Validation and Evaluation of the Stable Isotope Marking Technique in the Lake Sturgeon, *Acipenser fulvescens*

by

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Abstract

The Lake Sturgeon is an endangered species throughout much of its range, consequently substantial effort has been made to restore and/or sustain populations through stock enhancement. These efforts however require a reliable indicator for monitoring success or failure. In this thesis the enriched stable isotope marking technique was examined to determine its validity and effectiveness in marking of Lake Sturgeon fin rays. The enriched stable isotope marking technique uses stable isotopes of elements that replace calcium in boney tissue. The ratio between these isotopes are fixed in nature, however in laboratory settings can be easily manipulated. Using laser ablation inductively coupled plasma mass spectrometry the ratios of naturally occurring isotopes can be determined from a small sample of the fin ray, thus allowing for the discrimination of fish stocks. Further, the thesis examined some of the underlying assumptions and mechanisms of uptake for strontium and barium into the Lake Sturgeon. For the marking technique to work, strontium and barium must be taken up from the environment and accumulated long-term in boney tissue. Net flux and tissue retention of strontium is reported and the research has demonstrated that enriched stable isotopes can be successfully used to mark the fin ray of Lake Sturgeon for long-term (> 500 days) stock discrimination.

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Chapter 1: General Introduction

Lake Sturgeon, *Acipenser fulvescens,* populations are declining throughout their natural range and are considered endangered, threatened or a species of conservation concern by USA management groups (U.S. Fish and Wildlife Service 2003). In Canada Lake Sturgeon populations are listed as endangered, threatened or of special concern (http://www.cosewic.gc.ca/). Because of the Lake Sturgeon's status, conservation effort is being put into all facets of Lake Sturgeon biology including protection of existing populations and habitat and the use of stock enhancement with hatchery-reared juveniles (Hay-Chmielewski and Whelan 1997; Auer 2003).

Measurement of the success, or failure, of stock enhancement programs is largely examined at the system level, and in simplistic terms can be assessed as an increase, or decrease, in the non-lethal catch per unit effort (number of fish caught over standardized time). While this provides an indication of the success or failure of the stock enhanced fishery, unless fish are marked there is no way of knowing their origin and therefore the contribution to the overall population of hatchery-reared fish. An effective marking technique allows researchers to track the success of hatchery-reared fish and determine the proportion of the hatchery-reared fish to the overall population. Marking techniques also inform us about the survival of hatchery-reared Lake Sturgeon as well as determine if there are differences between the natural and stock enhanced progeny in terms of life history traits. With this knowledge stock enhancement programs can be 'fine tuned' for optimal success.

Stock enhancement is considered any use of management practices, including aquaculture technology to increase the population of a given species in a given geographical range (Lorenzen 2014). These technologies can be simply raising fish in the hatchery setting but

may also include the introduction of artificial habitats, feeding, or predator control (Lorenzen 2014). All of these techniques serve to increase the population in species where population size is reduced, in many cases due to anthropogenic activities. However these enhancements are not always successful, and research has shown that some stock enhancements have caused greater damage to the ecosystem or the species of interest. Raising fish in the hatchery can impact fitness of the organism. Olla et al. (1998) showed that juvenile Coho Salmon, *Oncorhynchus kisutch,* and Atlantic Cod, *Gadus morhua*, raised in the hatchery setting lacked appropriate anti-predator behaviour when compared to wild reared fish. This deficit in natural behaviour would lead to higher predation rates and as such a lower survival rate. It has also been observed that enhancement programs can destroy natural populations. In Pink Salmon, *Oncorhynchus gorbushcha*, hatchery raised fish completely replaced the wild stock in Prince William Sound, Alaska (Hilborn and Eggers 2000). This replacement of wild stock decreases the genetic diversity of the population and can be problematic, especially in cases like the ones observed in Olla et al. (1998) where hatchery raised fish have less fitness due in to lack of appropriate anti-predator behaviour (Lorenzen 2014).

Problems may also arise from stock enhancement when interspecific interactions are examined. Stock enhancement of predatory fish may negatively effect populations of prey species (Lorenzen 2014). Levin and Williams (2002) observed a decrease in the survival of wild Chinook Salmon, *Oncorhynchus tshawytscha,* in response to release of hatchery-reared Steelhead Trout, *Oncorhynchus mykiss irideus*.

Despite the risks and examples of stock enhancement going poorly, the literature also shows a great deal of stock enhancement programs used effectively to rescue at risk species. This includes the stocking program of Red Drum, *Sciaenops ocellatus,* in Texas (McEachron et al.

1995), Chum Salmon, *Oncorhynchus keta*, in Japan (Hilborn 1998) and record harvests of both wild and hatchery-reared salmon in Alaska (Heard 2012). Success here in Manitoba with Lake Sturgeon stock enhancement programs has also begun to be realized (McDougall et al. 2014). However, the risks associated with stock enhancement programs, dictates they must be closely monitored to ensure that the goals of the program are met, consequently cost effective marking of large numbers of fish can prove useful in assessing the merits of individual stock enhancement programs.

Marking or tagging fish is an important tool that allows long term identification and study of individuals and groups (Thorrold et al. 2001) in addition to insight into population estimates and dynamics (Garrott et al. 2012), determination of natal areas or the origin of migrants (Jones et al. 1999). There are many different marking techniques and devices, each with distinct advantages and disadvantages depending on the research objectives of any given study. The three main types of tags or marks are passive, electronic and biological (Drenner et al. 2012). Passive tags are defined as tags that do not contain a battery, whereas electronic tags do and as such have the ability to store and/or transmit data but also have a finite life span. Biological tags are considered as marks or characteristics that naturally occur or natural characteristics that are manipulated and allow us to distinguish an individual or group (Drenner et al. 2012). A further review of the three main types of tags follows to help understand which marking technique will be ideal for use in this project.

Passive Tags

Some examples of passive tags include: fin clipping (Matala et al. 2012), passive integrated transponder tags (PIT) (Skalski et al. 2001), coded wire tags (CWT) (Sharma and Quinn 2012) and dye marking of individuals (Jones et al. 1999). Fin clipping is where part of a

fin is removed; it is a simple and cost-effective technique that allows users to mark large amounts of hatchery-raised fish. Fish can be identified rapidly as marked by eye and as such this is a non-terminal technique (Skalski et al. 2009). In some cases fins are removed completely, like adipose fins in salmonids but some species have the ability to regenerate lost fins (Armstrong 1949; Shetter 1951; Johnson and Ugedal 1988; Coombs et al. 1990). However, evidence suggests that clipping the adipose fin may have negative fitness impacts on fish swimming (Reimchen and Temple 2004). The size of the fish also limits the use of adipose fin clipping; fish must reach a fork length of at least 50mm before the use of this technique is applicable (Skalski et al. 2009). Another major issue with fin clipping is the inability to distinguish individuals from one another (Skalski et al. 2009).

Passive integrated transponders or PIT tags have a unique code to each tag that can be easily read by scanning the tag with the appropriate reader, similar to a bar code reader at a grocery store. This allows for a unique individual code to every fish that is marked which is a distinct advantage over fin clipping. Like fin clipping PIT tagging is non-terminal. Also like fin clipping PIT tagging requires that fish are a minimum fork length of 50 mm as the abdominal cavity of the fish must be large enough to accommodate the tag (Skalski et al. 2009). Skalski et al. (2009) concluded because of the ease of applying PIT tags, it is an excellent choice to mark large quantities of fish. However PIT tags require the handling of each fish to be tagged, which can lead to increased stress of the animals and also imposes a cost in time to handle and apply the PIT tag. For large amounts of fish the time required to tag the fish might limit the applicability of PIT tags. PIT tags are generally very stable in the environment due to the fact that they are typically inserted in the abdominal cavity of the fish (Skalski et al. 2009). However, as much as a 30% loss of PIT tags has been reported in the Brown Trout, *Salmo trutta* (Acolas et

al. 2007). Skalski et al. (2009) suggest that retention success of the PIT tag is primarily due to the skill of the user and not necessarily the rejection of the tag by the fish. Another possible downside to PIT tags is evidence to suggest that PIT tags can influence both growth and survival of fish although this is equivocal. Soula et al. (2012) found a significant decrease in mass and specific growth rate (SGR) 15 days after implantation in red porgy, *Pagrus pagrus*, with an average mass of 4 g. Conversely, Peterson (1994) found that Coho Salmon as small as 2.8 g were found to have no significant decrease in overwinter survival or overwinter growth when implanted with PIT tags. Skalski et al. (2009) suggest mortality and other negative effects from using PIT tags may be influenced by factors such as size of the fish, the species of the fish and the skill of the user implanting the tag.

Coded wire tags, or CWT's are also internally implanted and like PIT tags can be used to mark large numbers of fish. However, unlike PIT tags and fin clipping, they require destructive sampling in order to identify the fish. CWTs can however be implanted in fish weighing less than 2.1g, which is a distinct advantage over PIT and fin clipping techniques (Skalski et al. 2009). Another important aspect to consider is cost when comparing the most appropriate tag to use in any given study. CWT's cost between $0.09 and $0.16 United States Dollars (USD) per tag (http://www.nmt.us/products/cwt/cwt.shtml), whereas PIT tags cost between $2.00 and $5.00 USD per tag depending on the size of the PIT tag (http://www.oregonrfid.biz).

Dye marking, can include immersion, injection or ingestion of the dye. This technique can be targeted to a specific area as well as a broad non-targeted approach. For instance immersion in oxytetracycline causes the otolith of immersed fish to fluoresce under UV light, whereas immersion in dyes can temporarily change the colouration of the body. There are many similarities between dye marking and fin clipping. Both techniques do not allow users to

distinguish between individuals; it only allows users to determine marked versus unmarked (Skalski et al. 2009). Also large amounts of fish can be dye marked with minimal effort (Jones et al. 1999). This technique has been used successfully on a variety of species, including the saltwater Coral Reef Damselfish, *Pomacentrus amboinensis,* (Jones et al. 1999), Chinook Salmon (Gaines and Martin 2004), and Sockeye Salmon, *Oncorhynchus nerka* (Carlson et al. 1998). Dye marking can be immediately detected either by eye or by a proprietary reader. Dye marking often does not normally require the sacrifice of the animal, but has been found to have a variable amount of stability in the wild (Skalski et al. 2009). The stability of the dye mark in fish is influenced by many factors from environmental pH and temperature to the fish species it is applied to (Skalski et al. 2009). It is also important to note that special consideration is needed when using dyes to ensure the levels that fish are exposed to do not have a detrimental effect on the fish or other organisms that may encounter the dye or consume the fish. Fish must be at least 50 mm in fork length before the use of this technique can be effective (Skalski et al. 2009). A further disadvantage to dye marking can be quite subjective and what one individual would deem as marked may not be considered marked by another individual.

Despite the advantages of some passive tags being low cost and easy to mark large numbers, most of these techniques have the disadvantage of handling each individual to mark them. Furthermore, each passive tag examined here had specific limitations such as the inability to detect the tag immediately, inability to determine the identity of an individual, fitness impacts and most importantly destruction of the animal to read the tag that limits the use of these tags to specific applications.

Electronic Tags

Electronic tags provide real time tracking data of tagged animals and depending on the precision of the receiving equipment can position the fish within 1 m accuracy in three-dimensional space. This undoubtedly provides far greater spatial and temporal resolution of fish movement but is also very costly. The main types of electronic tags are: archival tags, acoustic or radio tags, pop-off satellite tags (PSATs) and smart position or temperature transmitting tags (SPOTs).

As the name suggests archival tags archive data on fish movement and require recapture and recovery of the fish to retrieve them. They have mainly been used to record data about the environment an individual is in along with positional data. They also are size limited and the tags should not exceed 1.25% of the fish's body weight (Sutton and Benson 2003). Also due to cost, archival tags are not suitable for mass marking of fish (Drenner et al. 2012).

Radio or acoustic transmitters are also size limited and should not exceed 1.25% of the fishes body mass. However unlike archival tags, radio or acoustic tags transmit a signal that allows users to locate fish in real time and do not require the recapture of the fish. Radio tags have limited use in saltwater as the signal attenuates (Drenner et al. 2012) and must be considered when interpreting the data as signals from both devices can be subject to noise pollution (Drenner et al. 2012). Radio and acoustic tags are also limited by cost and are therefore not appropriate for mass marking of fish.

PSATs and SPOTs both archive and transmit information to a satellite that can be accessed from a desktop computer. However, these technologies are costly which again limits the use of these types of tags for mass marking fish despite the large amounts of data these tags can obtain (Drenner et al. 2012).

