Papadopoulou et al. 1980; Hanson and Zdanowicz 1999). Information from such analysis could potentially be used as a means for stock identification (Spencer et al. 2000). However, as noted with chemical marking, not all elements are incorporated into the otoliths as readily as others; therefore, not all elements associated with pollution will necessarily be good indicators in otoliths (Campana 1999; Hanson and Zdanowicz 1999).

Analysis of the chemical composition of the calcified structures of fish living in the freshwater environment has been used in a number of applications, despite the

limited number of published studies. Applications have included: pollution or lake acidification indication (e.g., Moreau et al. 1983), stock identification (e.g., Bronte et al. 1996), and natal tributary or geographic origin indicator (e.g., Thorrold et al. 1998a).

Johnson (1989) investigated the use of scales as an indicator of pollution. He analyzed the scales of lake trout *Salvelinus namaycush* and smallmouth bass *Micropterus dolomierui* collected over 4 decades to determine if concentrations of elements reflected trends in the inputs of metal contaminants into the lake. However, this study met with limited success because despite elevated concentrations of some of the elements in the otoliths, they did not correspond to the input events. More recently, Saquet et al. (2002) analyzed the otoliths of six species of freshwater fish to determine if otolith composition could be used to indicate dispersal of mine tailing effluent. However, like Johnson (1989), this study met with limited success, because the concentration of most elements measured remained constant.

Elements indicative of pollution have also been used with moderate success as a means of stock identification. Bronte et al. (1996) analyzed the elemental concentrations of whole otoliths from lake herring *Coregonus artedi* and were able to correctly classify an individual fish to its stock 12 to 86% of the time.

Stock identification is an important aspect of fisheries management, especially for commercially important species such as salmon. As mentioned previously, one of the earliest studies (Fisheries Agency of Japan 1967) investigated this very question with some species of Pacific salmon. Mulligan et al. (1983) successfully used the chemical composition of whole vertebrae, opercula, and otoliths to identify individual sockeye

salmon *O. nerka* from five Alaskan stocks and three Vancouver Island stocks with 80 to 95% accuracy. Lapi and Mulligan (1981) analyzed the freshwater region of sockeye salmon scales with ED-EM and were able to correctly identify lake of origin of sockeye salmon with 63% accuracy. In stocked Atlantic salmon *Salmo salar*, Kennedy et al. (1997; 2000) found that Sr isotope ratios in otoliths and vertebrae could be used to identify rearing stream. In other studies it has been suggested that strontium isotope ratios of stocked could be used to reconstruct movements of Atlantic salmon within the stream over small temporal and spatial scales (Kennedy et al. 2002). Recently, Wells et al. (2003) demonstrated that they could correctly reclassify individual westslope cutthroat trout *O. clarki lewisi* to their tributary of origin with 100% accuracy. Other studies have also indicated that composition of otoliths can be used as an indicator of geographic origin of fish in freshwater including Halden et al. (1996), Friedland et al. (1998), Ingram and Weber (1999), and Weber et al. (2002).

Chemical Composition of Otoliths

A number of calcified structures have been analyzed in microchemical studies, including otoliths, scales, and vertebrae. Otoliths are the most commonly used structure for these types of studies. Otoliths are mineral structures located in the inner ear of fish and are associated with detecting position (orientation) and motion. They are composed primarily of aragonitic calcium carbonate (> 90%) in an organic matrix (< 8%) with the remainder (ca. 2%) trace and minor elements (Campana 1999). It is the concentration of the trace and minor elements and their isotopes that offer the basis for use of otolith composition in fisheries studies. Otoliths begin forming embryonically, are acellular,

and are not susceptible to resorption or metabolic reworking once the material is laid down (Campana and Thorrold 2001). Trace and minor elements are incorporated from the endolymph onto the surface of the otoliths as the fish grows (Campana and Neilson 1985; Campana 1999; Markwitz et al. 2000). These properties make otoliths ideal for microchemical studies. However, one of the main disadvantages is that the fish need to be sacrificed in order to collect the otoliths for analysis.

The chemical composition of other calcified structures have been examined with the aim of using them for similar applications as otoliths (e.g., Belanger et al. 1987; Wells et al. 2003). Scales and fin-rays are advantageous because, unlike otoliths, they do not require sacrifice of the fish to collect the sample. But, the temporal stability of these structures has not been adequately tested and thus far has not gained wide-acceptance. Scales are dermal in origin and consist of an inner protein layer covered by a mineralized bony layer (Coote et al. 1991). As with otoliths, trace and minor elements, such as Sr, are incorporated into the structure. However, under certain circumstances the minerals can be reabsorbed and remineralized (Bilton 1974; Gauldie and Nelson 1990).

Over 30 elements have been detected in otoliths but most occur at low concentrations (<100 ppm, Campana 1999). Which elements are incorporated into the otoliths and their concentrations are influenced by a number of factors, including geology, water chemistry, temperature, and physiology (Arslan and Paulson 2003). Elements that make the best stock delineators or recorders of the environment are those whose incorporation into the otolith consistently reflect the water chemistry the fish inhabit and are not under strict physiological or biological control. For these reasons, Sr

is the most commonly used element. Sr and Ca are both alkaline earth metals and are chemically similar. Because of similar valance, ionic radius, and crystal structure, Sr readily substitutes for Ca in the aragonitic crystal lattice of otoliths (Radtke and Shafer 1992; Farrell and Campana 1996). However, it has been found that it is not the absolute concentration of the element of interest, but the ratio of the element to Ca that is the most relevant measure of relative uptake (Lehtonen et al. 1992; Rieman et al. 1994; Campana 1999). Studies have shown that the uptake of some elements in otoliths, such as Sr, is a function of the available Ca in the ambient water (Campana 1999). Strontium, unlike Ca, is not subject to strict physiological regulation, so the Ca biochemical processes cannot distinguish between Ca and Sr; thus the concentrations in otoliths are more likely to reflect the concentrations in the environment (Campana 1999; Markwitz et al. 2000).

Though Sr:Ca ratio has been the most widely used elemental ratio in otolith microchemistry studies, a number of different elements also have been used. Other alkaline earth and alkaline metals, such as Mg, Ba, and Li, are found in otoliths in concentrations of a few parts-per-million and have been successfully used in otolith microchemistry studies (Arslan and Paulson 2003). For example, Ba, forms a carbonate that is isostructural with aragonite and can replace the Ca in the crystal lattice of the otolith, just as Sr does, and its use in otolith microchemistry studies is becoming more common. Controlled studies have shown that the incorporation of many alkaline earth metals into the otoliths is directly related to the ambient water, thus demonstrating their

potential utility (Bath et al. 2000; Wells et al. 2000; Elsdon and Gillanders 2003; Wells et al. 2003).

The rare earth elements (REE), such as La, Ce, and Sm, are another group of elements that could potentially prove useful for otolith microchemistry studies; however, there have been few studies that have investigated their use (Arslan and Paulson 2003). Ennevor (1994) and Ennevor and Beames (1993) successfully experimented with using REEs for mass marking juvenile coho salmon *O. kisutch* by increasing concentrations of these elements in the water in which the fish were reared. Concentrations of REEs in otoliths are related to their concentrations in the ambient water (Arslan and Paulson 2003); therefore, REEs could potentially be useful environmental indicators.

Transition metals (e.g., Zn, Mn, Fe, Cu, Cd, and Hg) also appear to have application to otolith studies (Campana 1999). Though some studies suggest the concentrations in otoliths for some of these elements reflect their availability in the environment (Papadopoulou et al. 1980; Johnson 1989; Campana 1999), data on uptake regulation for others are limited and they might not be incorporated into otoliths as well as into muscle or bone (Campana 1999).

Elements such as Na, K, S, P, and Cl are commonly found in otoliths at concentrations > 100 ppm, and have often been measured because they are easily detected by most analytical techniques (Campana et al. 1995). However, these elements are all under physiological control and have been found to vary little among populations; therefore, their concentrations in the otolith may not reflect availability in the

environment thus limiting their usefulness for certain applications (Kalish 1989; Fowler et al. 1995b; Proctor and Thresher 1998).

Stable isotope ratio analysis has also been proven to be useful for a variety of otolith microchemistry applications. The measurement of stable isotopic variation in biogenic carbonates (e.g., corals and bivalves) has been used extensively in paleoclimate studies and studies of seasonality (Patterson et al. 1993) and are useful indicators of temperature, salinity, and trophic level (Campana 1999). The isotopes studied most extensively have been O and C, although the isotopes of Sr and S have also been used (Campana 1999). For oxygen ($^{18}O/^{16}O$, expressed as $\delta^{18}O$) and strontium ($^{87}Sr/^{86}Sr$, expressed as $\delta^{87}Sr$) isotope ratios, water is the primary source and studies have show that these isotopes are incorporated into otoliths in ratios close to the ratio of the water (Kalish 1991a; Kennedy et al. 1997; Thorrold et al. 1997a). However, carbon ($^{13}C/^{12}C$, $\delta^{13}C$) isotope composition is primarily derived from the diet (Gannes et al. 1997). Differences in $\delta^{18}O$ in the water are related to sea surface temperatures, evaporation rates, and freshwater inputs (Edmonds et al. 1999). Variation in $\delta^{13}C$ is a result of different metabolic processes that incorporate C, so plants with different photosynthetic pathways, and food derived from marine versus freshwater sources will have different carbon isotopic composition (Gannes et al. 1997). Sulfur isotopic ratios ($^{34}S/^{32}S$, $\delta^{34}S$) also vary between marine and freshwater food webs because of differences in synthesis of S-bearing compounds, but also vary with lithology, anthropogenic inputs, and atmospheric levels (Nriagu et al. 1991; Weber et al. 2002). Strontium isotope ratio ($\delta^{87}Sr$) in water will also vary with lithology and age of the rock of the watershed (Åberg

et al. 1989; Kennedy et al. 1997) and are independent of physiological regulation (Kennedy et al. 2002). This means that Sr isotope ratio analysis has the potential to be a more sensitive indicator of movement through environments than elements or other isotopic markers (Kennedy et al. 2002).

Although only a relatively few freshwater otolith microchemistry studies have been published (this includes a few studies interested in the freshwater growth region of the otolith of anadromous species), a variety of elements and isotopes have already been found to be useful markers in the freshwater environment. As mentioned previously, Wells et al. (2003) were able to correctly reclassify juvenile westslope cutthroat trout *O. clarki lewisi* into the stream from which they were collected with 100% accuracy, using Sr:Ca, Ba:Ca, and Mg:Ca. These same elements, plus Mn, were used to correctly identify river of origin of American shad *Alosa sapidissima* with over 80% accuracy (Thorrold et al. 1998a). Lapi and Mulligan (1981) used Zn and Ba to predict the lake of origin of adult sockeye salmon by analyzing the freshwater growth region of their scales. However, Sr was not useful because of low concentrations, which precluded accurate measurements given the analytical technique used (Lapi and Mulligan 1981). Bronte et al. (1996) used 10 elements to discriminate among spawning concentrations of lake herring in Lake Superior. The elements used included seven transition metals: Zn, Cd, Hg, Co, Cu, Ni, and Cr, plus Pb, As, and Se. The studies by Kennedy suggest that Sr isotope ratios in the vertebrae and otoliths of Atlantic salmon *Salmo salar* otoliths are effective natural markers that can be used to identify tributary of origin and movement within watersheds (Kennedy et al. 1997; Kennedy et al. 2000; Kennedy et al. 2002).

Ingram and Weber (1999) also found Sr isotope ratios to be potentially useful in identifying origin of chinook salmon *O. tshawytscha* in the Sacramento–San Joaquin river system, California. Sulfur isotope ratios also show promise as an indicator of hatchery versus wild origin, migration, and reconstruction of nutrition sources (Weber et al. 2002).

Measurement Methods

The analytical technique and its inherent properties (i.e., accuracy, sensitivity, precision, and resolution) has a great influence on which elements and isotopes can be measured in the otolith sample and therefore, to which applications the generated data can be applied. The variety of instruments that have been used to analyze the chemical composition of otoliths (at least 17) is a result of the development of new analytical techniques and the diversity of the objectives of otolith microchemistry studies. The instruments used in otolith microchemistry studies can be classified into two main categories: bulk analysis (or solution based) and microprobes. Inductively coupled plasma mass spectrometry (ICPMS), specifically isotope dilution-ICPMS (ID-ICPMS), is the most commonly used instrument in the bulk analysis category. The most commonly used microprobe instruments are wavelength dispersive electron microprobe (WD-EM), laser ablation-ICPMS (LA-ICPMS), and proton induced X-ray emission analysis (PIXE) (Campana et al. 1997). Secondary ion mass spectrometry (SIMS) was identified by (Campana et al. 1997) as one of the newer analytical methods that could be useful for the study of otolith microchemistry.

Bulk Analysis

Instruments that require dissolving of the sample were some of the first to be used in otolith microchemistry (e.g., atomic absorption spectrometry, Gauldie and Nathan 1977). Although these bulk analysis, or solution-based, instruments tend to have higher sensitivity, accuracy, and precision (Campana and Gagné 1995; Campana et al. 1997; Campana 1999), they lack the spatial resolution of microprobes because the whole otolith is typically dissolved and analyzed (Halden et al. 1995). Thus, elements of interest are averaged over the life of the fish and details of environmental history, such as detection of movement between different water bodies, cannot be retrieved. Bulk analysis, however, has additional advantages, including ease of sample preparation, ability to simultaneously detect multiple elements and their isotopes, and the availability of accurate and precise protocols (Edmonds et al. 1992; Campana 1999). Therefore, whole otolith analysis is particularly useful for stock discrimination when one is interested in detecting overall differences between groups (Campana 1999). With the development of ICPMS, which has detection limits in the ppb range (Fassett and Paulsen 1989), many more elements present in otoliths can be detected than was previously possible (Campana and Gagné 1995). The ability to detect a suite of elements simultaneously is an important aspect of this analytical technique, as it allows development of elemental signatures useful for classifying fish by stock or group with a potentially high degree of accuracy (e.g., Campana et al. 1994; Campana and Gagné 1995; Campana et al. 1995). Another analytical technique – ID-ICPMS – differs from traditional ICPMS in that the samples are spiked with known quantities of a stable

isotope for each of the elements of interest. Over 60 elements with more than one stable isotope can be measured with ID-ICPMS and concentrations can be measured with 0.25% accuracy and precision (Fassett and Paulsen 1989). Therefore, ID-ICPMS is more accurate, precise, and sensitive than electron microprobes (Campana et al. 1995). Thermal ionization mass spectrometry (TIMS) is another variant of isotope dilution mass spectrometry that is accurate to the parts-per-billion (Fassett and Paulsen 1989). TIMS has successfully been used to measure isotope abundances in the vertebrae and otoliths of fish (e.g., Kennedy et al. 1997; Kennedy et al. 2000; Kennedy et al. 2002).

The inability to take advantage of the temporal information stored in otoliths is a major disadvantage of bulk analysis techniques, especially when the goal is to gather information on the environmental history of the fish, identify the tributary or estuary of origin, or detect elemental marks in returning hatchery fish. In response to this limitation, several recent studies have sampled portions of the structures using micromilling apparatuses and the resulting samples were then analyzed using solution-based instruments. Portions of the otolith, or hard part, are sampled by milling very small amounts of otolith material from specific growth bands (e.g., Patterson et al. 1993; Smith and Patterson 1994; Gao 1999; Weidman and Millner 2000; Kennedy et al. 2002). In these studies the drilled paths ranged between 2 and 40 μm wide and as little as 10 to 40 μg of material was removed for analysis. This amount of material represented anywhere from 2 weeks to 6 months of the fish's life depending upon the growth rate of the fish at the time the section sampled was deposited (Smith and Patterson 1994). Typically 5 to 10 mg is the minimum amount of material needed for analysis but if the

analyzer is properly equipped, as little as 0.3 mg is needed (Campana 1999). Micromilling enables one to gather information on specific periods of a fish's life while taking advantage of the accuracy and precision of solution-based instruments. For example, Kennedy et al. (2002) were able to characterize the various life-history phases (hatchery rearing, stream residence, and marine residence) in Atlantic salmon and showed fine-scale movement of the fish within the watershed during the stream residence phase.

Microprobes

Many otolith microchemistry applications are well suited for using microprobes. Microprobes are beam-based instruments that are able to analyze samples in their solid form. Therefore, information can be collected from a specific area or from a series of points from the core to the edge of the structure. The changes in chemistry can then be correlated with the age and growth information stored in the otoliths in order to determine the timing of specific life history events such as migration to the marine environment (Howland et al. 2001) or physiological changes (Otake et al. 1994; Otake et al. 1997). A number of papers have described the various instruments (see Gunn et al. 1992; Radtke and Shafer 1992). These instruments use narrow (< 10 μm) electron, proton, laser, or ion beams that are relatively non-destructive. These beams either ablate material or produce X-ray emissions from the elements in the sample. The small diameter of the beam allows for the precise analysis of a given area. Microprobe analysis typically requires more sample preparation than bulk analysis. Otoliths must be mounted and sectioned or ground so that the nucleus and the concentric growth rings are

exposed just as is done for age and growth analysis. The following briefly describes some of the most commonly used instruments and discusses their relative advantages and limitations for otolith microchemistry applications.

Electron Microprobe. Electron microprobes were some of the first beam-based instruments used for otolith microchemistry studies due to their wide availability. As a result, they are perhaps the most critically evaluated analytical technique for otolith microchemistry studies (e.g., Gunn et al. 1992; Toole and Nielsen 1992; Zimmerman and Nielsen 2003). Electron microprobes use a focused beam of electrons to analyze its composition. The elements in the sample absorb the energy and change their electron state, thereby radiating element-specific X-rays (Coutant 1990). Major advantages of electron microprobes include a high spatial resolution, the ability to analyze many elements (atomic numbers from 11(Na) to 92 (U)), and because they can be relatively nondestructive to the sample surface.

There are two types of electron microprobes, energy dispersive (ED-EM) and wavelength dispersive (WD-EM). Both types of electron microprobes have spatial resolutions of about 3-μm. ED-EMs are multi-channel analyzers, able to acquire information on a broad range of elements simultaneously, whereas WD-EMs are single channel analyzers so the number of elements that can be analyzed at a time is dependent upon the number of detectors (typically 3 to 4, Gunn et al. 1992).

WD-EM is the more precise electron microprobe with minimum detection limits of a few hundred ppm whereas ED-EM has minimum detection limits of a few thousand ppm (Gunn et al. 1992). Therefore, electron microprobes are not sensitive or precise

enough to reliably detect all of the trace elements known to be present in otoliths (Coutant 1990; Kalish 1990; Gunn et al. 1992; Fowler et al. 1995a). Gunn et al. (1992) concluded that ED-EM is not useful for trace element analysis and life history transects of otoliths. Analyses using WD-EM are effectively limited to the analysis of the major elements and the few minor elements found in otoliths in concentrations greater than 100 ppm (Campana 1999). However, WD-EM has successfully been used in many studies where differences in trace elemental concentrations are large, such as in the differentiation of salmon of resident migratory and migratory origin (e.g. Rieman et al. 1994).

Laser Ablation Inductively Coupled Plasma Mass Spectrometry. LA-ICPMS combines the spatial resolution of beam-based instruments with the analytical capabilities of solution-based ICMPS (Campana et al. 1994). A high-powered pulsed laser is aimed at the sample and the photon energy is converted into kinetic energy (Thorrold et al. 1997b). The kinetic energy causes part of the sample to vaporize and the ionized sample is swept into a mass analyzer. LA-ICPMS is more sensitive than electron microprobes, typically having a detection limit in the ppb range. Another advantage of LA-ICPMS is that it requires little sample preparation when compared to other beam-based methods. The sample surface does not have to be polished as smoothly as is required for analyses using electron or proton microprobes. LA-ICPMS is also able to simultaneously detect the elements and their isotopes with atomic weights from 3 (Li) to 92 (U). This technique is locally destructive in that it leaves a pit where the laser beam hits the otolith. When this instrument was first developed, minimum

beam width was 30 μm (Wang et al. 1994; Fowler et al. 1995a); too large for detailed environmental history work. However, beam diameters are now as small as 10 μm (Thorrold et al. 1997b). The main disadvantage of LA-ICPMS is that the saline nature of otoliths reduces the sensitivity and capabilities of the ICPMS in measuring trace element concentrations (Arslan and Paulson 2003). It is also difficult to measure Ca at the same time as the minor trace elements because it is so abundant. In addition, there is a lack of primary standards on which to base absolute elemental concentrations (Wang et al. 1994).

LA-ICPMS has been used in otolith microchemistry studies that span all environment types (marine, estuarine, and freshwater) and has been used to identify tributary of capture (Wells et al. 2003), natal homing in marine and estuarine species (Thorrold et al. 2001), and stock identification (Campana et al. 1994).

Proton Induced X-ray Emission Analysis. Like electron microprobes, PIXE instruments analyze the energy spectra of characteristic X-rays emitted from a sample that has been bombarded with a particle beam. However, PIXE uses a beam of protons instead of electrons to bombard the sample (Braun 1987). The advantages of PIXE are that it is able to simultaneously analyze all elements present in the sample at detectable concentrations, data acquisition is fast, and PIXE is highly sensitive, with detection limits of less than 1 ppm (Johansson and Campbell 1988). PIXE is able to achieve beam widths as small as 10 μm (Gauldie et al. 1994). Micro-PIXE, a variant of PIXE, is able to analyze samples with beam widths as small as 1 to 5 μm (Johansson and Campbell 1988; Sie and Thresher 1992; Halden et al. 1995; Campbell et al. 2002). The sensitivity

of this instrument requires that great care be taken not to introduce contaminants (Sie and Thresher 1992). However, narrow beams (Campbell et al. 1995) can heat the otolith and result in specimen damage, plus if the sample is too thin (< 50 μm) the potential exists to inadvertently analyze the substrate on which the otolith is mounted (Campbell et al. 1995). Another disadvantage is that high count rates of major elements can negatively effect detection limits of trace elements (Campbell et al. 1995). To adjust for this, the low energy X-rays produced by major elements, such as Ca, are attenuated using aluminum foil (Coote et al. 1991; Halden et al. 1996). Consequently, most otolith microchemistry studies using this instrument report absolute concentrations of the elements measured and not element:Ca ratios.

PIXE has been used in studies ranging from pollution detection (Saquet et al. 2002), discrimination of resident from migratory individuals (Howland et al. 2001), and identification of different life history/migration strategies (Howland et al. 2001; Limburg et al. 2001). For example, Limburg et al. (2001) used elemental mapping with PIXE to document a variety of migration strategies in anadromous Baltic sea trout *Salmo trutta*; including the surprising discovery of a life history form that does not have an obligatory freshwater phase for reproduction.

Secondary Ion Mass Spectrometry. Secondary ion mass spectrometry (SIMS) is one of the newer instruments and has been identified by Campana et al. (1997) and Radtke and Shafer (1992) as an analytical technique that could be useful for the study of otolith composition. SIMS uses an ion beam instead of electrons or protons to analyze the sample. The ion beam strikes the sample and causes ions and neutral particles to

sputter off the surface of the sample; the ions are then swept into a mass analyzer and counted (Braun 1987). The data can be displayed as an image of the spatial distribution of a particular element or isotope, a depth profile of a single ion as a function of analysis time, or a mass spectrum of a single spot (Radtke and Shafer 1992). Sample preparation for analysis with SIMS is similar to the preparation required for electron and proton microprobe analysis, in that the surface of the sample requires careful polishing. The sample also needs to be mounted on a conductive surface, such as a silicon wafer, or covered with a thin coat of conductive material (e.g., Au or C) because otoliths are non-conductive and would otherwise charge during analysis and result in reduced ion counts. There are two main classes of SIMS instruments: dynamic SIMS and static time-of-flight SIMS (ToF-SIMS). Dynamic SIMS has greater sensitivity than ToF-SIMS and quantification of sample composition is easier because it uses a higher energy beam and ablates a relatively large amount of material compared to ToF-SIMS, which only samples from the top two atomic layers of the sample (Seah and Briggs 1992). Dynamic SIMS has been used by Friedland et al. (1998) to measure Sr:Ca ratios when investigating growth and maturation in Atlantic salmon and by Weber et al. (2002) to demonstrate that S isotope ratios could be used to reconstruct chinook salmon life histories. Also, Seyama (2003) and Seyama et al. (1991; 1992) have demonstrated the ability of fast atom bombardment SIMS (a dynamic SIMS variant) to measure the chemical composition of otoliths. But to my knowledge, static ToF-SIMS has not been used to analyze the microchemical properties of otoliths.

The features of ToF-SIMS that would make it useful for otolith microchemistry are its ability to detect a large number of elements and their isotopes simultaneously (H to U), its sensitivity, and high spatial and mass resolutions that can be obtained. Both positive and negative ions can be studied using ToF-SIMS; therefore, most elements in the periodic table can be detected (Radtke and Shafer 1992). Detection limits are typically in the parts per million and can be in the parts per billion range for some elements (Jede et al. 1992; Radtke and Shafer 1992; Campana et al. 1997). SIMS could potentially detect elements with concentrations in the parts per billion, such as Cd, Sn, Rb, and U, which are difficult to detect using LA-ICMS or PIXE (Campana et al. 1997). ToF- SIMS is capable of higher spatial resolution (smaller beam diameters) than electron microprobes (Radtke and Shafer 1992) or even dynamic SIMS (Schueler 1992; Seah and Briggs 1992). Beam diameters as small as 0.5 to 1 μm can be achieved depending upon the primary ion used (Braun 1987; Radtke and Shafer 1992). ToF-SIMS also has better mass resolution (separation of intensity peaks), and mass determination accuracy than dynamic SIMS (Schueler 1992; Seah and Briggs 1992) Although ToF-SIMS has a high spatial resolution and highly sensitive, there is a tradeoff between the two (Radtke and Shafer 1992). The sensitivity of ToF-SIMS can vary widely between different elements and even with the same element in different matrices (Braun 1987). These matrix-dependent sensitivities make it difficult to quantify elemental concentrations using ToF-SIMS; therefore, it is difficult to convert the ion count measurements into absolute concentrations as with other analytic techniques such as LA-ICMPS. Nonetheless, ToF-

SIMS has the potential to be successfully applied to otolith microchemistry studies including investigations of life history variation and stock structure.

Additional Considerations

Often it is the environmental effect on the composition of otoliths that is of interest to otolith microchemistry researchers; however, there are a number of other factors that contribute to "noise" in the measurement making it more difficult to detect the "signal" of interest. Sources of variation include physiological and ontogenetic effects, storage and handling of otoliths, and measurement error associated with the analytical technique used.

In addition to the ambient water chemistry, incorporation of elements into otoliths is regulated by a combination of factors such as temperature, pH, metabolic rate, and other physiological changes. One of the better documented examples is the change in Sr:Ca in the otoliths of eels (*Anguilla* spp. and *Conger myrisaster*) as they metamorphose from leptocephali to glass eels (Otake et al. 1994; 1997; Arai et al. 1999). For example, Otake et al. (1997) found that Sr:Ca in the otoliths of leptocephali decreased at the onset of metamorphosis, despite the stable environmental conditions of the costal waters where these fish were collected. Otake et al. (1994; 1997) note that eel leptocephali contain elevated amounts of gelatinous extracellular matrix that is composed of Sr-philic sulfated glycosaminglycans (GAGs), which contributes to high Sr content in the endolymph. As the GAGs breakdown during metamorphosis, the body Sr content decreases, leading to a decrease in otolith Sr:Ca (Otake et al. 1997).

Stress, change in growth rate, or onset of reproductive activity, may be related to levels of Ca-binding proteins in the blood plasma and affect otolith composition (Kalish 1989). Friedland et al. (1998) found that Sr:Ca in mature Atlantic salmon otoliths peaked prior to spawning migration and attributed it to maturation. They also found an inverse relation between growth rates and Sr:Ca. This relation was also reported by Sadvoy and Severin (1992; 1994) for white grunt *Haemulon plumieri* and red hind *Epinephelus guttatus*.

Once the otoliths are collected for microchemical analysis, methods of storage and handling are an important consideration before starting any study. Milton and Chenery (1998) and Proctor and Thresher (1998) found that the element concentrations in otoliths can be affected handling, storage, and preparation methods used. This can be of concern when otoliths are collected or prepared by multiple organizations or laboratories, or when obtained from archived samples. It has commonly been assumed that the composition measured is the true composition of the structure being analyzed and that the elemental composition remains the same even after the fish is sacrificed (Proctor and Thresher 1998). Otoliths are often left in the fish for a period before being removed and are stored frozen or in ethanol. Milton and Chenery (1998) reported that of the seven elements detected in the otoliths of *Tenualosa toli*, using LA-ICPMS, Li, Mn, and Sr did not differ among treatments (e.g., freezing, storing in alcohol, storing dry) but Na, Mg, Co, and Ba did. Proctor and Thresher (1998), using electron microprobes, found elements associated with the organic matrix (Na, K, S, and Cl) were more affected by handling and storage than Ca and Sr, which are associated with the aragonite.

Handling and storage treatments in this study included immediate extraction and delayed extraction of otoliths, storage in chilled seawater and ethanol, and short-term immersion in tap water and distilled water. Preparation of otoliths for analysis (sectioning, grinding, and polishing) can also have an effect on otolith composition, but as with other treatments, Ca and Sr were found to be robust to common methods of otolith preparation (Proctor and Thresher 1998). However, care must still be used when handling, storing, and preparing otoliths for microchemical analysis, to minimize post-mortem artifacts because there are many steps in the processing of samples that can lead to contamination through addition, depletion, or re-distribution of elements in the otoliths (Proctor and Thresher 1998). To this end, great care is taken in handling and preparing otoliths for analysis. Precautions include using only non-metallic, acid-washed tools and glassware, using only ultra-pure water and chemicals to wash or dissolve otoliths, and preparing otoliths under a positive flow fume hood (e.g., Fowler et al. 1995b).

The measurement precision and accuracy of the analytical technique is also of great importance, even more so as otolith microchemists push the capabilities of the instruments to detect very small differences in chemical composition of otoliths and other hard parts. Campana et al. (1997) were concerned about the proliferation of instruments used, their precision and accuracy, and the ability to make comparisons among the studies. In a blind experiment using both artificial and actual otoliths, they found that none of the four most popular beam-based techniques (ED-EM, WD-EM, LA-ICMPS, and PIXE), were clearly superior, because they each performed well with different subsets of elements (Campana et al. 1997). They found that trace elements

(e.g., Fe, Ni, Cu, and Ba) were best measured with LA-ICMPS and PIXE, while electron microprobes performed better at measuring K and Na concentrations. Campana et al. (1997) also noted that there was considerable variation in results from different laboratories using the same technique, which was likely due to laboratory-specific operation conditions and data reduction methods.

To reduce such variation in otolith microchemistry studies, Campana (1999) suggested that samples be analyzed in a random order to reduce the effect of instrument drift and Campana et al. (1997) proposed that a matrix-matched certified reference material (CRM) be developed to ensure accuracy in the measurement of elemental concentrations and consistency of measurements. Instrument drift (or change in sensitivity) can be a significant concern for most analytical techniques, even if standards are used (Campana 1999). Therefore, Campana (1999) suggested that samples be analyzed randomly so that the analysis of any particular group is spaced over the entire analysis period. In addition, most studies use some means of calibrating the instrument not only to be able to convert detector counts to concentrations, but to also ensure internal consistency (i.e., repeatability of measurements) within and between session, and for correcting for instrument drift within sampling sessions. There has been a push to develop a matrix-matched CRM made of otoliths. Using a matrix-matched standard is important because detection limits of various elements can vary depending on other elements present and the matrix of the sample being analyzed (e.g. ICMPS, and ToF-SIMS).

I examined 50 papers published since the experiment by Campana et al. (1997) to assess the use of standards (particularly matrix-matched standards) in otolith microchemistry studies. Between 1999 and 2003, 12 studies (24%) did not explicitly state if a standard was used, nor did they reference the methodology used for calibration. Of the studies that did not report using standards, 10 were studies that used one of the microprobe techniques. Studies that employed PIXE were least likely to report if a standard was used (Table 2.1). In the remaining 38 studies, a variety of standards were used. The most common method of calibration for the solution-based techniques (ICPMS and ID-ICPMS) was addition of known quantities to dissolved otolith samples. A number of studies used the CRM made from red emperor *Lutjanus sebae* otoliths that was produced by Yoshinaga et al. (2000). Studies that analyzed isotope ratios – isotope ratio mass spectrometry (IRMS) and TIMS, used NIST standard reference materials (SRM) to calibrate the instruments (although the exact standard used for the IRMS studies were not always specified). Standards used to calibrate the microprobes ranged from ion implantation of specific elements into actual otoliths (Gauldie and Markwitz 2002) to steel (Campbell et al. 2002). Standards used in LA-ICPMS studies included bone meal, aragonite solid, and liquid standards. A number of the studies referenced the procedures of Rosenthal et al. (1999), which alleviate the need for matrix matching with solid samples (Wells et al. 2003). WD-EM studies consistently used NIST strontianite and calcite standard reference materials to calibrate the instruments, although two studies did not reference the use of any standards.

Many of the standards used were not developed for otolith microchemistry and may therefore be of questionable value. Jackson et al. (1993) cited this as a problem in the field of geology. While exact matrix matches may not always be required (Wang et al. 1994), ideally primary standards need to be developed with matrix properties that match the sample of interest. Otoliths, in particular, have distinctive organic matrices that could affect the analysis of the chemical composition. The CRM produced by Yoshinaga et al. (2000) has been used in a number of studies (e.g., Yoshinaga et al. 1999; Arslan and Paulson 2003), but these have all been bulk analysis techniques. Campana et al. (1997) attempted to produce artificial otoliths, with known concentrations of elements, to be used as standards for microprobes, but had limited success because of the glass used to bind the otolith powder. An alternative to using a binder, such as glass, is to press the ground otolith powder into a disc to produce an artificial otolith (see Chapter 3). A new and promising technique to produce solid, matrix-matched otolith standards is through a process called ion implantation. Gauldie and Markwitz (2002) demonstrated the feasibility of this technique by implanting a known number of Sr atoms into an otolith section, and measuring the concentration of Sr in the otolith with an estimated error of 10%. Ion implantation has an advantage over compressed otolith powders in that the matrix and crystal structure in the otolith is preserved (Gauldie and Markwitz 2002).

Discussion

The field of otolith microchemistry is growing and continually evolving and it is clear that there is still much to be learned. Many of the applications have yet to reach

their full potential (Campana 1999). The constant development and improvement of analytical techniques, in the areas of accuracy, sensitivity, and spatial resolution, has played an important role in the advancement of the field and will continue to do so into the future. As new instruments are employed, it is important that their efficacy for analyzing otoliths be tested and compared to existing technologies in order to determine if the technique should be abandoned or reevaluated, as viewed in the context of the research and development model of Jones (Figure 2.1). However, Thresher (1995) cautioned otolith microchemistry researchers to be cognizant of several potential problems that need to be acknowledged. He said that otolith microchemistry researchers must realize that: 1) "biologist are not physicists" and that otolith microchemical analyses are not as simple as "otoliths in, composition out"; 2) the analytical and statistical aspects of this type of research are complex and as biologists many of us are novices; and 3) we need to recognize that otoliths are not perfect records of an individual fish's environmental and physiological history, hence considerable variation is likely to be encountered. In order to avoid these problems Thresher recommends that methodological studies need to be done and the analytic and statistical techniques used need to be critically evaluated. In addition, researchers need to communicate amongst each other so we do not independently make the same mistakes.

Only a few studies have critically evaluated the use of specific instruments to analyze the chemical composition of otoliths. Electron microprobes were evaluated by Gunn et al. (1992), and Toole and Nielson (1992) tested their precision. More recently, Zimmerman and Nielsen (2003) revisited the subject because a standard protocol for

measuring Sr in otoliths using WD-EM does not exist and the methods used vary among laboratories. Similar studies should be conducted for the any instrument used to analyze the chemical composition of otoliths and there should be emphasis placed on the standardization of methods.

Table 2.1. Summary of standards used for the calibration of microprobe instruments. Information is from 16 out of 26 papers published between 1999 and 2003 that reported using a standard.

Analytical method	Standard used	*n*	References
LA-ICPMS	NIST-SRM 612 (glass)	2	Elsdon and Gillanders (2003, 2002)
	NIST-SRM 1486 (bone)	1	Veinott et al. (1999)
	Aragonite solid	1	Thorrold et al. (2001)
	Liquid standard	1	Wells et al. (2003)
PIXE	Ion implantation into otolith	1	Gauldie and Markwitz (2002)
	Basalt standard	1	Howland et al. (2001)
	NIST-SRM 1155 (steel)	1	Campbell et al. (2002)
WD-EM	NIST SRMs strontianite and calcite	7	Zimmerman and Nielsen (2003) Benson and Fitzsimons (2002) Jessop et al. (2002) Zimmerman and Reeves (2002) Arai and Miyazaki (2001) Limburg et al. (2001) Arai et al.(1999)
FAB-SIMS	Pressed $CaCO_3$ powder	1	Seyama (2003)

The research has shown that otolith composition is not controlled by a single factor and that the combination of factors involved may be species specific. However, we still lack adequate understanding of the factors that influence the incorporation of elements into the calcified structures of fish and this is limiting progress in several areas (Campana 1999). For example, we do not fully understand the incorporation of elements into the otolith protein, or why there is preferential uptake of certain elements over others. A better understanding of these processes will help researchers determine which elements are better environmental indicators (Campana 1999). In addition, it will help in separating the chemical signal from the noise.

The otolith microchemistry studies to date have demonstrated the vast potential of this field of research and have already made valuable contributions to fisheries science. Already, there are studies whose findings suggest that fisheries scientists need to rethink some previously held beliefs. For example, Sr:Ca analysis of otoliths of maturing Japanese eels *Anguilla japonica* and Atlantic eels *A. anguilla* suggest that the freshwater migration phase of the life history of these species is not obligatory (Tsukamoto et al. 1998). Other studies have also demonstrated the power of otolith microchemistry over genetic analysis for stock identification. Both Bronte (1996) and Thorrold et al. (2001) were able to identify separate spawning stocks in lake herring and weakfish, respectively indicating spawning site fidelity. However, corresponding genetic studies of the same assemblages were not able to differentiate among the stocks. Campana (1999) posits that the strength in otolith microchemistry research lies in the application of this technology with other complementary tools, such as genetic analysis

or tagging. However, this requires a concerted effort to conduct comprehensive evaluations of the analytic techniques used and a better understanding of the role of the environment and physiology on the concentration of the different elements in order to effectively unlock the information stored in the calcified tissues of fish and exploit it to its full potential.

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CHAPTER 3

APPLICATION OF TIME-OF-FLIGHT SECONDARY ION MASS SPECTROMETRY TO THE STUDY OF FRESHWATER FISH OTOLITHS

Introduction

Fish otoliths have unique chemical and chronological properties that provide a permanent record of the life history of individual fish including age, growth rate, and environmental history – information critical to understanding the ecology of fish and for the effective management of fisheries (see Campana and Thorrold 2001 for a review). Sagittal otoliths are complex biogenic mineral structures composed primarily of aragonitic calcium carbonate (> 90%) in an organic matrix (< 8%) with the remainder (2%) being trace and minor elements (Campana 1999). Trace and minor elements are incorporated from the surrounding endolymph fluid onto the surface of the otoliths as the fish grow (Campana 1999). Because changes in the aquatic environment influence the composition of the endolymph, changes in the environmental conditions such as salinity, temperature, and water chemistry are thereby reflected as changes in the otolith microchemistry (Kalish 1991; Campana 1999).

The microchemical analysis of otoliths has been used in a number of areas in fisheries science. Stock (or population) identification and structure have been one of the main applications (Edmonds et al. 1989). Other applications have included determination of the environmental and early life histories of fish. For example, discrimination between anadromous and non-anadromous (resident) salmonids (Rieman

et al. 1994; Howland et al. 2001), reconstruction of migration history (Secor 1992; Limburg 1995; Arai and Miyazaki 2001; Limburg et al. 2001), reconstruction of salinity (Limburg 1995) and temperature histories (Weidman and Millner 2000), and identification of tributary of origin in anadromous fish (Kennedy et al. 1997; Kennedy et al. 2002). To date, much of the research has focused on marine and anadromous species because freshwater generally has lower concentrations of elements than saltwater and, until recently, instruments with the sensitivity to accurately determine the distribution and variation of trace concentrations of elements in otoliths have not been available.

A variety of analytic techniques have been used in otolith microchemistry studies. This is due to the diversity of objectives in otolith microchemistry studies, the fact that this is a developing area of research with few comprehensive studies on the accuracy and precision of the techniques being used, and little standardization of methods (Gunn et al. 1992). The instruments that have been used fall in two categories: bulk analysis methods (e.g., inductively-coupled plasma mass spectrometry; ICP-MS) and microprobes. Bulk analysis, or solution-based, techniques are advantageous in that sample preparation is relatively easy, a suite of elements can be detected simultaneously, and these techniques tend to be accurate and precise (Edmonds et al. 1992; Campana 1999). However, the temporal information stored in the otoliths is lost because these instruments require the otolith material to be dissolved (typically the whole otolith); therefore, the concentrations of the elements of interest are averaged, or integrated, over the life of the fish. This is problematic when the goal is to gather information on the environmental history of the fish (e.g., migration patterns), when trying to identify its

origin (i.e., natal stream, lake, or estuary), or determining the timing of life history events such as age at migration. Bulk analysis methods have been used to develop some temporal/life-history information through "micro-milling" techniques where material is dissected or "milled" from a specific region in the otolith, collected, and then digested for analysis with standard solution-based techniques. These methods require extensive preparation, and are limited when used for otoliths of small fish because of the amount of material required for analysis.

Microprobes are advantageous for applications with a temporal component because specific areas in a cross-section of an otolith can be analyzed and two-dimensional elemental maps can be obtained, thus providing information on elemental composition over the entire life of the fish. This compositional information can then be correlated with the chronological information in the otoliths to provide unique insights into the environmental history of individual fish. A number of microprobes, employing a variety of beam types (e.g., laser, electron, proton, and ion), have been used in otolith microchemistry studies. The beams in these instruments are relatively nondestructive and often can focus to less than 10 μm. The small diameter of the beams allows for precise analysis of a given area such as the otolith nucleus, or along a growth axis of the otolith, and can provide high-resolution temporal information (otoliths usually grow 1-5 μm/day, Markwitz et al. 2000). Electron microprobes, specifically wavelength dispersive electron microprobes (WD-EM), have been commonly used due to their relatively low cost, ease of use, accessibility, well-characterized standards, and data reduction analytical protocols. The WD-EM technique has been thoroughly examined

for use in otolith microchemistry studies (e.g., Gunn et al. 1992; Toole and Nielsen 1992; Zimmerman and Nielsen 2003) and is well-suited for some applications. However, the sensitivity of WD-EMs (100 ppm range) effectively restricts analyses to the major elements and a few minor elements found in otoliths and limits their use in detecting trace (< 100 ppm) elements (Campana 1999).

Secondary ion mass spectrometry (SIMS) has been identified as a method that could be useful for the study of otolith microchemistry (Radtke and Shafer 1992; Campana et al. 1997). Some studies (e.g., Friedland et al. 1998; Weber et al. 2002) have used dynamic SIMS (also known as ion microprobes), or fast atom bombardment SIMS (Seyama et al. 1991; Seyama et al. 1992), but to my knowledge, static time-of-flight SIMS (ToF-SIMS) has not been used to analyze the microchemical properties of otoliths. The ability of ToF-SIMS to quasi-simultaneously measure peak intensities over a virtually unlimited mass range, its high spatial resolution, mass resolution, and mass determination accuracy, lends itself to applications not possible with conventional dynamic SIMS (Schueler 1992; Seah and Briggs 1992). However, dynamic SIMS typically has greater sensitivities than ToF-SIMS and quantification of sample composition is easier (Seah and Briggs 1992). The features of ToF-SIMS that make it useful for otolith microchemistry are: its ability to non-destructively sample a specimen (allowing repeated sampling of a site); detection of a large number of elements and their isotopes simultaneously (H to U); high spatial and mass resolution, and sensitivity. Both positive and negative ions can be studied; therefore, most elements in the periodic table can be detected (Radtke and Shafer 1992). With sub-micron beam diameters, SIMS can

achieve higher spatial resolution than electron microprobes (Radtke and Shafer 1992). Detection limits are typically in the parts per million, and in the parts per billion range for some elements (Jede et al. 1992; Radtke and Shafer 1992; Campana et al. 1997) – sensitivity much greater than WD-EM, laser ablation ICP-MS (LA ICP-MS), or proton induced X-ray emission (PIXE). However, sensitivity can vary among different elements and even the same element in different matrices (Braun 1987). The matrix dependent sensitivities, and uncertainties related to standardization of surface compositions, make it difficult to use ToF-SIMS for quantitative studies. Nonetheless, ion counts obtained from ToF-SIMS have the potential to be successfully applied to otolith microchemistry studies.

In this study, I evaluated the precision and accuracy of ToF-SIMS for use in otolith microchemistry studies. My objectives were to: 1) test precision using an otolith "standard" with a matrix that matched real otoliths to be used to test the reproducibility of results (i.e., relative counts of the elements of interest) from session to session; 2) test whether ToF-SIMS results were comparable to a quantifiable analytic technique (WD-EM) that is generally accepted for use in otolith microchemistry studies; and 3) compare otolith composition with water samples collected from the same location as the fish in order to evaluate if differences observed in the otoliths were directly related to the chemistry of ambient water.

Methods

Otolith Preparation

Otoliths used in the ToF-SIMS analyses were collected from several species of fish and from several locations. These included otoliths from 19 westslope cutthroat trout *Oncorhynchus clarki lewisi* from four tributaries of the Coeur d'Alene River, Idaho; two sockeye salmon *O. nerka* from the Snake River, Idaho that had spent time in saltwater and were captured returning to their natal stream to spawn; 24 rainbow trout *O. mykiss* from the Missouri River and three of its tributaries, north of Helena, Montana; and 30 lake trout *Salvelinus namaycush* from three lakes in Yellowstone National Park (YNP), Wyoming (Chapter 4). The sagittal otoliths were extracted from the fish, cleaned as described by Fowler et al. (1995), and stored dry in acid-washed polyethylene vials. Thin sagittal sections of the otoliths were prepared using standard methods (Secor et al. 1992). Otoliths were mounted sulcus side up on glass microscope slides using thermoplastic polymer (Crystalbond 509), ground just past the sulcal groove with 2,000-grit wet/dry sandpaper, then polished with 0.3-µm aluminum oxide polishing powder. The other side of the otoliths was then ground and polished to the nucleus (Figure 3.1). Sample slides were cut to 1×1 cm around the otolith, in order to fit in the ToF-SIMS sample holder, and cleaned in baths of analytical grade hexane and methanol. The otoliths were sputter-coated with 15 nm of Au-Pd to give the samples a conductive surface and reduce sample charging during ToF-SIMS analysis. A final cleaning with hexane and methanol removed any surface contaminants. Clean methods were used throughout otolith extraction, preparation, and analysis. This included using only non-

metallic acid washed instruments, wearing latex gloves when handling the samples, and using Milli-Q water to clean the samples and equipment between grinding and polishing stages and between samples.

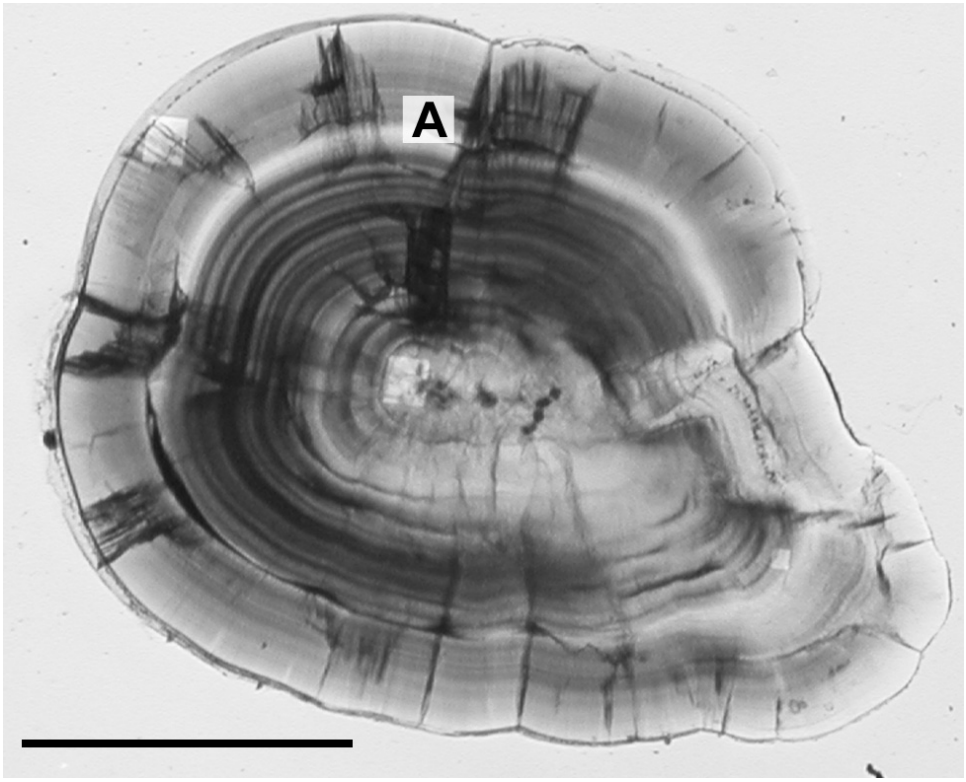


Figure 3.1. Thin sagittal cross-section of a 1 year-old westslope cutthroat trout otolith. Primordia (dark central spots), nucleus (immediately surrounding area), sub-annual growth bands, and annual mark (A) are visible. Bright squares in the nucleus and at the edge are the 60×60-µm areas where the Au-Pd coating was removed for ToF-SIMS analysis. The early growth zone is the area adjacent to the nucleus. Scale bar = 0.5 mm.

ToF-SIMS Analysis

Analyses were conducted in a Phi-Evan's triple focusing time-of-flight secondary ion mass spectroscopy system at the Image and Chemical Analysis Laboratory (ICAL) of the Montana State University Physics Department. Schueler (1992) gives a detailed description of the ToF-SIMS system used in this study. Once an area of interest on the

sample was selected, the Au-Pd coating was removed by sputtering a 60×60 μm area for 4 min. The sputtering also had the effect of removing surface contaminants. Positive ion spectra were collected using a pulsed energetic (15keV) primary Ga^+ ion beam, focused to 9×9 μm and aimed in the center of the cleared area. Analyses were run in high mass resolution mode. Adjusting the buncher, buncher delay, and sample potential while maintaining the beam focus maximized mass resolution. Even though the buncher potential degrades the focus of the incident Ga^+ beam it still stays within the sputtered area during spectral acquisition while maintaining a high mass resolution. Acquisition time for each analysis was 5 min. The length of acquisition was necessary in order to detect an adequate number of ions of the minor and trace elements present in the otoliths.

Several zones within each of the otoliths were analyzed. These included the nucleus/primordia (otolith center) in the cutthroat trout and sockeye salmon otoliths, the early growth zone (just adjacent to the nucleus) for the rainbow and lake trout otoliths, and the otolith edge for all fish. In addition, zones of freshwater growth and marine growth were analyzed in the two sockeye salmon otoliths. Variation within the otolith zones was estimated by analyzing multiple sites (2 to 4) within a zone (i.e., nucleus, otolith edge) for a randomly selected subset of otoliths in each group. In addition, measurement precision was estimated by replicating analyses at the same site (2 to 4 times) for randomly selected sites in a subset of the otoliths.

For each analysis, a full mass spectrum was produced and stored on disk, which allowed for determination of the ion counts for elements of interest at a later date with

WinCadence v.3.3 software (Physical Electronics, Inc.). I was mainly interested in measuring Ca and Sr in otoliths, in particular the ratio of Sr to Ca (Sr:Ca). Sr:Ca is commonly used in otolith microchemistry studies because Sr is physically and chemically similar to Ca and can substitute for Ca in the crystal lattice of the otolith (Radtke and Shafer 1992). The resulting strontium carbonate (strontianite) is isostructural to the aragonite in the otolith (Lippmann 1973). There is also a strong correlation between Sr:Ca in the otolith and in ambient water because Sr is not under strict physiological regulation and is thus more likely to reflect the environment than other elements such as Na and P, which are physiologically regulated (Campana 1999). In addition, normalization of Sr to Ca aids in reducing inter-sample variation (Campana et al. 1997) and compensates for variations in analytical conditions (Allison 1996). Other elements that may substitute for Ca in otoliths are also of interest to otolith microchemistry researchers. These elements include other alkali earth metals such as Ba or elements that form divalent cations such as Fe, Mn. I did not include such elements in this study because they were not measured as part of the water chemical analyses or the WD-EM analyses; therefore comparisons with the ToF-SIMS data could not be made.

Otolith 'Standard'

Examination of preliminary data revealed large variation among sessions in the relative ion counts within the same otolith. Therefore, a homogenous 'otolith' was required in order to be used in each session to ensure internal consistency of the measurements. Otoliths from about 500 hatchery-raised rainbow trout were extracted and cleaned as described previously. The otoliths (0.2 g) were ground into a fine powder

using an aluminum oxide mortar and pestle. The otolith powder was then mixed and sifted through 5-µm mesh nylon cloth. An artificial otolith was made by pressing a small portion of the otolith powder between two pieces of acid-washed acetate in an infrared spectroscopy press. The disc was mounted on glass slides like actual otoliths and polished with 0.3-µm polishing powder. The polish removed any loose otolith material and smoothed the sample surface. The sample was cleaned and sputter-coated with Au-Pd as previously described.

A series of spectra were collected when the otolith 'standard' was first made in order to establish a baseline Sr:Ca ratio. Analyses included four sites within the artificial otolith that were analyzed over three sessions. Two of these sites were analyzed multiple times within a single session. Two-factor analysis of variation (ANOVA) was used to determine if there were significant site (homogeneity of sample) or session (consistency between sessions) effects.

Once the baseline value was established, the otolith 'standard' was used at the beginning of each ToF-SIMS session. If the Sr:Ca fell outside of acceptable levels (± 2 SD), the ToF-SIMS settings were re-checked and adjustments were made until precision was within accepted levels.

Comparison of ToF-SIMS to WD-EM

As a means of testing the efficacy of ToF-SIMS for the microchemical analysis of fish otoliths, I compared the Sr:Ca of pairs of otoliths where one otolith was analyzed with ToF-SIMS and the other with WD-EM. A comparison to WD-EM was chosen because it is one of the beam-based instruments commonly used in otolith

microchemistry studies (Campana et al. 1997) and such data were available. The samples that were used for comparison included the otoliths from 19 westslope cutthroat trout and two sockeye salmon.

Preparation and elemental analysis of the otoliths analyzed using WD-EM followed the methods of Rieman et al. (1994). Preparation of the otoliths was similar to that for ToF-SIMS analysis except that 20 nm of carbon was used as a conductive coating instead of Au-Pd. Microchemical analyses were done with a Cameca SX-50 wavelength dispersive microprobe at Oregon State University and followed the procedures outlined by Toole and Nielsen (1992). A 1-kv, 50nA, 5-µm diameter beam was used for the analyses and the counting time was 40 s. Standards for Sr and Ca were strontianite ($SrCO_3$-USNM R10065) and calcite ($CaCO_3$-USNM 136321). The Sr:Ca for the WD-EM analyses are reported as normalized mole fractions.

The same zones within the otoliths were analyzed with WD-EM as were analyzed with ToF-SIMS. These included the nucleus/primordia and edge zone for all of the fish, plus the freshwater growth and saltwater growth zones for the sockeye salmon. Similar to ToF-SIMS analyses, multiple sites were analyzed within the different otolith zones and multiple analyses were done within a small area. Unlike ToF-SIMS, WD-EM creates a pit at the analysis spot; therefore, the beam had to be moved in order to replicate the analysis. In the case of the WD-EM analyzed otoliths, the nucleus was analyzed 10 times and the three locations on the edge of the otoliths were analyzed five times each, for each otolith.

The mean Sr:Ca within each zone was calculated for each otolith analyzed and compared with the mean Sr:Ca of the corresponding otolith of the pair, which was measured with ToF-SIMS. Simple linear regression was used to characterize the relation between Sr:Ca determined by the two methods. In addition, the variation within each zone and site with replicate samples were determined and the coefficients of variation (CV) were compared with the corresponding data from the ToF-SIMS analyses with Wilcoxon rank-sum tests.

Relation of Otolith Sr:Ca to Water Chemistry

In addition to comparing Sr:Ca from ToF-SIMS analyses to results from WD-EM analyses, I wanted to test if the differences in otolith Sr:Ca, determined using ToF-SIMS, were related to the Sr:Ca of the water that the fish were from, as has been shown in other studies (e.g., Campana 1999; Bath et al. 2000; Wells et al. 2003). Data for this comparison included westslope cutthroat trout otolith and water samples from four Coeur d'Alene River tributaries, rainbow trout otolith and water samples from the Missouri River and three of its tributaries, and lake trout otolith and water samples from three YNP lakes. In addition, the otolith Sr:Ca from the saltwater growth zones of the sockeye salmon was included in this comparison.

Water chemistry data for the Sr and Ca concentrations came from several sources. The Coeur d'Alene River samples (2 per stream over 2 years) and Missouri River samples (3 to 6 per stream, collected three times in 1 year, Chapter 5) were collected by taking surface samples from various locations within each of the tributaries and the mainstem river where fish were collected. The samples were processed in the

field according to standard protocols for elemental analysis (American Public Health Association 1998). Elemental concentrations were determined using standard protocols (American Public Health Association 1998) with a Fison's Instruments Accuris ICP optical emission spectrometer (analytic wavelengths for Ca and Sr were 317.93 and 407.77, respectively). Yellowstone National Park water samples from two of the three lakes were surface samples (4 samples per lake, Chapter 3). These samples were collected and processed in the field the same as the other water samples. Water samples from Yellowstone Lake, the third lake, were collected at various locations and depths around the lake over two years (n = 71) (Balistrieri et al. in press). Elemental concentrations for all YNP water samples were measured with a Perkin-Elmer Sciex Elan 6000 ICP-MS (Lamothe et al. 1999). The Sr:Ca of seawater (for comparison with the sockeye salmon) was water based on published concentrations of Sr and Ca in Pacific Ocean surface waters (Whitfield and Turner 1987).

Because the data for the otolith and water Sr:Ca were not paired for each sample (i.e., water samples were collected independently from when the otoliths were formed), I calculated the mean water Sr:Ca and the mean otolith Sr:Ca of all fish analyzed for each of the bodies of water sampled and plotted these data. Simple linear regression was used to characterize the relation between otolith Sr:Ca and the water Sr:Ca. In addition, distribution coefficients, the ratio of Sr:Ca in the otolith relative to the Sr:Ca in the water (Kinsman and Holland 1969), were calculated for each species of fish.

Results

Calcium Isotopes

Initial examination of the data suggested that because of the length of data acquisition and the predominance of Ca in otoliths, the mass analyzer was being "swamped" with too many ^{40}Ca ions, the major Ca isotope. That is, ion counts were underestimates of actual number of ^{40}Ca ions because of dead time of the analyzer. Because of this loss of ion counts, there was a non-linear relation between count rate and Ca abundance as the limits of the detector were approached (Figure 3.2). The relative abundances of the naturally occurring isotopes of Ca are stable and a linear relation would be expected between the ion counts of pairs of these isotopes with a slope equal to the ratio of their relative abundances, if the isotopes were present in the otoliths in their natural abundances and equally likely to be detected by the mass analyzer. For example, the slope of the ^{44}Ca ion count to ^{40}Ca should be constant at 0.022 (i.e., ^{44}Ca:^{40}Ca = 2.086/96.941). However, the relation between ^{44}Ca and ^{40}Ca ion counts was not linear and the proportion of ^{44}Ca ions to ^{40}Ca ions was greater than 0.022. Comparison of ^{42}Ca to ^{40}Ca ion counts show a similar relation. Furthermore, there appears to be a maximum number of ^{40}Ca ions (about 3.3×10^{6}) that can be counted (Figure 3.2). Therefore, ^{40}Ca counts could not be used to calculate Sr:Ca.

There was, however, a strong linear relation between ^{44}Ca and ^{42}Ca ion counts (Figure 3.3). Specifically,

$$^{42}\text{Ca} = (0.327 \pm 0.0004\ \text{SE}) \times {}^{44}\text{Ca} - (215.176 \pm 44.239\ \text{SE})$$

$$(r^2 = 0.999,\ P < 0.001).$$

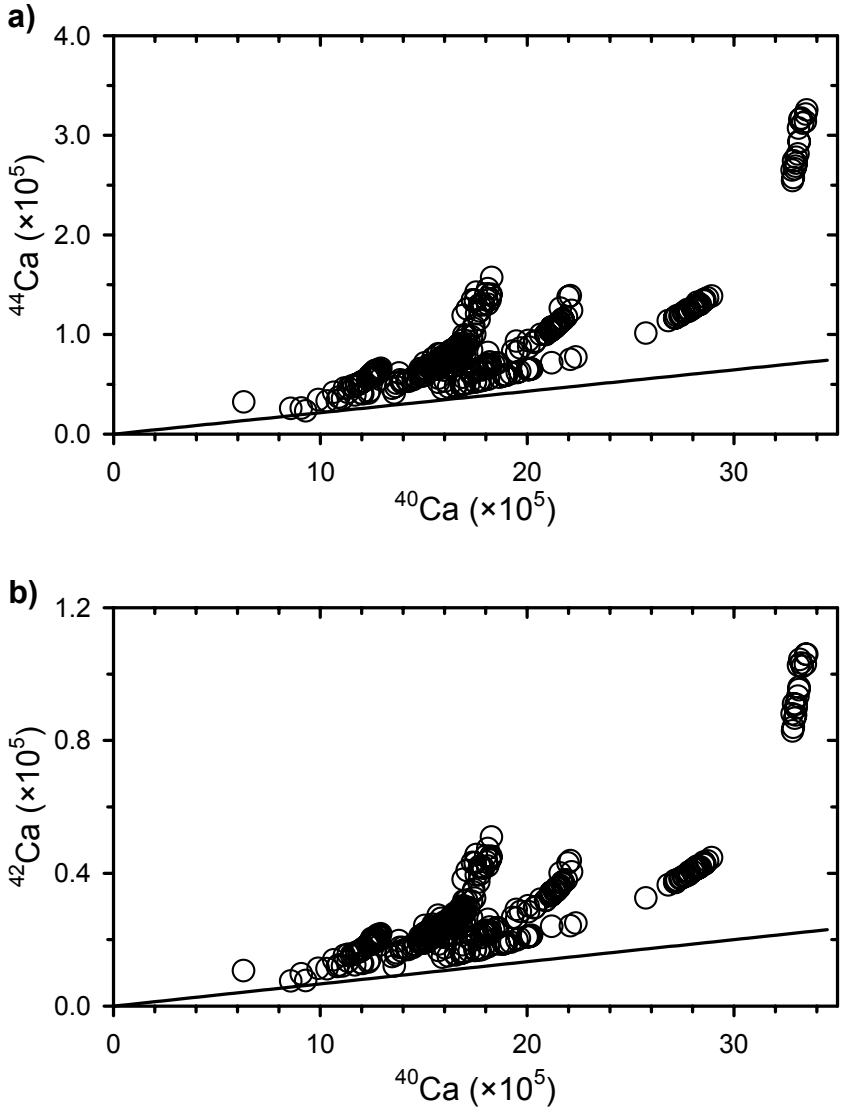


Figure 3.2. Comparison of ^{40}Ca ion counts to a) ^{44}Ca and b) ^{42}Ca ion counts from ToF-SIMS analyses of otoliths. Solid lines are the theoretical relation between ^{40}Ca and each of the other two isotopes based on relative abundances of these naturally occurring Ca isotopes. The slopes of the lines are the ratio of the relative abundance of each of the isotopes to ^{40}Ca (^{44}Ca:^{40}Ca = 0.0215, ^{42}Ca:^{40}Ca = 0.0067). n = 435.