Biological Tags

Biological tags rely on either natural or unnatural biological variables that researchers can use to distinguish identity or where the organism has migrated. Two main biological tagging techniques include genetic markers (Letcher and King 1999) and microchemistry of hard structures such as the otolith, vertebrae, scales or fin rays (Thorrold et al. 2001; Tillet et al. 2011; Adey et al. 2009; Smith and Whitledge 2011).

Genetic tagging is the use of the organisms' own genetic code to determine their identity (Andreou et al. 2012). The added advantage of genetic tags is this technique can also be used to determine the relatedness between individuals. Relatedness can only be determined if the genotype of all possible parents are known, which can be a huge task depending on the population size. One disadvantage with genetic marking is identification of an individual is not immediate as with most of the passive tags. Conversely there are many advantages to genetic marking such as it requires no manipulation and the mark is permanent in the organism (Skalski et al. 2009). Secondly, identification from genetic tagging does not require sacrifice of the animal, as a small fin clip will provide ample material for analysis (Skalski et al. 2009) and as such the negative implications on fitness are minimal to the organism. Genetic marking as a stock enhancement assessment tool has been successfully employed in a number of different species including, the Common Dace, *Leuciscus leuciscus* (Andreou et al. 2012), the Lemon Shark, *Negaprion brevirostris* (Feldheim et al. 2002), the Pallid Sturgeon, *Scaphirhynchus albus* and Lake Sturgeon (DeHaan et al. 2008; McQuown et al. 2003).

In the present study stable isotope marking was selected due to the requirements of the application in Lake Sturgeon rearing. In short, isotopic marking of the fin ray using stable isotopes of barium or strontium was selected as it is nonterminal, cost effective and entire

cohorts of hatchery raised fish can be marked with minimal effort, as it does not require individual handling. Fish can be marked in large quantities simply by adding the stable isotopes into the water the fish are being kept in, where as many other marking techniques require handling of each individual fish (Skalski et al. 2009).

Stable isotopic marking is where the user either finds a natural mark that distinguishes a group or individuals from others or the user manipulates the environment to create a mark to distinguish the group or individuals from others. An example of a natural mark is the strontium to calcium ratio in bony tissue. Typically, increased strontium to calcium ratios in bony tissue are used to indicate life in increased saline waters (Hamer et al. 2015; Campana 1999; Dregens et al. 1969), which allows users to determine if fish migrated or spent a significant amount of time in saltwater or freshwater. However, little is known about the metabolism and mechanism of uptake rate and deposition of barium and strontium in fish. Understanding movements of these ions into the organism alongside information on incorporation of the ions in various tissues and turnover rates in these same tissues will substantially improve inference made on life history events in wild fishes based on the elemental signature of hard structures such as fin rays or otoliths. At present it is assumed that strontium and/or barium enter and exit an organism using the same transport mechanisms as calcium. This is based on the hypothesis that barium and strontium ions behave chemically similar to calcium ions because all three elements are found in the same group in the periodic table. Two crucial similarities are the ionic size and ionic charge. Calcium, barium and strontium when in ionic form are found predominantly as divalent cations and are similar in ionic radius, with calcium being the smallest followed by strontium then barium as the largest.

Success or failure, of current stock enhancement efforts of Lake Sturgeon in the province of Manitoba is largely unknown. The research conducted in this thesis examined the efficacy of the stable isotope marking technique in the fin ray of Lake Sturgeon. In chapter 2 flux rates of strontium alongside turnover rates of strontium and barium in a variety of tissues were determined to provide a stronger understanding of the physiological metabolism of these ions in the Lake Sturgeon. In chapter 3 the efficacy of transgenerational marking of fin rays with barium or strontium stable isotopes is compared with immersion of larval fish. Further, length of immersion in the stable isotope is examined for marking efficiency and finally duration of the isotopic signature in the fin ray is assessed.

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Chapter 2: Determination of Whole Body Strontium Influx, Efflux, Net Flux, Tissue Accumulation and Tissue Turnover Rates in the Juvenile Lake Sturgeon, *Acipenser fulvescens*

Abstract

To build complex tissues and organs, organisms need building blocks from the environment. Elemental building blocks exist in ionic form in the aqueous environment and require particular transport mechanisms for uptake into the organism by transcellular or paracellular routes. Each element will have a specific rate of uptake and excretion of its ionic form known as influx and efflux respectively that is dependent on a number of factors including water chemistry and life phase of the organism of interest. Once inside the organism the ions can either be incorporated or excreted from the organism. Incorporation of any ion is not necessarily permanent and resorption can occur, usually at a rate dependent on concentration gradients. The persistence of an ion within a tissue can be described as the biological half-life of the ion in the selected tissue. Using radioactive strontium (^{85}Sr) influx and efflux rates of strontium were determined in juvenile Lake Sturgeon to be 3.02 ± 0.364 pmol.$h^{-1}g^{-1}$ and 0.04 ± 0.007 pmol.$h^{-1}g^{-1}$ respectively. Furthermore, short-term accumulation of strontium in a variety of tissues was assessed and found to be greatest in the fin ray. Subsequent experiments using stable isotopes of strontium (^{86}Sr) and barium (^{137}Ba) estimated the biological half-life for strontium and barium in muscle, vertebrae and fin ray. Half-life of both stable isotopes was found to be shortest in muscle, intermediate in vertebrae and longest in fin ray. The data provide the initial physiological parameters for the uptake and partitioning of strontium and barium in Lake Sturgeon and are discussed in the context of using these elemental signatures to infer life history events in this group of fishes.

Introduction

Elements are the building blocks required for complex molecules that make up organisms and allow life. Homeostatic regulation of these elements in an organism is a crucial process to ensure survival and propagation of the organism. Strontium is an element that is a member of the alkaline earth metal group in the second column of the periodic table and as such shares chemical similarities with beryllium, magnesium, calcium, barium and radium. As members of the alkaline earth metal group these elements form divalent cations when in an aqueous environment and have similar ionic radii. Of the alkaline earth elements, only magnesium and calcium are thought to be essential for life.

Strontium has been shown to exhibit similar biological effects as calcium including roles in physiology; while much less potent than calcium, supra-physiological concentrations of strontium inhibited the release of the calcium regulatory hormone parathyroid hormone (PTH) in bovine parathyroid cells (*Bos taurus*) (Brown et al. 1990), furthermore, muscular contraction and blood clotting was also stimulated but to a lesser extent by strontium (Nielsen 2004). The high concentrations and reduced ability to stimulate blood clotting and muscular contraction suggests that strontium mimics calcium albeit less effectively. However there is evidence that strontium can affect physiological processes. Jensen et al. (1997) observed a correlation between strontium content in bone and bone compression strength in mammals. Further, studies have shown that strontium administration decreases symptoms of osteoporosis, or bone weakening in humans (Shorr and Carter 1952; McCaslin and Janes 1959; Marie 1996; Grynpas et al. 1996) by decreasing bone resorption and increasing bone ossification.

Despite the potential for strontium specific biological actions there appears to be no homeostatic control over strontium concentrations in biological fluids in mammals at least

(Nielsen 2004). Furthermore, there is no reported correlation between strontium deficiency and osteoporosis (Zhang et al. 2002) nor are there examples of negative health effects of strontium deficiency in mammals (Nielsen et al. 2004). Finally, to date there is no evidence to suggest that strontium is an essential element in vertebrates (Chowdhury and Blust 2012). Thus, due to the ability of strontium to replace calcium in physiological processes, the current hypothesis is that no separate uptake mechanisms for strontium exists, rather strontium uptake is facilitated by epithelia cell calcium channels and pumps. This in concert with the overall greater abundance of calcium in comparison with strontium in the environment and in extracellular fluids, (in mammals extracellular calcium levels are often in the millimolar range where strontium concentrations are in the micromolar range (Nielsen 2004)), leads to their being no evolutionary pressure to develop unique transport and homeostatic mechanisms specifically for strontium (Nielsen 2004).

The majority of calcium uptake in fish occurs across mitochondria rich cells and pavement cells of the gills (Flik et al. 1995), however, there is some evidence of calcium uptake across the gastrointestinal tract of fish (Allen et al. 2011; Flik and Verbost 1993; Flik et al. 1990). Current models for calcium transport across epithelial cells in fish are similar to those described in mammals where there is a transcellular and paracellular pathway (Hoenderop et al. 2005). Briefly, in the transcellular pathway calcium is allowed to enter the cell from the environment via transient receptor potential channels (TRPV5 or TRPV6) (Hoenderop et al. 2002; Peng et al. 2003). Calcium then travels across the epithelia cell through the cytosol from the apical membrane to the basolateral membrane through two hypothesized mechanisms. The first is the facilitated diffusion model, where the driving force of calcium moving across the cell is powered by the rates of uptake and removal of calcium, which creates a concentration gradient across the

cell driving calcium towards the basolateral membrane (Hoenderop et al. 2005). The calcium either passes freely or is bound to intracellular carrier proteins such as calbindin, which deposits calcium at the basolateral membrane (Hoenderop et al. 2005).

The second proposed model of calcium transport through the cell is known as the vesicular model. In this, the calcium entering the cell on the apical membrane causes a disruption in the actin filaments of the cytoskeleton. This disruption causes the formation of a vesicle; some of the vesicles are transported to a lysosome and then merge with the lysosome. The lysosomes and the vesicles that do not merge with the lysosomes are then taken to the basolateral membrane via the microtubule skeleton where vesicles merge with the membrane and calcium is deposited outside of the cell (Larsson and Nemere 2002). Calcium is then transported out of the cell across the basolateral membrane into the plasma via either the sodium calcium exchanger (NCX) or plasma membrane calcium ATPase (PMCA) (Van Baal et al. 1996; Flik et al. 1990; Hildmann et al. 1982). NCX is a secondarily active transporter, driven by the inward sodium gradient created by sodium potassium ATPase (Na^+/K^+ ATPase). NCX has been observed to be the driving force of intracellular calcium removal in teleost enterocytes and mammalian kidney tissue (Flik et al. 1990; Van Ball et al. 1996; Bindels et al. 1991). In contrast to PMCA, a primary active transporter, which uses ATP to move calcium against its own concentration gradient and the chief driver in the removal of intracellular calcium in mammalian enterocytes (Hildmann et al. 1982). The paracellular pathway is where the calcium moves across the epithelia layer between cells.

The mechanism of calcium uptake has not been fully confirmed in the present study species, the Lake Sturgeon. However, several of the proteins involved in mammalian and teleost fish epithelial calcium transport have been shown to play a role in calcium uptake across the

spiral valve of Lake Sturgeon. Genz et al. (2013) found with the use of specific protein inhibitors that when TRPV5, NCX and PMCA were inhibited there was a decrease in calcium influx in the spiral valve of the Lake Sturgeon.

At the time of writing there were no reports of whole body strontium flux in any member of the acipenseriformes (sturgeons and paddlefish) and very few in teleosts. In rainbow trout, *Oncorhynchus mykiss*, strontium influx across the gills was shown to be lower than calcium (Schiffman 1965), however, this was attributed to the substantially higher environmental calcium concentration compared to strontium. Further, kinetic studies report a higher V_{max} for strontium and barium influx in mammalian nerve cells than the V_{max} values for calcium (Nachshen and Blaustein 1982). The V_{max} of an ion is the theoretical fastest uptake rate of the ion by a given cell, tissue or organism. This value is related to the K_M of an ion, which is the concentration of the ion in the external environment that will produce a rate of uptake that is one half of V_{max} and the reciprocal of which can be viewed as the affinity of a transport for a particular ion or molecule. These data suggest that despite the potential for strontium to replace calcium transport, affinity for calcium remains substantially higher than strontium in this cell type.

Following uptake any particular ion may be incorporated in any number of tissues or organs. Dependent on the tissue type a particular ion may face several barriers before it is incorporated. For instance for a strontium ion to be incorporated into the otolith of a fish it must pass through the epithelial layer of the gills or intestine into the plasma. From the plasma the ion must be transported into the endolymph and finally it must be taken into the matrix of the otolith (Campana et al. 1999). These barriers allow for the potential for differential accumulation of ions in different tissues and fluids that is largely dependent on the functionality of the tissue and thus presence or absence of transport proteins for that ion. These functional and molecular

characteristics will greatly affect the chemical constituents of a selected tissue or fluid and can be seen by comparing the chemical profiles of tissues such as bone and scales in fish. Miller et al. (1992) illustrate the different chemical compositions of liver, muscle and bone in White Sucker, *Catostomus commersonii*, that clearly indicates ion selectivity and preferential incorporation into different tissues.