The mean ^{42}Ca:^{44}Ca ratio was 0.324 (SD = 0.006, CV = 1.77%), equivalent to the slope of the regression forced through the origin. The slope of the relation between ^{42}Ca and ^{44}Ca was significantly different than the predicted slope of 0.310 (^{42}Ca:^{44}Ca =

0.647/2.086, *t*-test, $P < 0.001$). However, the linear relation holds true for the range of ion counts that were recorded. Therefore, ^{44}Ca ion counts could be used to calculate the Sr:Ca ratios. Furthermore, I could also estimate the expected ^{40}Ca counts and use those to calculate Sr:Ca ratios that would be comparable (in magnitude) to those obtained using other analytical techniques. Expected ^{40}Ca counts ($^{40}Ca_{adj}$), based on the ^{44}Ca ion counts, were thus calculated using:

$$^{40}Ca_{adj} = {}^{44}Ca \Big/ \left(\text{relative abundance of } {}^{44}Ca \Big/ \text{relative abundance of } {}^{40}Ca \right)$$

$$= {}^{44}Ca \Big/ \left(2.087/96.941 \right) = {}^{44}Ca \Big/ 0.0215.$$

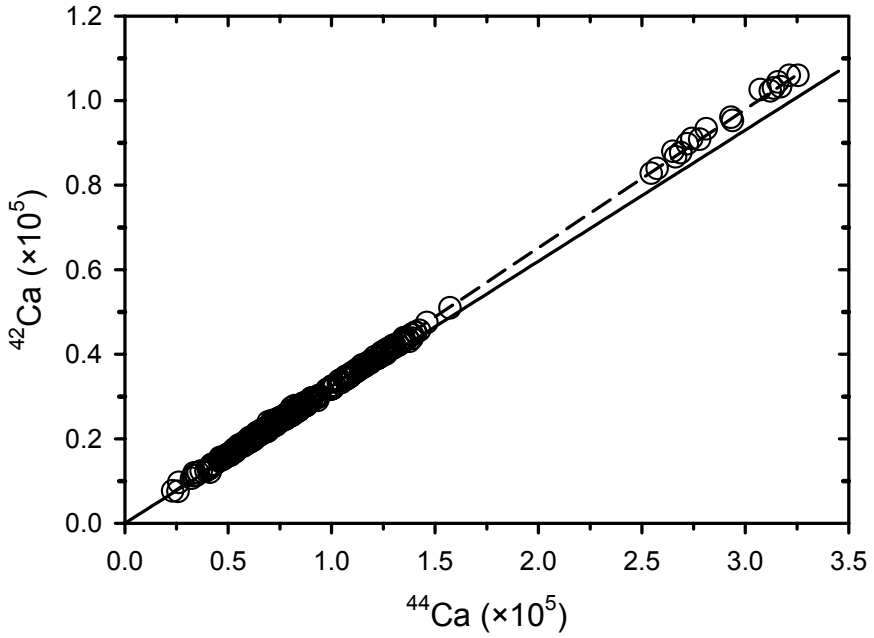


Figure 3.3. Comparison of ^{44}Ca and ^{42}Ca ion counts from ToF-SIMS analysis of otoliths. The dashed line is the simple linear regression line fitted to the data. The solid line is the theoretical relation between ^{44}Ca and ^{42}Ca based on the relative abundance of these naturally occurring isotopes of Ca ($^{42}Ca/^{44}Ca = 0.310$). $n = 435$.

Otolith 'Standard'

A total of 13 analyses were used to estimate the baseline Sr:Ca of the artificial otolith. The two-way ANOVA results indicated that the Sr:Ca ratios of the sites analyzed were not significantly different ($F_{3,7} = 0.28$, $P = 0.84$) and there was no significant session effect ($F_{2,7} = 3.18$, $P = 0.13$). In addition, the interaction was not significant ($F_{2,7} = 1.04$, $P = 0.42$). The overall mean Sr:Ca of all assays of the artificial otolith was 0.336×10^{-3} (SE = 0.006×10^{-3}). The mean square error of the model was 0.00039×10^{-3}, meaning that the coefficient of variation, or overall precision, was 5.8%.

The Sr:Ca of the standard fell within the ± 2 SD of the baseline mean after initial setup 63% (12 of 19 sampling sessions) of the time (Figure 3.4). After further adjustments of the instrument, the Sr:Ca was generally within the two standard deviation criteria (sessions 4, 6, 8, and 20). The number of sessions that yielded unacceptable results declined as experience with the ToF-SIMS increased.

Comparison of ToF-SIMS to WD-EM

There was a significant positive linear relation between Sr:Ca ratios obtained using ToF-SIMS and WD-EM (Figure 3.5). However, the edge zone Sr:Ca of four of the five cutthroat trout otoliths from a single stream strongly influenced the fit of the regression. The Sr:Ca ratios from the WD-EM analyses for the edge zone of these fish were about twice that obtained using the ToF-SIMS. When these samples were removed from the analysis, the amount of variation explained by the regression increased from 79 to 97% and the slope of the regression increased from 0.58 to 0.91, indicating a near 1:1 relation between Sr:Ca obtained with ToF-SIMS and WD-EM.

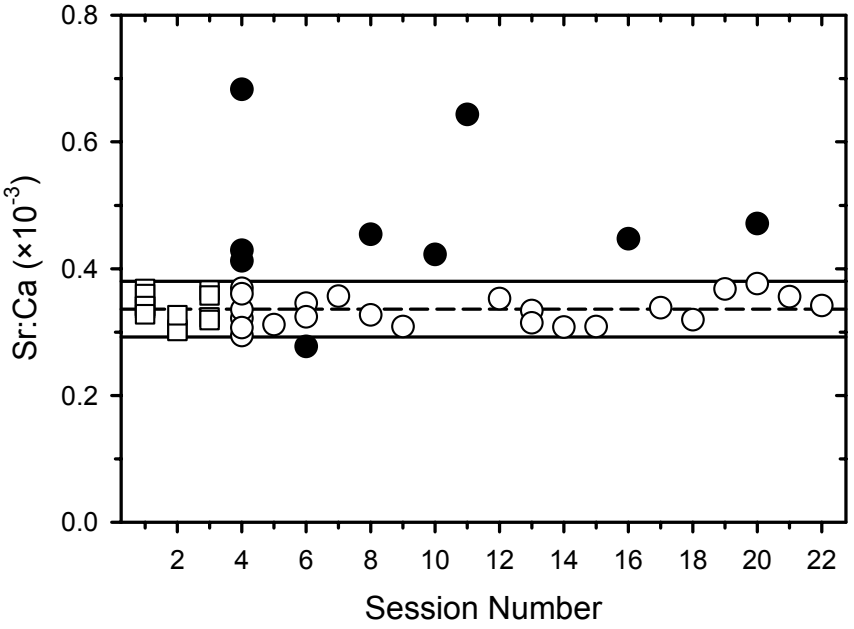


Figure 3.4. Artificial otolith Sr:Ca ratios used to check the consistency of ToF-SIMS from session to session. Baseline Sr:Ca (squares) was established over three sessions and used to calculate mean Sr:Ca (dashed line). If the Sr:Ca (filled circles) was outside the range of acceptability (± 2 SD, solid lines), adjustments were made to the instrument until the Sr:Ca was within specifications (open circles).

The relation between ToF-SIMS and WD-EM Sr:Ca with all of the data can be described by the linear equation:

$$Sr{:}Ca_{ToF\text{-}SIMS} \times 10^{-3} = (0.583 \pm 0.046\ SE) \times [Sr{:}Ca]_{WD\text{-}EM} + (0.153 \pm 0.082\ SE)$$

$$(r^2 = 0.787,\ P < 0.0001).$$

Removal of the high-leverage points resulted in the following relation:

$$Sr{:}Ca_{ToF\text{-}SIMS} \times 10^{-3} = (0.906 \pm 0.031\ SE) \times [Sr{:}Ca]_{WD\text{-}EM} - (0.0982 \pm 0.040\ SE)$$

$$(r^2 = 0.957,\ P < 0.0001).$$

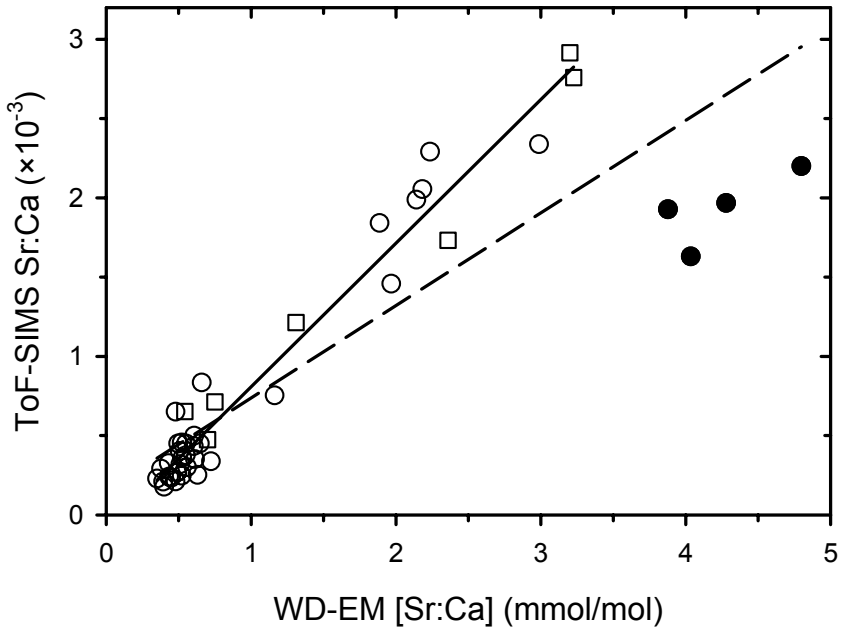


Figure 3.5. Comparison of mean ToF-SIMS Sr:Ca to mean WD-EM Sr:Ca from pairs of otoliths. Otoliths analyzed were from 19 westslope cutthroat trout (circles), and two anadromous sockeye salmon (squares). The dashed line is the fitted least square regression using all of the data. The solid line is the fitted regression of all of the data except for the edge zones of four cutthroat trout from a single stream (filled circles). n = 45 otolith-zone combinations.

Comparison of the replicate analyses of the cutthroat trout otoliths indicated that variation, measured as CVs, in the otolith zones were similar (Wilcoxon rank-sum test, P = 0.18) between the two analysis techniques (Table 3.1). However, site replication was significantly less (Wilcoxon rank-sum test, $P < 0.0001$) for the ToF-SIMS data than the WD-EM data.

Table 3.1. Comparison between ToF-SIMS and WD-EM of repeat sampling precision (measured as CV) within otolith zones and within otolith sample sites for westslope cutthroat trout from the Coeur d'Alene River system.

	Zone variation (%)					Site replication (%)				
	n	Mean	Median	Min	Max	*n*	Mean	Median	Min	Max
ToF-SIMS	15	12.46	7.23	0.54	42.36	19	7.10	5.68	0.55	29.07
WD-EM	38	15.36	14.46	2.88	47.82	58	19.18	19.24	2.88	45.67

Comparison to Water Chemistry

There was a strong linear relation between the Sr:Ca in the otoliths and in the water (Figure 3.6). The relation between otolith and water Sr:Ca can be described by the regression equation:

$$Sr{:}Ca_{otolith} \times 10^{-3} = (0.3234 \pm 0.02SE) \times [Sr{:}Ca]_{water} - (0.1285 \pm 0.07SE)$$

$$(r^2 = 0.9677, P < 0.0001).$$

The distribution coefficient of Sr:Ca between otolith and ambient water for all species of fish was 0.27 (SE = 0.015, n = 12). The distribution coefficient for westslope cutthroat trout from the Coeur d'Alene River system was 0.32 (SE = 0.027, n = 4), and 0.24 (SE = 0.011, n = 4 and 3 respectively) for rainbow trout from the Missouri River system and lake trout from YNP. The Sr:Ca for the water from Tom Lavin Creek (Coeur d'Alene) had the highest Sr:Ca at 6.17 mmol/mol, which was almost as high as the Sr:Ca of ocean water (8.9 mmol/mol), indicating that freshwater Sr:Ca can vary widely and can be almost as high as saltwater.

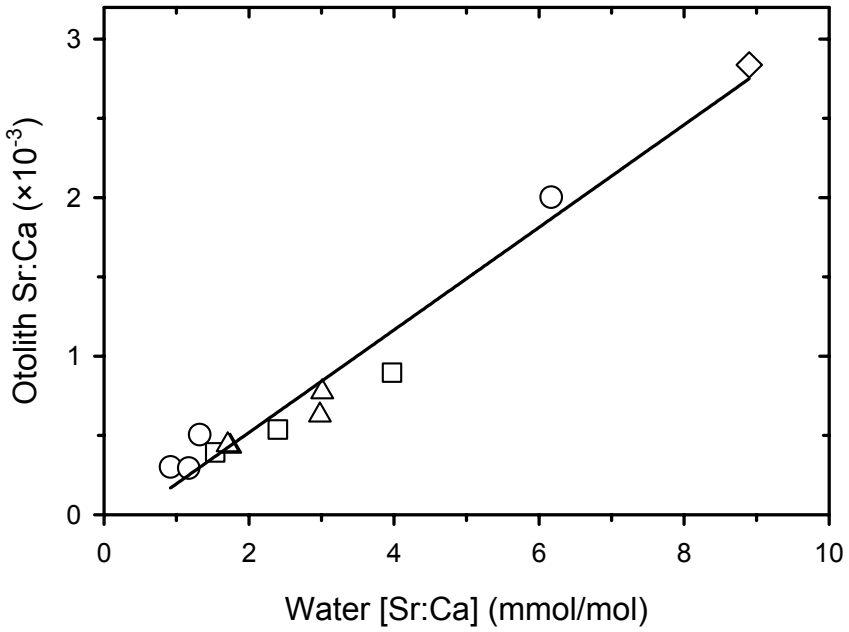


Figure 3.6. Relation of mean otolith Sr:Ca to mean water Sr:Ca. Data include ToF-SIMS analyses of westslope cutthroat trout from four tributaries of the Coeur d'Alene River (circles), lake trout from three lakes in Yellowstone National Park (squares), rainbow trout from the Missouri River and three of its tributaries (triangles), and the marine growth zone of two sockeye salmon (diamonds).

Discussion

The results presented in this work demonstrate the efficacy of ToF-SIMS for analyzing the composition of fish otoliths. This initial study focused on the measurement of Sr:Ca because of the relation between otolith Sr:Ca and the availability of these elements in ambient waters, plus the predominant use of this ratio in otolith microchemistry studies. One of the most important findings of this study, was the need to use ^{44}Ca or ^{42}Ca isotopes instead of ^{40}Ca to normalize Sr ion counts, when using ToF-SIMS for the analysis of otoliths. The non-linear relation of the less abundant isotopes to ^{40}Ca indicated that I was experiencing count loss, which would have lead to over

estimation Sr:Ca ratios especially when ^{40}Ca counts were nearing the maximum 3.3×10^{6}. The count loss is due to a high yield (> 1) of ^{40}Ca ions (i.e., more than one secondary Ca^{+} ion is generated per primary Ga^{+} pulse) and ToF-SIMS is designed such that only a single ion of particular mass can be detected per incident Ga^{+} pulse. This single ion count mode is not a problem for most ToF-SIMS applications because typically each Ga^{+} pulse generates much less than one ion of a particular mass (yield < 1). The linear relation of ^{42}Ca and ^{44}Ca indicated that the yield was < 1 for these isotopes and would therefore be useful for normalizing trace element counts. Normalizing Sr to a less abundant isotope of Ca when analyzing carbonates is not uncommon. For example, Allison (1996) normalized trace element count rates to ^{44}Ca to compensate for variations in analytical conditions for analyses of coral aragonite with an ion microprobe and Wang et al. (1994) normalized Sr count rates to ^{48}Ca in LA ICP-MS analysis of a fish scale.

Testing the efficacy of ToF-SIMS, analysis of the otolith 'standard' showed that I could reproduce Sr:Ca measurements from one session to the next with less than 6% variation. Because quantification in ToF-SIMS is difficult, internal consistency of the measurements is important in order to lend confidence in the interpretation of results. Matrix matched standards (or certified reference material) are essential for analytical quality assurance and their lack of availability has hindered research in this field (Yoshinaga et al. 2000). Among the many otolith microchemistry studies, very few have used matrix-matched samples for calibration or to assess instrument performance (e.g., Campana et al. 1997; Gillanders 2001; Gillanders 2002; Weber et al. 2002). Our results suggest that session-to-session variation in the sensitive instruments used for otolith

microchemistry is an important consideration. Similar to our study, Gillanders (2002) used ground otolith material during each session to assess the consistency of measurements.

These findings further showed that ToF-SIMS ion count ratios were strongly correlated to WD-EM analyses of otoliths from the same fish. The near 1:1 relation between the ion count ratios of ToF-SIMS analyses and molar concentration ratios of the WD-EM analyses indicated that Sr:Ca ratios determined by ToF-SIMS are an accurate index of the Sr:Ca concentrations in the otoliths. Therefore, with care, as outlined in this chapter, one can reproduce analytical results obtained by a microprobe. It is unclear if the higher measurement precision of ToF-SIMS, compared to WD-EM, is due to true higher precision or differences in beam diameter [wider beam diameters average the otolith composition over a greater time period, thus reducing variation in measurements (Markwitz et al. 2000)]. Campana et al. (1997) found significant variation in accuracy and precision in otolith elemental analyses both among instruments and between laboratories using the same analysis technique. Slight differences in the Sr:Ca between the two techniques used in this study were to be expected. However, it is difficult to explain the large difference in the edge zone Sr:Ca of the four westslope cutthroat trout where the Sr:Ca determined by ToF-SIMS was about half of the WD-EM results. One possibility is that the areas analyzed in these otoliths were areas where the normal aragonite of the otolith was replaced by vaterite, a rare polymorph of calcium carbonate. Vaterite has a hexagonal unit cell structure as opposed to the orthorhombic symmetry of aragonite (Lippmann 1973). Otoliths with vaterite have been found to have significantly

less Sr than aragonitic otoliths (Gauldie 1986, 1996; Brown and Severin 1999). Typically, otoliths with vaterite replacement are identified by eye, and as in the case of this study removed from the sample set prior to any further analysis. Re-examination of the otoliths that were in question did not reveal any obvious vaterite replacement in the areas analyzed, but it can sometimes be difficult to visually distinguish vaterite from aragonite (Gauldie et al. 1997).

Otolith composition can be affected by a number of potential factors including water chemistry, temperature, diet, and fish growth and reproductive condition. However, it has been shown that the majority of Sr (88.0%) and Ca (75.5%) in otoliths are derived from ambient waters. In this study, I found a strong relation between otolith Sr:Ca and water Sr:Ca, suggesting that the ambient water strongly influences otolith composition in freshwater salmonids. The distribution coefficients of Sr incorporation into the otoliths varied among the species examined, but were within the range of variation that might be anticipated from the environmental and biological factors that can influence the incorporation rate. These data therefore suggest that the process for incorporation of Sr into fish otoliths has been largely conserved across the species examined. In the freshwater environments that I examined, Sr:Ca water concentrations varied sixfold, suggesting that freshwater elemental concentrations vary more than expected, and therefore there is a high probability that analysis of chemical signatures incorporated into the otoliths of freshwater fishes holds great potential for studies of movement, life history, and stock delineation.

Otolith microchemical analysis has many applications to freshwater systems; however, there is the need to establish accurate techniques that are precise and sensitive enough to detect the often-small differences in the trace element concentrations found in otoliths. ToF-SIMS offers the advantage of non-destructive surface analysis of otoliths (which allows for repeat sampling), high spatial and mass resolution, sensitivity in the ppm to ppb range, and the ability to simultaneously measure all elements. A non-destructive analytical technique is important when the application requires correlating elemental distribution to the growth structure of the otolith. Our study demonstrated the efficacy of ToF-SIMS in addressing important questions regarding fisheries management especially in freshwater systems. For example, ToF-SIMS has successfully been applied as a forensics tool to determine the origin and timing of the illegal introduction of lake trout in Yellowstone Lake, YNP (Chapter 4). Other elements that may be incorporated into otoliths in relation to their availability in the environment, such as Zn, Pb, Mn, Ba, and Fe (Campana 1999), need to be examined in a similar manner as Sr was in this study. In addition, the development of matrix-matched standards with varying concentrations of the elements of interest, either from raising fish under controlled condition or by ion implantation (e.g., Gauldie and Markwitz 2002) should be pursued. This study has demonstrated that with care, ToF-SIMS can reproduce analytical results that conform to the known relations that exist in the field of otolith microchemistry.

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CHAPTER 4

NATURAL CHEMICAL MARKERS IDENTIFY SOURCE AND DATE OF INTRODUCTION OF AN EXOTIC SPECIES: LAKE TROUT IN YELLOWSTONE LAKE

Introduction

Exotic species pose one of the most pervasive threats to freshwaters worldwide (Hall and Mills 2000; Rahel 2000; Kolar and Lodge 2002). Dramatic changes in species abundance and energy flow have been observed following the establishment of a single new species, even in large lakes (Zaret and Paine 1973; Vander Zanden et al. 1999). Though many exotic species have been intentionally introduced for commercial or recreational purposes, unauthorized transplants and invasions have also contributed substantially to exotic species expansion (McMahon and Bennett 1996; Fuller et al. 1999; Rahel 2000).

When a new exotic species is first discovered, questions about where it originated and when it was transplanted or invaded are frequently difficult to answer with confidence (Radtke 1995; McMahon and Bennett 1996; Hebert and Cristescu 2002). This uncertainty hinders possible management actions for reducing future occurrences and, in some instances, raises questions as to whether a presumed invader is in fact native, or else has resided in the system longer than suspected but at low abundance (Kaeding et al. 1996; Waters et al. 2002). Recent investigations of freshwater zooplankton illustrate the utility of genetic markers as a forensic tool for studying invasion biology (Cristescu et al. 2001; Hebert and Cristescu 2002). In this

study, I use natural chemical markers in fish otoliths to identify the probable source and date of introduction of an exotic fish species.

Exotic lake trout (*Salvelinus namaycush*) were first discovered in Yellowstone Lake, Yellowstone National Park, Wyoming, USA, in 1994 (Kaeding et al. 1996). This 250,000 ha high-elevation lake near the headwaters of the Yellowstone River drainage is one of the largest relatively intact lake ecosystems in the U.S. and is the primary remaining habitat for Yellowstone cutthroat trout (*Oncorhynchus clarki bouvieri*) (Gresswell and Varley 1988). The clear, deep, cold waters and abundant prey base of Yellowstone Lake provide prime habitat for piscivorous lake trout, and by 1996, their population was estimated at several thousand, including individuals as large as 91 cm (Ruzycki et al. 2003). Development of an abundant lake trout population was anticipated if left unchecked, with the resultant high predation pressure causing a significant decline in the cutthroat trout population (Varley and Schullery 1995; Ruzycki et al. 2003). Cutthroat trout generally evolved in the absence of competing top predators (Behnke 1992), and similar declines have been documented for several western North American lakes following lake trout introduction (Cordone and Frantz 1966; Marnell 1988; Behnke 1992; Donald and Alger 1993). Consequently, an aggressive lake trout removal program was initiated in Yellowstone Lake in 1995 to protect a valuable recreational fishery and the integrity of the lake's terrestrial and aquatic foodwebs, which are heavily dependent on cutthroat trout (Varley and Schullery 1995; Koel et al. 2003).

The origin of the lake trout in Yellowstone Lake is unknown. Though lake trout from the Great Lakes were introduced into Yellowstone National Park's Shoshone and Lewis lakes in the late 1800s and later spread to Heart Lake, these lakes are in the Snake River (Pacific) drainage and lack connection to the Yellowstone River (Atlantic) drainage (Figure 4.1) (Varley and Schullery 1983). Prior to 1994, no lake trout had been reported in Yellowstone Lake despite extensive population sampling and angler survey records dating back more than 50 years (Gresswell and Varley 1988; Kaeding et al. 1996). Based on the age and size of lake trout when they were first discovered in 1994 (≤ 5 years and 43 cm), it was estimated that lake trout had reproduced in Yellowstone Lake since at least 1989, but when the original transplant occurred was unknown (Kaeding et al. 1996).

I used chemical analysis of lake trout otoliths to estimate where they originated and when they were transplanted into Yellowstone Lake. Because trace elements of ambient waters are incorporated into otoliths as a fish grows, analysis of natural chemical markers in otoliths can be used to reconstruct environmental history, including timing of movements and stock origins (Campana 1999; Limburg et al. 2001; Thorrold et al. 2001). However, to my knowledge, there have been no previous reports of the use of otolith microchemistry to assess introductions of exotic species. Sr:Ca ratio has been the most widely used chemical marker in otolith microchemistry studies because of the strong correlation between otolith and ambient water ratios; substitution of Sr for Ca in the otolith's crystalline structure; and because Sr is more apt to reflect environmental

concentrations than other elements due to a lack of physiological regulation (Campana 1999).

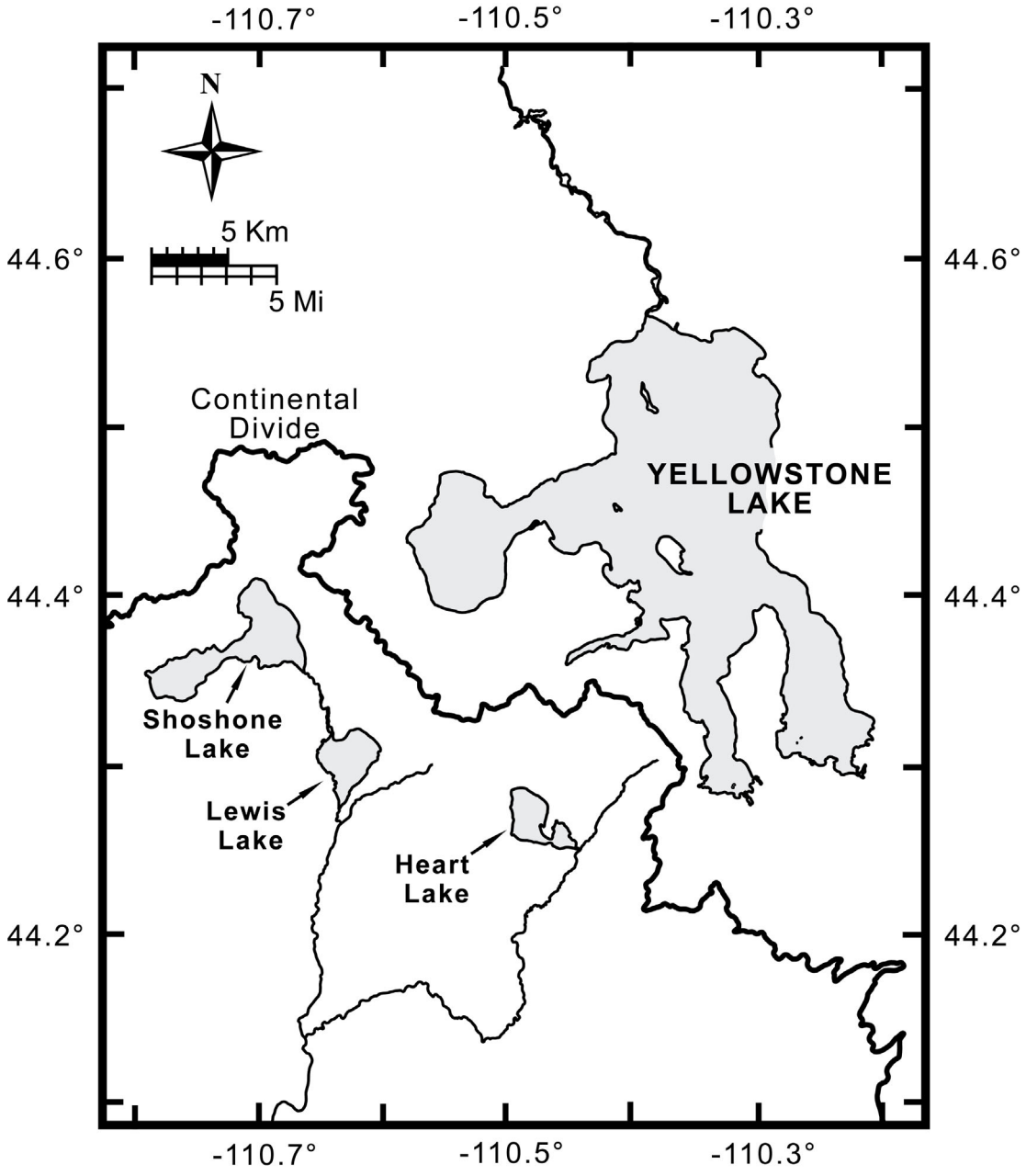


Figure 4.1. Map of the major lakes in the study area, Yellowstone National Park, Wyoming, USA.

I compared the Sr:Ca ratios in otoliths from suspected transplants to: 1) otoliths of lake trout from more recent year-classes, thought to have been spawned and reared in Yellowstone Lake; and 2) otoliths of lake trout from two likely source lakes from within Yellowstone National Park (Lewis and Heart lakes). I hypothesized that lake trout reared in a single lake would have similar otolith Sr:Ca ratios throughout their lives, from the early-growth zone near the nucleus to the outer edge. In contrast, I predicted that lake trout transplanted into Yellowstone Lake would have a significantly different chemical composition between the two zones, reflective of a change in environmental history, and that the Sr:Ca ratio of the early-growth zone could be used to identify the probable source lake of the transplant. I further surmised that among suspected transplants, the timing of the change in Sr:Ca ratio in relation to the age of the fish could provide an estimated date of when transplantation occurred.

Materials and Methods

Otolith Collection and Preparation

Otoliths from suspected transplants into Yellowstone Lake were obtained by randomly selecting 20 pairs from archived otolith samples of the largest fish (> 70 cm total length) that had been collected during early stages of the lake trout removal program (Table 4.1). These samples included 10 otoliths from the 109 lake trout > 70 cm collected in 1996 and 10 otoliths from the 55 lake trout >70 cm collected in 1997. Otoliths from lake trout of 'known origin'—suspected offspring of the original founding population in Yellowstone Lake (year-classes 1988-1991), and lake trout of various ages from Heart and Lewis lakes—were randomly selected from fish gillnetted in 1999 (n =

10 for each lake). Otoliths were extracted, cleaned, and stored in polyethylene vials soon after collection. One otolith from each fish was sectioned, ground, and polished to expose the nucleus following the techniques of Secor et al. (1992). Prior to microchemical analysis, otolith sections were cleaned in a series of baths of Milli-Q water, analytical grade hexane, and analytical grade methanol, to remove impurities, then sputter-coated with 15 nm of Au-Pd alloy to reduce the build up of an electrical charge on the sample during analysis.

Table 4.1. Summary of length, age, and year-class of the 'known-origin' lake trout and suspected transplants collected from three lakes in Yellowstone National Park, Wyoming, in 1999 and 1996/1997, respectively.

			Total length (mm)		Age (years)		Year-class
Group	Lake	*n*	median	range	median	range	range
'Known-origin'							
	Heart	10	490	(327-691)	15	(8-22)	1977-1991
	Lewis	10	503	(416-949)	14	(9-26)	1973-1990
	Yellowstone	10	720[a]	(700-767)	9	(8-11)	1988-1991
Suspected transplants							
	Yellowstone	20	805	(765-890)	18	(10-32)	1965-1987

[a] Length data was not available for 5 of the 'known-origin' Yellowstone Lake fish.

Otolith Chemistry

Otolith chemical composition was measured with a Phi-Evans time-of-flight secondary ion mass spectrometer (ToF-SIMS) (Schueler 1992) at the Image and Chemical Analysis Laboratory, Montana State University. Positive ion spectra were

collected in high-mass resolution mode using a pulsed 15 keV primary Ga^+ ion beam focused to 9×9 μm. Ion counts for ^{88}Sr and ^{44}Ca were measured from the spectra and reported as Sr:Ca ratios. A lab standard, consisting of ground and pressed otoliths from hatchery reared rainbow trout (*O. mykiss*), was analyzed at the start of each session to assess instrument precision, and the ToF-SIMS was recalibrated if the Sr:Ca ratio was > 2 SD of the base-line mean of the standard (Chapter 3). Within-site sampling variation was estimated by repeat sampling of the same site on 10 randomly selected otoliths. Prior to sampling, a 60×60 μm area was sputtered for 4 min to remove the alloy coating. Ion counts were made at the center of the sputtered area.

For each otolith, ion counts were measured at two sites, the early-growth zone near the nucleus, and a zone near the outer edge (Figure 4.2). For a subset of otoliths from each group (suspected transplants into Yellowstone Lake, $n = 5$; offspring of transplants, $n = 1$; Heart Lake, $n = 2$; Lewis Lake, $n = 1$) additional ion counts were measured at three equidistant points between the early-growth and edge sample sites. If large changes were detected between adjacent sample sites, further sites were sampled to pinpoint the location of temporal changes in Sr:Ca ratio along the otolith axis (Figure 4.2). Abrupt changes in Sr:Ca ratio have been correlated to rapid changes in water chemistry (e.g., Limburg et al. 2001). The date of a large change in Sr:Ca, indicative of a movement to new waters, was estimated by comparing the change in relation to the age of the fish. Annuli were identified using ageing criteria developed for lake trout otoliths by Sharp and Bernard (1988). Distances from the otolith nucleus to each sample site and to each annulus were measured using a compound microscope, drawing attachment, and

digitizing pad. Back calculation was used to estimate length at age and growth rate (DeVries and Frie 1996).

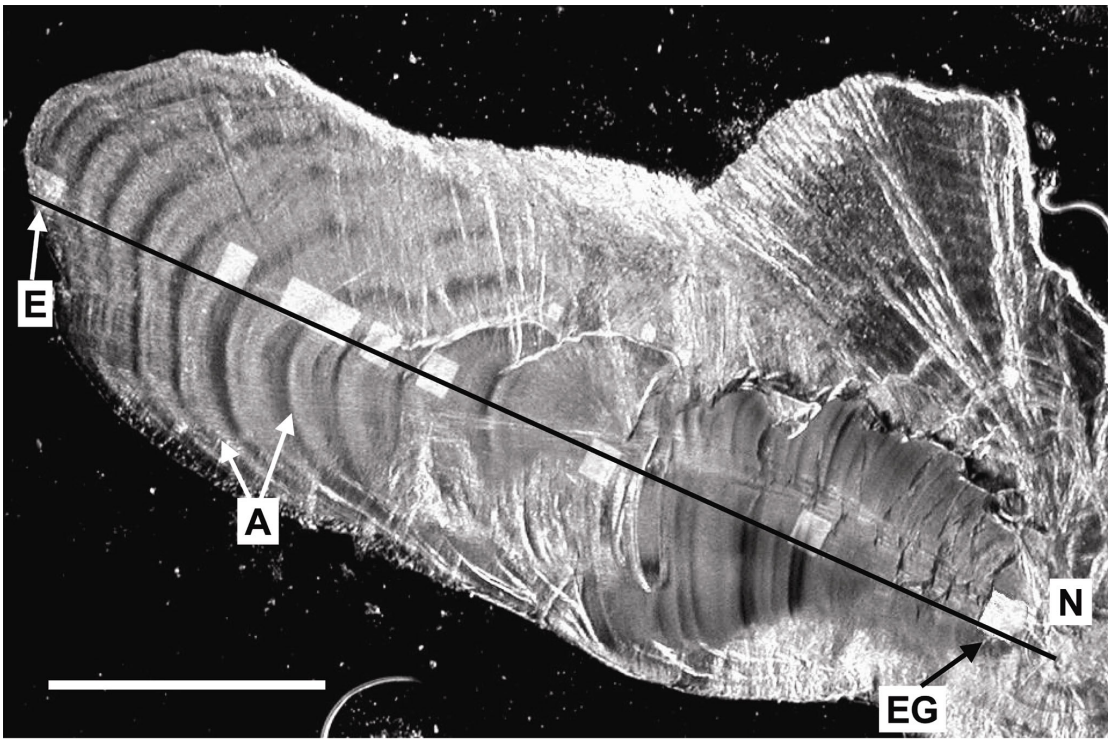


Figure 4.2. Transverse section of a lake trout (85 cm, 18 yrs) otolith from a suspected transplant into Yellowstone Lake. Microchemical analysis sites for ^{88}Sr and ^{44}Ca ion counts were located in the early-growth zone (EG), near the nucleus (N), and the edge zone (E) in each otolith. Sr:Ca ratios data were obtained from a 9×9 μm area within the areas cleared of the Au-Pd coating (white squares). Analyses along a transect (solid line) were used to pinpoint the location of any temporal changes in Sr:Ca ratios. A = annuli. Scale bar = 0.5 mm.

Water Chemistry

Sr:Ca ratios of water from each of the three study lakes was measured to assess if geochemical differences existed among the lakes and the degree to which these differences were imparted to lake trout otoliths. Surface water samples were collected from Heart and Lewis lakes in 2000 (n = 4 per lake) following standard protocols

(American Public Health Association 1998). Yellowstone Lake water samples were collected at different depths in the water column in four areas of the lake (Southeast Arm, West Thumb, Mary Bay, and Stevenson Island) in 1997 (n = 30) and 1998 (n = 41) (Balistrieri et al. in press). Sr and Ca molar concentrations were measured with a Perkin-Elmer Sciex Elan 6000 inductively coupled plasma mass spectrometer (Lamothe et al. 1999).

Statistical Analyses

Otolith Sr:Ca ratios among 'known-origin' lake trout from Heart, Lewis, and Yellowstone lakes were compared to determine if each lake imparted a unique chemical signature. Lake and otolith zone (early-growth and edge) were included as fixed factors, and fish, zone×fish interaction, and zone replication, as random factors in the analysis of variance (ANOVA) model. This resulted in the following mixed linear model:

$$y_{ijklm} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_{k(i)} + (\beta\gamma)_{jk(i)} + \delta_{l(jk(i))} + \varepsilon_{ijklm},$$

where

i = 1, …, 3,

j = 1, …, 2,

k = 1, …, 10,

l = 1, …, a_h ($1 < a_h < 4$),

m = 1, ..., n,

and

μ = the overall mean,

α_i = the effect of the i^{th} lake,

β_j = the effect of the j^{th} otolith zone,

$\alpha\beta_{ij}$ = the lake×otolith zone interaction,

$\gamma_{k(i)}$ = the effect of the k^{th} fish within the i^{th} level of the lakes,

$\beta\gamma_{jk(i)}$ = the otolith zone×fish within lakes interaction,

$\delta_{l(jk(i))}$ = the effect of the l^{th} site within the $jk(i)^{th}$ level of the zone×fish within lake interaction, and

ε_{ijklm} = random error due to sampling.

Type 3 *F*-statistics were used in the mixed model ANOVA and the Satterthwaite approximation was used to calculate the degrees of freedom for the statistical tests (Littell et al. 1996; PROC MIXED, SAS Institute 2000). Statistical correlation within the random effects was estimated using the restricted maximum likelihood method and compound symmetry covariance structure (i.e., constant variance and covariance). The normality assumption was assessed using residual analysis. Tukey's multiple comparison tests were used to compare pairwise differences of the least-square means of the fixed effects.

Nearest-neighbor discriminant analysis was used to determine the probable source of lake trout in Yellowstone Lake. Mean otolith Sr:Ca ratios for 'known-origin' fish, weighted by number of sites sampled in each otolith zone, was first used to construct a nonparametric discriminant model (*k* nearest neighbors = 9). Model accuracy was evaluated by cross-validation. The Sr:Ca ratios from lake trout otoliths of

suspected transplants were then classified using the discriminant model developed for the 'known-origin' data set.

Differences in lake water Sr:Ca ratio among the three study lakes was evaluated using a one factor ANOVA. Simple linear regression was used to evaluate the strength of the relationship between otolith and lake water Sr:Ca ratio.

Results

Lake water Sr:Ca ratios were significantly different among Heart, Lewis, and Yellowstone lakes (ANOVA, $F = 402.21$, df = 2, 76, $P < 0.001$) with differences in mean values among the lakes ranging from 160 to 270% (Figure 4.3). Otolith Sr:Ca ratios of 'known origin' lake trout from each lake showed a near 1:1 relationship with lake water Sr:Ca ratios ($r = 0.997$, $P = 0.04$, Figure 4.3), and were significantly different among the three lakes ($F = 28.84$, df = 2, 27.9, $P < 0.001$, Figure 4.3 and Figure 4.4). Mean otolith Sr:Ca ratios of Yellowstone Lake lake trout were highly significantly different than lake trout otolith Sr:Ca ratios from Heart Lake (Tukey's, $t = 5.10$, df = 28, $P < 0.001$) and Lewis Lake ($t = 7.43$, df = 28, $P < 0.001$). Differences in otolith Sr:Ca ratios between lake trout from Lewis and Heart lakes were moderately significant ($t = 2.33$, df = 27.8, $P = 0.068$).

Otolith Sr:Ca ratios between the early-growth and edge zones among known-origin lake trout from each lake varied little (ANOVA, $F = 1.17$, df = 1, 28.5, $P = 0.289$, Figure 4.4), the average percent difference ranging from 0.1-5.3%, despite a wide range in the age of fish sampled (8-26 years, Table 4.1). Within-site sampling variation was also low, averaging 3.92% ($n = 25$). Accordingly, there was no significant interaction

between lake and otolith zone (F = 0.15, df = 2, 28.5, P = 0.857). Additional samples taken between the early-growth and edge zones also revealed consistent Sr:Ca ratios across the otolith growth axis among lake trout sampled from the same lake (Figure 4.5a,b). Nearest-neighbor discriminant analysis correctly classified 90 to 100% of lake trout into their home lake (Table 4.2).

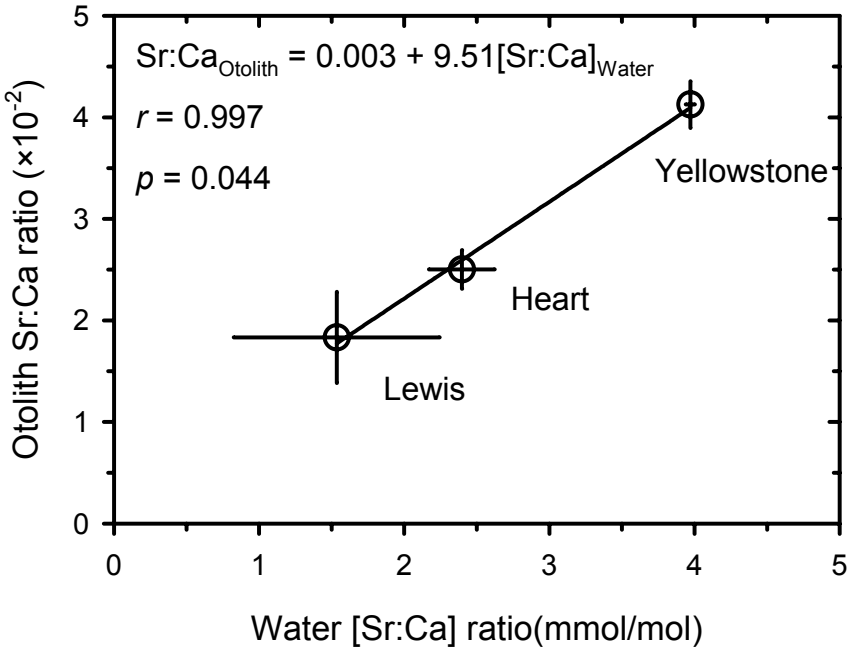


Figure 4.3. Relation between mean (±2 SE) lake water Sr:Ca ratios and mean (±2 SE) otolith Sr:Ca ratios of 'known-origin' lake trout from Heart, Lewis, and Yellowstone lakes, Yellowstone National Park, Wyoming.

In sharp contrast to lake trout of known origin, 18 of 20 suspected transplants, ranging in age from 13 to 32 years of age at the time of their collection in 1996 and 1997, exhibited substantial increases ($\bar{x}$ = 256%) between the early growth and edge zones (Figure 4.4). The two fish that did not show a large increase in Sr:Ca ratio were the youngest of the fish sampled (age 10 and 11). Eighty percent of the edge zones of

suspected transplants were classified by the discriminant model as Yellowstone Lake, whereas 90% of the Sr:Ca ratios measured in the early-growth zone were classified as Lewis Lake (Table 4.2). Among lake trout otoliths with large increases in Sr:Ca ratios between the early-growth and edge otolith zones, 100% of the early-growth zones were classified as Lewis Lake. Sampling along the otolith axis of a random subset (n = 3) of lake trout exhibiting the abrupt shift in Sr:Ca ratio, revealed Sr:Ca ratio increases occurred within a short time period (Figure 4.5c, d). Timing of the increase in Sr:Ca ratios was estimated to occur in 1989 for two fish (Figure 4.5c), and 1996 for one fish (Figure 4.5d) of the subset sampled.

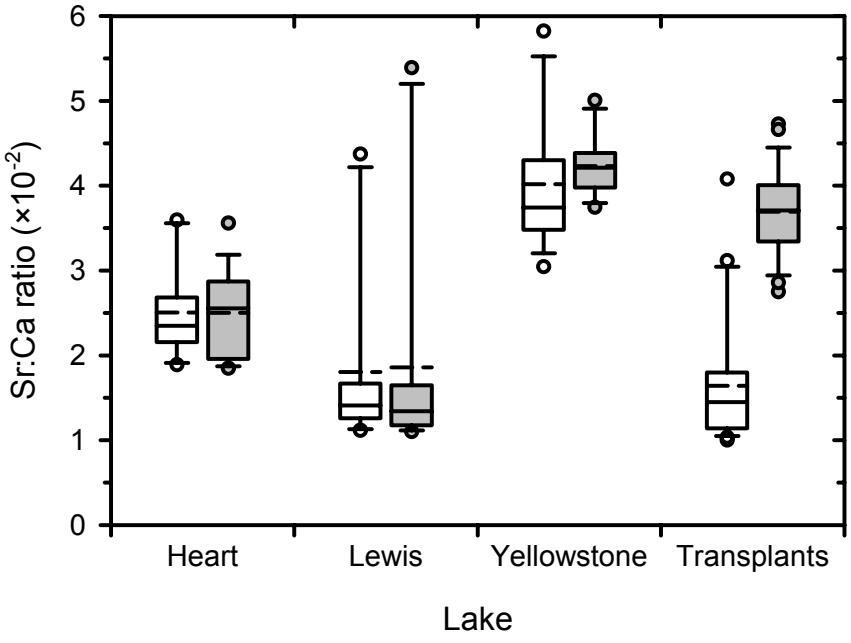


Figure 4.4. Comparison of Sr:Ca ratios of 'known-origin' lake trout otoliths and otoliths of suspected transplants. Two zones were analyzed for each otolith: early-growth (open boxes) and edge zones (shaded boxes) for both 'known-origin' lake trout from Heart, Lewis, and Yellowstone lakes (n = 10 per lake), and suspected transplants netted from Yellowstone Lake (n = 20). Boxes show the mean Sr:Ca ratios (dashed line), median (central solid line), 1st and 3rd quartiles (box edges) and individual outliers (circles) outside the 10th and 90th percentiles (whiskers).

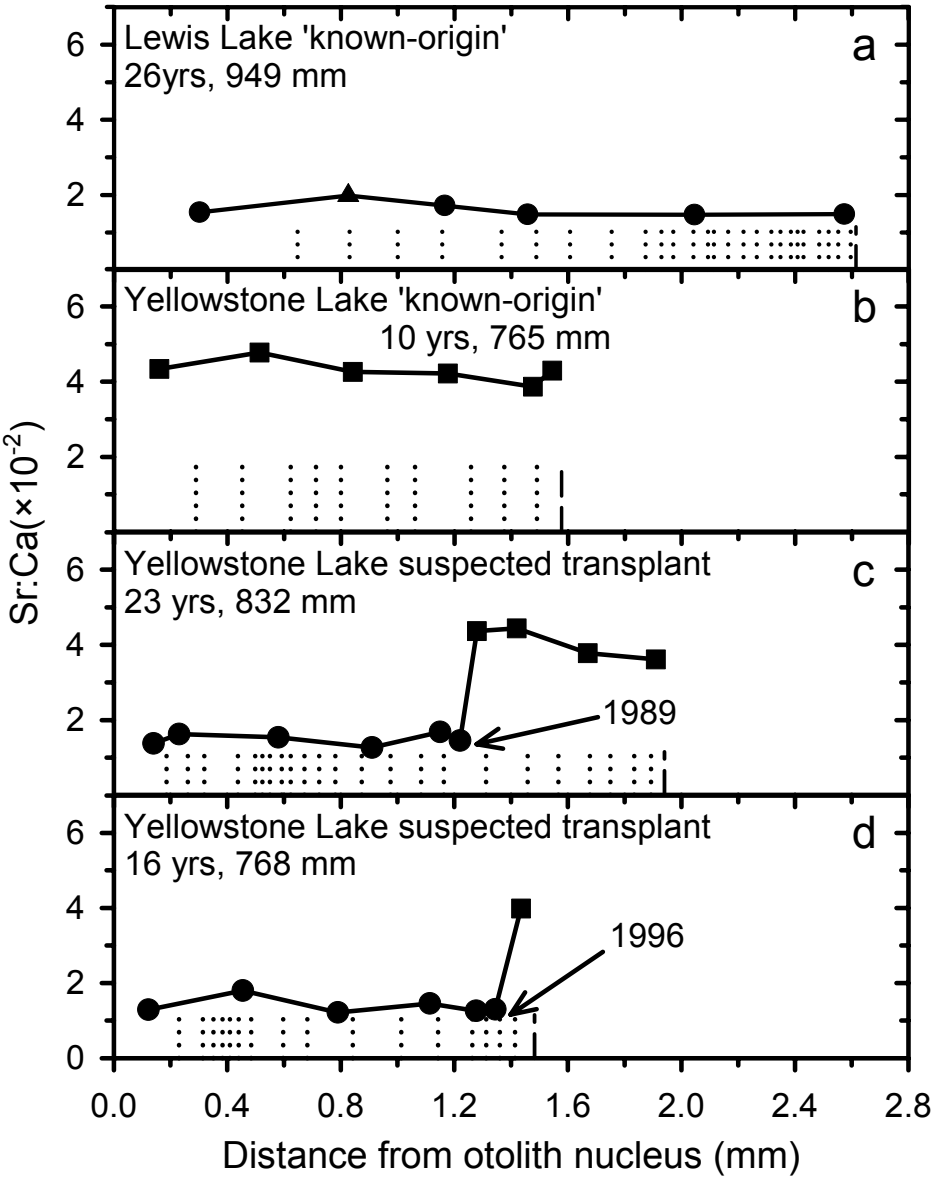


Figure 4.5. Typical patterns of Sr:Ca ratios measured along otolith axis of lake trout from the study lakes. 'Known-origin' lake trout from Lewis (a) and Yellowstone (b) lakes illustrating the low variation in Sr:Ca ratios during their life span. Suspected transplants to Yellowstone Lake show rapid increase in Sr:Ca ratios corresponding to transplant dates of 1989 (c) and 1996 (d). Analysis sites were classified by discriminant analysis: ● Lewis Lake; ■ Yellowstone Lake; ▲ Heart Lake. Dotted lines show location of annuli and dashed line the otolith edge. Arrows mark the estimated year that the increase in Sr:Ca ratios occurred. Sr:Ca ratios of a third suspected transplant (18 yrs, 850 mm) showed an identical pattern as in panel c.

Table 4.2. Classification of lake trout into probable source lakes. Cross-validation results for the 'known-origin' lake trout calibration data set (n = 10 fish/lake) were used to assess classification accuracy. Early-growth and edge zones of the otoliths from the group of suspected transplants captured in Yellowstone Lake (n = 20) were classified into one of the three lakes. The probable origin of lake trout in Yellowstone Lake was based on the early-growth zone Sr:Ca ratios of suspected transplants.

	% classification into lake		
From Lake	Heart	Lewis	Yellowstone
'Known-origin'			
Heart	100.0	0.0	0.0
Lewis	0.0	90.0	100.0
Yellowstone	0.0	0.0	100.0
Suspected transplants			
Early-growth	5.0	90.0	5.0
Edge	20.0	0.0	80.0

Discussion

This work demonstrates that otolith chemical composition can be used to identify a probable source and date of exotic species introductions. In the three large lakes I studied, water chemistry differed significantly among lakes and these differences were directly imparted to lake trout otoliths. The low variation in Sr:Ca ratios of 'known-origin' lake trout along the otolith axis (from the early-growth to the edge zones), despite a wide range of ages, established that lake trout from each lake lived in a similar water chemistry throughout their lives. This temporal and spatial stability in otolith chemical

signatures was reflected by the high discriminatory power to classify lake trout by their home lake based on unique otolith Sr:Ca ratios. These findings corroborate previous work demonstrating 1) a strong association between chemical composition of water and otolith chemistry (Bath et al. 2000; Wells et al. 2003), and 2) that source waters, even in freshwater environments, can be identified with a moderate to high degree of precision based on otolith chemical composition (Thorrold et al. 1998; Wells et al. 2003). Both attributes of otoliths are important in determining stock origins, and our findings indicate that this is particularly relevant for exotic species where stock origin is frequently unknown.

Unlike known-origin lake trout, the large and rapid change Sr:Ca ratio along the otolith axis of suspected transplants demonstrates these fish experienced a rapid change in water chemistry. The magnitude of the change in otolith Sr:Ca ratio among suspected transplants (256%) mirrors that shown by anadromous fish migrating from freshwater to seawater. For example, Limburg (1995) found otolith Sr:Ca ratios of age-0 American shad (*Alosa sapidissima*) increased by 250 to 620% during movement from freshwater to seawater. Such a large and rapid change in otolith chemistry among lake trout from older year classes provides strong support for the hypothesis lake trout that they had been transplanted to Yellowstone Lake. All Yellowstone Lake lake trout from younger age classes (≤ 11 years) at the time of collection in 1996-1999, had similar early growth and edge zone Sr:Ca ratios, indicating a constant environmental history, whereas all lake trout from older year classes had a marked increase in Sr:Ca ratios between the early growth and edge zones indicating these fish had reared in waters of distinctly different

water chemistry during their life span. These results therefore support the assertion that initial transplanting of lake trout into Yellowstone Lake occurred as early as the late 1980s (Kaeding et al. 1996). Moreover, the dating of transplants as occurring in 1989 and 1996, suggests that multiple transfers may have occurred. The classification of 90% of the early-growth zone Sr:Ca ratios of the suspected transplants into Lewis Lake by discriminant analysis suggests this is a likely source of transplanted lake trout (Table 4.2). Lewis Lake, unlike Heart Lake, is accessible by road, which may have facilitated the unauthorized transfer of lake trout into Yellowstone Lake.

Change in Sr:Ca ratios with age, growth, or maturation (Friedland et al. 1998) are possible alternatives to the transplant hypothesis. Inverse relationships between otolith Sr:Ca ratios and growth have been observed in some cases (e.g., Sadvoy and Severin 1994; Friedland et al. 1998) but I found no correlation between Sr:Ca ratio and growth rate of lake trout ($r = 0.09$, $P = 0.49$). This lack of relation between Sr:Ca and growth has also been observed in other studies (e.g., Kalish 1989; Gallahar and Kingsford 1992). An age- or maturation-induced Sr:Ca ratio increase was also unlikely as lower Sr:Ca ratios would be expected in the early growth zone and among earlier ages for all lake trout. However, the pattern of increased Sr:Ca ratio was only observed in suspected transplants and not in the early-growth zone or among earlier age groups of other lake trout sampled from Yellowstone Lake (Figure 4.3, Figure 4.5b) nor from any of the lake trout sampled from Heart and Lewis lakes (Figure 4.3).

Another possible explanation for the increase in Sr:Ca ratios in the otoliths of the suspected transplant group of lake trout is spatial or temporal variation in Sr:Ca ratios of

water within Yellowstone Lake. The increase in otolith Sr:Ca ratios in 1989 observed in some lake trout coincided with the intense wildfire in Yellowstone National Park in 1988 that altered dissolved ion concentrations of some streams in Yellowstone National Park (Minshall et al. 1997). Although Sr was not measured, other dissolved ions in the lake, however, showed only minor changes in concentration after the fire (Lathrop 1994); therefore major changes in lake-water Sr:Ca ratios were unlikely. In addition, Yellowstone Lake has many hydrothermal vents that may be a source of local enrichment of Sr and Ca (Balistrieri et al. in press). However, I found that lake water Sr:Ca ratios varied little (< 3.5%) with depth or among lake subbasins; therefore, it is unlikely that the increase in otolith Sr:Ca ratios was a result of fish inhabiting different areas within Yellowstone Lake with different Sr:Ca ratios.

These results should be interpreted with two important caveats. First, the long life span of lake trout facilitated a long-term retrospective analysis of their environmental history. Detection of such unique chemical marks would have been more difficult in species with higher turnover rates or species that migrate extensively between waters of differing chemical signatures. Second, although Lewis Lake was identified as the source lake for transplanted lake trout with a high degree of probability, not all ambient waters have unique Sr:Ca signatures (Gillanders et al. 2001; Wells et al. 2003, Chapter 5). Therefore, I cannot eliminate the possibility that lake trout were transplanted from some other (unknown) lake with a Sr:Ca ratios similar to Lewis Lake. In future studies, use of isotopes or other elements in addition to Sr, could enhance the accuracy of identifying source waters (e.g., Kennedy et al. 2002; Wells et al. 2003).

There is growing appreciation for just how extensive introductions of exotic species have been and the formidable problem they present for aquatic ecosystem management (Hall and Mills 2000; Rahel 2000; Kolar and Lodge 2002). For instance, in Montana alone, 375 cases of unauthorized introductions of fishes have been documented in 224 different waters comprising 45 different species (Vashro 1995). Discovery of a new species often poses questions about when the invasion occurred and the geographic origin of the exotic, but thus far, few tools have been available to answer such key questions (Radtke 1995; McMahon and Bennett 1996; Hebert and Cristescu 2002; Waters et al. 2002). Better knowledge of where exotic species originated and the relative risks they pose is essential for design of educational and regulatory programs aimed at stemming the tide of future unauthorized introductions (McMahon and Bennett 1996; Kolar and Lodge 2002). Genetic markers have recently been shown to be a useful forensic tool for studying invasion biology (Cristescu et al. 2001; Hebert and Cristescu 2002; Waters et al. 2002). This study demonstrates how chemical analysis of otoliths can provide a novel forensic tool to estimate geographic origin and timing of exotic fish introductions. Because chemical and genetic analysis techniques each have distinct advantages and limitations (Cristescu et al. 2001, this study; Thorrold et al. 2001; Hebert and Cristescu 2002), a combination of both tools could provide important new insights into the study of invasion biology.

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CHAPTER 5

DETERMINATION OF NATAL ORIGIN AND AGE AT MIGRATION IN WILD MISSOURI RIVER RAINBOW TROUT

Introduction

Freshwater salmonids typically display one of four life history migration patterns: fluvial, fluvial-adfluvial, lacustrine-adfluvial, and allucustrine (Chapter 1, Varley and Gresswell 1988; Northcote 1997). For fluvial-adfluvial stocks in particular, spawning, rearing, and feeding habitats are frequently separated by long distances (Northcote 1997). Therefore, proper management and conservation of salmonids requires knowledge of the range of life history types present as well as the areas important to recruitment. However, determination of natal origin and life history type can be problematic because of a lack of effective and efficient techniques that permit ready identification of these population characteristics. Traditionally, natal origin and life history type have been identified directly through the use of tags, wherein individuals or groups recruiting from an area or hatchery are tagged as juveniles and later recovered as adults. However, this technique is often limited by a combination of restricted tagging options for small fish due to tag loss and biotic effects (Buckley and Blankenship 1990; Bergman et al. 1992; Mourning et al. 1994; McMahon et al. 1996), and the large number of fish required to be tagged and recovered to make the studies cost-effective (Kaill et al. 1990) and statistically robust (Seber 1982; Heimbuch et al. 1990). For example, Kaill et al. (1990) coded wire tagged (CWT) over 0.5 million pink

salmon fry *Oncorhynchus gorbuscha* and recovered < 0.5% of the tagged fish a year later. Similarly, Munro et al. (2003), CWTed 12,304 juvenile rainbow trout *O. mykiss* during their outmigration from natal tributaries to the Missouri River, Montana, and recovered only 1% as adults in the mainstem river, despite three years of intensive sampling.

Chemical analysis of fish otoliths or other calcified structures offers a novel approach for characterizing natal origin and life history patterns (Campana 1999; Campana and Thorrold 2001). As a fish grows, unique chemical signatures of ambient waters are incorporated into the otolith, thereby leaving a record of environmental history. Retrospective analysis of adult otoliths therefore offers the potential for indirectly determining natal origin, rearing location and period, and contribution of specific life history types or different waters to overall recruitment (e.g., Kennedy et al. 1997; Thorrold et al. 1998; Ingram and Weber 1999; Wells et al. 2003).

The age at outmigration in samples of adult rainbow trout and steelhead is commonly determined by examining changes in circuli spacing in the scales. Narrowly spaced circuli are indicative of growth in the relatively less productive natal stream and wider spaced circuli are indicative of the more productive river, lake, or marine environment to which the fish migrated (Rosenau 1991; Tsiger et al. 1994; Bernard and Myers 1996). Studies have shown that the length of stream rearing in rainbow trout and steelhead can vary from <1 to 5 yrs and that while a particular age at migration may be predominant, several outmigration strategies may be present within a population (Kwain 1971).

The objectives of this study were: 1) to assess the effectiveness of otolith microchemistry for identifying the natal tributary in Missouri River rainbow trout; 2) use scale patterns to determine the range of outmigration strategies and estimate length of the fish at outmigration; and 3) measure the relative contribution of the spawning tributaries and outmigration strategies to adult recruitment in the wild rainbow trout population inhabiting the Missouri River, Montana. This population has several desirable characteristics for evaluating these techniques. Adult recruitment to the mainstem fishery is maintained by spawning and rearing from several tributaries, and the area is characterized by a heterogeneous lithography (see Study Area) that could impart different chemical signatures in the otoliths of juvenile fish that could then be potentially recognizable in adult otoliths. Scales of some of the adult rainbow trout show a distinct growth 'check', which is believed to correspond to the time of entry into the main river and a result of increased food availability and growth (S. Leathe and G. Liknes, Montana Fish, Wildlife and Parks (MT FWP), pers. obs.). In addition, whirling disease has recently spread to the population, severely reducing juvenile recruitment from at least one spawning tributary (Leathe et al. 2002b). Thus, knowledge of the relative contribution of spawning tributaries to overall recruitment is important for assessing the long-term effects of the disease on the fishery.

To test the effectiveness of otolith microchemistry, I first characterized the water chemistry of the main spawning tributaries and the Missouri River to assess the degree of differences in Sr:Ca among tributaries that would be imparted to the otoliths. I then developed a discriminant function model for otolith Sr:Ca using juvenile rainbow trout

of known origin, and applied the model to adults of known origin to estimate the accuracy of characterizing natal origin using adult otoliths. Based on these results, I also describe a method for evaluating the feasibility of microchemical analysis for determining natal origin among fish populations in other locales using analysis of water chemistry.

To determine if the 'checks' in Missouri River rainbow trout scales correspond to outmigration from the natal tributary to the mainstem river, I examined the scales of adult rainbow trout that had been CWTed as outmigrating juveniles and later recaptured in the mainstem (Munro et al. 2003) for the presence of a 'migration' check. I then estimated the proportion of fish with migration checks in subsamples of scales from rainbow trout caught in the Craig section of the Missouri River between 1997 and 2000 to determine if whirling disease affected the composition of outmigration strategies recruited to the fishery. These samples covered the years before the whirling disease impacted the production of rainbow trout from Little Prickly Pear Creek (1997 and 1998) and after an impact was observed (1999 and 2000).