Previous research using radioactive strontium (^{90}Sr) in male guppies of the genus *Lebistes* demonstrated that accumulation of ^{90}Sr was highest in the head region of the guppies (Rosenthal, 1957), furthermore, while the percentage values of accumulation of radioactive strontium and calcium were significantly different from one another the trend in distribution of the two isotopes was consistent (Rosenthal 1957), with the exception of the viscera of the fish that was shown to accumulate 13.5% of the radioactive strontium and only 7.3% of the radioactive calcium (Rosenthal 1956; Rosenthal 1957). Finally, half-life, of ^{90}Sr was estimated at 8 days for the viscera and 600 days for the spine and cranium (Rosenthal 1957) indicative of fast turnover of calcium in soft tissues and storage of calcium in hard tissues.

Using the radioactive isotope ^{85}Sr the present study aimed to examine the net flux of ^{85}Sr under environmental calcium conditions equivalent to those found in the Winnipeg River, Manitoba. Furthermore, accumulation of ^{85}Sr in both hard and soft tissues of juvenile Lake Sturgeon was examined following a six-hour immersion of fish in ^{85}Sr labeled water. Finally, accumulation and turnover of the non-radioactive stable isotopes ^{86}Sr and ^{137}Ba in hard and soft tissues was examined following injection of juvenile Lake Sturgeon with ^{86}Sr or ^{137}Ba and subsequent sampling 3, 7 and 30 days post injection. Based on previous whole body calcium flux studies in juvenile Lake Sturgeon (Allen et al. 2011) I hypothesised that the net movement of ^{85}Sr would be in an inward direction; further the isotope would be preferentially incorporated in

hard tissues such as vertebrae and fin rays and that the turnover of both ^{86}Sr and ^{137}Ba would be highest in the hard tissues.

Materials and Methods

Determination of whole body influx

Eight age 0+ Lake Sturgeon, total mass 13.7 ± 0.7 g, total length 19.5 ± 0.4 cm were used to determine whole body strontium influx. Experimental fish were held at 15°C on a 16h:8h (light: dark) schedule and fed daily with blood worm (Sally's bloodworm) in flow through dechlorinated City of Winnipeg tap water. On the day of experiment fish were captured by dip net and quickly dried and weighed by electronic balance before being placed in a 10:1 (volume:mass of fish) flux chamber. Flux chambers were constructed from two Ziploc® sandwich bags (16.5 cm x 14.0 cm) (SC Johnson Canada, Brantford, ON), with one bag placed within the other. The inner bag was filled with water identical to holding conditions with one exception, addition of 0.75 $kBq.mL^{-1}$ of ^{85}Sr (^{85}Sr; Specific Activity 723.23 $MBq.mg^{-1}$) (Perkin Elmer, Waltham, MA, USA). ^{85}Sr was used to determine influx and efflux rates as such measurements require detection of changes in isotope concentration over a short time period that is not possible to detect using non-radioactive isotopic forms of the element. The outer bag was present to ensure no leakage of radioactive material into the water bath. When fish were introduced into the flux chamber a 1.75 mL water sample was taken as t=0. Hourly water samples (1.75 mL) were then taken for the following 6 hours and radioactivity was measured in each sample to determine the loss of radioactivity from the environment every hour.

During the experiments the flux chambers were held at a constant temperature of 15 °C by immersing the chambers in a water bath (Julabo, Allenton, PA, USA). Chambers were aerated with forced air during the experiment and the bags remained open for the duration of the

experiment for gas equilibration. A lid was placed on the water bath to reduce the amount of ambient light that the experimental fish were exposed to.

After six hours fish were removed from the flux chambers and rinsed in tracer free water for 1 minute. Fish were then placed in an overdose of anaesthetic containing 300 $mg.L^{-1}$ ethyl 3-aminobenzoate methanesulfonate (MS-222) buffered with an equal mass of $NaHCO_3$. Total length was taken and a blood sample was then drawn from the caudal sinus using a heparinized 27g needle and 1 mL syringe. Blood was centrifuged at 13,000 g and 4 °C (Fisher Scientific Accuspin Micro 17R, Fisher Scientific, Ottawa, ON, Canada) to separate plasma and red blood cells. Plasma and water samples were retained for analysis of radioactivity in triplicate. Between 20-50 µL of plasma, depending on the amount of plasma obtained from the fish, and 0.5 mL water samples were added to 7 mL vials and counted on a gamma counter for 5 minutes (Wizard 2, Perkin Elmer, Waltham, MA, USA).

Fish carcasses were then frozen and at a later date processed for whole body ^{85}Sr by digesting in an acid bath, 2.5:1 (volume:mass) of 2 M HNO_3 (Fisher Scientific, Ottawa, ON, Canada). Radioactivity in the resultant digest was measured as described above and used to determine influx rate using a modified equation from Allen et al. (2011):

$$Influx = CPM_{total}/(SA_{Sr} \times CE \times T \times M \times 60 \times A)$$

Where influx ($pmol.g^{-1}.h^{-1}$) was determined by CPM_{total} as the calculated amount of radioactivity in counts per minute (CPM) for the entire fish; SA_{Sr} was the specific activity of ^{85}Sr; CE was counting efficiency for ^{85}Sr; T was the duration of the experiment in hours and M was the mass of the fish in grams; A factor of 60 was used to convert from CPM to Becquerel; and A represents the atomic mass of the selected element.

A pilot study was also conducted to determine the effectiveness of using the disappearance of radioactivity from the water bath to determine ^{85}Sr influx rates as previously described in Wood (2011). Determination of strontium influx via this method is a less time intensive method and was calculated using a modified equation from Wood (2011):

$$\text{Influx} = ((CPM_i - CPM_f) \times V_i) / (SA_{Sr} \times CE \times T \times M \times 60 \times A)$$

Influx rate ($pmol.g^{-1}h^{-1}$) was the amount of ^{85}Sr disappearing from the environment and into the fish; CPM_i was the starting measured CPM from each experimental flux chamber; CPM_f was the final CPM for each experimental flux chamber; V_i was the volume of the flux chamber at the beginning of the experiment; SA_{Sr} was the specific activity of ^{85}Sr; CE was the counting efficiency for ^{85}Sr; T was the duration of the experiment in hours; M was the mass of the fish in grams; a factor of 60 was used to convert CPM to Becquerel; and A was the atomic mass of the radioisotope used. The pilot study demonstrated a high correlation (R^2=0.9379) between the two measurement techniques therefore the disappearance method was used to determine strontium influx.

Determination of whole body efflux

Five age 0+ Lake Sturgeon, total mass 20.5 ± 4.0 g, total length 21.2 ± 0.2 cm were used to determine the efflux rate of strontium. The fish were held in identical conditions as the fish used in the influx study. Fish were captured by dip-net, dried and quickly weighed. A Lake Sturgeon Ringer's solution was made according to Allen et al. (2009) as follows: NaCl 108 mM, KCl 1.8 mM, Na_2HPO_4 2.1 mM, KH_2PO_4 0.2 mM, $MgSO_4 \cdot 7H_2O$ 0.8 mM, $CaSO_4$ 1.6 mM, $NaHCO_3$ 8.0mM pH adjusted to 7.6. ^{85}Sr was added to the Ringer's solution at time of injection to a calculated dose of 5.5 $kBq.g^{-1}$ of fish mass. Volume of dose was also controlled and was calculated to be 7.5 $\mu L.g^{-1}$ of fish mass. Fish were injected with the ^{85}Sr labelled Ringer's in the

intraperitoneal cavity using a 27g needle and 1 mL syringe. The fish were then placed into a 20 L tank with tracer free water and held for 48 hours to allow efflux rates to equilibrate. During this time fish were held in identical conditions as stated before with the exception that they were not fed. On the day of experiment fish were captured via dip-net and placed in flux chambers constructed from Glad® LockWare™ Small Food Storage Containers (473 mL) (Glad, Orangeville, ON, Canada). Flux chambers were filled with 10:1 (volume:body mass) tank water. Immediately after introduction of the fish to the flux chamber a 1.75 mL water sample was taken at t=0. Flux chambers were once again aerated as described above and placed in a water bath maintained at 15°C, as described above. Fish remained in the flux chambers for 6 hours with water samples (1.75 ml) taken every hour and radioactivity was determined to provide the appearance of radioactivity every hour.

At 6 hours fish were removed from the flux chambers and rinsed in tracer free water for 1 minute then placed in an overdose of anaesthetic as described above. Total length was measured and a blood sample was taken from the caudal sinus using a heparinized 27g needle and 1mL syringe. Blood was then centrifuged to separate plasma from red blood cells. The plasma and water samples were then analysed for radioactivity as previously described as the same volumes for counting were used. The measured amount of radioactivity in each sample was then used to calculate the efflux rate using a modified version from Allen et al. (2011).

$$\text{Efflux} = ((CPM_i - CPM_f) \times V_i) / (SA_{Sr} \times CE \times T \times M \times A \times 60)$$

Where efflux ($pmol.g^{-1}.h^{-1}$) was the rate of strontium leaving the organism; CPM_i was the calculated amount of CPM for the entire flux chamber at the beginning of the experiment; CPM_f was the calculated amount of CPM for the entire flux chamber at the end of the experiment; a factor of 60 was used to convert CPM to Becquerel; V_i was the volume of the flux chamber at the

beginning of the experiment; SA_{Sr} was the specific activity of ^{85}Sr; CE was the counting efficiency of ^{85}Sr; T was the time period in hours; A was the atomic mass of the radioisotope used; and M was the mass of the fish in grams.

Determination of strontium partitioning using the radioisotope ^{85}Sr

Strontium partitioning was run concurrently with determination of whole body strontium influx experiments. Spiral valve, fin ray, skull, skin, vertebrate, skeletal muscle, gill and brain tissue were carefully removed from each fish used in the influx experiments (see above). Following dissection each tissue was weighed and placed into 7ml vials where a 2.5:1 volume to mass of distilled water was added to the vial. After the addition of distilled water the samples were sonicated at 40% intensity (I) for 15 pulses at 2 seconds each to lyse all cells within the tissue sample (Fisher Scientific Ultrasonic Dismembrator 150T, Fisher Scientific Canada, Toronto, Ontario, Canada) . The resultant fluid was then analysed for radioactivity in the same fashion as the plasma and water samples. The counts per minute were then transformed to mass of strontium present per mass of tissue using the following formula.

$$SPM= (((CPM/(CE \times 60))/M)/SA)/A$$

Where SPM ($nmol.g^{-1}$) was the strontium present per mass of tissue and CPM was the measured counts per minute; CE was the counting efficiency for ^{85}Sr; M was the mass of tissue; A denotes the atomic mass of the radioisotope; and SA was the specific activity of the isotope. The factor of 60 was used to convert from CPM to Becquerel. The values were then averaged by tissue type.

Determination of tissue specific half-life of strontium and barium using ^{86}Sr and ^{137}Ba

In this series of experiments the stable isotopes ^{86}Sr and ^{137}Ba were used. Use of the radioactive isotope, ^{85}Sr, was not possible due to the duration of the experiment and logistical issues associated with long term holding of radioactively labeled fish. Age-1 Lake Sturgeon were

held at the University of Manitoba, Winnipeg, Canada and separated into 3 groups (n=24 for each group) (Strontium Injected: 224.2 ± 13.7 g, Barium Injected: 242.5 ± 18.3 g, and Control: 217.1 ± 10.8 g). Individual fish were injected intraperitoneally using a sterile 1 mL syringe and 23g needle with one of three treatments: 2 $mg.kg^{-1}$ $^{137}BaCO_3$ in Lake Sturgeon Ringer's, 2 $mg.kg^{-1}$ $^{86}SrCO_3$ in Lake Sturgeon Ringer's and Lake Sturgeon Ringer's only as a control. All doses were volume controlled at 0.02 $mL.g^{-1}$ of body mass. Prior to injection the fish were lightly anaesthetised by immersion in a bath of 50 $mg.L^{-1}$ of MS-222. Eight fish from each treatment group were sacrificed by immersions in an overdose of MS-222 as described previously at 3, 7 and 30 days post injection. Skeletal muscle, fin ray and vertebrae were removed from each fish and flash frozen in liquid nitrogen. When required metal scissors were used during the dissection but use was minimised and when possible alternative plastic tools were used to minimise trace metal contamination of the tissue samples as much as possible. Tissue samples were thawed, rinsed in MilliQ water, dried and weighed. The cleaned tissue was then acid digested by adding 10 mL of 70% Nitric Acid (Trace Metal Grade, Fisher Scientific, Ottawa, ON, Canada) and left for two days, then 2 mL of 30% Hydrogen Peroxide (Trace Metal Grade, Fisher Scientific, Ottawa, ON, Canada) was added and the fluid was left for another two days. The resultant fluid was filtered with 0.1 mm filters and then analysed for $^{88}Sr/^{86}Sr$ and $^{138}Ba/^{137}Ba$ ratios using solution based inductively coupled plasma mass spectrometry (SO-ICP-MS) (Thermo Finnigan Element 2, Thermo Fisher Scientific Inc., Mississauga, ON, Canada). The isotopic ratio for control fish did not differ regardless of sampling time or tissue and thus was considered background for each tissue type in comparison to the tissue samples from the experimental fish (see Figures 2.3 – 2.8). Consequently, isotopic ratios from the treatment fish

were subtracted from the control values to provide the amount of ^{86}Sr or ^{137}Ba above baseline following:

$$R_N=R_C-R_T$$

Where R_N was the transformed ratio and R_C was the ratio of the control group and R_T was the ratio of the treatment group.