Study Area

The 56.2 km section of the Missouri River between Holter Dam and Cascade, Montana (Figure 5.1) is rated a Class I/blue ribbon trout fishery and is one of the top wild trout fisheries in Montana. In 1995, prior to detection of whirling disease in the system, this section of the Missouri River was ranked second, statewide, for amount of fishing pressure (McFarland and Hughes 1997). Despite a drop in ranking to fourth in 1997 (the year after whirling disease was detected in the system), this section of the

Missouri currently receives the most fishing pressure (123,472 angler days) statewide (McFarland and Meredith 1999, 2002). Flows in this section of the Missouri River are regulated through a series of dams beginning with Canyon Ferry Reservoir, south of Helena, Montana. Mean annual discharge from 1946 to 2000 was 155.1 m^3/s (range: 89.2-237.7 m^3/s), measured at the USGS gauging station below Holter Dam, near Wolf Creek, Montana. Spawning occurs primarily in three main tributaries – Little Prickly Pear Creek, the Dearborn River, and Sheep Creek. Spawning is limited in the mainstem river (Leathe et al. 1988).

Little Prickly Pear Creek enters the Missouri River at river kilometer 4.3 below Holter Dam. The upper stretches of Little Prickly Pear Creek are underlain by fine-grained clastic sediments of the Greyson Shale and Spokane Shale Formations of the Precambrian Belt Supergroup (Bregman 1981; Lewis 1998) and the lower stretches traverse dominantly felsic volcanic rocks of the Upper Cretaceous Two Medicine Formation and Quaternary sediments (Schmidt 1972). Mean annual discharge of the stream is 2.5 m^3/s (range: 0.9-5.2 m^3/s, from 1963 to 2000). In 1988, an estimated 15,000 rainbow trout spawned in the lower 32 km (Leathe et al. 1988). Two tributaries to Little Prickly Pear Creek, Lyons and Wolf creeks, are also actively used by spawning rainbow trout (Grisak 1999). Wolf Creek is underlain by clastic volcanic rocks and ash-flow tuffs of the Upper Cretaceous Two Medicine Formation and Quaternary sediments (Schmidt 1972). Information on the underlying geology of Lyons Creek was not available.

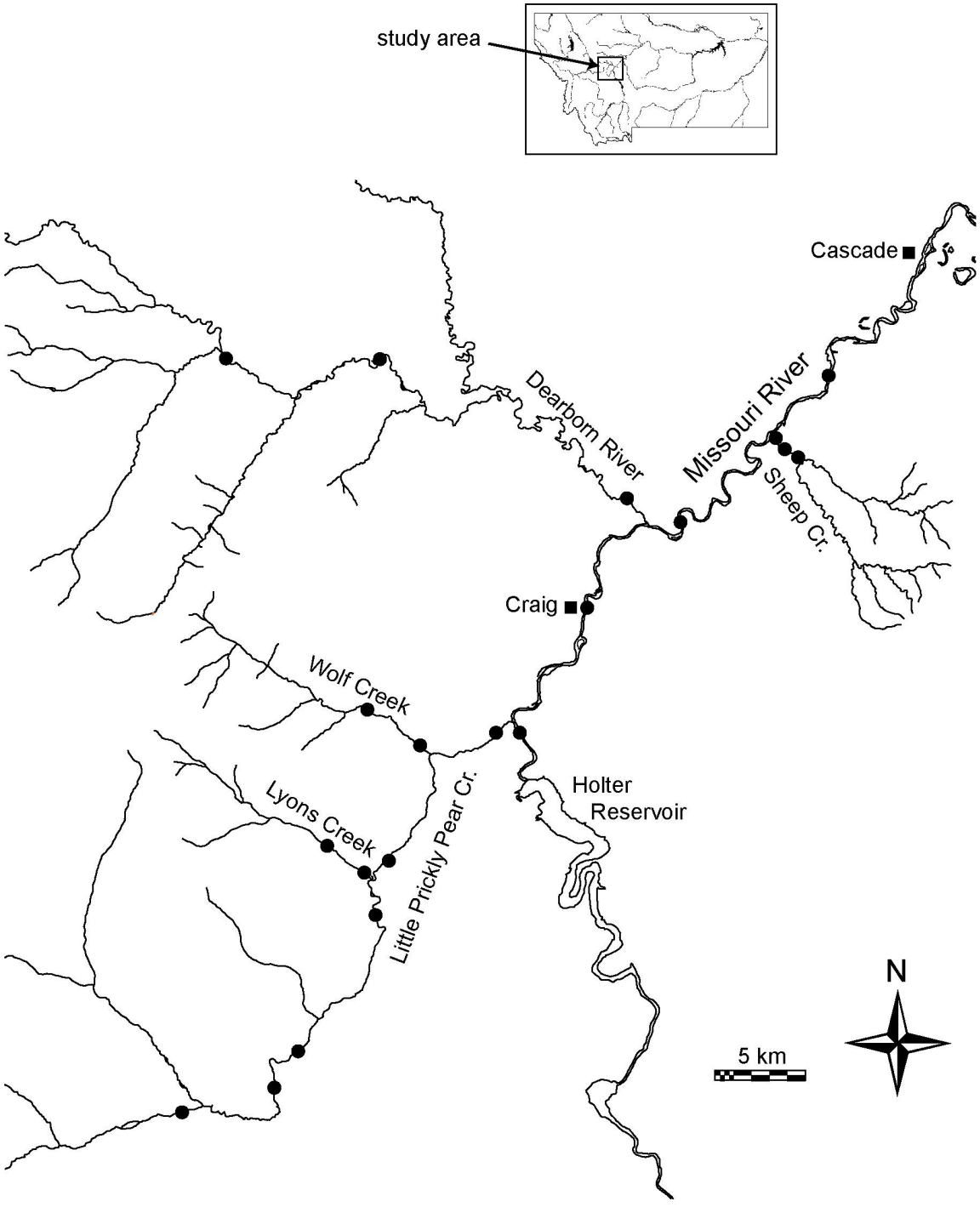


Figure 5.1. Map of study area on the Missouri River, Holter Dam to Cascade, Montana. Location of water sampling locations (●) in the upper Missouri River and tributaries.

The Dearborn River is a large 113-km long river that enters the Missouri River at river kilometer 21.6, near the town of Craig. Alkalic, felsic volcanic, and volcaniclastic rocks of the Cretaceous Adel Mountain Volcanics underlie the lower reaches of the Dearborn River. Fine-grained clastic sediments of the rocks of the Upper Cretaceous Two Medicine Formation underlie the upper part of the drainage. Only in the headwaters does the drainage pass through Precambrian clastic sediments of the Spokane and Empire Formations of the Belt Supergroup. There are minor limestone units reported in this area (Mudge et al. 1983; Vuke 2000). Mean annual discharge is 5.9 m^3/s (range: 1.6-10.1 m^3/s, 1946-2000). A 1988 helicopter survey of the drainage estimated that 20,000 rainbow trout migrated up the Dearborn River to spawn, primarily between river kilometers 16 to 64 (Leathe et al. 1988).

Sheep Creek enters the Missouri River at river kilometer 37.3 below Holter Dam. The creek is 3.4 km long from the mouth to the confluence of the North and South forks of Sheep Creek, which are 13.0 and 16.1 km, respectively. Sheep Creek is almost entirely underlain by alkalic, felsic volcanic, and volcaniclastic rocks of the Cretaceous Adel Mountain Volcanics. The North Fork Sheep Creek has headwaters in the fine-grained clastic sediments of the rocks of the Upper Cretaceous Two Medicine Formation. The lower section of the creek passes through Quaternary sediments, mostly stream channel deposits (Soward 1975; Vuke 2000). Mean daily discharge from March to October, 1999, was estimated at 0.34 m^3/s (range: 0.9-3.6 m^3/s). Despite its small size, about 1,000 rainbow trout redds have been counted in the lower 3.4 km the drainage (Leathe et al. 2001).

Overall there are three major geologic units that the Missouri River tributaries pass through: the Cretaceous Adel Mountain Volcanics; the Upper Cretaceous Two Medicine Formation, and the Precambrian Belt Supergroup. The alkalic rocks of the Adel Mountain Volcanics would be expected to have the highest Sr:Ca (whole rock analysis), the Two Medicine Formation, which is reworked volcanic rocks, would be expected to have an intermediate level of Sr:Ca, and rocks of the Belt Supergroup would have the lowest Sr:Ca of the three geologies (D. Mogk, Montana State University, personal communication). Therefore, it would be expected that rainbow trout otoliths from Sheep Creek would have higher Sr:Ca than fish from either Little Prickly Pear Creek or the Dearborn River because of the predominance of Adel Mountain Volcanics. The Sr:Ca of rainbow trout otoliths from Little Prickly Pear Creek and the Dearborn River drainages would be expected to be similar to each other, but lower that Sheep Creek fish, because their drainages are both dominated by the Two Medicine Formation and the Belt Supergroup formations.

There are three rainbow trout population-monitoring sections on the mainstem Missouri River within the study area. From south to north, these sections are referred to as Craig, Hardy and Cascade. The Craig section extends from the Wolf Creek Bridge to Cascade, Montana (8.2 km). Little Prickly Pear Creek flows into the Missouri River within this section. The Hardy section extends from the Mid Canon fishing access site downriver for 9.0 km. The confluence of the Dearborn River is about 3.3 km upriver of the start of the Hardy section. The Cascade section is 6.6 km long, beginning about 0.6 km downriver from the mouth of Sheep Creek.

Methods

Water Chemistry

To assess spatial and temporal differences in strontium and calcium concentrations of water in the mainstem and tributaries that could be imparted to rainbow trout otoliths, water samples were collected in 1999 from 20 different sites during three time periods. Tributaries sampled were: Little Prickly Pear Creek and two of its tributaries (Lyons and Wolf creeks), the Dearborn River, and Sheep Creek (Figure 5.1). To assess spatial variation of water chemistry within a tributary, water samples were collected from several sites: near the mouth, in sections with different geology, and below the confluence of secondary tributaries. On the mainstem Missouri River, water samples were collected at four sites between Holter Dam and the town of Cascade (Figure 5.1). To assess temporal differences in water chemistry, water samples were obtained from the same sites on three different occasions: in March during the late winter, low discharge period before runoff; in May during spring runoff; and in October to characterize the summer low flow period. Duplicate samples were collected for a subset (75%) of the sites during each sampling period to assess within-site variation.

Water samples were collected and processed according to standard protocols for elemental analyses (American Public Health Association 1998). Water was collected in 1-L polyethylene acid-washed bottles from areas of flowing water and 100 ml of the sample was immediately filtered through a 0.45-μm-pore membrane filter. The filtered water was transferred to opaque acid-washed polyethylene bottles, preserved with 1 ml of analytical grade concentrated nitric acid, and refrigerated.

Strontium and calcium concentrations (mg/L) were measured with an Fison's Instruments Accuris ICP-optical emission spectrometer at the Soil, Plant, and Water Analytical Laboratory, Montana State University, Bozeman, Montana. Standard analysis protocols were used with analytical wavelengths of 317.93 and 407.77 for Ca and Sr, respectively (American Public Health Association 1998). Concentrations of Sr and Ca were converted to molar concentrations (μM) and the ratio of Sr to Ca (Sr:Ca) was used in statistical analyses, a standard approach in otolith microchemistry studies (Rieman et al. 1994; Campana et al. 1997, Chapter 2).

Variation in duplicate samples was estimated using a one-way analysis of variance (ANOVA) with sample site modeled as a random effect (PROC GLM, SAS Institute 2000). The Sr:Ca among the tributaries was compared using a three factor ANOVA with stream, sample occasion, and site (nested within stream) modeled as fixed effects (PROC GLM, SAS Institute 2000). The following model was used in the analysis:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \gamma\alpha\beta_{k(ij)} + (\alpha\beta)_{ij} + \varepsilon_{ijk}$$

where

$i = 1, \ldots, 6,$

$j = 1, \ldots, 3,$

$k = 1, \ldots, a_h \ (2 \le a_h \le 6),$

and

$\mu =$ the overall mean,

$\alpha_i =$ effect of i^{th} stream,

β_j = effect of the j^{th} sample occasion,

$\gamma\alpha\beta_{k(ij)}$ = effect of the k^{th} site nested within the i^{th} and j^{th} level of the streams,

$(\alpha\beta)_{ij}$ = the stream×sample occasion interaction, and

ε_{ijk} = random error due to sampling.

Type 3 *F*-statistics were used in the analysis and normality assumptions were assessed using residual analysis. The assumption of independence of the samples was not met for sites within streams because of autocorrelation of samples taken from the same stream. Tukey's multiple comparison procedure was used to compare all pairwise differences of the means. For all significance tests, $\alpha = 0.05$.

Otolith Microchemistry

Two sets of rainbow trout otoliths were collected for microchemical analysis. The first set of otoliths was from juveniles collected from the three spawning tributaries (Little Prickly Pear Creek, the Dearborn River, and Sheep Creek) as the fish outmigrated to the mainstem Missouri River (Figure 5.1). It was assumed that these fish had spent their entire lives within the tributary where they were captured; therefore, the chemical composition of their otoliths would be representative of all fish from the same tributary. Fish were captured in rotary screw traps near the mouths of Little Prickly Pear Creek and the Dearborn River, and weir traps near the mouth of Sheep Creek. Samples of migrating juvenile rainbow trout were taken from each trap every other week during peak outmigration and less often at other times. A maximum of 10 fish for every 10-mm length-class present in the live-wells of the traps were randomly subsampled and kept for

otolith analysis. Total length (to the nearest mm; TL) was measured, date of capture, and trap location was noted for each fish sampled. Fish were stored in individual plastic bags and frozen until the otoliths were extracted. Otoliths from 10 fish from each of the three spawning tributaries were selected for microchemical analysis (age-1, mean TL = 87 mm, SD = 11.6). Half of the fish from each tributary were randomly selected from fish collected in May 1998 and the rest were randomly selected from fish collected in May 1999.

The second set of otoliths was from adult rainbow trout of known origin. These fish had been batch-tagged with CWTs as young-of-the-year (YOY) or yearlings as they migrated from the tributaries and were later recaptured in the Missouri River during the annual fall population estimates (Munro et al. 2003). The tags were placed in different body locations to indicate the tributary of origin. As with the group of juvenile fish, TL was recorded and the fish were frozen until the otoliths could be extracted. The otoliths of 9 of these fish (3 from each tributary) were randomly selected for chemical analysis. All of these fish had been tagged in 1998 and were recaptured 5 to 29 months after tagging.

Upon extraction, otoliths were cleaned as described by Fowler et al. (1995), and stored dry in acid-washed polyethylene vials. Thin sagittal sections of the otoliths were prepared using standard methods described by Secor et al. (1992). Clean, dry otoliths were mounted sulcus side up on glass microscope slides using thermoplastic polymer (Crystalbond 509). Otoliths were ground just past the sulcal groove with 2,000-grit wet/dry sandpaper then polished with 0.3-µm aluminum oxide polishing powder. The

other side of the otoliths was then ground and polished to the nucleus. Sample slides were cut to 1×1 cm around the otolith, in order to fit in the sample holder for chemical analysis with time-of-flight secondary ion mass spectrometry (ToF-SIMS; Chapter 3), and ultrasonically cleaned in a series of baths of analytical grade hexane and methanol. The otoliths were sputter-coated with 15 nm of Au-Pd alloy to give the samples a conductive surface and reduce sample charging during ToF-SIMS analysis. A final ultrasonic cleaning in hexane and methanol baths removed surface contaminants. Clean methods were used throughout otolith extraction, preparation, and analysis. This included using only non-metallic acid-washed instruments, wearing latex gloves when handling the samples, and ultrasonically cleaning the samples and equipment in Milli-Q water between the grinding and polishing stages and between samples.

Otolith microchemical analyses were conducted in a Phi-Evan's triple focusing time-of-flight secondary ion mass spectroscopy system at the Image and Chemical Analysis Laboratory, Physics Department, Montana State University (Schueler 1992 , Chapter 3). Positive ion spectra were collected from the areas of interest on the otoliths using a 9×9 Ga^{+} beam as described in Chapter 3. Ion counts for ^{88}Sr and ^{44}Ca were measured from the spectra and Sr:Ca ratios were calculated using adjusted ^{44}Ca ion counts that estimated ^{40}Ca counts (see Chapter 3). Internal consistency (i.e., precision of measurements from session to session) of Sr:Ca measurements with the ToF-SIMS was checked at the start of each session using an otolith 'standard' made of ground otoliths as described in Chapter 3. Ion counts for additional elements were also measured from the spectra. These elements included the alkali and alkali earth metals Li, Na, Mg, and Ba;

the transition metals Zn, Mn, Fe, and Cd; plus As, Sb, and Se. As with Sr, the elements were normalized to Ca counts for analysis. However, only Fe, and Se were consistent in elemental ratios (element:Ca) from session to session, like Sr:Ca (see Appendix). This indicates that either these elements were not homogeneously distributed within the otolith 'standard', they were more sensitive to sample preparation and storage methods, or there was inherent variability in the measurements because of matrix effects.

To characterize the composition of the otoliths of juvenile fish from the different tributaries, two to five sites were analyzed within each otolith. This accounted for variation within the otoliths. Variation within an analysis site was accounted for by sampling the same site two to three times for four randomly selected otoliths per tributary.

Otolith elemental ratios of the outmigrating juvenile rainbow trout were compared among the three tributaries with a series of univariate ANOVAs. The model used to analyze the data included two fixed factors: the natal stream (Little Prickly Pear Creek, the Dearborn River, or Sheep Creek) and year collected (1998 or 1999). In addition to the fixed factors, two random factors (fish, and otolith replication) were included in the model to account for variation from fish to fish and variation within a given otolith. This resulted in the following linear mixed model:

$$y_{ijklm} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_{k(ij)} + \delta_{l(k(ij))} + \varepsilon_{ijklm},$$

where

i = 1, …, 3,

j = 1, …, 2,

k = 1, …, 5,

l = 1, …, a_h ($2 < a_h < 5$),

m = 1, ..., n,

and

μ = overall mean,

α_i = effect of the i^{th} stream,

β_j = effect of the j^{th} year,

$(\alpha\beta)_{ij}$ = stream×year interaction,

$\gamma_{k(ij)}$ = effect of the k^{th} fish within the i^{th} level of stream and j^{th} level of year,

$\delta_{l(k(ij))}$ = effect of the l^{th} site within the k^{th} fish within the i^{th} level of stream and j^{th} level of year,

ε_{ijklm} = random error due to sampling.

Type 3 *F*-statistics were used in the ANOVA and the Satterthwaite approximation was used to calculate the degrees of freedom for the statistical tests (PROC MIXED, Littell et al. 1996; SAS Institute 2000). Statistical correlation was estimated using the restricted maximum likelihood method (REML) and compound symmetry covariance structure (i.e., constant variance and constant covariance). The normality assumption was assessed using residual analysis. Tukey's multiple

comparison procedure was used to compare all pairwise differences of the least-square means of the stream effect. For all significance tests $\alpha = 0.05$.

Nearest-neighbor discriminant analysis (PROC DISCRIM, SAS Institute 2000) was used to estimate classification accuracy of natal origin using otolith composition. Three different variable selection procedures (forward selection, backward elimination, and stepwise selection) (PROC STEPDISC, SAS Institute 2000) were first used to determine if all of the elements were necessary for effective discrimination and which elements were the best discriminators (Johnson 1998). The forward selection and stepwise selection procedures selected only Sr. The backwards elimination procedure selected Sr, Ba, Mg, Zn, Fe, Cd, Sb, and Se elemental ratios. For the forward selection procedure, entry of a variable into the discriminating set was contingent on its having a significant ($\alpha < 0.15$) F value in an analysis of covariance (ANCOVA), with the variables already in the set modeled as covariates. For the backwards elimination procedure a variable remained in the discriminating set as long as its F value was significant ($\alpha < 0.15$) in an ANCOVA, again with the other variables modeled as covariates. The stepwise procedure added and retained variables in the discriminating set using the same levels of significance as the other two procedures (Johnson 1998; SAS Institute 2000).

The otolith elemental data from the 30 juvenile outmigrant rainbow trout were then used to build non-parametric discriminant models – one model with Sr:Ca as the only discriminator, and the other with the subset of elemental ratios selected by the backwards selection procedure as the discriminators. For both models, the mean otolith

element:Ca ratio for each fish, weighted by number of samples in both zones, was used to construct the discriminant rule.

To determine the accuracy of the discriminant models in predicting natal tributary in adult rainbow trout, elemental ratios were measured for one to five sites within the first year of growth of the recaptured known-origin adults. The mean elemental ratios of the 'tributary growth' for each of the fish was then classified into one of the three spawning tributaries using the discriminant criterion developed with the juvenile outmigrant dataset for both the Sr:Ca and multi-element models. Given the results of these analyses (see 'Results'), I did not pursue the identification of natal origin in Missouri River rainbow trout by otolith microchemistry any further.

Sample Size Estimation for Otolith Microchemistry Analyses

Sampling the appropriate number of experimental units is an important aspect to any study in order to achieve a desired level of detection among populations. However, in the case of freshwater otolith microchemistry there are few studies on which to base estimates of the distribution parameters (mean and variance) for the otolith composition of fish from a given stream or river. It is, therefore, difficult to estimate the appropriate sample size. But given the well documented relation between water Sr:Ca and otolith Sr:Ca (see, Campana 1999; Bath et al. 2000; Elsdon and Gillanders 2003; Wells et al. 2003, Chapter 3), one can predict the expected mean otolith composition of fish from a given stream based on preliminary water samples, which are easily collected and relatively inexpensive to analyze. An approximate minimum number of fish to sample from each stream can then be calculated based on the desired level of detection. A

fisheries manager or researcher can then determine, based on initial water sampling, whether or not it would be feasible to differentiate among fish from the different locations of interest, and if so, they can budget appropriately.

I illustrate this method of sample size estimation using the Missouri River spawning tributary water and otolith Sr:Ca data from this study and the equation for the relation between water Sr:Ca and otolith Sr:Ca (Figure 3.6, Chapter 3). First, approximate 95% confidence intervals for the mean water Sr:Ca for each of the streams (Little Prickly Pear Creek, Dearborn River, and Sheep Creek) were calculated using the formula:

$$\bar{x}_{stream_i} \pm 2\frac{s_{stream_i}}{\sqrt{n_{stream_i}}} \quad (5.1)$$

The upper and lower bounds of the confidence interval were then used to interpolate the expected upper and lower confidence bounds for the otolith composition of fish from that stream (Figure 5.2). Minimum sample sizes needed to detect the desired level of difference between pairs of streams were then approximated from the solution for n_1 and n_2 in Equation 5.2.

$$\hat{\bar{y}}_1 - \hat{\bar{y}}_2 = \delta \geq 2\left(\frac{s_1^*}{\sqrt{n_1}} + \frac{s_2^*}{\sqrt{n_2}}\right) \quad (5.2)$$

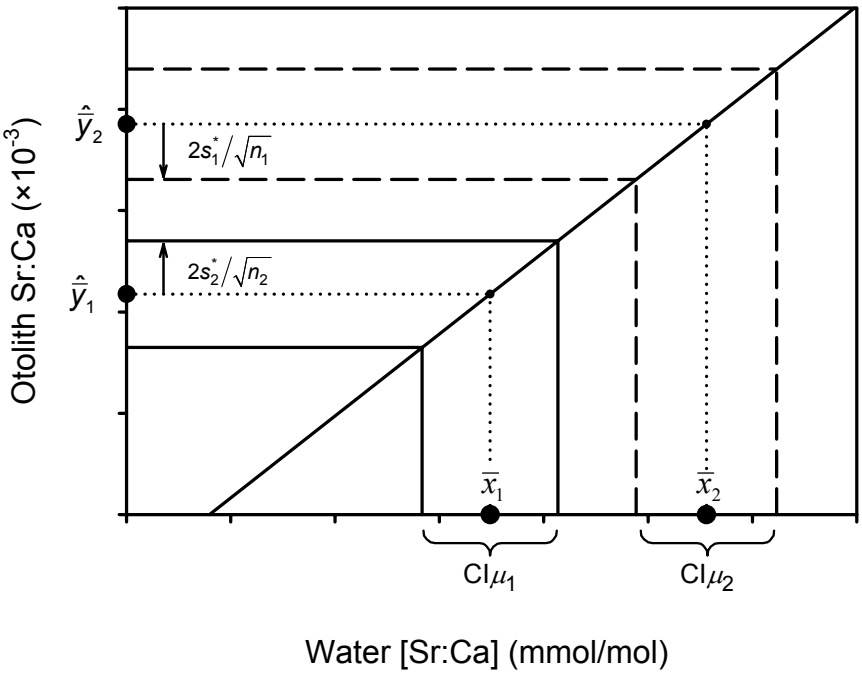


Figure 5.2. Diagram illustrating estimation of mean and variance of otolith Sr:Ca based on water Sr:Ca in order to estimate approximate minimum sample sizes.

Where $\hat{\bar{y}}_1$ and $\hat{\bar{y}}_2$ are the estimated mean otolith Sr:Ca of the two streams being compared, δ is the difference between the estimated mean otolith Sr:Ca of the two streams (or the desired detectable difference; e.g. 0.01×10^{-3}), s_1^* and s_2^* are estimates of the sample standard deviations, and n_1 and n_2 are the number of fish sampled from each stream. This procedure is essentially estimating the maximum widths of nonoverlapping approximate 95% confidence intervals for the two population means. Minimum sample sizes needed were then estimated for every stream pair combination, under the restriction that $n_1 = n_2$ in each calculation. For comparison, I also estimated the minimum sample size needed to detect differences among the streams using the observed otolith Sr:Ca.

Scale Growth Pattern

In order to use scale patterns to estimate contribution of different juvenile outmigration strategies, I first needed to verify that 'checks' observed in the scales of Missouri River rainbow trout were indeed migration checks and could be used to determine age at migration. To do this I examined the scales of rainbow trout whose age at migration was known. Scale samples from rainbow trout that had been CWTed as outmigrating juveniles and later recaptured in the mainstem river as adults (see Munro et al. 2003) were examined for the presence of a migration check and its location in relation to the annuli. Juvenile fish were tagged from 1998 to 2000 and a different code was used each year so that with the age of the fish the age at migration could be determined. Scales were cleaned of skin, dirt and mucus, and at least three scales that were not damaged or regenerated were selected for each fish. If no scales for an individual fish met these requirements, then that individual was removed from further analysis. Impressions of the selected scales were then made on acetate sheets. Scales were examined at 40 to 100× magnification using a compound microscope. Identification of annuli to estimate age was done using standard ageing criteria (Jearld 1983). Identification of occurrence, and location, of a migration check was done using criteria typically used to identify the freshwater growth zone in anadromous salmon and trout and to separate hatchery from wild fish (e.g., Mosher 1969; Seelbach and Whelan 1988; Bernard and Myers 1996; Marcogliese and Casselman 1998). Scale circuli formed during tributary rearing were characterized as being thinner and more closely spaced (Figure 5.3) than circuli formed in the mainstem Missouri River (cf, Mosher 1969; Davis

et al. 1990; Rosenau 1991). To check for variability in formation of the migration check, all scales from the recaptured CWTed adult rainbow trout were aged three separate times, with at least one month between each age estimate. For every scale, radius and distance from the focus to each annulus and migration check (if present) were measured using a compound microscope equipped with a drawing attachment and digitizing pad connected to a computer with Calcified Structure Age and Growth Data Extraction Software (Casselman and Scott 1991). Measurements were made on all pressed scales independent of type of tag code (i.e., age at migration).

To assess recruitment of the different juvenile life histories into the mainstem rainbow trout population I then examined rainbow trout scale samples from Missouri River annual population estimates (supplied by MT FWP). A subset of acetate scale impressions from the 1997 to 2000 Craig section samples were examined for the presence of a migration check. The Craig section extends from Wolf Creek Bridge to Cascade (8.2 river-km) and 86% (24/28) of the CWT fish recaptured in this section were from Little Prickly Pear Creek (see Results: Coded Wire Tag Recaptures). At least 50 samples were randomly selected from fish that were ≤ 400 mm (age-1 to age-2) for each year. This meant that age classes 1995 through 1999 were examined. Two readers (myself and P. Hamlin, MT FWP, Great Falls) independently aged all fish. One scale from each fish was selected and scale radius and distance to each annulus and migration check were measured as described above.

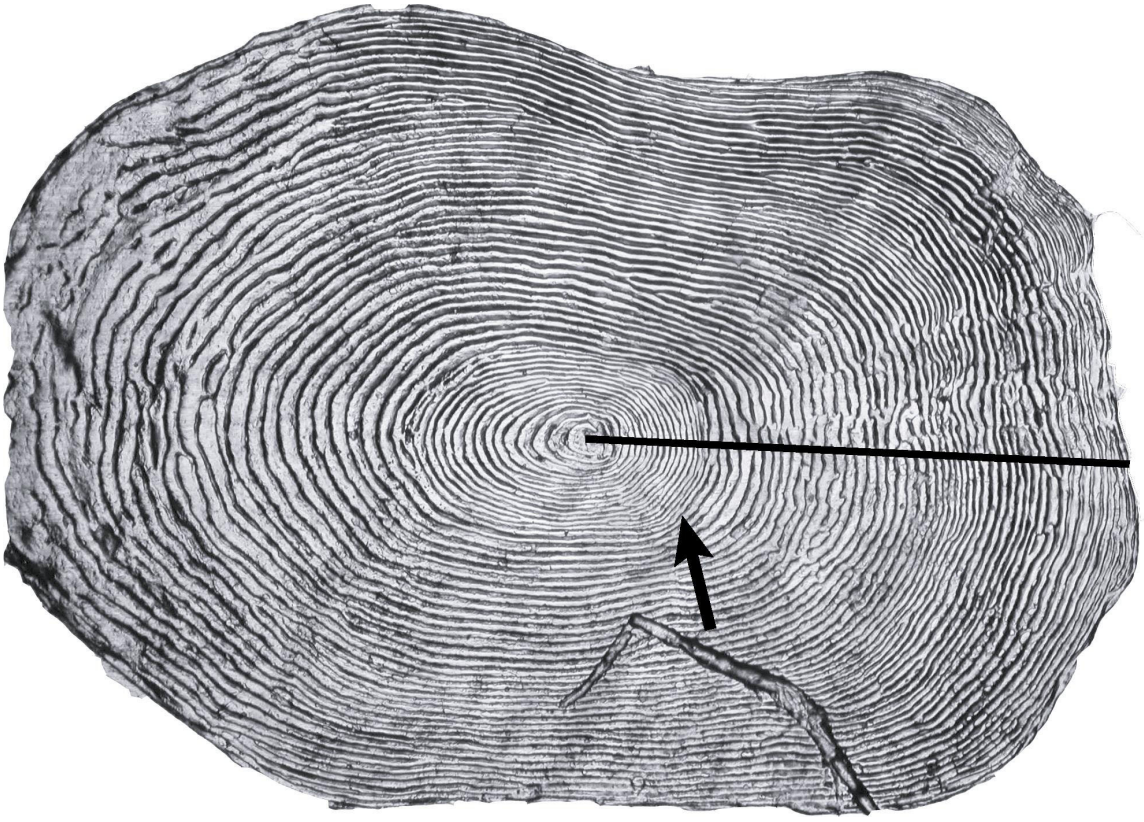


Figure 5.3. Scale of a 3 year-old rainbow trout tagged as an yearling migrant from Sheep creek in 1998 and recaptured in 2000 in the mainstem Missouri River. Arrow marks location of the migration check and first annulus. Line indicates measurement axis.

In addition to age at migration, I estimated the length at migration to determine if it had changed over time. The Fraser-Lee method of back-calculation (Ricker 1975) was used to estimate length at migration for the subsample of scales from the Craig section of the Missouri River. Weighted least-squares regression was used to estimate the intercept of the fish body length to scale radius relation, which is used in the Fraser-Lee equation. The scale samples from the Craig section, plus the CWT recaptured fish, and a sample of 55 scales from outmigrating juvenile rainbow trout were used to define this relation.

Juvenile outmigrants > 45 mm were used in this relation because the initial scale platelet is typically not fully formed in smaller fish (Ward et al. 1989).

Coded Wire Tag Recaptures

As mentioned in the introduction, tagging fish is a commonly used approach to estimate contribution of various sources to a population. While the relatively small number of recaptured CWT rainbow trout precludes a rigorous statistical analysis, these fish do provide insight into the dynamics of the rainbow trout population in the Missouri River. This is because location of the tag and tag code provided information on the natal origin and outmigration life history of each of the recaptured fish. I compared the composition of outmigration strategies in the sample of recaptured adult rainbow trout to the proportion of YOY and yearling outmigrants that were tagged between 1998 and 2000 to assess if certain juvenile life history strategies were more successful than others. In addition, the section of river where the CWTed fish were recaptured was used as an index of the contribution of the various spawning tributaries to the population monitoring sections of the Missouri River (Craig, Hardy, and Cascade).