Data analysis

Differences in accumulated ^{85}Sr and ^{137}Ba were compared using analysis of variance followed by Tukey's post-hoc test. Mean isotopic ratios from treated fish were compared against the mean ratios from control fish using an unpaired student's t-test with the Holm-Bonferroni correction for multiple comparisons with significance set at $\alpha=0.05$.

R_N values were plotted against the days post injection (DPI) and data were analysed using a exponential decay analysis on Graphpad Prism constraining the plateau value to 0 from the correction as no ratio should be higher than the natural or control value. Exponential decay is when the rate of decay is proportional to the amount of material remaining (i.e.: more material has higher rate of decay and vice versa.). The value was constrained to zero, as a zero would equate the control ratio value or a return to the natural state. From this output the half-life for each treatment and tissue was determined. To ensure the appropriateness of the one phase decay the data from $\ln[R_N]$ vs. DPI was fit with a linear line of best fit with linear regression. Using the natural logarithm of the isotopic ratio, half-life was calculated based on the slope of the line of best fit. All data were analysed in GraphPad Prism (GraphPad, La Jola, CA, USA).

Results

Strontium influx was determined as 3.02 $pmol.h^{-1}g^{-1}$ ± 0.364 $pmol.h^{-1}g^{-1}$ and efflux as 0.04 ± 0.007 $pmol.h^{-1}g^{-1}$ resulting in a net flux of 2.98 $pmol.h^{-1}g^{-1}$ (Fig. 2.1). Measureable values

of ^{85}Sr were obtained from all tissues sampled following immersion for six hours in radioisotopic strontium. Accumulation of ^{85}Sr in the fin ray was significantly higher than all other tissues sampled (Fig. 2.2). While there was a trend for an increased accumulation in all hard tissues sampled (fin ray, skull, vertebra) ^{85}Sr values for the skull and vertebra were not significantly different from all the soft tissues sampled.

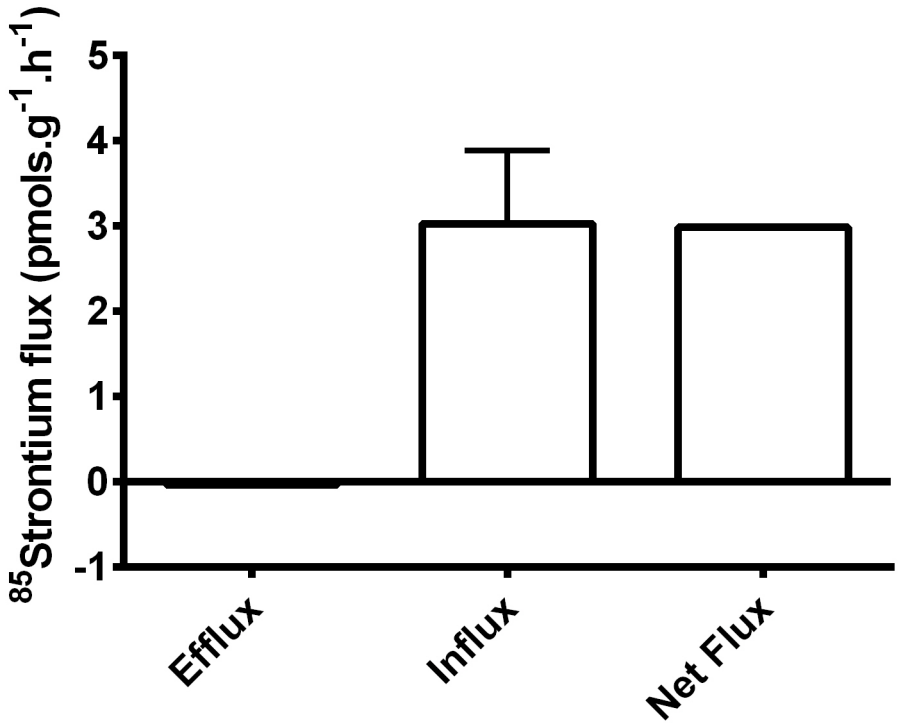

Figure 2.1. Whole-body ^{85}Sr influx, efflux and net flux in juvenile Lake Sturgeon, *Acipenser fulvescens*, at 15°C. Data are means ± 95% C.I. (Influx N=8; efflux N=5).

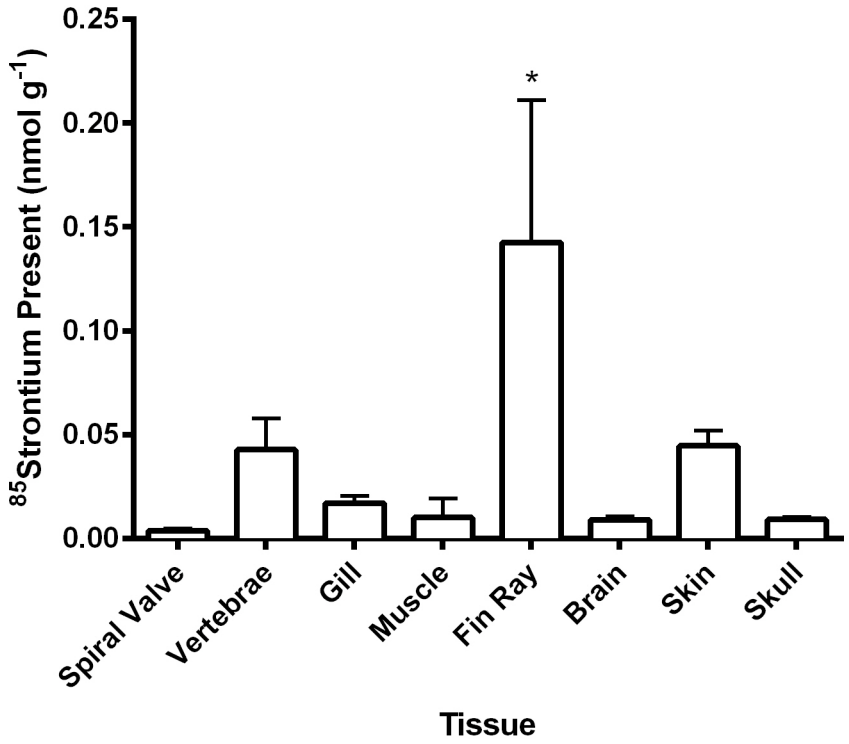

Figure 2.2. The ^{85}Sr present in selected tissues in juvenile Lake Sturgeon, *Acipenser fulvescens* after 6 hours immersion in 0.75 kBq.mL^{-1} at 15°C. Data are means ± 95% C.I. (N=8). Significant differences between tissues are indicated by an asterisk (*) (ANOVA, $P<0.05$, Tukey's post hoc test).

Isotopic ratios of ^{138}Ba/^{137}Ba in control fish did not differ between sampling time-points (3, 7 and 30 days post injection) regardless of tissue sampled. However, the isotopic ratio of ^{138}Ba/^{137}Ba was significantly lower in all tissues for all time points following injection of 2 mg.kg^{-1} ^{137}Ba (Figures 2.3, 2.4 & 2.5). Similar trends were observed following injection of 2 mg.kg^{-1} dose of ^{86}Sr, with the exception that the ^{88}Sr/^{86}Sr ratio in muscle was not significantly different from control fish 30 days after the injection (Figures 2.6, 2.7 & 2.8). Further the ^{88}Sr/^{86}Sr ratio in control fish was lower than the predicted natural ratio in muscle and vertebrae but similar to the natural ratio in the fin ray.

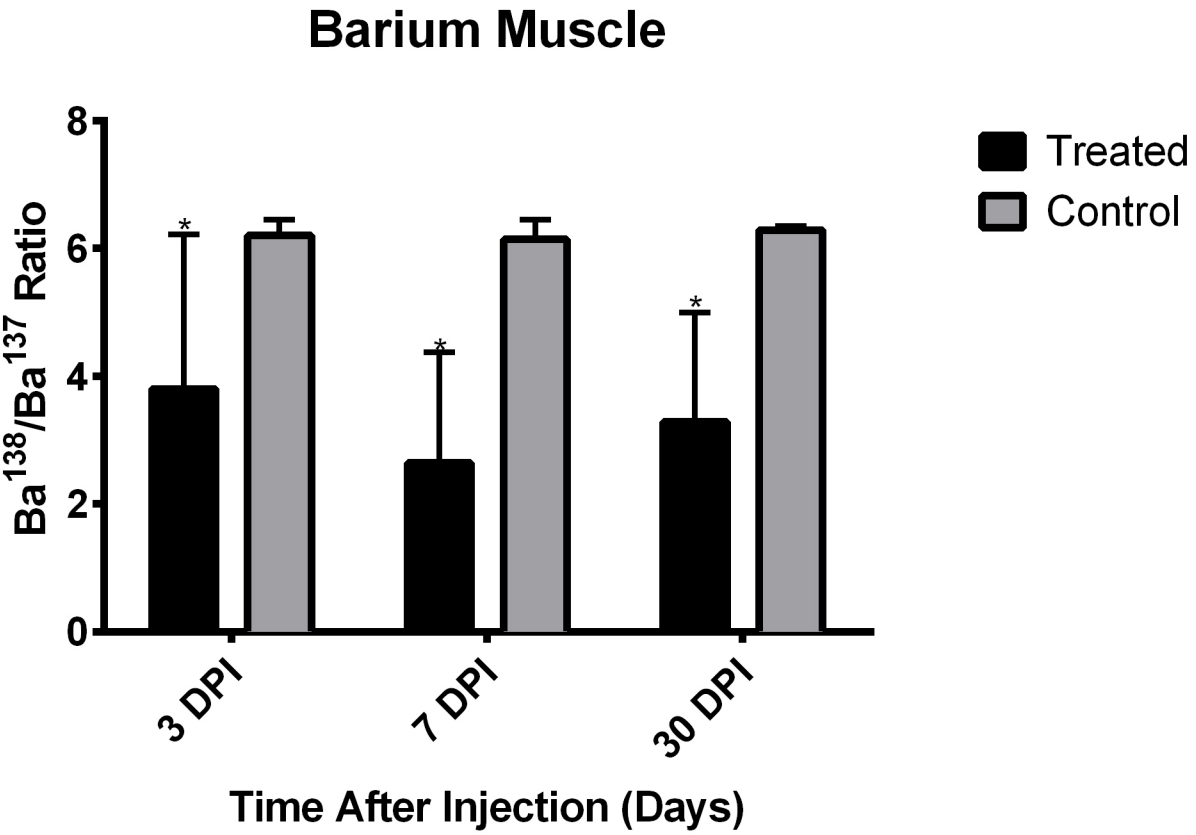

Figure 2.3. $^{138}Ba/^{137}Ba$ ratio in juvenile Lake Sturgeon muscle, *Acipenser fulvescens*, from fish injected with 2 $mg.kg^{-1}$ of ^{137}Ba in Lake Sturgeon Ringer's represented by black bars, or Lake Sturgeon Ringer's only (control) represented by grey bars. Tissue was removed at 3, 7 or 30 days post injection and digested in nitric acid and then hydrogen peroxide. $^{138}Ba/^{137}Ba$ ratio was determined from solution based inductively coupled plasma mass spectrometry on the resultant fluid digests. Data are means ± 95% C.I. (N=6). Significant differences between treatment and control groups are indicated by an asterisk (*) (Student's unpaired t-test, corrected for multiple comparisons using the Holm-Bonferroni method, $P<0.05$).