Results

Water Chemistry

The estimated standard deviation of Sr:Ca in duplicate water was 0.01 mmol/mol. The percent difference for each pair of samples averaged 3.49% (range: 0.00-19.98%). Only 4 of 45 paired-samples differed by more than 10%.

Water Sr:Ca ratios differed significantly among streams ($F = 881.58$; df = 5, 46; $P < 0.0001$), varying by a factor of about two from the lowest to the highest Sr:Ca values (Figure 5.4). Tukey's multiple comparison tests indicated that the streams fit into three groups whose mean Sr:Ca ratios were similar within each group ($P > 0.05$) but significantly different between the groups. The mean Sr:Ca ratios of Sheep Creek (2.97×10^{-3}), Lyons Creek (3.23×10^{-3}), and the Missouri River (3.01×10^{-3}) were around two times higher than Little Prickly Pear Creek (1.65×10^{-3}), and the Dearborn River (1.74×10^{-3}) (Table 5.1). Wolf Creek Sr:Ca (2.17×10^{-3}) was significantly different than all other streams.

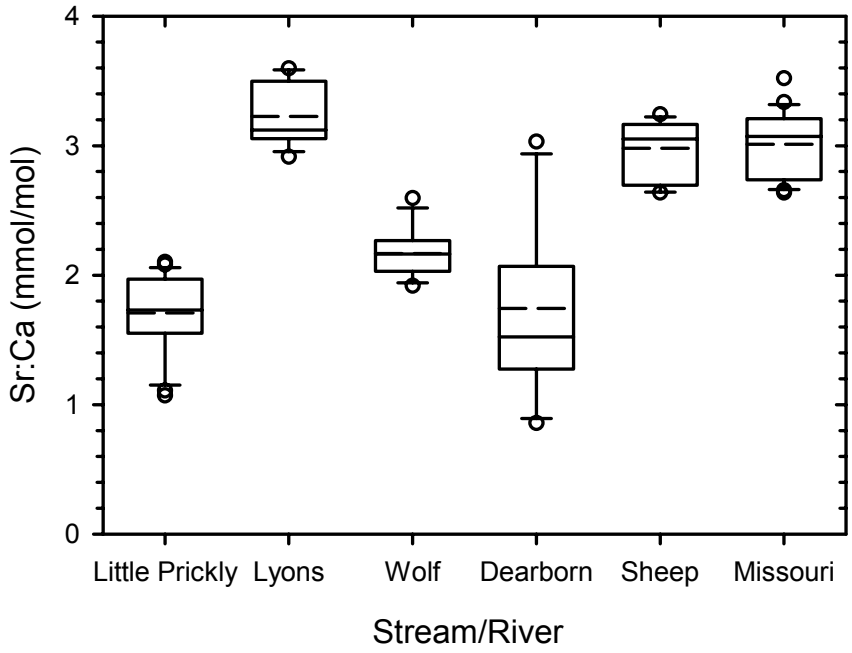


Figure 5.4. Mean Sr:Ca ratios of water samples collected from the Missouri River and spawning tributaries on three occasions during 1999 (late winter, spring runoff, and late summer) from two to six sites within each water body. Boxes show the mean Sr:Ca ratios (dashed line), media (central solid line), 1st and 3rd quartiles (box edges) and individual outliers (circles) outside the 10th and 90th percentiles (whiskers). Total n = 106.

Within-tributary variation was also significant in some streams ($F = 21.34$; df = 12, 62; $P < 0.0001$) (Figure 5.5). In Little Prickly Pear Creek, Sr:Ca of two major tributaries, Lyons Creek and Wolf Creek, were significantly higher than the Little Prickly Pear mainstem. In addition, longitudinal variation in Sr:Ca was also found within Little Prickly Pear Creek and the Dearborn River, with significant increases in Sr:Ca ratios among downstream samples (Little Prickly Pear Creek: correlation coefficient (ρ) = 0.91, $P < 0.0001$; Dearborn River: $\rho = 0.83$, $P < 0.0001$; Figure 5.5). In contrast, there was no relation between Sr:Ca and sample location for Sheep Creek ($\rho = -0.01$; $P = 0.98$), or the Missouri River ($\rho = -0.28$; $P = 0.18$) suggesting that fish residing throughout these water bodies experienced similar Sr:Ca ratios.

Table 5.1. Summary of water Sr:Ca ratios (means and ranges) and mean daily discharge for Little Prickly Pear Creek, two of its tributaries (Lyons and Wolf creeks), the Dearborn River, Sheep Creek, and the Missouri River for the three sets of samples collected in 1999. All discharge data are from the USGS gaging stations, except for Sheep Creek, which is estimated from a stream gage at the fish trap site.

		March			May			October		
Stream	*n*	Discharge	Sr:Ca (mmol/mol)		Discharge	Sr:Ca (mmol/mol)		Discharge	Sr:Ca (mmol/mol)	
		(m^3/s)	mean	Range	(m^3/s)	mean	range	(m^3/s)	mean	range
Little Prickly	9	1.46	1.785	(1.161-2.103)	7.95	1.727	(1.108-2.053)	1.23	1.611	(1.070-1.975)
Lyons	4	—	3.517	(3.369-3.598)	—	3.073	(3.042-3.135)	—	3.092	(2.915-3.260)
Wolf	4	—	2.247	(2.162-2.339)	—	2.222	(2.058-2.595)	—	2.031	(1.918-2.194)
Dearborn	6	1.43	1.992	(1.483-2.632)	18.53	1.312	(0.857-1.826)	1.54	1.925	(1.271-3.032)
Sheep	4	0.11	3.042	(3.010-3.071)	3.62	3.185	(3.136-3.244)	0.18	2.669	(2.638-2.699)
Missouri	8	193.93	3.228	(3.083-3.520)	188.33	2.976	(2.637-3.212)	125.65	2.829	(2.655-3.338)

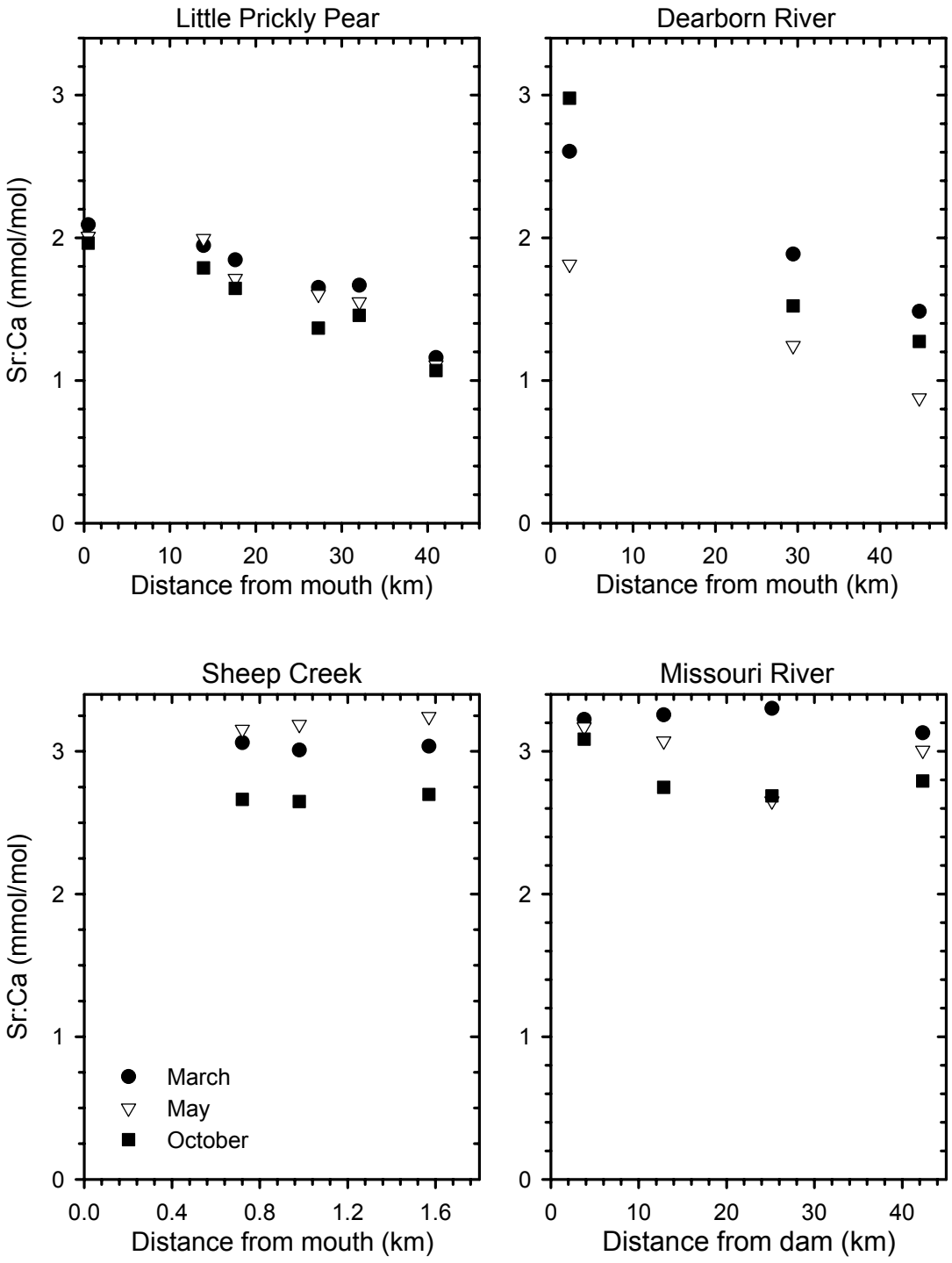


Figure 5.5. Water Sr:Ca ratios of Missouri River spawning tributaries and mainstem river. Note that the distances between samples sites (x-axis) are not all on the same scale.

Water Sr:Ca differed significantly among sampling occasions in some of the streams ($F = 66.44$; df = 2, 46; $P < 0.0001$) and there was a significant sampling occasion×tributary interaction ($F = 22.83$; df = 10, 46; $P < 0.0001$). However, there was no consistent pattern between the Sr:Ca ratio and the season sampled. The Sr:Ca ratios were lowest in the spring samples (high-discharge) for the Dearborn River and Lyons Creek, while it was the opposite for the Missouri River (highest Sr:Ca was associated with highest discharge). These results suggest that for the Dearborn River and Lyons Creek, Sr:Ca levels are diluted by surface runoff in the spring and the Sr:Ca of the Missouri River is influenced by the release of water from Holter Reservoir, which possibly has a higher Sr:Ca ratio than the other sources of water for this section of the Missouri River. An explanation for the pattern of Sr:Ca in Sheep Creek is more difficult because the spring sample (highest discharge) and the late winter sample (lowest discharge) were associated with the two highest Sr:Ca ratios (Figure 5.6). Sr:Ca was similar among the seasons sampled for both Little Prickly Pear and Wolf creeks.

Despite the significant variation in Sr:Ca among sampling occasions within some of the streams, the mean CV of replicate samples collected in different seasons was low (9.7%). The Dearborn River had the highest variation (21-25%). The other streams all had CVs < 10% (similar to the paired duplicate samples). This suggests that the temporal variation was not as important as within and between stream variations, except for the Dearborn River.

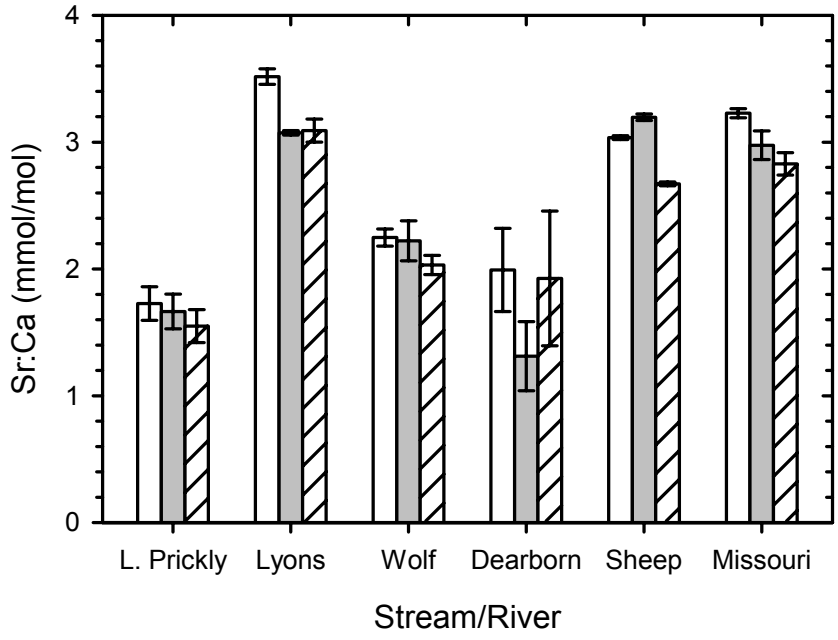


Figure 5.6. Variation among three sampling occasions of mean water Sr:Ca (± SE) collected from the Missouri River and spawning tributaries. Open bars are late winter (March 1999), shaded bars are spring runoff (May 1999), and hatched bars are late summer (October 1999).

Otolith Microchemistry

Juvenile Outmigrant Rainbow Trout. Otolith Sr:Ca of juvenile rainbow trout from the three Missouri River spawning tributaries was significantly different (F = 21.50, df = 2, 25, $P < 0.0001$, Figure 5.7). Mean otolith Sr:Ca (0.63×10^{-3}, SD = 0.115×10^{-3}) of Sheep Creek fish was significantly higher (Tukey's, $P < 0.0001$) than Little Prickly Pear Creek (0.45×10^{-3}, SD = 0.093×10^{-3}) and Dearborn River fish (0.44×10^{-3}, SD = 0.101×10^{-3}). However, the otolith Sr:Ca of Little Prickly fish was not significantly different from that of Dearborn River rainbow trout ($P = 0.99$). Year was not a significant main effect ($F = 2.22$, df = 1, 25, $P = 0.149$), but the interaction between stream and year was significant ($F = 4.87$, df = 2, 25, $P = 0.016$), due to the

significant difference between the otolith Sr:Ca of Sheep Creek fish in 1998 and 1999 Sheep Creek fish (Tukey's, $P = 0.023$).

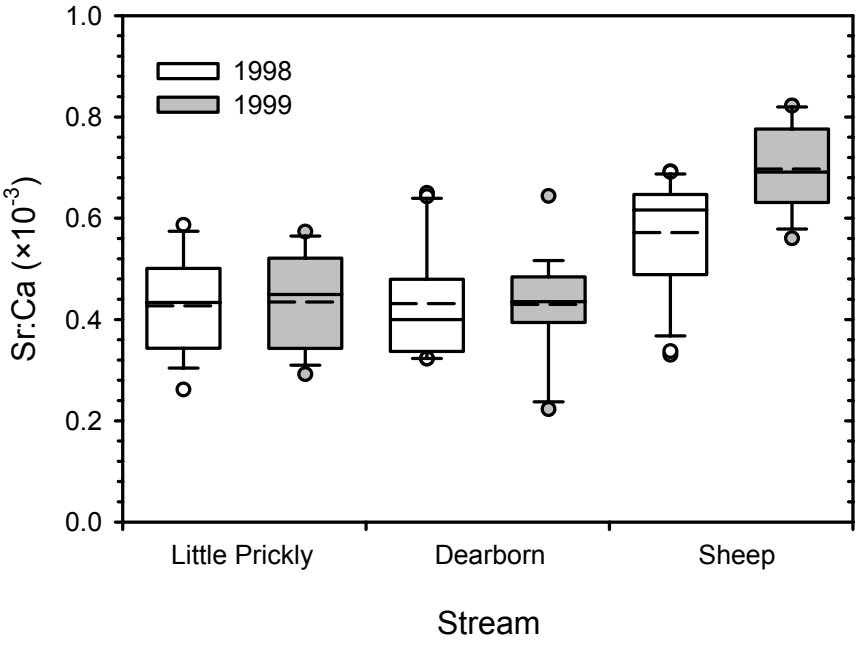


Figure 5.7. Comparison of otolith Sr:Ca of juvenile rainbow trout from three Missouri River spawning tributaries. Fish were collected from Little Prickly Pear Creek, Dearborn River, and Sheep Creek in 1998 (open boxes) and 1999 (shaded boxes) - 5 fish/stream/year ($n = 110$ analyses). The boxes show the mean Sr:Ca (dashed line), median (central solid line), 1st and 3rd quartiles (box edges) and individual outliers (circles) outside the 10th and 90th percentiles (whiskers).

In addition to Sr:Ca ratios, I measured other elemental ratios (element:Ca) in the otoliths juvenile rainbow trout from the Missouri River spawning tributaries in order to determine if any of these elements improved differentiation among rainbow trout from the different spawning tributaries. The elements measured included the alkali and alkali earth metals Li, Na, Mg, and Ba; the transition metals Zn, Mn, Fe, and Cd.; plus As, Sb, and Se. However, unlike Sr:Ca, the were no significant differences in the mean elemental ratios among the tributaries for any of these elements (Table 5.2). This was

not surprising given the variability in the measurement of many of these elements in the otolith "standard" (see Appendix).

Table 5.2. Results of mixed model analysis of variance (ANOVA) comparing elemental ratios in juvenile rainbow trout otoliths collected from three Missouri River spawning tributaries (Little Prickly Pear Creek, Dearborn River, and Sheep Creek) in 1998 and 1999. Type 3 *F* tests were used to test for the fixed effects of tributary, collection year, and tributary×year interaction.

	Tributary		Year		Tributary×Year		
Element	*F*	Pr > *F*	*F*	Pr > *F*	*F*	Pr > *F*	df[1]
Sr	21.50	< 0.0001	2.22	0.15	4.87	0.02	25
Ba	0.87	0.42	2.54	0.11	3.90	0.02*	93
Mg	1.37	0.27	0.00	0.96	0.86	0.44	25
Li	0.72	0.49	1.06	0.31	1.60	0.22	24
Na	0.43	0.66	7.79	0.01	0.49	0.62	24
Zn	1.28	0.30	5.81	0.02*	1.26	0.30	23
Mn	0.09	0.91	0.09	0.76	0.19	0.83	24
Fe	0.26	0.77	0.30	0.59	1.09	0.35	23
Cd	0.17	0.84	0.14	0.71	0.12	0.88	32
As	0.27	0.76	0.39	0.53	0.53	0.59	26
Se	0.49	0.62	8.12	0.01*	0.85	0.44	23
Sb	0.31	0.74	0.00	0.99	0.17	0.84	24

[1] Numerator degrees of freedom for tests of tributary, year, and tributary×year effects were 2, 1, 2, respectively.

* Significant effects.

As in the ANOVA, nearest-neighbor discriminant analysis using only Sr:Ca indicated that Sheep Creek juvenile rainbow trout were different than Little Prickly Pear Creek and the Dearborn River fish, and the fish from the latter two spawning tributaries are more difficult to distinguish from each other. The overall correct classification rate of the cross-validation procedure was 73%. Juvenile rainbow trout from Sheep and Little Prickly Pear creeks were 90% correctly reclassified (Table 5.3). However, correct reclassification of the Dearborn River fish was only 50%. The Dearborn River fish that were incorrectly reclassified were predicted to have come from both Little Prickly Pear and Sheep creeks. The reason for the low correct reclassification rate of Dearborn River fish is that the mean Sr:Ca ratios were similar to that of Little Prickly Pear Creek fish, resulting in posterior probabilities that favored classification into Little Prickly Pear Creek, whereas other Dearborn River juvenile rainbow trout had high Sr:Ca (similar to Sheep Creek fish) and were therefore classified as to more likely belonging to Sheep Creek (Figure 5.7).

The multi-element discriminant analysis model did not improve the re-classification accuracy of juvenile rainbow trout into their natal tributary over the Sr:Ca model. Similar to the Sr:Ca discriminant analysis, overall reclassification accuracy was 73%, and 90% of the Little Prickly Pear and Sheep creek fish were correctly reclassified (Table 5.4). Reclassification accuracy of the Dearborn River fish decreased to 40%, but inclusion of the additional elements eliminated the misclassification of fish into Sheep Creek.

Table 5.3. Classification of juvenile rainbow trout outmigrants into their natal tributary using Sr:Ca. Cross-validation results for the juvenile rainbow trout dataset (n = 10 fish/tributary) were used to assess classification accuracy.

	% classification into tributary			
From tributary	Little Prickly Pear	Dearborn	Sheep	Tie[1]
Little Prickly Pear	90.0	10.0	0.0	0.0
Dearborn	30.0	50.0	20.0	0.0
Sheep	0.0	0.0	90.00	10.0
Total	40.0	20.0	36.7	3.3
	Error count estimates for tributary			
Rate	0.10	0.50	0.10	

[1] Highest posterior probability was the same for more than one group.

Table 5.4. Classification of juvenile rainbow trout outmigrants into their natal tributary using Sr, Ba, Mg, Zn, Fe, Cd, Sb, and Se. Cross-validation results for the juvenile rainbow trout dataset (n = 10 fish/tributary) were used to assess classification accuracy.

	% classification into tributary			
From tributary	Little Prickly Pear	Dearborn	Sheep	Tie[1]
Little Prickly Pear	90.0	0.0	0.0	10.0
Dearborn	50.0	40.0	00.0	10.0
Sheep	10.0	0.0	90.00	0.0
Total	50.0	13.3	30.0	6.7
	Error count estimates for tributary			
Rate	0.10	0.60	0.10	

[1] Highest posterior probability was the same for more than one group.

Known-origin Adult Rainbow Trout. Accuracy of the Sr and multi-element discriminant analysis functions were tested using an independent dataset of known-origin adult rainbow trout. Overall classification accuracy of the Sr discriminant analysis model was lower for the test dataset (known-origin adults) than the cross-validation accuracy using the calibration data set of juvenile rainbow trout (67% vs. 73%). Whereas Sheep Creek fish had a 100% correct classification rate (mean Sr:Ca = 0.94×10^{-3}), classification accuracy of Dearborn River and Little Prickly Pear Creek fish was low, especially for Little Prickly Pear fish (Table 5.5). Of particular note was the low classification accuracy (33%) of the Little Prickly Pear Creek known-origin adult fish compared to the 90% re-classification of the juvenile outmigrant fish. The mean Sr:Ca of the known-origin adult fish that were misclassified into Sheep Creek was 0.59×10^{-3}, compared to 0.45×10^{-3} in the juvenile outmigrant otoliths. The reason for the higher Sr:Ca ratios in the tributary growth of the adult fish compared to that of the juvenile fish is unclear. Perhaps it is a result of these fish having been spawned in Lyons or Wolf Creek (both tributaries of Little Prickly Pear Creek), which have significantly higher Sr:Ca in the water and would therefore be expected to have higher Sr:Ca in the otoliths.

Classification accuracy of the known-origin adult rainbow trout in the multi-element discriminant analysis model was lower than that of the Sr discriminant analysis model (55 vs. 67%). As with the Sr:Ca only model, all Sheep Creek fish were correctly classified into their natal tributary, and one Little Prickly Pear fish was correctly classified. However, with Dearborn River fish, only one was correctly classified into its

natal tributary, and the other two were misclassified as to having come from Little Prickly Pear Creek.

Table 5.5. Classification of known-origin adult rainbow trout into their natal tributary using Sr:Ca. These classification results for the known-origin adult rainbow-trout dataset (n = 3 fish/tributary) were used as an independent assessment of the discriminant model accuracy.

From tributary	% Classification into tributary		
	Little Prickly Pear	Dearborn River	Sheep Creek
Little Prickly Pear	33.3	0.0	66.7
Dearborn	33.3	66.7	0.0
Sheep	0.0	0.0	100.0
	Error count estimates for tributary		
Rate	66.7	33.3	0.0

Sample Size Estimation. Predictions of otolith Sr:Ca mean and standard deviation based on water chemistry samples appeared to produce reasonable results (Figure 5.8). Both the predicted mean and approximate 95% confidence interval of otolith Sr:Ca based on water samples for Little Prickly Pear Creek were similar to the observed mean and confidence interval of otolith Sr:Ca from Little Prickly Pear Creek fish. The predicted mean otolith Sr:Ca for the Dearborn River was similar to the observed value, but the approximate confidence intervals were wider. This is due to the fact the variability of the water chemistry in the Dearborn River and the limited number of sites that could be accessed to sample (increasing the number of samples would

narrow the interval estimate). The predicted approximate confidence interval for the otolith Sr:Ca Sheep Creek was similar to the observed; however, the predicted mean was higher than the observed value. The reason for this is that the observed otolith Sr:Ca for Sheep Creek fish was below the fitted regression line (Figure 3.6).

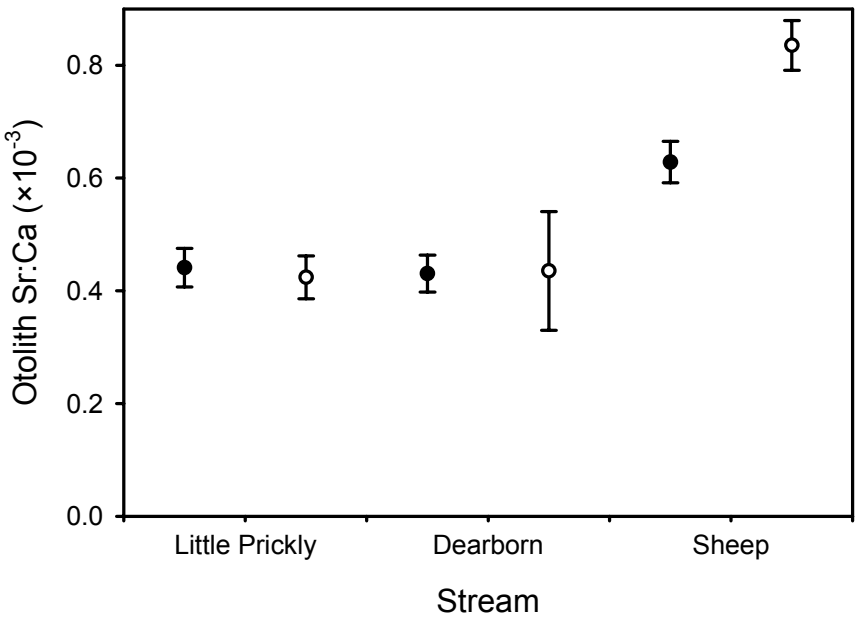


Figure 5.8. Comparison of observed (closed circles) and predicted (open circles) mean (±95% CI) otolith Sr:Ca for the three main spawning tributaries in the Missouri River. Predicted mean and confidence interval were calculated from the regression equation for the water/otolith relation (Chapter 3) and predicted Sr:Ca based on water.

Based upon the predicted 95% confidence interval for Little Prickly Pear Creek and Dearborn River rainbow trout otoliths, I conservatively estimated that a minimum of 4,158 otoliths from each stream would need to be analyzed in order to detect a difference (δ) of 0.01×10^{-3} between these two streams. In contrast, a sample size of only 4 fish from each stream would be needed to detect differences between fish from Little Prickly Pear and Sheep creeks ($\delta = 0.18\times10^{-3}$). To detect differences between fish from the

Dearborn River and Sheep Creek fish a minimum of 10 fish from each stream would need to be analyzed ($\delta = 0.19\times10^{-3}$). The δs used in each analysis were based on the observed differences between the mean otolith Sr:Ca of the stream pair in the analysis.

Using the observed Sr:Ca values from the juvenile outmigrant rainbow trout and the same values for δ, the minimum number of samples needed to detect differences between Little Prickly Pear Creek and Dearborn River fish was still very large and was estimated to be 1,505 (compared to the estimated 4,505 fish based on water Sr:Ca). For comparisons between Little Prickly Pear and Sheep creeks and the Dearborn River and Sheep Creek the estimated number of fish needed to detect differences were closer to the numbers estimated from the water chemistry (3 and 4, respectively). Thus indicating that estimating minimum sample sizes based on water samples is a reasonable (but conservative) approach that would be beneficial in the initial stages of study design.

Scale Growth Pattern

The scales of rainbow trout whose age at migration was known were first examined to determine if a migration check was present and if its location, in relation to the annuli could be used to determine the age at migration. Between 1998 and 2000, 126 adult CWTed rainbow trout were recaptured in the mainstem Missouri River during the MT FWP fall population estimates. Seven of the fish were removed from further analysis because the fish were released after detecting the CWT or because all of the scales sampled were regenerated. Recognition of the presence or absence of a migration check in the scales of a fish was consistent (96%); suggesting that the presence or

absence of such a mark is consistent among the scales typically sampled from the rainbow trout.

Migration checks were positively identified in the scales of 94.8% of the known yearling migrants. In two of the fish, the migration check was located at the second annulus, indicating that these fish may have been late yearling outmigrants or remained in the tributary for an additional winter after being tagged. There was not a distinct migration check in the scales of any of the known YOY migrants (2 fish). These results suggest that the presence of a migration check is a good indicator of migration and age at migration.

To estimate contribution of outmigration strategies I examined 213 scales from samples of adult rainbow trout (≤ 400 mm, ages 1 and 2) collected from the Craig section of the Missouri River between 1997 and 2000. Overall, the yearling outmigration strategy contributed the most to the range of outmigration strategies found in age-1 and age-2 rainbow trout from the Craig section of the Missouri River (87.8%). In addition, the contribution of the various outmigration strategies did not appear to change over time, (Figure 5.9). This is in light of the fact that whirling disease in Little Prickly Pear Creek had started to show signs of impacting the production of juvenile rainbow trout from that stream, starting with the 1998 year-class (Leathe et al. 2001) Yearling outmigrants were predominant in the samples between 1997 and 2000 (74.6 to 95.4%) and YOY outmigrants made up 4.7 to 15.1% of the samples. The predominance of the yearling outmigration strategy is in contrast to the outmigration trapping data from 1998 and 1999 (Figure 5.10). The 1998 year-class from Little Prickly Pear Creek was

comprised of 65.4% YOY outmigrants and 35.4% yearling migrants (Leathe et al. 2001). These data suggest that the YOY life history is heavily selected against in the mainstem Missouri River.

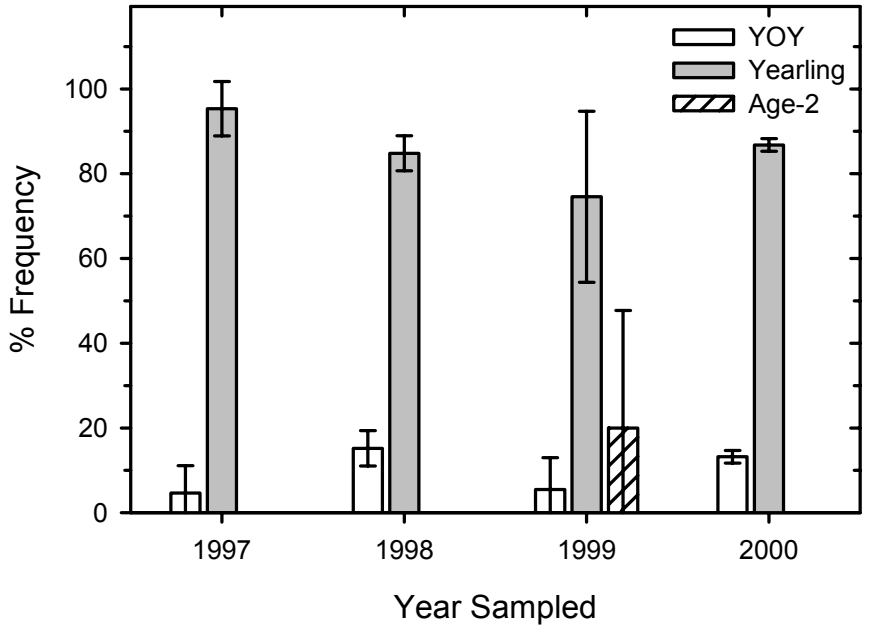


Figure 5.9. Comparison of proportions of outmigration strategies among years (1997 to 2000) for rainbow trout collected from the Craig section of the Missouri River. Age at out migration was estimated from scale growth patterns. Fish were age-1 and age-2 (n = 213). Open bars are YOY outmigrants, shaded bars are yearling outmigrants, and hatched bars are age-2 outmigrants. Error bars are 95% confidence intervals.