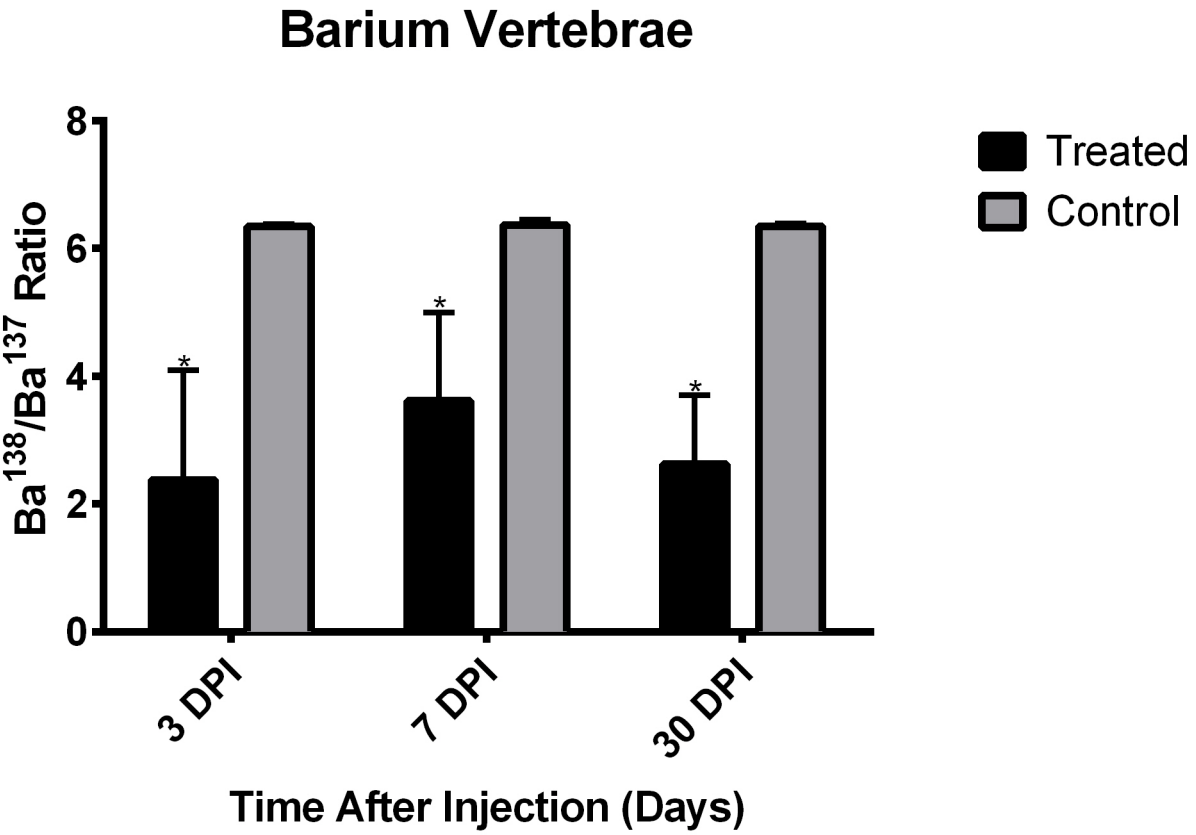

Figure 2.4. $^{138}Ba/^{137}Ba$ ratio in juvenile Lake Sturgeon vertebrae, *Acipenser fulvescens*, from fish injected with 2 $mg.kg^{-1}$ of ^{137}Ba in Lake Sturgeon Ringer's represented by black bars, or Lake Sturgeon Ringer's only (control) represented by grey bars. Tissue was removed at 3, 7 or 30 days post injection and digested in nitric acid and then hydrogen peroxide. $^{138}Ba/^{137}Ba$ ratio was determined from solution based inductively coupled plasma mass spectrometry on the resultant fluid digests. Data are means ± 95% C.I. (N=6). Significant differences between treatment and control groups are indicated by an asterisk (*) (Student's unpaired t-test, corrected for multiple comparisons using the Holm-Bonferroni method, $P<0.05$).

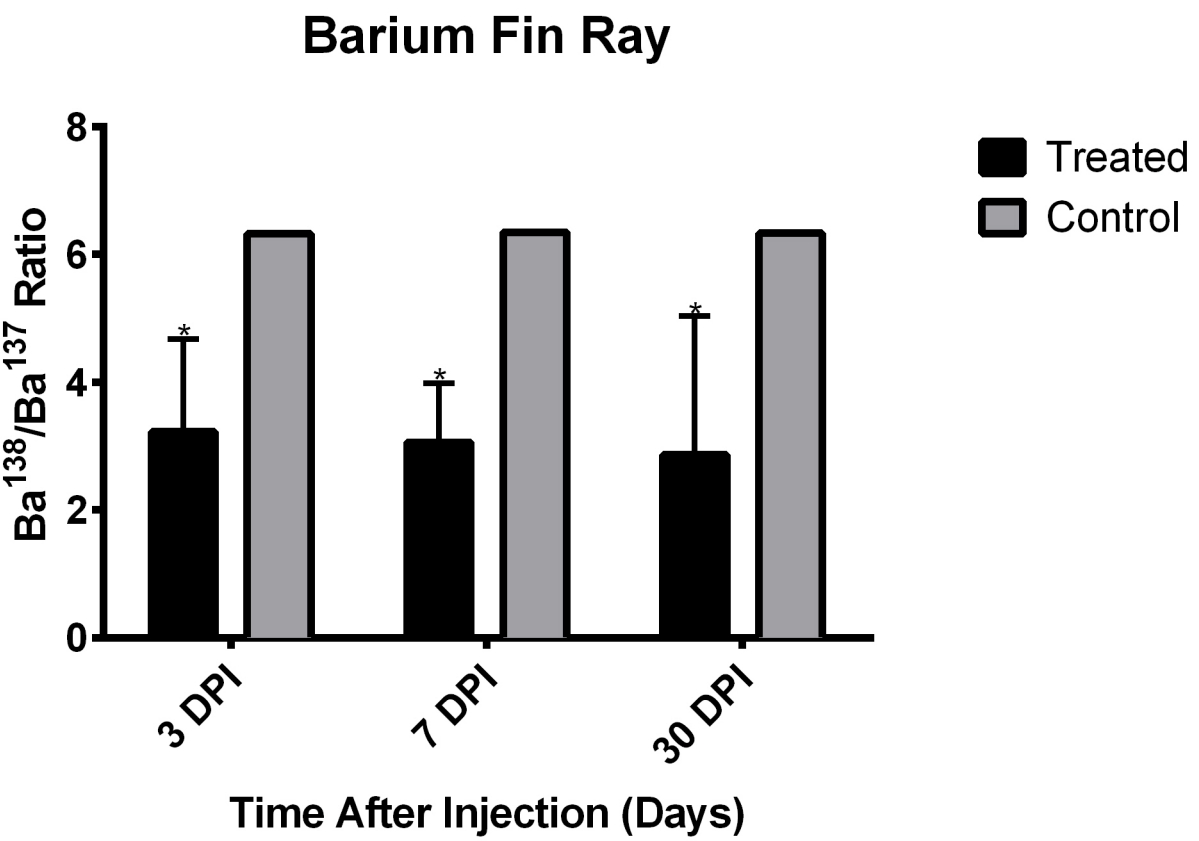

Figure 2.5. $^{138}Ba/^{137}Ba$ ratio in juvenile Lake Sturgeon fin ray, *Acipenser fulvescens*, from fish injected with 2 $mg.kg^{-1}$ of ^{137}Ba in Lake Sturgeon Ringer's represented by black bars, or Lake Sturgeon Ringer's only (control) represented by grey bars. Tissue was removed at 3, 7 or 30 days post injection and digested in nitric acid and then hydrogen peroxide. $^{138}Ba/^{137}Ba$ ratio was determined from solution based inductively coupled plasma mass spectrometry on the resultant fluid digests. Data are means ± 95% C.I. (N=6). Significant differences between treatment and control group are indicated by an asterisk (*) (Student's unpaired t-test, corrected for multiple comparisons using the Holm-Bonferroni method, $P<0.05$).

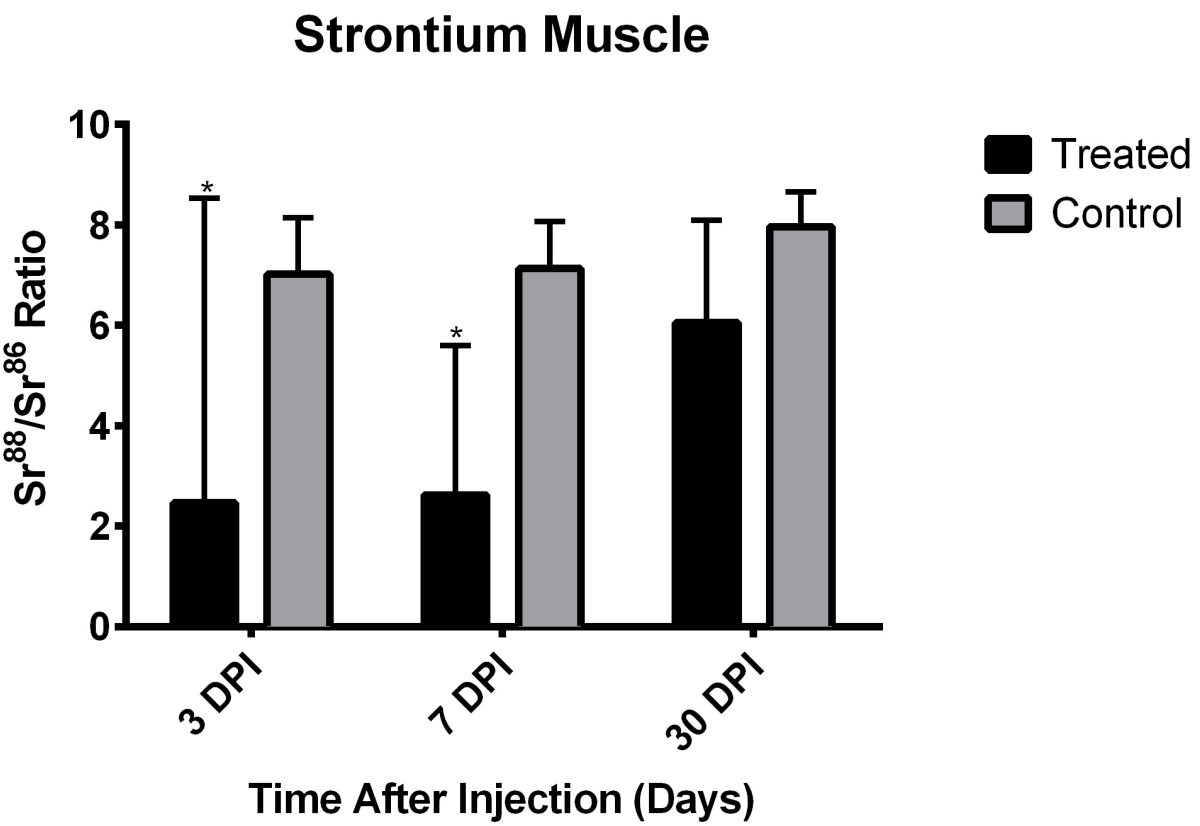

Figure 2.6. $^{88}Sr/^{86}Sr$ ratio in juvenile Lake Sturgeon muscle, *Acipenser fulvescens*, from fish injected with 2 $mg.kg^{-1}$ of ^{86}Sr in Lake Sturgeon Ringer's represented by black bars, or Lake Sturgeon Ringer's only (control) represented by grey bars. Tissue was removed at 3, 7 or 30 days post injection and digested in nitric acid and then hydrogen peroxide. $^{88}Sr/^{86}Sr$ ratio was determined from solution based inductively coupled plasma mass spectrometry on the resultant fluid digests. Data are means ± 95% C.I. (N=3-6). Significant differences between treatment and control group are indicated by an asterisk (*) (Student's unpaired t-test, corrected for multiple comparisons using the Holm-Bonferroni method, $P<0.05$).

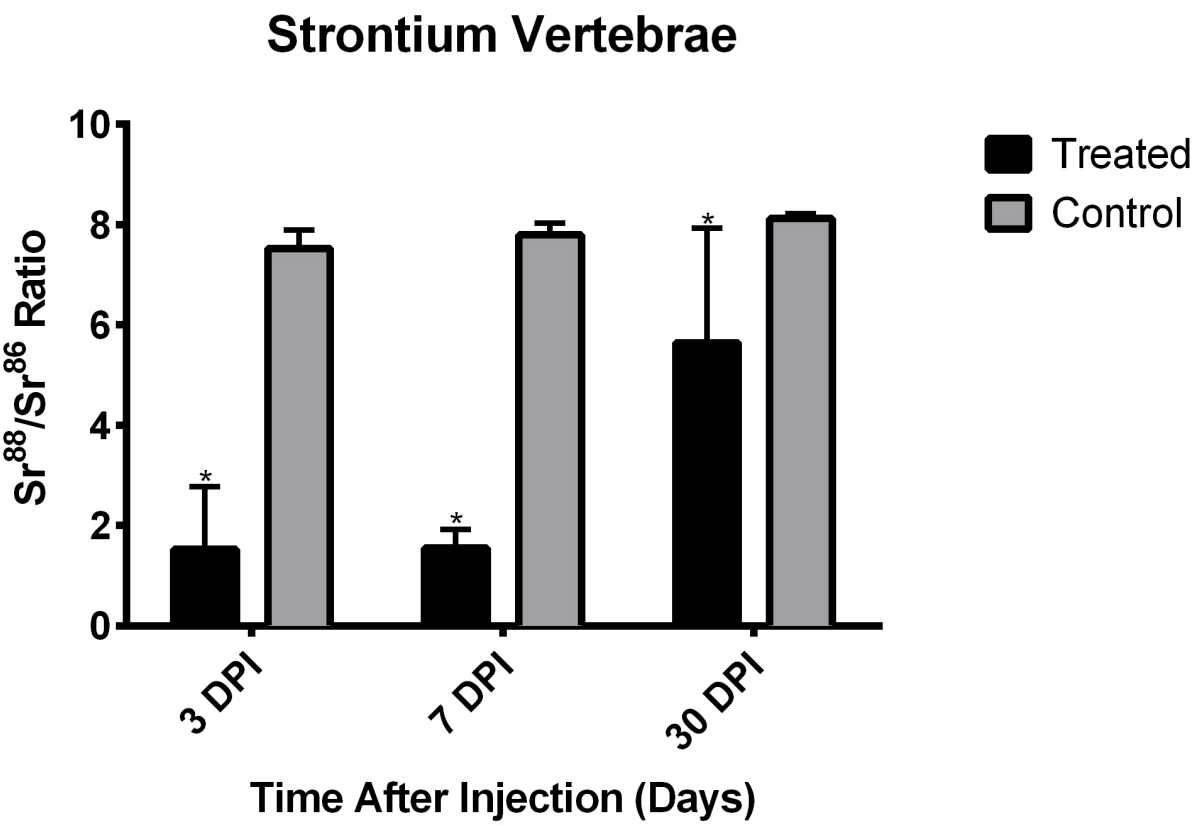

Figure 2.7. $^{88}Sr/^{86}Sr$ ratio in juvenile Lake Sturgeon vertebrae, *Acipenser fulvescens*, from fish injected with 2 $mg.kg^{-1}$ of ^{86}Sr in Lake Sturgeon Ringer's represented by black bars, or Lake Sturgeon Ringer's only (control) represented by grey bars. Tissue was removed at 3, 7 or 30 days post injection and digested in nitric acid and then hydrogen peroxide. $^{88}Sr/^{86}Sr$ ratio was determined from solution based inductively coupled plasma mass spectrometry on the resultant fluid digests. Data are means ± 95% C.I. (N=6). Significant differences between treatment and control group are indicated by an asterisk (*) (Student's unpaired t-test, corrected for multiple comparisons using the Holm-Bonferroni method, $P<0.05$).

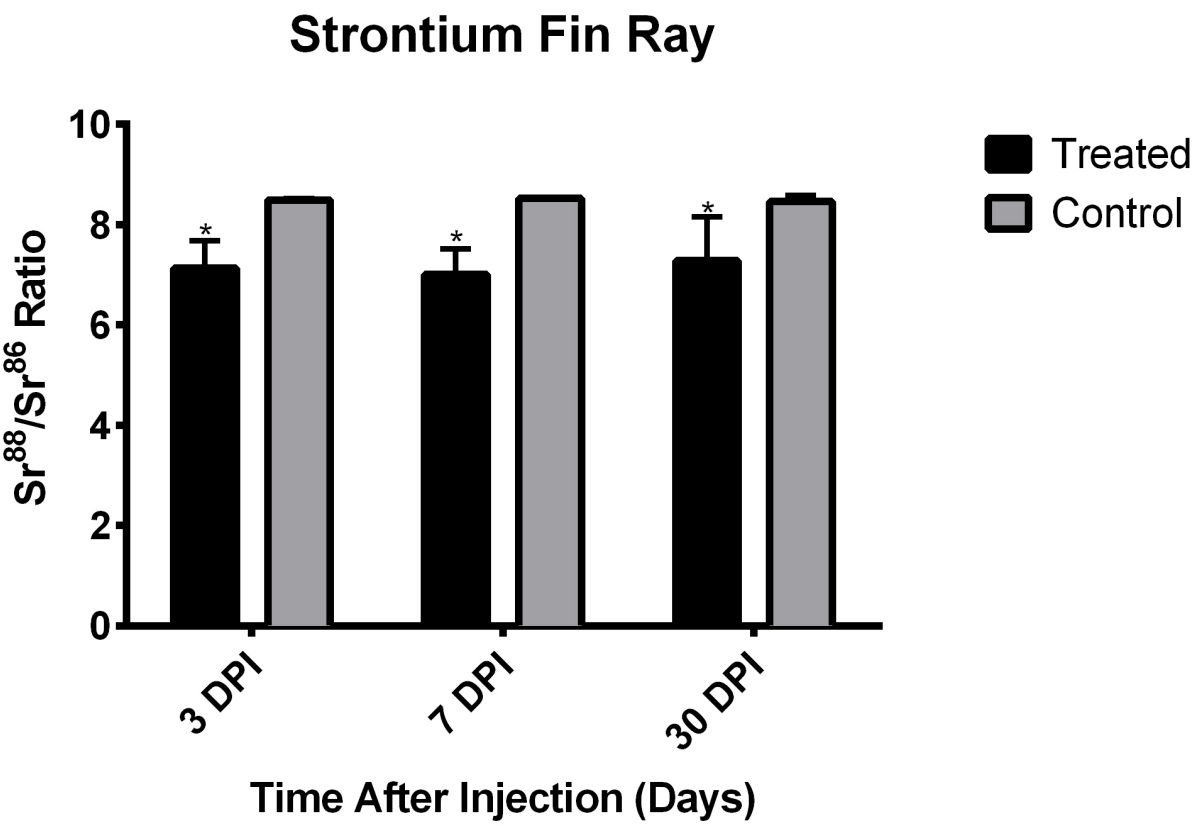

Figure 2.8. $^{88}Sr/^{86}Sr$ ratio in juvenile Lake Sturgeon fin ray, *Acipenser fulvescens*, from fish injected with 2 $mg.kg^{-1}$ of ^{86}Sr in Lake Sturgeon Ringer's represented by black bars, or Lake Sturgeon Ringer's only (control) represented by grey bars. Tissue was removed at 3, 7 or 30 days post injection and digested in nitric acid and then hydrogen peroxide. $^{88}Sr/^{86}Sr$ ratio was determined from solution based inductively coupled plasma mass spectrometry on the resultant fluid digests. Data are means ± 95% C.I. (N=6). Significant differences between treatment and control group are indicated by an asterisk (*) (Student's unpaired t-test, corrected for multiple comparisons using the Holm-Sidak method, $P<0.05$).

The observed biological half-life for ^{137}Ba in muscle, vertebrae and fin ray was determined to be 1.98×10^{12} days, 5.15×10^{13} days and 3.17×10^{15} days respectively. There was a strong negative relationship between time and concentration of ^{86}Sr in muscle ($r^2=0.9868$) and vertebrae ($r^2=0.9822$) (figs 2.9 & 2.10) and half-life of ^{86}Sr was determined to be 15.05 days in muscle, 18.00 days in vertebrae 121.9 days in fin ray.

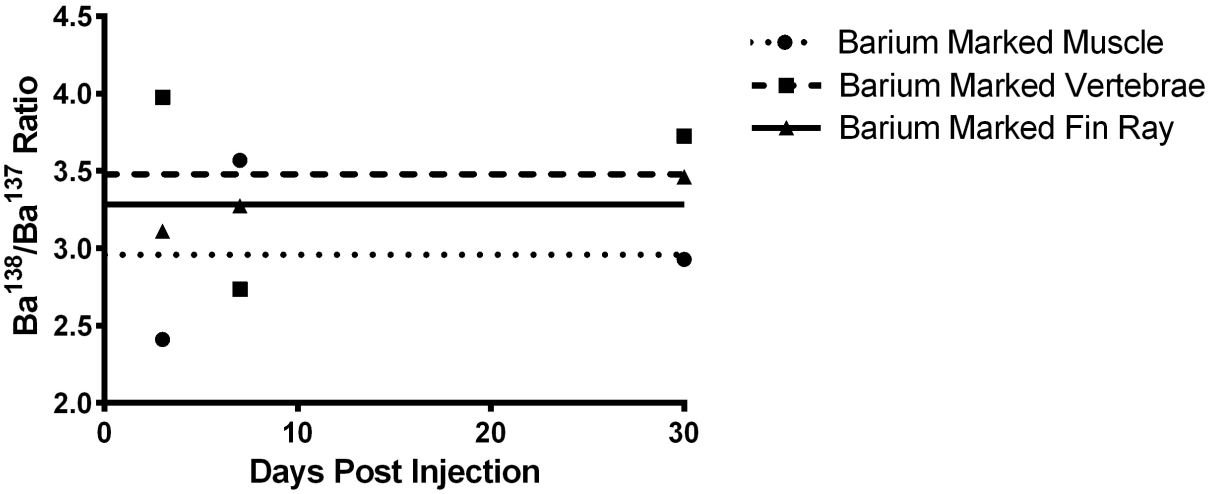

Figure 2.9. The relationship between the $^{138}Ba/^{137}Ba$ ratio and time after injection in juvenile Lake Sturgeon (*Acipenser fulvescens*) from fish injected with $2mg.kg^{-1}$ of ^{137}Ba in Lake Sturgeon Ringer's. Tissues analyzed were muscle: represented by circles, vertebrae: represented by squares, and fin ray: represented by triangles. Tissue was removed at 3, 7 or 30 days post injection and digested in nitric acid and then hydrogen peroxide. $^{138}Ba/^{137}Ba$ ratio was determined from solution based inductively coupled plasma mass spectrometry on the resultant fluid from digestions. $^{138}Ba/^{137}Ba$ ratio presented was transformed by subtracting the control ratio from the equivalent time point. Data are means (N=6) and curves fit by one phase decay analysis using GraphPad Prism.

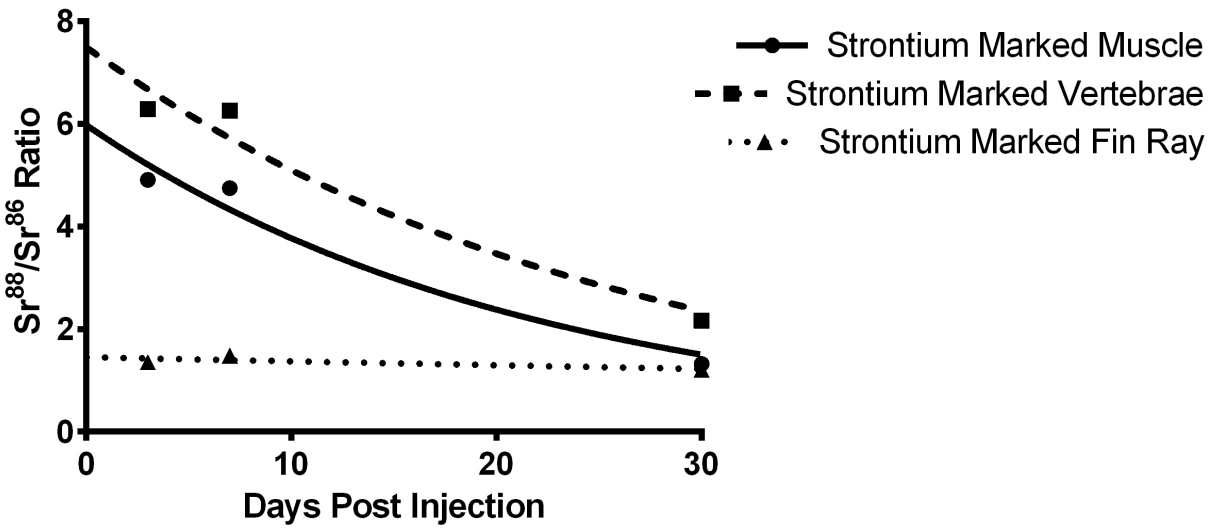

Figure 2.10. The relationship between the $^{88}Sr/^{86}Sr$ ratio and time after injection in juvenile Lake Sturgeon (*Acipenser fulvescens*) from fish injected with $2mg.kg^{-1}$ of ^{86}Sr in Lake Sturgeon Ringer's. Tissues analyzed were muscle: represented by circles, vertebrae: represented by squares, and fin ray: represented by triangles. Tissue was dissected out at 3, 7 or 30 days post injection and digested in nitric acid and then hydrogen peroxide. $^{88}Sr/^{86}Sr$ ratio was determined from solution based inductively coupled plasma mass spectrometry on the resultant fluid from digestions. $^{88}Sr/^{86}Sr$ ratio presented was transformed by subtracting the control ratio from the equivalent time point. Data are means (N=3-6) and curves fitted with one phase decay analysis with GraphPad Prism.