Length at Migration

A linear relation between fish length and scale radius was found with the weighted least-squares regression (r = 0.96, Figure 5.11) and the relation was described by the equation:

Total Length = (185.37 ± 1.66 SE) × Scale radius + (27.92 ± 1.68 SE)

(r^2 = 0.925, P < 0.0001).

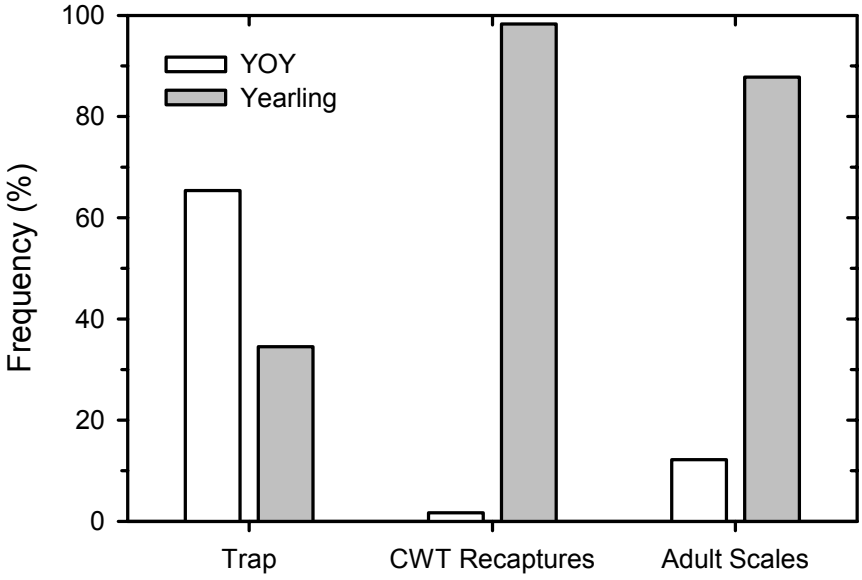


Figure 5.10. Comparison of proportion of YOY to yearling outmigrants between outmigrant trapping data, coded wire tagged (CWT) recaptured rainbow trout of known migration strategy, and mainstem river scale samples. Outmigrant trap data is for the 1998 year class from Little Prickly Pear Creek; n = 20,080; (Leathe et al. 2001), the adult scale estimate is based on samples of the 1996 to 1999 year classes in the Craig section of the Missouri River (n = 213). n = 126 for CWT recaptures

The average back-calculated length at migration for CWT recaptured fish was 99 mm (SD = 19.2 mm) and ranged from 65 mm to 273 mm. For year classes 1996 to 1999, the average back-calculated length at migration was 113.21 mm (SD = 33.22). Back-calculated length at migration was unimodally distributed and skewed to the right for most year classes and minimum length at outmigration was 50 mm (Figure 5.12). The mean length at outmigration was significantly different between years (ANOVA, F = 2.91, df = 3, 182, P = 0.036), but Tukey's multiple comparison procedure indicated that mean length at outmigration only differed between the 1996 and 1998 year class, with the 1998 fish being on average larger than the 1996 fish.

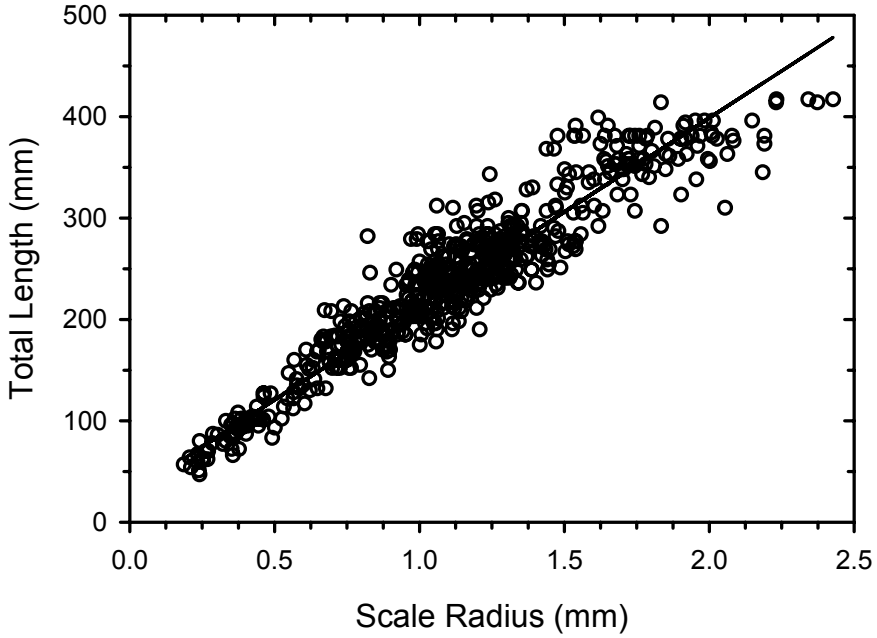


Figure 5.11. Relation between total body length and scale radius for rainbow trout from the Missouri River (n = 651).

Coded Wire Tag Recaptures

Overall recapture rate of tagged fish was 1.02% (12,304 tagged, 126 recaptured) (Munro et al. 2003). There was no significant difference in the proportion of recaptured fish among the three tributaries (0.92-1.09%, Fisher's exact test, P = 0.65); suggesting that there was no difference in mortality of the outmigrants among the tributaries. This assumes that mortality due to tagging was equal among all CWT locations (see Munro et al. 2003) and that there was equal probability of recapture regardless of natal origin. Both YOY and yearling rainbow trout were tagged, in proportion to their being captured in the traps, as they outmigrated from their natal tributaries. The 12,304 tagged fish that were released was comprised of 23% YOY outmigrants and the remaining yearling migrants (Leathe et al. 2001). However, in the sample of 126 recaptured CWT rainbow

trout only 2 (1.6%) of the fish were YOY migrants, suggesting as with the scale growth pattern analysis, that the YOY outmigration strategy contributes relatively little to adult recruitment (Figure 5.10).

The section of river where the tagged fish were recaptured provided interesting information on the relative contribution of the spawning tributaries to the mainstem population and movement within the Missouri River. In the Craig section, Little Prickly Pear Creek is the spawning tributary from which rainbow trout are predominantly recruited (86%, Figure 5.13). Little Prickly Pear Creek enters the Missouri River in the Craig section and the other spawning tributaries are > 16 km downriver. Four Dearborn River fish (ages 2 and 3) were found to have moved upstream into the Craig section. The Hardy section rainbow trout were predominantly recruited from the Dearborn River (82%). Rainbow trout moving down river from Little Prickly Creek were also recruited in the Hardy section, but there is no evidence of Sheep Creek fish moving upstream. Despite Sheep Creek entering the Missouri River < 1 km above the Cascade section, the rainbow trout recruited in the Cascade section are predominantly from the Dearborn River (61%), although Sheep Creek fish do contribute significantly (32%) to the rainbow trout population in this section. Overall, it appears that rainbow trout age-3 and younger tend to remain in the section of the Missouri River closest to their natal stream. However, there is some significant downstream movement by Little Prickly Pear Creek and Dearborn River rainbow trout but very little upstream movement into another section.

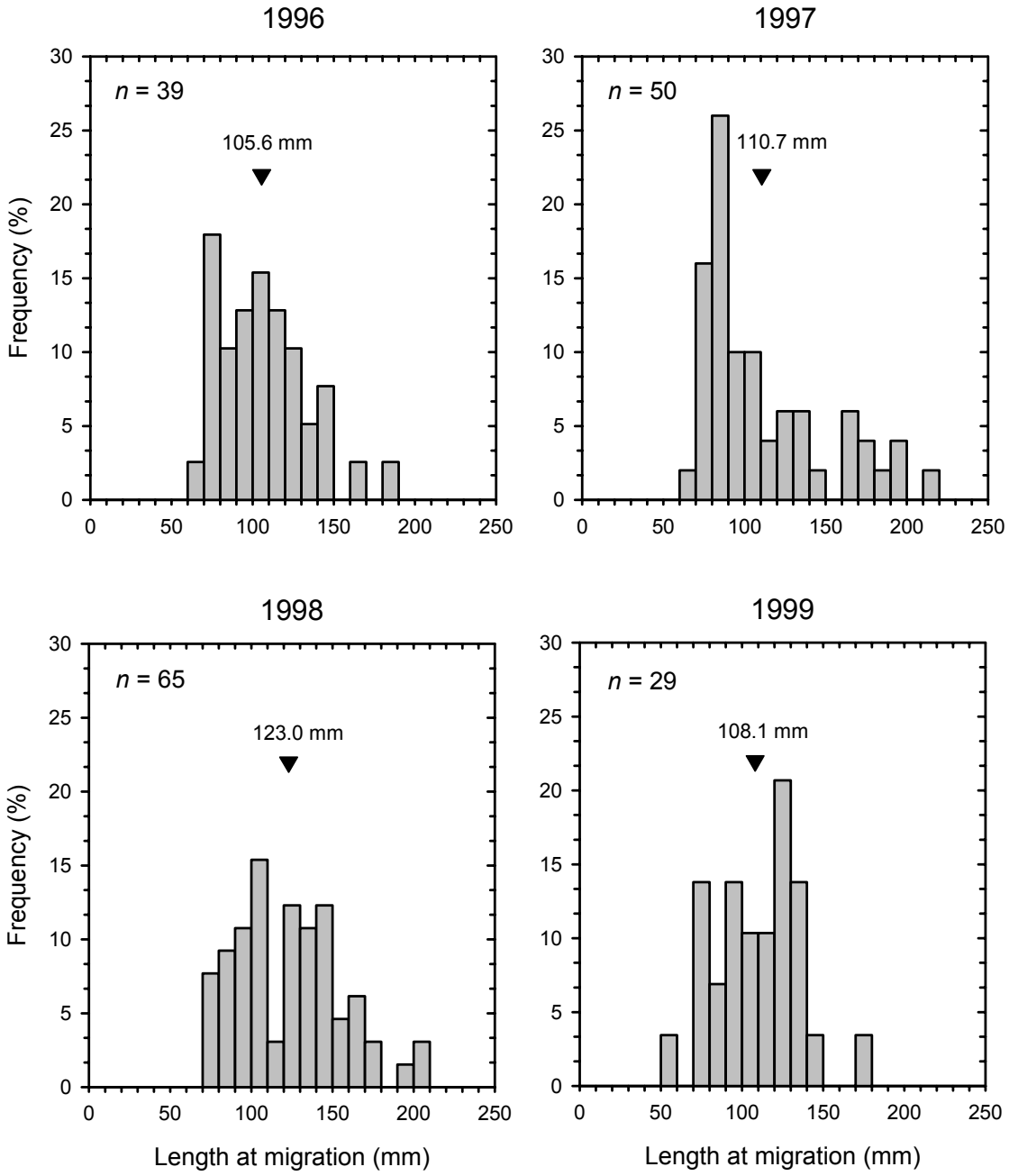


Figure 5.12. Frequency distributions of back-calculated length at migration from age-1 and 2 rainbow trout from the Craig section of the Missouri Rive for year classes 1996-1999. Triangles indicate mean length at migration.

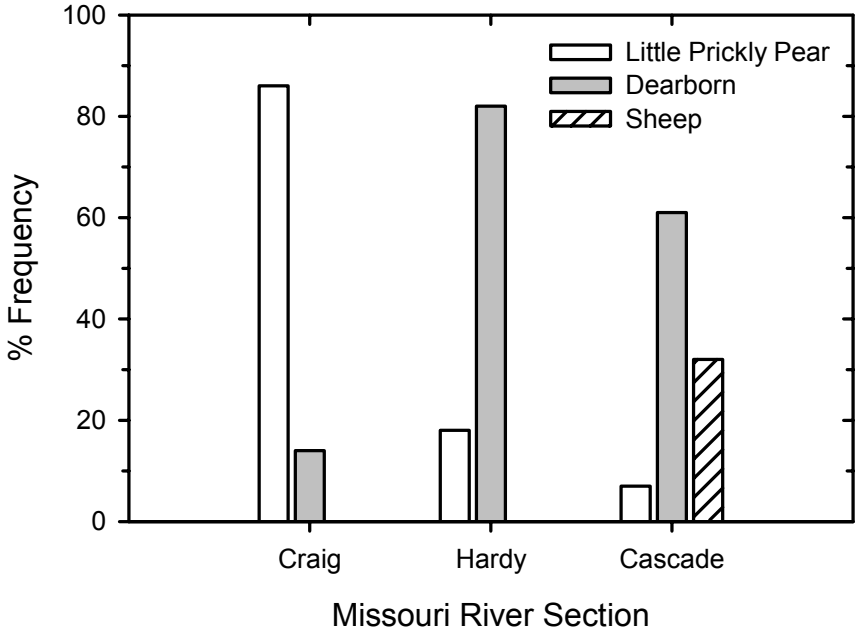


Figure 5.13. Summary of the contribution of the three Missouri River spawning tributaries to the three MT FWP population monitoring sections on the mainstem river (based on coded wire tag recaptures, $n = 126$). Open bars indicate proportion of Little Prickly Pear rainbow trout recaptured in a given section; shaded bars indicate proportion of Dearborn River rainbow trout; and hatched bars indicate proportion of Sheep Creek fish.

Discussion

Identification of natal origin and age at migration in wild freshwater salmonids through the use of otolith microchemistry and scale growth pattern analysis holds promise. However, this study illustrates some of the potential limitations in these techniques, particularly with otolith microchemistry. Although I was able to identify natal origin of rainbow trout from Sheep Creek with a high degree of accuracy, correctly identifying fish from Little Prickly Pear Creek and the Dearborn River proved to be more difficult. Therefore, estimating the contribution of the three major spawning

tributaries in the Missouri River based on otolith microchemistry alone was not possible, especially because Dearborn River and Little Prickly Pear Creek supplied the vast majority of outmigrants to the Missouri River and because whirling disease has had the greatest impact on the production of juvenile rainbow trout from Little Prickly Pear Creek (Leathe et al. 2002a). Analysis of the scale growth patterns of rainbow trout with known outmigration strategies indicate that a distinct migration check is clearly visible in over > 94% of the scales of fish that rear in their natal stream for one year or more, and was therefore useful in examining the variation in juvenile outmigration strategies and the relative contribution the strategies to the adult population in the Craig section of the Missouri River. In addition, the recapture data from CWT rainbow trout was useful in providing insight into the relative composition of the adult rainbow trout population, with regards to both juvenile outmigration strategy and which tributaries contribute fish to the various sections of the Missouri River.

Otolith Microchemistry

The ability to use otolith microchemistry to predict natal origin in Missouri River rainbow trout was met with mixed success. Within tributary variation in otolith Sr:Ca was similar for all streams (18-23%), but only Sheep Creek fish were significantly different from the other tributaries when analyzed with both ANOVA and discriminant analysis. A multi-element approach was not more effective. Little Prickly Pear Creek and the Dearborn River fish could not consistently be distinguished from each other, but it is these two tributaries that produce the bulk of the rainbow trout in the Missouri River and to date whirling disease has had the greatest impact on rainbow trout production in

Little Prickly Pear Creek (Leathe et al. 2002a). Therefore, assessing the contribution of the different spawning tributaries to the mainstem river through the use of otolith microchemistry was not feasible.

There are several potential reasons as to why identifying the natal origin of rainbow trout in the Missouri River proved to be difficult. These reason include: composition of the otoliths does not reflect the composition of the ambient environment, measurement error in the analytic technique, movement of the fish between locations, and homogeneity of the environment (Thresher 1999; Gillanders et al. 2001; Gillanders 2002). Another reason would be large variations within locations that mask differences between locations.

The lack of influence of the environment on the composition of the otoliths is unlikely for a number of the elemental ratios examined, particularly Sr:Ca. Numerous studies have found a relation between otolith and water Sr:Ca (see Chapter 2 and 3). Similar relations have also been found for Ba and Mg (e.g., Thorrold et al. 1998; Bath et al. 2000; Wells et al. 2003). It has also been suggested that the concentration of Fe and Li in otoliths may be related to their availability in the ambient water (Campana 1999). Thorrold et al. (1998) did not find a relation between otolith and the water for Mn and it is known that Na is physiologically regulated (Campana 1999). Of the remaining elements examined, there is no evidence in otolith microchemistry studies to suggest whether or not Cd, As, Sb, or Se concentrations in otoliths are influenced by the ambient environment.

Error associated with measurement is another explanation as to why otolith chemistry was not significant between Little Prickly Pear and the Dearborn River. Measurements of Sr:Ca were precise (CV = 5.8%, see Chapter 3) as were measurements of Fe and Se (<11%, see Appendix). Measurement variation for all of the other elements examined ranged from 42 to 95% (see Appendix). This would mean that much larger differences in these elements would be needed to differentiate among groups (Gillanders et al. 2001). Therefore, measurement error is likely not a concern for Sr, Fe, and Se, but may contribute to the lack of significant differences among streams in the ANOVAs and the lack of improved discrimination by the multi-element discriminant analysis model.

Movement between locations is an unlikely explanation for the inability to correctly identify the natal tributary of Little Prickly Pear Creek and Dearborn River rainbow trout. All of the juvenile fish used in the microchemistry analysis were age-1 and < 120 mm, and were collected as they were outmigrating from their natal streams, which are located 16 to 33 river-km apart. Therefore, it is most probable that these fish had spent their entire life in the drainage in which they were captured.

Perhaps the most likely explanation as to why there was not a significant difference between Little Prickly Pear Creek and Dearborn River rainbow trout, but Sheep Creek fish were different, is the geological similarity between the Little Prickly Pear Creek and Dearborn River drainages. The spatial variation in water Sr:Ca observed within and among the tributaries coincided with the underlying geologies. The upper reaches of both Little Prickly Pear Creek and the Dearborn River pass through the Belt Supergroup and the lower reaches of Little Prickly Pear Creek and the mid-section of the

Dearborn River pass through the reworked volcanic rocks of the Two Medicine Formation. Given the similar geologies of Little Prickly Pear Creek and the Dearborn River, the spatial variability within the two streams would be expected as well as the similarity between the two streams with regard to mean water and otolith Sr:Ca ratios. In contrast, the Sheep Creek drainage is dominated by the Adel Mountain Volcanics. Therefore, it is not unexpected that the water chemistry, and thus the otoliths of Sheep Creek rainbow trout, were significantly higher than the other two tributaries.

The inability to differentiate fish from different locations, however, is still an important result because there are few studies that have reported the inability to detect differences in fish from different locations (Gillanders et al. 2001). For example, Gillanders et al. (2001) found little variation in the otolith composition of two-banded bream *Diplodus vulgaris* among locations in the south-west Mediterranean Sea. Otolith microchemistry studies that show negative results are important because they can be helpful in determining when the application of these techniques may be useful (Gillanders et al. 2001), or when otolith microchemistry may not be an appropriate tool. In addition, negative results are important because they add balance to the field, may indicate when an area of research is not fully developed or clearly defined, or indicate that current methodologies are inadequate (Browman 1999).

However, the inability to detect differences between all of the spawning tributaries in this section of the Missouri River does not mean that otolith microchemistry is not a useful technique for investigating sources and life history strategies in freshwater species. Otolith microchemistry has worked well in certain

situations when differences among water bodies are sufficiently large. For example, I was able to identify the likely source of illegally introduced lake trout *Salvelinus namaycush* (Chapter 4). Wells et al. (2003) were able to correctly reclassify individual westslope cutthroat trout *O. clarki lewisi* from three tributaries of the Coeur d'Alene River system to their tributary of origin with 100% accuracy based on Sr:Ca, Ba:Ca, and Mg:Ca ratios. However, examination of the water chemistry from 36 tributaries in the Coeur d'Alene River system indicated that not all stream chemistries are sufficiently different from one another and therefore identification of fish to specific stream of origin would not always be possible. Nearest-neighbor cluster analysis indicated that there were about seven clusters of streams that would have high probability of being discriminated (Wells et al. 2003). Similarly, Kennedy et al. (2000) identified 14 distinct Sr isotope ratios among 29 river sites.

Differences in otolith composition between groups of fish at the scale of interest (e.g., at the level of individual stream) may be small. Therefore, in the initial stages of study design, it is important to anticipate such difficulties and to select an appropriate sample size. The technique for minimum sample size estimation that I presented, is similar to the approach of Wells et al. (2003), in that water chemistry is used as a preliminary step in determining under what conditions one would expect to be able to detect differences in the otolith composition of fish from different locations. However, with the method that I propose, one is able to estimate the sample sizes that would be needed to be able to detect differences and therefore adjust sample sizes and budgets accordingly. Prediction of otolith Sr:Ca mean and variance from the water chemistries

using the relation between otolith and water Sr:Ca presented in Chapter 3 did a reasonable job. Incorporation of information from future studies would improve the predictive abilities of this method. In the Missouri River example presented in this chapter there was about a two-fold difference in minimum sample size estimated between observed and predicted otolith microchemistry. However, it did demonstrate that in either case an impractical number of samples would be required to detect differences between Little Prickly Pear Creek and Dearborn River rainbow trout using Sr:Ca.

Scale Growth Pattern

Missouri River rainbow trout scales typically have a distinct inner-zone of closely spaced circuli that appears to represent tributary rearing in the natal streams followed by an outer-zone of more widely spaced circuli, indicative of mainstem river growth (Figure 5.3). The presence of this pattern in the scales of 94% of the known yearling outmigrants strongly suggest that, as reported in Kwain (1971), the change in circuli spacing I used to identify the migration check is a result of a change in feeding and growth conditions. Although only two known YOY outmigrants were recaptured, neither fish had a distinct inner-zone of closely spaced circuli that could be identified as tributary growth and thus no migration check. Therefore, YOY migrants could only be identified by lack of a migration check.

Although scales of known YOY outmigrants did not have a migration check, the lack of a migration check is not confirmation of a YOY outmigration strategy. A distinct migration check was not present in 5% of the known yearling migrants and,

although not sampled, fish that were spawned in the mainstem river would likely not have a migration check. Therefore, the estimate of 11% YOY outmigrants in the mainstem population is likely an over-estimation. A possible reason why there is no migration check present in YOY outmigrants may be because of the timing and size at outmigration of YOY from their natal tributaries. Between 80 and 90% of the YOY outmigration was between July and August and the length of outmigrants was 25 to 30 mm in July and 70-90 mm by September (Leathe et al. 2001). Scale platelets typically are not fully formed in rainbow trout until the fish are at least 45 mm (Ward et al. 1989). In addition, the widths of the first 3 circuli are wider than other circuli laid down during tributary rearing (personal observation). Therefore, many of the YOY would not have scales, or many useful circuli, formed at the time of migration so a distinct migration check is not formed in the scales. The few YOY outmigrating in September or later are > 80 mm and would have enough circuli on the scales to form a distinct tributary growth zone of narrow circuli. However, at this time of year the fish are likely entering the slow winter growth period and therefore it would be difficult to distinguish tributary growth from mainstem growth at this time of the year.

Contribution to Adult Recruitment

Coded wire tag recaptures and scale growth pattern analysis of samples from the Craig section of the Missouri River indicated that rearing in the natal tributary for 1 year is the dominant life history strategy in this system. The proportion of yearling outmigrants (87%) in the Craig section samples was lower than the 93% in the CWT recaptured fish, although this was not a significant difference (Fisher's exact test, P =

0.13). In contrast, the outmigration trapping data from 1998 and 1999 indicates that the predominant strategy in Missouri River rainbow trout is the YOY outmigration. For example, for the 1998 year class, it was estimated that 13,136 (95% CI = 10,551-17,647) rainbow trout outmigrated as YOY and 6,944 (95% CI = 5,602-9148) outmigrated the following year as yearlings (Leathe et al. 2001). This suggests that YOY outmigrants do not contribute substantially to adult recruitment in the mainstem river.

Rainbow trout in the Missouri River appear to require a protracted period of rearing in their natal tributary. The average back-calculated length at migration was 113.6 mm for rainbow trout in the Craig section of the Missouri River. The majority (95%) of yearling outmigrants were estimated to have been between 86 and 178 mm, although fish that outmigrated as small as 50 mm were observed. The range of estimated length at migration of yearling outmigrants was similar to range in length of yearling outmigrants caught in the Little Prickly Pear Creek trap (mean TL = 80-110 mm, MT FWP, unpublished data, Leathe et al. 2001). A period of stream rearing is important for many populations in order for the fish to reach a minimum size to ensure survival in the river lake or ocean (Rosenau 1991). Biette et al. (1981) found the length at smolting for rainbow trout in the Great Lakes was between 64 and 244 mm (slightly greater than the range in length of the Missouri River rainbow trout). The mean length at migration for the rainbow trout populations studied by (Rosenau 1991) was 140 mm; few fish < 94 mm at the time of outmigration survived to be recruited to adulthood. Ward and Slaney (1988) also reported that there was a minimum size of smolts that survive to adulthood in Keogh River steelhead. However, a protracted period of stream

rearing might not be necessary for all populations (Hayes 1988) and the optimal size for outmigration will depend upon local conditions (Rosenau 1991).

The composition of outmigration strategies in Missouri River rainbow trout appears to be fairly unique in that the range in duration of stream residence is predominantly less than 1 year and that over 90% of the mainstem population is comprised of yearling outmigrants (Table 5.6). Studies have shown that rainbow trout exhibit a great amount of plasticity with regards to the duration of stream rearing (Kwain 1971). While a predominance of yearly outmigrants in the population is common (8 out of 17 locations,), only Van Velson (1974) reported > 90% contribution of yearling migrants in a population. However, the YOY outmigration strategy was not reported to be present in this reservoir population. The YOY outmigration strategy is not a common strategy found in adult populations (Table 5.6), but both Rosenau (1991) and Hayes (1995) found that YOY outmigrants can contribute significantly to the adult population. For example, in Lake Alexandrina, New Zealand the 30 to 64% of the adult population from the three spawning streams were YOY migrants (Rosenau 1991).

Although I was unable to estimate the contribution of natal tributaries to the Missouri River rainbow trout fishery using otolith microchemistry, coded wire tag recapture data suggests that the composition of the different population monitoring sections recruit predominantly from the closest upstream spawning tributaries. While there was no evidence of upstream movement of age-1 rainbow trout in the mainstem river, four Dearborn River fish were recaptured in the Craig section, 17 km upstream. These fish age-2 and 3 and ranged in length from 366 to 450 mm. This suggests that

there may be some upstream movement in larger, older fish. This could explain the continued normal-to-above-average population estimates of age-2 and older rainbow trout in the Craig section of the Missouri River in recent years, despite the dramatic decline in the number of yearling rainbow trout being recruited to that section of river due to whirling disease (Leathe et al. 2002a).

In summary, Missouri River rainbow trout appear to exhibit a limited number of juvenile outmigration life history strategies. Scale growth pattern and coded wire tag recaptures indicate that Missouri River rainbow trout that are recruited to the fishery are predominantly yearling migrants (> 80%). Rainbow trout that migrated as YOY and age-2 make only minor contributions to the mainstem population. The data also suggest that the proportion of YOY to yearling outmigrants in the Craig section has not changed despite Little Prickly Pear Creek being heavily infected with whirling disease since 1998. Outmigrant trap data from Little Prickly Pear Creek indicates that the majority of rainbow trout outmigrate during their first summer as YOY and the remaining migrate the following spring as yearlings. In addition, this study suggests that Missouri River rainbow trout require a protracted period of rearing, which has not changed significantly since whirling disease was detected in Little Prickly Pear Creek and began to affect the production of juvenile rainbow trout. The estimated mean length at migration of rainbow trout in the Craig section of the Missouri River was 114 mm with a minimum length of 50mm. Rainbow trout are most susceptible *M. cerebralis* in the first 9 weeks/756 degree days of development post hatch or approximately 40 mm (Ryce 2003). Therefore, it would appear that smaller, younger rainbow trout outmigrating to

the Missouri River have to overcome both whirling disease infection risk during their most vulnerable period and the low probability of survival once in the mainstem river.

Table 5.6. List of results from studies that investigated the duration of stream rearing in rainbow trout and steelhead, illustrating the variation in life history strategy in this species[1]. Results are presented as % composition of population.

		Years of stream rearing					
		0	1	2	3	4	5
Author	Location	Steelhead					
Maher & Larkin 1954	Chilliwack R., BC	–	2.0	62.1	35.4	0.5	–
Shapovalov & Taft 1954	Waddell Cr., CA	–	40.0	19.0	1.0	–	–
Chapman 1958	Alsea R., OR	–	1.4	80.9	17.5	0.2	–
Withler 1966	Southern, BC	–	1.8	39.9	54.9	3.4	–
Narver 1969	Babine R., BC	–	–	2.0	82.0	15.0	1.0
Ward & Slaney 1988	Keogh R., BC	–	–	30.0	60.2	9.8	–
Ward et al. 1989	Keogh R., BC	–	–	28.6	62.8	8.6	–
Daugherty et al. 2003	Carp R., MI	–	14.6	84.2	1.2	–	–
		Rainbow trout					
Hartman 1959	Grout Br., NY	–	13.5	62.5	–	–	–
	Cold Br., NY	–	24.7	49.2	–	–	–
	Catherine Cr., NY	–	73.0	0.0	–	–	–
Northcote 1969	Pothole L., BC	6.1	54.3	31.8	7.8	–	–
Dodge & MacCrimmon 1970	Bothwell, Cr. ON	–	–	99.0	–	–	–
Kwain 1971	Batchawana Bay, ON	–	38.2	58.1	3.7	–	–
Stauffer 1972		–	64.0	34.0	2.0	–	–
Van Velson 1974	McConaughy Res., NE	–	92.0	8.0	–	–	–
Kwain 1981	Stokely Creek, ON	–	41.0	53.0	6.0	–	–
Rosenau 1991	Waimarino R, NZ	29.8	70.2	–	–	–	–
	Tokaanu R., NZ	50.6	49.4	–	–	–	–
	Hinemaiaia R., NZ	63.8	36.2	–	–	–	–
Present study	Craig section, Missouri R., MT	11.3	87.8	0.9	–	–	–

[1] Table adapted from Kwain (1971).

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CHAPTER 6

CONCLUSIONS

The purpose of this research was to develop methods for identifying natal origin and life history variation (outmigration strategies) in a wild rainbow trout population, specifically to determine if there were life history strategies in Missouri River rainbow trout that limited their exposure to the nonnative parasite *Myxobolus cerebralis*. I also wished to estimate the contribution of the different spawning tributaries and life history types to the mainstem river and determine the changes in composition of these factors as whirling disease spread through the system. To accomplish these objectives, I used otolith microchemistry to identify natal origin and scale growth patterns to identify age at migration. However, there have been a limited number of studies that have applied otolith microchemistry techniques to freshwater systems and ToF-SIMS, the analytic technique used, had never been applied to the analysis of otoliths. Therefore, this research focused heavily on the development of otolith microchemistry analysis techniques with ToF-SIMS and assessing its efficacy for freshwater applications.

Otolith Microchemistry

The field of otolith microchemistry has grown rapidly in the last 10 to 15 years and has been used in a variety of applications in fisheries science. To analyze otoliths, researchers have used a number of different instruments. This is a reflection of the range of applications, improvement of existing technologies, and development of new analytic

techniques. However, I concluded in my review of current procedures and instruments in Chapter 2 that there has been, for the most part, a lack of critical evaluation of many of the instruments and standardization of methods and operating conditions, including use of matrix-matched standards for instrument calibration. While the decision to use bulk or beam-based techniques is typically based on the application, the specific instrument used for analyses often depends upon availability.