Discussion

The results of the present study provide novel baseline data for the metabolism of both barium and strontium in Lake Sturgeon and are to the authors' knowledge the first to examine movement and accumulation of these ions in a chondrostean. Stable isotopes have been used as a marking technique, in Lake Sturgeon and a number of other fish species either by immersion in the labeled water or transgenerationally by injection of the mother just prior to ovulation (Smith and Whitledge 2011; Walther and Thorrold 2006; Munro et al. 2008). However, few data are available regarding the metabolism of either barium or strontium in fishes.

The first assumption in stable isotope marking is that the fish takes up the isotope used to mark the fish. The present study clearly demonstrates a strong inward net flux of strontium, which was dominated by an influx that was approximately two orders of magnitude higher than

the measured efflux. It is important to note that the environmental conditions used in the present study were constant for both influx and efflux measurements, however previous research has demonstrated the effect of changing environmental variables such as calcium concentration, pH and temperature can significantly influence uptake rate of strontium in fishes (Chowdhury et al. 2000; Chowdhury and Blust 2001a; Chowdhury and Blust 2001b). It has previously been demonstrated that calcium flux rate in juvenile Lake Sturgeon is significantly impacted by environmental calcium concentration (Genz et al. 2013; Allen et al. 2011). Further Chowdhury et al. (2000) observed as environmental calcium concentrations increased V_{max} for strontium decreased. This shows that higher environmental calcium concentrations have a negative relationship with strontium influx, suggesting that increased environmental calcium may result in a less efficient uptake of strontium and therefore less effective marking of hard tissues. Clearly similar experiments as described by Chowdhury et al. (2000) are warranted in Lake Sturgeon to determine the validity of this technique in waters of higher calcium concentration.

As neither barium nor strontium is considered an essential metal it is presumed that these ions enter the fish via calcium transport mechanisms albeit at a lower rate. For example, in similarly aged juvenile Lake Sturgeon calcium influx was reported to be between 110-130 $nmol.h^{-1}.g^{-1}$ (Allen et al., 2011) at least five orders of magnitude higher than the strontium influx observed in the current study (3.02 ± 0.364 $pmol.h^{-1}.g^{-1}$). The efflux rate for strontium ($0.04 \text{ x} \pm 0.007$ $pmols.h^{-1}.g^{-1}$) was also at least five orders of magnitude lower than the previously reported calcium efflux rate (9-35 $nmol.h^{-1}.g^{-1}$) in Lake Sturgeon (Allen et al. 2011). However despite the large differences in magnitude between the influx and efflux of calcium and strontium, they both are strongly inward. The differences in rate are likely the result of concentrations of the two ions with the concentration of calcium being at least two orders of magnitude higher in the

environment and even more so in the internal body fluids. However, differences in physiological requirement of the two ions will also explain the observed differences in flux rates. Kinetic properties of the calcium transport proteins that are related to the substrate (calcium or strontium) are not understood in Lake Sturgeon but such studies would be useful in describing the selectivity of calcium transport proteins for alternative alkaline earth metals such as strontium and barium. Chowdhury et al. (2000) reported a K_M for calcium of 28.5 μM in the common carp, *Cyprinus carpio*, which was more than three times lower than the K_M for strontium (96 μM) suggesting that the affinity for calcium uptake is approximately three times greater than strontium in the carp.

Interestingly, strontium flux rates in Lake Sturgeon are much lower than those found in the common carp, which was observed to have a net flux of strontium of approximately 25 $nmol.g^{-1}.h^{-1}$ under similar environmental conditions (Chowdhury et al. 2000). This is approximately four orders of magnitude higher than the strontium flux observed in the current study and more recently strontium flux rates reported for Common Carp (2.74 $\mu mol.g^{-1}.h^{-1}$); Silver Carp, *Hypophthalmichthys molitrix* (1.26 $\mu mol.g^{-1}.h^{-1}$); Bighead Carp, *Hypophthalmichthys nobilis* (1.47 $\mu mol.g^{-1}.h^{-1}$); Grass Carp, *Ctenopharyngodon idella* (1.47 $\mu mol.g^{-1}.h^{-1}$); Prussian carp, *Carassius gibelio* (1.79 $\mu mol.g^{-1}.h^{-1}$); European perch, *Perca fluviatilis* (0.74 $\mu mol.g^{-1}.h^{-1}$) and Northern Pike, *Esox lucius* (0.95 $\mu mol.g^{-1}.h^{-1}$) (Belyayev et al. 2011) were all in the micromolar range, again substantially higher than those estimated in the present study for Lake Sturgeon. The increased influx rates reported for the teleost fish in previous studies is likely due to the differences in calcium metabolism between teleost and chondrostean fishes, for instance chondrosteans have one of the lowest reported circulating levels of calcium among the fishes. Furthermore, differences in basal metabolic rate between the

Lake Sturgeon in this and previous studies may also influence flux rates. These observed differences between species in strontium flux rates highlight the need for empirical physiological studies of alkaline earth metal metabolism in fishes if they are to be used as indicators of fish movement or life history in the wild.

The present study demonstrated rapid preferential accumulation of strontium in hard tissue especially the fin ray after exposure to ^{85}Sr and ^{86}Sr. These observations are novel in chondrosteans, however, are well supported by similar studies on teleosts. In the Toadfish, *Opsanus tau*, uptake of ^{45}Ca was initially fastest in the vertebrae compared to the jaw but uptake rate stabilised between the two tissues over time (Simmons et al. 1970). Preferential uptake of ^{90}Sr and ^{45}Ca was also shown in *Lesbistes sp.* where the skull had relatively greater accumulation of ^{90}Sr and ^{45}Ca compared to the viscera and muscle after exposure to both radioisotopes for 5 and 10 days (Rosenthal, 1957). The accumulation of strontium in calcium rich tissue was further confirmed by Martin and Goldberg (1962) who report that after 256 days 95% of ^{90}Sr was eliminated from the Pacific mackerel, *Scomber australasicus*, however 80% of the remaining ^{90}Sr was found in calcareous tissues. In the freshwater Brown Bullhead, *Ameiurus nebulosus*, and Northern Pike 95% of strontium was accumulated in hard tissues (Yankovich 2009) and strontium accumulation has been shown to be highest in the hard tissues of the Common Carp (Chowdhury and Blust 2012).

^{86}Sr and ^{137}Ba half-lives were determined for muscle, vertebrae and fin ray. As predicted muscle tissue had the shortest half-life (16.9 days for ^{86}Sr) indicative of a fast turnover rate for metabolically active tissue, whereas fin ray had the longest (131.5 days for ^{86}Sr) indicative of a slower turnover in metabolically less active tissue. Interestingly the ^{88}Sr/^{86}Sr ratio in muscle and vertebrae of control fish was lower than the predicted natural ratio and in the fin ray was similar

to the predicted natural ratio of the Sr isotopes. This may be the result of mass dependent fractionation of isotopes of strontium in tissues of different density, however, further research is required to support this notion. Comparison of calculated half-lives between ^{86}Sr and ^{137}Ba revealed that the half-life of ^{86}Sr was consistently and substantially lower than the half-life for ^{137}Ba in all tissues examined ($>10^{12}$ days for ^{137}Ba). This difference in retention of the two isotopes was unexpected and is at present difficult to explain particularly given the assumption that both barium and strontium utilize calcium transport mechanisms and cellular processes in a similar way and neither are considered essential elements. One possible explanation of the difference in biological half-life between strontium and barium in tissue maybe due to a higher affinity for calcium and strontium in comparison to barium by the cellular uptake and excretion methods. The same concentration of barium and strontium were used in the immersion trials described in chapter 3, however, the amount of barium detected in the fin rays was consistently lower than the amount of strontium in marked areas (B. Carriere, unpublished). It is unknown if this is proof of an underlying affinity or a different phenomena, however, it may explain the differences in the biological half-life. There is some evidence to support this hypothesis, as Campana (1999) estimates that 25% of barium from the endolymph fluid is crystalized into the otolith, whereas 50% of strontium from the endolymph fluid is crystalized into the otolith. This suggests a preferential deposition of these two ions, however the mechanism is unclear. Vennekens et al. (2000) has observed that barium and strontium have similar permeability through ECaC in human kidney cells. However it is unknown if Lake Sturgeon ECaC behaves in the same way as human kidney ECaC. Further research should focus on the differential permeability of ions that use the calcium uptake mechanism, including barium, strontium and magnesium and if it affects the biological half-life of the ions in tissue.

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Chapter 3: Marking Study

Abstract

To ensure success of fisheries conservation efforts appropriate and effective monitoring procedures assessing the success or failure of stock enhancement are essential. Use of stable isotopes to mark individual or groups of fish relies on the uptake of the isotope from the water and subsequent deposition of this isotope in hard structures such as the otolith or fin ray. In this study the effectiveness of using the rare but stable isotopes of barium (^{137}Ba) and strontium (^{86}Sr) to isotopically mark the fin ray of Lake Sturgeon by transgenerational or immersion techniques was assessed. Marking success was dependent on isotope used and marking technique. Immersion of juvenile Lake Sturgeon in enriched concentrations of ^{137}barium (^{137}Ba) led to an immediate marking success of 93% whereas immersion of a similar age class in enriched concentrations of ^{86}Strontium (^{86}Sr) led to an immediate marking success of 91%. Transgenerational marking was ineffective for ^{137}Ba in the present study and only 33% successful for ^{86}Sr. Timing and length of immersion in either ^{137}Ba or ^{86}Sr affected the marking success where improved marking success was observed in fish 100 days post fertilization (dpf) compared with 12 dpf. Finally, marking success was positively correlated to length of immersion in either ^{137}Ba or ^{86}Sr for 100 dpf fish, with the most persistent and successful isotopic mark being in 100dpf fish immersed in ^{86}Sr for 10 days.

Introduction

Isotopic marking

Isotopic marking is a relatively recent technique where the ratio of elemental isotopes can be used to infer life history traits or identify groups of fish from either a geographically or environmentally distinct location. Isotopes are atoms of the same element but vary in the number

of neutrons in their nucleus. The difference in neutrons causes the atoms to have different atomic masses, however, chemically these isotopes behave similarly. Internal concentrations of isotopes of several elements deposited in aragonite crystal and bone of fish have been shown to correlate with environmental concentrations of their respective isotopes (Dregens et al. 1969). The presence of these isotopes can be in low concentrations in the environment so specialized and emerging techniques such as microchemical analysis of the material are needed. This involves the analysis of trace amounts of elements in the biological material and a number of techniques have been used, with laser ablation inductively coupled plasma mass spectrometry (LA-ICP MS) receiving considerable attention in recent years (Walther and Limburg 2012; Boehler et al. 2012; Friedrich and Halden 2011; Reguir et al. 2010).

There are two key aspects required so that inferences can be made between elemental deposition and life history traits of fish. The first is that the biological material being analysed needs to have a solid matrix so that material from these matrices can be ablated in a predictable and reliable way. In fish, microchemical analysis has been conducted on otoliths, vertebrae, scales and fin rays (Swanson et al. 2010; Tillet et al. 2011; Adey et al. 2009; Smith and Whitledge 2011). The second is that the turnover time for elements, once laid down in the chosen structure, is relatively low. Absorption of all of the above structures can occur to a greater or lesser extent and may be species specific. To date there is no evidence that the aragonite crystal matrix in otoliths is resorbed therefore otoliths have been shown to act as stable indicators throughout the life of the fish (Campana et al. 1995). However scales, vertebrae and fin rays may be subject to reabsorption during the lifetime of the fish, which can cause the loss of the trace elements laid down in the structure. The possibility of the loss of trace elements means that scales, vertebrae and fin rays are more variable in their ability to act as an indicator when

compared to otoliths. That said each of the above structures has been used to infer life history traits in fish. Another aspect to consider is the loss of these bony elements. Many fish species have deciduous scales, or they lose and regrow their scales at least once in their lifetime. Also chance events like predatory attempts may cause loss in scales as well as fin rays. A further review of the use of microchemical analysis on these structures is required to determine the appropriateness of these techniques.

Otolith Microchemistry

Otolith microchemistry serves as an excellent way to determine life history through examination of the ratios of elements or isotopes present in the otolith (Sturrock et al. 2012; Skalski et al. 2009; Thorrold et al. 2006). Otolith microchemistry does not require fish to exceed a particular size or age, although one major disadvantage is that removal of the otolith is a terminal procedure (Thorrold et al. 2006). However, a distinct advantage is that the otolith is permanent and is not reabsorbed and the deposition of the aragonite crystal in the otolith matrix is seasonal (Dregens et al. 1969) such that ring structures or annuli provide us with the opportunity to age the fish and therefore allow for reasonably accurate determination of when and what type of an environment the fish may have inhabited.