For my research I required a sensitive, beam-based analytic technique. Secondary ion mass spectrometry was identified by Campana et al. (1999) and Radtke and Shafer (1992) as a potentially useful technique for otolith microchemistry analysis and a ToF-SIMS was readily available at the Image and Chemical Analysis Laboratory, MSU. However, to my knowledge, ToF-SIMS had never been used to analyze the chemical composition of otoliths. Therefore, I needed to test and establish the efficacy of ToF-SIMS for use in otolith microchemistry studies. In Chapter 3, I showed that Sr:Ca ratios could be measured consistently, using an otolith 'standard', which was made of ground otoliths from rainbow trout raised under constant conditions. Measurements of Sr:Ca of the otolith 'standard' were precise, varying < 6%. I also established that Sr:Ca ion count ratios measured by ToF-SIMS were comparable to the molar concentration ratios determined using WD-EM – a technique that has been widely accepted for otolith chemical analysis. The relation between the count ratios of ToF-SIMS and the quantitative measurement of WD-EM was important because it illustrated the quantitative capabilities of ToF-SIMS, which was commonly considered a qualitative technique. Finally, I showed that otolith Sr:Ca was highly correlated to the Sr:Ca of

ambient water. This relation established that, like other instruments used to analyze the chemical composition of otoliths, otolith Sr:Ca measurements by ToF-SIMS could be used as an environmental marker in fish, particularly the freshwater environment.

Development of the ToF-SIMS technique for otolith microchemistry highlighted the need to fully and rigorously establish the efficacy of a technique prior to it being adopted for widespread use and application. In the scheme of the research paradigm described by Jones (1995), there are aspects of ToF-SIMS analysis of otoliths that need further investigation. Of utmost importance is the rigorous testing of the measurement of other elements that, like Sr, could potentially be used as environmental markers, such as other alkaline earth metals and the rare earth elements, so that the most important capabilities of ToF-SIMS – its high mass resolution and ability to measure most elements simultaneously – can be utilized effectively and accurately. Attempts to use multiple elements, however, met with limited success. Apart from Sr, Fe, and Se, I found high variation in element:Ca ratios in the otolith 'standard'. The reason for these results is unclear, especially because with other techniques relations between otolith and water composition have been established for some of these elements (e.g., Ba, and Mg). Therefore, it is important to determine whether the observed variation was due to inherent variation in the otoliths, sample preparation, storage methods, or measurement error associated with the ToF-SIMS technique for these elements in otoliths.

To further establish the use of ToF-SIMS for otolith microchemistry, I believe the next step should be to run controlled laboratory experiments with known elemental concentrations in the water that cover the range of concentrations expected to be found

in the environment (e.g., Bath et al. 2000). This approach would also allow us to use the relations between elemental concentrations in the otolith and water for estimating minimum sample sizes required in natural systems (Chapter 5). Finally, I believe that establishment of matrix-matched certified reference materials, such as that produced by Yoshinaga et al. (2000), should be a high priority, especially for beam-based techniques. These standards should be used routinely to ensure the validity of the data collected. I found that intersession variability with these sensitive instruments could be significant – an issue that has not been fully addressed in the many of otolith microchemistry studies to date.

I used otolith microchemistry in two different freshwater applications. The first was a new application of otolith microchemistry as a forensics tool to identify the likely source and timing of the illegal introduction of lake trout into Yellowstone Lake. The second application was to identify the natal origin of wild rainbow trout in the Missouri River. The lake trout study was important for two reasons: 1) it illustrated that otolith microchemical analysis with ToF-SIMS can be a powerful tool in discriminating fish from different locations and identifying their origin; and 2) otolith microchemical analysis can be used in this novel way to investigate the invasion or illegal introduction of exotic species. The method could be used to answer the often intractable questions regarding the source and timing of invasion or introduction of an exotic species.

Using otolith microchemistry, I was able to show that introductions of lake trout into Yellowstone Lake began as early as the mid-1980s as was presumed, based on the age of juvenile lake trout caught in the lake. I also showed that the illegal introductions

likely continued into the mid-1990s, after the initial discovery of lake trout in Yellowstone Lake. This suggested that there was a concerted effort to establish lake trout in the lake.

The introduction of exotic species is a formidable problem for aquatic environments. The ability to determine the geographic origin of an exotic species and its relative risk to the environment is critical to the development of educational and regulatory programs aimed at stemming the tide of future unauthorized introductions (McMahon and Bennett 1996; Kolar and Lodge 2002). Determination of the source and date of introduction of exotic species is often beset with many alternative possibilities (McMahon and Bennett 1996; Waters et al. 2002). I have shown that otolith microchemistry can be an important new tool for confirming the source and timing of such introductions.

The results of the otolith microchemical analysis of Missouri River rainbow trout indicated that the chemical analysis of otoliths might not be suitable for every situation. Analyses of Sr:Ca and multiple elements indicated that it was difficult to correctly identify juvenile fish from either Little Prickly Pear Creek or the Dearborn River and hence to identify natal origin of fish with a high degree of confidence. The lack of well-defined differences among all spawning tributaries in the Missouri River was due primarily to similarity of the water chemistry of Little Prickly Pear Creek and the Dearborn River. Both streams had similar water chemistries (Sr:Ca). Consequently, the juvenile rainbow trout rearing in these streams exhibited similar chemical composition of their otoliths. However, this negative result, while limiting the ability to identify natal

tributary of origin and contribution of the tributaries to the mainstem river, illustrated that the usefulness of otolith microchemistry depended on the variability of the water chemistry. This is not a result unique to the Missouri River or ToF-SIMS. Previous studies have also shown that differences among fish from different locations using a variety of analytic techniques and elemental and isotopic markers, despite their great sensitivity, are not always successful (e.g., Kennedy et al. 2000; Gillanders et al. 2001). However, I demonstrated that water chemistry could be used as an initial, low cost survey tool to determine if otolith microchemistry could be used profitably and cost effectively to assess life history and stock differences.

The variability in the geology and number of locations of interest can determine the discriminatory power within a system (Kennedy et al. 2000). In addition, temporal variation in water chemistry, variations in the otoliths due to physiologic and ontogenetic changes in the fish, and the sensitivity and accuracy of the analytic technique will determine the level of noise that needs to be separated from the underlying signal. It is important to understand the geological characteristics of a system that influence the water chemistry and subsequently otolith composition. Therefore, the potential power and limitations of the technique for a given system and spatial scale of interest (i.e., individual streams or groups of streams within a basin) can be determined prior to collection and analysis of otoliths (Schneider 1994; Wells et al. 2003). A preliminary investigation of the underlying geology or a synoptic water chemistry analysis would aid in understanding the variation of water chemistry in the system. This information then could be used to predict and define the spatial scale at which one would find high

discriminatory power (Kennedy et al. 2000; Wells et al. 2003). Alternatively, one could use the relation between otolith and water chemistries to estimate minimum sample sizes needed to detect a desired difference (Chapter 5). This would be most beneficial to managers and researchers as a means to quickly, and relatively inexpensively, estimate a feasible spatial scale to detect differences among groups of fish and estimate sample sizes necessary to detect a desired level of difference. What I demonstrated could easily be applied to elements other than Sr, such as Ba and Mg, where relationships between otolith and water chemistries have been found. I believe using empirical data from preliminary water samples from locations of interest is an important aspect to any freshwater otolith microchemistry study. A better understanding of the spatial and temporal variation of the environmental chemistry in the preliminary stages of study design will lead to better designed and executed studies.

Overall, the results of my work are consistent with other otolith microchemistry studies (both marine and freshwater) as they demonstrate the efficacy of a technique to identify the origin of fish from freshwater systems. When identifiable differences do exist, otolith microchemistry can be used to estimate rates of migration and dispersal within a river system, assist in defining critical habitat, evaluate habitat restoration efforts, and estimate relative contribution of various sources to a population (Kennedy et al. 2000; Wells et al. 2003). In the future, the full potential of the otolith microchemistry technique will be realized through careful and concerted efforts to properly evaluate the analytic techniques used, a better understanding of the role of the environment and physiology on the composition of otoliths, and combining techniques such as elemental

and stable isotope analyses (e.g., Edmonds et al. 1999). In addition, the strength of otolith microchemistry research lies in the application of otolith microchemistry techniques with other complimentary tools such as genetic analysis (Campana 1999).

Natal Origin and Life History Variation

I used a combination of otolith microchemistry, scale growth patterns, and coded wire tagging to identify natal origin and age at migration in Missouri River rainbow trout. Otolith microchemistry was not an effective technique in this system. Recaptured CWT adult rainbow trout indicated that rainbow trout are recruited into the mainstem population in relative proportion to juvenile production from the tributaries. Dearborn River fish comprised the majority of recaptured fish, Little Prickly Pear fish were the next abundant, and Sheep Creek the least abundant. Juvenile outmigration trapping by Montana Fish, Wildlife and Parks, in a companion study, indicated that the Dearborn River and Little Prickly Pear Creek produced the majority of juvenile rainbow trout (approximately 105,000 and 47,000 fish/year, respectively) (Leathe et al. 2001). This is in comparison to the approximately 25,000 juvenile rainbow trout/year produced by Sheep Creek – the third significant spawning tributary in the system (Leathe et al. 2001). Furthermore, locations of the recaptured CWT fish indicated juvenile rainbow trout were recruited primarily in the section of river closest to their natal stream. The upper portion of the Missouri River (Craig section) was predominantly comprised of fish recruited from Little Prickly Pear Creek, whereas the lower portion (Hardy and Cascade sections) was dominated by Dearborn River rainbow trout. Also, there was indication of upstream movement by age-2 and 3 Dearborn River fish into the Craig section (~ 9.0 river-km).

However, a similar upstream movement of Sheep Creek fish into the Hardy section (~ 3.4 river-km) was not observed. This difference in upstream movement between the three sections of river may be a result of reduced recruitment from Little Prickly Pear Creek because of whirling disease.

Missouri River rainbow trout exhibited a limited number of juvenile outmigration strategies. Scale growth patterns and recaptured CWT adult rainbow trout indicated that rearing in the natal tributary for 1 year was the dominant life history strategy in the Missouri River system. Young-of-the-year and age-2 outmigrants contributed < 20% to the adult rainbow trout population in the Craig section. This is in contrast to the outmigration trap data, which suggested that the larger portion of juvenile outmigrants from Little Prickly Pear Creek is YOY outmigrants (> 65%).

Recaptured CWT adult rainbow trout from all three spawning tributaries indicated that the yearling outmigration strategy was most successfully recruited. Of the 126 recaptured fish, 98.4% were yearling migrants, even though 76.8% of the tagged juvenile outmigrants were yearling migrants. Potamodromous rainbow trout population with two or three outmigration strategies, with one being dominant, is common (see Table 5.6). Length at migration may be a more important factor in determining successful recruitment into the adult population as has been observed in a number of other studies (e.g., Van Velson 1974; Rosenau 1991).

Implications to Whirling Disease

The dynamics of whirling disease are complex. In order to develop effective management plans to contain the spread and limit the effects of the disease on wild trout

populations, we need to better understand the biology and ecology of the parasite and its two hosts (Kerans and Zale 2002). The Missouri River rainbow trout fishery offered a unique opportunity to study the dynamics of whirling disease because the parasite was detected before there were any major effects on the rainbow trout population. The goals of my study and several companion studies were to link information on rainbow trout juvenile life history and natal stream production, infection dynamics, and mainstem population response to assess the spread of whirling disease in the Missouri River system, determine the relative contribution of different juvenile life histories and natal streams in light of the establishment of whirling disease in the system, and to measure adult population response to areas with heavily infected tributaries (McMahon et al. 1998; Leathe et al. 2001; Leathe et al. 2002).

Whirling disease infected rainbow trout were first detected in Little Prickly Pear Creek in 1996. Since then the extent and severity of the disease have increased. Currently, *M. cerebralis* is found in two of the three main spawning tributaries in the Missouri River. Little Prickly Pear Creek has been highly infected since 1998 (Leathe et al. 2002). Sentinel rainbow trout exposure studies (see Vincent 2000; Downing et al. 2002 for description of methodology) indicate > 95% infection rate and infection severities between 3 and 4 (on a 0-4 lesion scale, Baldwin et al. 2000). However, tributaries to Little Prickly Pear have different levels of infection. For example, Wolf Creek is heavily infected (> 3), but Lyons Creek is still whirling disease free (E.R. Vincent, MT FWP, pers. comm.). Whirling disease was not detected in the Dearborn River until 2001 (Leathe et al. 2002). However, since then it has spread rapidly with the

highest infection in the South, Middle, and North forks of the river, while infection severity in the main river remains moderate (2-3 near the mouth, E R. Vincent, pers. comm.). To date, Sheep Creek is still whirling disease free. Infection in the mainstem river is spatially and temporally variable. Infection severity in the Craig section is high (> 3, Leathe et al. 2002) but the lower sections are typically <1 with occasional reports of 2 in some sites (E.R. Vincent pers. comm.).

In addition to the spatial variability within the Missouri River, whirling disease infection severity in the Missouri River system shows a seasonal pattern similar to other sentinel fish exposure studies (Baldwin et al. 2000; Downing et al. 2002; MacConnell and Vincent 2002). Peak infection and severity in sentinel fish were found to extend from mid-April to mid-July in Little Prickly Pear Creek, while in the mainstem river it is not as protracted – only extending from mid-April to late-June (Leathe et al. 2002).

What are the implications of these findings to continued spread of whirling disease in the Missouri River? The Little Prickly Pear Creek drainage appears to have reached the limit of infection (other than in Lyons Creek). Peak spawning in Little Prickly Pear Creek occurs around mid-April; and it is estimated that peak emergence of rainbow trout fry occurs around late-May (Grisak 1999) – during peak whirling disease infection risk. Given that rainbow trout are most susceptible to *M. cerebralis* in the first 9 weeks (759 degree-days) post-hatch (Ryce 2003) and the ubiquitous presence of *M. cerebralis* in the stream, it is highly likely that rainbow trout in Little Prickly Pear Creek are at high risk of becoming infected as soon as they emerge, regardless of their outmigration strategy. The probability of avoiding infection is likely to be very small

considering that even short-term exposure to *M. cerebralis* can cause severe whirling disease (Markiw 1991; Ryce et al. 2001).

Continued production of juvenile rainbow trout from Little Prickly Creek and recruitment of age-1 fish in the Craig section suggest that some individuals can pass through the infection gauntlet and survive. Lyons Creek, a tributary to Little Prickly Pear Creek is still *M. cerebralis* free (E. R. Vincent, pers. comm.). Lyons Creek has the highest density of rainbow trout redds in the drainage (Grisak 1999), but little is known about age and timing of outmigration of juvenile rainbow trout from this tributary. It is possible that if juveniles over-winter in this tributary, or at least remain in the locale until after the peak infection period, their risk of severe infection leading to mortality would be greatly reduced.

The Dearborn River is in the early to mid stages of whirling disease infection. It will be interesting to see how the disease progresses within the drainage and manifests itself within the rainbow trout population. The Dearborn River is different from Little Prickly Pear Creek in that percentage of organic material in the sediments, ammonia, and conductivity are lower, as is the abundance of *T. tubifex* (McMahon et al. 1999). Will the Dearborn River become uniformly infected similar to Little Prickly Pear Creek, or will infection risk be spatially and temporally variable like the Madison River? Less is known about timing of peak spawning of rainbow trout, fry emergence, and timing of peak infection in the Dearborn River than in Little Prickly Pear Creek. However, if infection risk in the Dearborn River were to become as uniform as Little Prickly Pear Creek, we could expect a similar scenario, with rainbow trout fry emerging into waters

of high infection risk. On the other hand, if infection risk is not ubiquitous within the drainage, there may be refugia where exposure of fry to *M. cerebralis* would be low, reducing the infection risk. Consequently, production of juvenile rainbow trout from the Dearborn River would be maintained, albeit at lower than current levels. However, Downing et al. (2002) suggested that movement of fry over even short distances (<100 m) could increase their infection risk in a system with a wide variation in disease severity.

Already whirling disease is affecting the Missouri River rainbow trout population. Since 1998, there has been a decline in the number of yearling outmigrants caught in the Little Prickly Pear rotary screw trap, from approximately 7,400 in 1998 to approximately 850 in 2001 (Leathe et al. 2002). Similarly, since 1999 there has been a significant decline in the number of age-1 fish in the Craig section, which monitors recruits primarily from Little Prickly Pear Creek (according to the CWT recapture data). The density of age-1 rainbow trout in the Craig section had declined from a mean of 1,325 fish/km (SD = 178) between 1994 and 1998 to a mean of 328 fish/km (SD = 116); this is despite continued record high numbers of age-2 and older rainbow trout in the section (Leathe et al. 2002). The estimated densities of age-1 rainbow trout in the Cascade section have also shown a decline, but not as prevalent as the Craig section (Leathe et al. 2002), which might be a result of the heavy recruitment of rainbow trout from the Dearborn River and Sheep Creek. Continued production of rainbow trout from the Dearborn River has likely offset severe population declines in adult rainbow trout in the Missouri River thus far, but continued spread of the parasite throughout this tributary

could lead to a collapse in recruitment. Scale pattern analysis suggests that smaller, YOY migrants are heavily selected against in the mainstem river and that outmigrating as yearlings is the most successful life history strategy. Therefore, age-0 fish from whirling disease infected streams have two obstacles to overcome: they are at a high risk of infection if they remain in their natal stream, but if they leave the stream too soon and manage to avoid severe infection, their chance of survival in the mainstem river is still very low. Therefore, continued production and recruitment of rainbow trout in the Missouri River will be from juveniles that emerge and rear in areas of low infection during their most vulnerable stage. Currently, these areas include Lyons Creek, Sheep Creek, the mainstem river, and possibly parts of the Dearborn River.

Whirling disease mortality will exert a strong selective force on the rainbow trout population. However this selection pressure against tributary spawned rainbow trout with life histories that overlap with times of high infection risk may reduce competition and lead to increased success of mainstem spawned fish where infection risk is currently much lower. This is comparable to the life history of rainbow trout in the Madison River where mainstem spawning is the predominant life history with limited tributary spawning (Downing et al. 2002).

Concern over the potential for an impending population crash led Montana Fish, Wildlife and Parks to adopt strict catch and release regulations on the Missouri River (Leathe et al. 2002). Ryce (2003) recommended that one way to manage whirling disease in the wild is for juveniles to rear in whirling disease free for 795 degree days post hatch or until they are about 40 mm. Therefore, protection and enhancement of

rainbow trout with life histories that fit this recommendation is another management option. This may involve stocking of fish with specific life histories, such as Madison River rainbow trout, which are primarily mainstem spawners.

Whirling disease in the Missouri River is still in a dynamic state so it is difficult to make any definitive conclusions about the future of the Missouri River rainbow trout fishery. Continued monitoring of the progress of the disease through the system and response of the rainbow trout population is essential. There is still much to be learned about the dynamics of whirling disease and its interactions with the salmonid and worm hosts. The Missouri River still provides a unique opportunity for a long-term case study of whirling disease in a wild rainbow trout fishery beginning at the early stages of infection.

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APPENDIX

SUMMARIES OF ELEMENTAL RATIO MEASUREMENTS IN OTOLITH 'STANDARD'

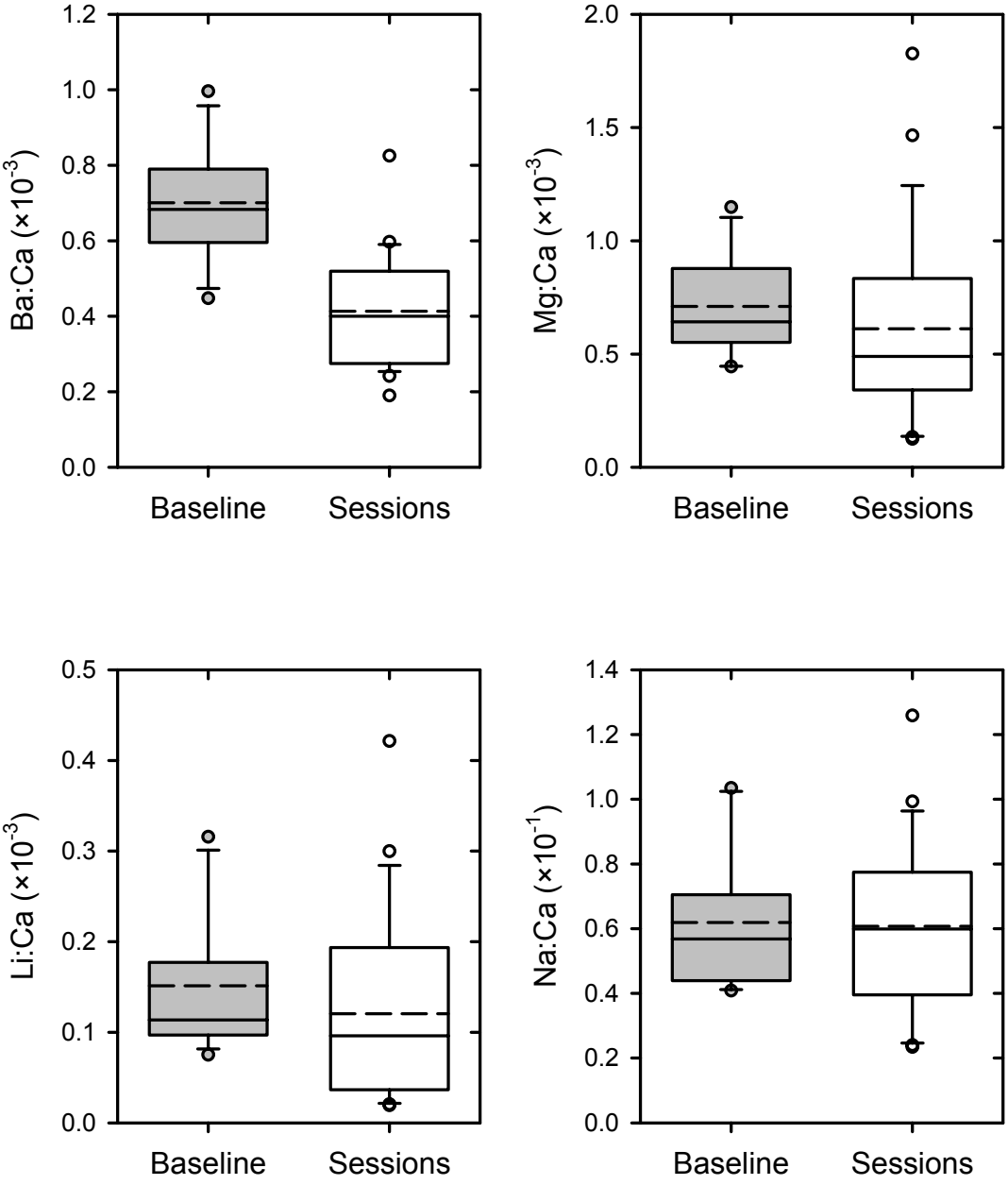


Figure A.1. Summary of baseline and session element:Ca ratios for the artificial otolith that was used to check the consistency of ToF-SIMS analyses from session to session. Elements examined (other than Sr) included: Ba, Mg, Li, Na, Zn, Mn, Fe, Cd, As,Sb, and Se. Baseline values (grey boxes) were established over three sessions (n = 13 assays). Session values (open boxes) were collected at the beginning of each session prior to analyses (n = 24 assays). The boxes show the mean element:Ca (dashed line), median (central solid line), 1st and 3rd quartiles (box edges) and individual outliers (circles) outside the 10th and 90th percentiles (whiskers).

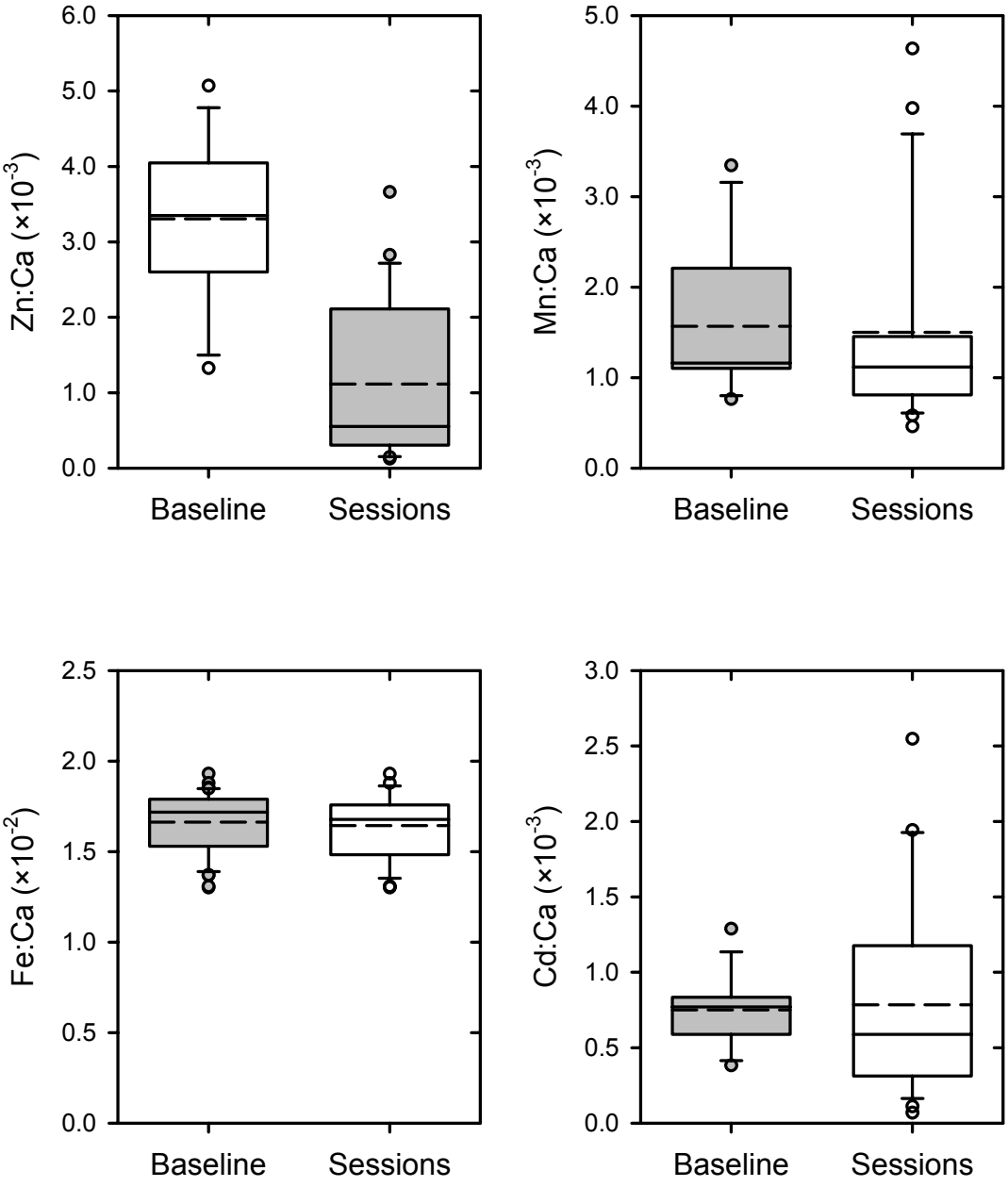


Figure A.1. Continued.

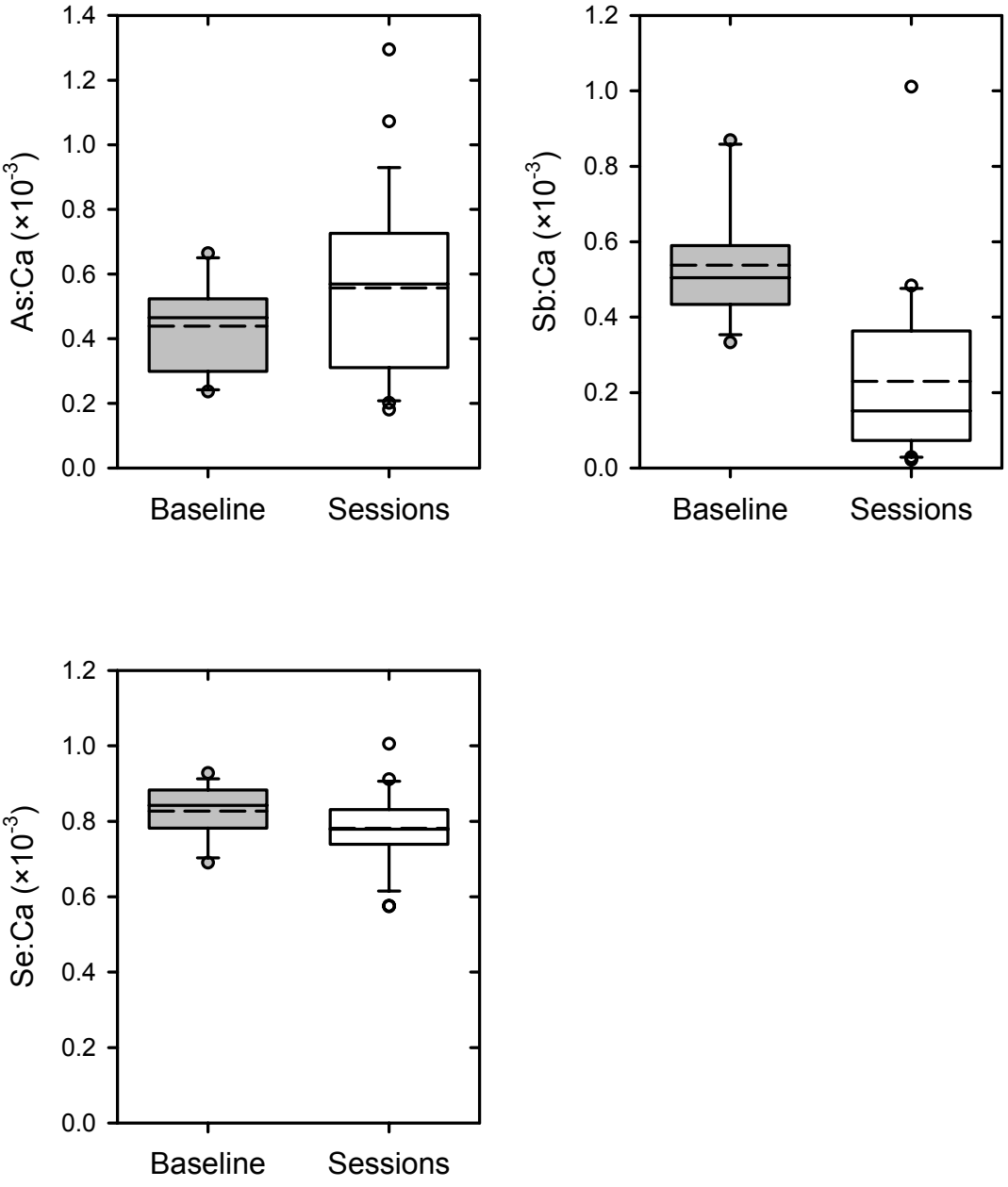


Figure A.1. Continued.

Table A.1. Summary of baseline and session measurements for the artificial otolith elemental ratios used to check the consistency of ToF-SIMS analyses from session to session. Elements examined included: Ba, Mg, Li, Na, Zn, Mn, Fe, Cd, As, Sb, and Se. Baseline values were established over three sessions (n = 13 assays). Session data include measurements over 19 sessions (n = 24 assays).

Element/Ca	Baseline			Sessions		
($\times 10^{-3}$)	$\bar{x}$	SD	CV	$\bar{x}$	SD	CV
Ba	0.7007	0.1502	21.44	0.2946[d]	0.1504	51.05
Mg	0.7098	0.2225	31.35	0.6053	0.4185[a]	69.14
Li	0.1514	0.0734	48.48	0.1235	0.1076	87.13
Na	61.9040	20.4110	32.97	60.4420	25.8190	42.72
Zn	3.3052	1.0601	32.07	1.1364[d]	1.0836	95.35
Mn	1.5674	0.8213	52.40	1.5320	1.1322	73.90
Fe	16.9150	1.4446	8.54	16.5040	1.7775	10.77
Cd	0.7502	0.2252	30.02	0.7342	0.6112[b]	83.25
As	0.4388	0.1372	31.27	0.5608	0.2784[a]	49.64
Sb	0.5376	0.1592	29.61	0.2365[c]	0.2244	94.88
Se	0.8270	0.0686	8.30	0.7901	0.0868	10.99

[a]significant difference at 0.05, [b]significant difference at 0.01, [c]significant difference at <0.001, [d]significant difference at <0.0001.
t-tests with df = 34 (if variances were not different and pooled for test), df = 33.9, 30.6, and 33.6 respectively for Mg, Cd, and As where variances were unequal and df were determined by Satterthwaite approximation.