Otolith formation is quite different than bone or scale growth, the otolith is bathed in endolymph fluid and is not in direct contact with epithelial tissue (Payan et al. 2004). Growth of the otolith is a regulated process where elements that are assimilated into the otolith must pass from the water across the branchial epithelia into the blood and then across the ear membrane and into the endolymph fluid and finally deposited onto the otolith. Because of the multiple steps across several epithelia, elements can be actively taken up, actively removed from a compartment or become trapped in a compartment and therefore the organism will have some

level of control over which elements are ultimately deposited in the otolith (Weiner 2008; Payan et al. 2004). It was originally thought that the incorporation of elements into the otolith was directly related to their abundance and availability in the environment (Sturrock et al. 2012). However, due to the mechanism of otolith growth this is not necessarily the case for the majority of the elements thus far examined (Sturrock et al. 2012). One possible reason for this is that an element may not be functionally relevant to the organism and is therefore actively removed before it can be incorporated. Further, many elements may be bound to blood proteins or are actively taken up by other tissues or organs prior to being taken into the otolith. Finally, basic osmoregulatory and iono-regulatory principles dictate that ions are actively taken up from the environment or excreted into the environment and as such concentration gradients exist between the internal and external environments and these gradients will change if for example a fish moves between environments such as anadromous fish moving from saline to fresh water or vice versa. In essence deposition of elements in hard structures of fish is a dynamic and regulated process that is not fully understood, however, strong inferences on life history can be predicted depending on the choice of element.

Sturrock et al. (2012) reported that to date there are 50 known elements in the otolith of fishes. However, only seven of the 50 are used routinely to provide an indication of life history traits of a number of different species. These elements are manganese (Mn), magnesium (Mg), copper (Cu), lithium (Li), zinc (Zn), Sr and Ba (Sturrock et al. 2012). It is now reasonably well accepted that Sr:Ca ratios in otoliths are a strong indication of the type of salinity a fish may have resided in, thus allowing for the determination of migration patterns in anadromous or catadromous fishes. A high Sr:Ca ratio in the otolith is indicative of residence in saline waters

whereas a low ratio suggests freshwater residence (Walther and Thorrold 2010; Panfili et al. 2012).

In addition to Sr, Ba has also been shown to have a more direct relationship between otolith concentration and the environment. Walther and Thorrold (2006) found that 83 % and 98 % of deposited Ba and Sr respectively came from the environment in which the organism is found in. As a consequence manipulation of environmental Sr and Ba is strongly reflected in the Sr and Ba signatures of the otolith. Therefore, introduction of rare stable isotopes of these two elements into the environmental medium may result in a predictable increase in the ratio of the rare and common isotope in the otolith and this has been demonstrated in Murray cod, *Maccullochella peelii,* otolith and Lake Sturgeon fin rays through uptake of the Ba and Sr in the medium for eggs, embryos or larval fish dependent on the technique used (Brown and Harris 1995; Smith and Whitledge 2011; Woodcock et al. 2011).

Pectoral Fin Ray Microchemistry

Pectoral fin ray microchemistry is much like otolith microchemistry, where concentrations of elements in the pectoral fin ray can provide information on the life history of the fish. However, unlike otolith microchemistry, pectoral fin ray microchemistry does not require the sacrifice of the animal. Clarke et al. (2007) found in the Arctic Grayling, *Thymallus arcticus,* that both the otolith and pectoral fin rays showed a correlation between the trace element concentrations in the environment and their concentrations in the otolith and pectoral fin ray. However, unlike the otolith, the fin ray is potentially prone to resorption, particularly during times of physiological challenges, such as calcium stress in females during vitellogenesis (Campana 1999).

Despite the possibility of resorption Veinott and Evans (1999) found that concentrations of ^{86}Sr in pectoral fin rays of white sturgeon, *Acipenser transmontanus,* did not change significantly over the span of two to six years in the wild. If pectoral fin ray elemental concentration stays relatively constant and incorporates trace elements in relation to their availability in the environment this would be an excellent alternative to otolith microchemistry as it has all the benefits of otolith microchemistry but allows users to conduct non-lethal sampling. This is beneficial to fisheries management programs, particularly when the species of interest is endangered or threatened, such as the Lake Sturgeon. Indeed, it has been shown that in Lake Sturgeon this technique is valid if the larvae are immersed in ^{86}Sr (Smith and Whitledge 2011). There is also evidence that confirms that this technique is effective using stable isotopes of both barium and strontium in immersion and transgenerational marking of teleost fish (Woodcock et al. 2011). Because of these advantages of stable isotope marking it was selected for evaluation in this study to determine its efficacy in Lake Sturgeon.

Marking methods

Typically the ratio between different isotopes is fixed in the natural aquatic environment, however, in a controlled setting, isotopic ratios can be manipulated through addition of the rare isotope to the water causing a significant deviation from natural isotopic ratios, producing ratios that can be used to uniquely mark the fish. This enriched stable isotopic marking technique has been demonstrated in a number of different species of fish Lake Sturgeon, Cinnamon Clownfish, Black Sea Bass, Coral Reef Grouper, Golden Perch and Murray Cod (Smith and Whitledge 2011; Thorrold et al. 2006; Williamson et al. 2009; Munro et al. 2009; Munro et al. 2008; Woodcock et al. 2011) either through immersion of the fish in the isotope or injection of the mother with the stable isotope just prior to spawning, known as transgenerational marking

(Smith and Whitledge 2011; Thorrold et al. 2006; Williamson et al. 2009; Munro et al. 2009; Munro et al. 2008; Woodcock et al. 2011)

Transgenerational marking

It is important to note the majority of these studies were conducted on the otolith of bony fishes and not the pectoral fin ray. However, available evidence suggests that these results would be consistent with the pectoral fin ray. The most common isotope of Ba is ^{138}Ba and the rare stable isotope most frequently used is ^{137}Ba. Thorrold et al. (2006) demonstrated that injection of an enriched ^{137}Ba isotope into a gravid female resulted in a novel Ba:Ca ratio in the otolith core of the progeny in Cinnamon Clownfish, *Amphiprion melanopus,* and the Black Sea Bass, *Centropristis striata*. Thorrold et al. (2006) also demonstrated that concentrations as low as 0.45 $\mu g.g^{-1}$ of ^{137}Ba were sufficient to produce significantly different Ba isotope ratios in the progeny. Building on the research by Thorrold et al. (2006), Williamson et al. (2009) investigated the possible effects on physiology, fertilization success and mortality of offspring in gravid Coral Reef Groupers, *Plectropomus leopardus*, when injected with an enriched $^{137}BaCO_3$, either 2 $mg.kg^{-1}$ or 4 $mg.kg^{-1}$ solution and no negative effects were found. Furthermore, no significant risk to organisms that ingested the marked fish were indicated as the concentrations in muscle and fat tissue were found to be lower than the acceptable levels determined by the U.S. Department of Health and Human Services (Williamson et al. 2009; ASTDR 2005). For transgenerational marking using enriched stable isotopes, timing of the administration appears to be critical to the success of marking the progeny. In the Golden Perch, *Macquaria ambigua*, females injected with ^{137}Ba at a dose of 20 $mg.g^{-1}$ of body mass just prior to administration of gonadotrophic releasing hormone (GnRH) (for induction of spawning) did not influence the $^{138}Ba/^{137}Ba$ ratio in the otolith of the progeny. However, administration of 20 $\mu g.g^{-1}$ of ^{137}Ba at

24h and 21 days prior to the induction of spawning produced a significantly lower $^{138}Ba/^{137}Ba$ ratio (Munro et al. 2009).

The amount of enriched stable isotope administered has also been shown to influence marking success of progeny (Munro et al. 2009). A dose of 40 $\mu g.g^{-1}$ of ^{137}Ba produced a significantly lower $^{138}Ba/^{137}Ba$ ratio when the fish were injected at 21 days, 24 hours and simultaneously during the induction of ovulation in the Golden Perch (Munro et al. 2009).

Immersion

There is also evidence that immersion in enriched $^{137}BaCO_3$ produces a significantly lower $^{138}Ba/^{137}Ba$ ratio. Munro et al. (2008) determined an optimal length and concentration for immersion in the Golden Perch and found 100% marking success when fish were immersed in 15 $\mu g.L^{-1}$ ^{137}Ba for 8 days. Woodcock et al. (2011) found that immersion in several different solutions each with a different enriched Ba isotope produced a significantly lower ratio of natural to rare Ba. The effects of immersion on the Ba isotopic signature in the otolith were observed after just one day of immersion in 90 $\mu g.L^{-1}$, however after 5 days the ratio decreased no further and immersion for longer than 5 days was deemed unnecessary (Woodcock et al. 2011).

Woodcock et al. (2011) found that immersion for 6 days in 250 $\mu g.L^{-1}$ of ^{88}Sr produced a significantly lower ^{86}Sr: ^{88}Sr ratio in the otolith of Murray Cod. Smith and Whitledge (2011) immersed juvenile Lake Sturgeon, mean total length 162.5 ± 0.89 mm, in one of 25, 50 or 100 $\mu g.L^{-1}$ enriched $^{86}SrCO_3$ solution for 10 days or 24 days and they found that there was a decrease in the ^{86}Sr:^{88}Sr ratio in the pectoral fin ray in all three concentration levels tested for both the 10 and 24 day immersions and the decreased ratios remained present for at least 120 days post immersion (Smith and Whitledge 2011). However, it is important to note that the concentration of enriched isotope influences marking success with the 25 $\mu g.L^{-1}$ treatment and the 50 $\mu g.L^{-1}$

treatment groups producing only 25-58% and 33-69% marking success respectively and the 100μg. L^{-1} treatment group producing 83-92% marking success in juvenile Lake Sturgeon (Smith and Whitledge 2011).

Previous Marking Studies on Lake Sturgeon

There have been many studies on Lake Sturgeon that have used different tracking and tagging devices including radio and acoustic transmitters (Auer 1999, Barth et al. 2009, Benson 2005), genetic markers (McQuown et al. 2003), PIT tags and CWTs (Barth et al. 2009; Barth et al. 2011; U.S. Fish and Wildlife Service 2005). However due to the described limitations of these techniques the present study examines the validity of using enriched stable isotopes to batch mark hatchery-reared fish. To the authors knowledge there is a single published account of marking Lake Sturgeon using enriched stable isotopes (Smith and Whitledge 2011). This study will expand on the work conducted by Smith and Whitledge (2011) by comparing the efficacy of ^{86}Sr in addition to ^{137}Ba as an isotopic mark on the fin ray and testing the transgenerational marking technique alongside the immersion technique. Further this study will assess if immersion time impacts on marking success and if the timing of the immersion affects marking success measured as numbers of marked fish in combination with the persistence of the mark in the fish.

Determining the efficiency and most cost-effective way of applying the isotopic marks will allow for improved use of the technique as it holds great promise as a tool for monitoring the success or failure of conservation efforts such as stock enhancement. I predict that the stable isotope marking technique in Lake Sturgeon will be viable, however, success will be variable with technique, timing and isotope all influencing success in regard to confidence of marking each fish and persistence of isotopic mark in the fin ray.

Materials and Methods

Gamete collection and transgenerational marking

Spawning adult Lake Sturgeon were captured using gill nets set overnight at the downstream side of Pointe du Bois generating station (est. 1909, 50º17'52N, 95º32'51W) in 2012 and 2013 on the Winnipeg River, Manitoba, Canada. Following capture, fish were either transported to the University of Manitoba animal holding facility in Winnipeg, Manitoba, Canada (2012) or maintained river-side in a large 2000L holding tank fed by a constant supply of river water using submersible pumps (2013). In both years spawning was induced in male and female fish following administration of gonadotropin releasing hormone (GnRH) in two doses (10 $mg.kg^{-1}$ followed by 5 $mg.kg^{-1}$) with the second dose being administered 24 hours prior to manually stripping eggs from the fish. In 2013 at the time of the second dose of GnRH two gravid females were randomly selected, one was injected intraperitoneally with a 2 $mg.kg^{-1}$ dose of enriched $^{137}BaCO_3$ solution and the other was injected intraperitoneally with a 2 $mg.kg^{-1}$ dose of enriched $^{88}SrCO_3$ solution for transgenerational marking of the progeny. Eggs were then collected and mixed with sperm from donor males for fertilisation. In 2013 eggs and sperm were collected riverside at Pointe du Bois and transported back to the animal holding facility at the University of Manitoba where the eggs were fertilized. In 2012 as the spawning adults were held in the animal holding facility egg and sperm collection and subsequent fertilisation occurred in the facility. Presumed fertilised eggs were then transferred to hatching jars for incubation in flow through aquaria with dechlorinated City of Winnipeg tap water maintained at 12 ± 1°C and a 16 hour light: 8 hour dark schedule for both the 2012 and 2013 cohorts. A similar procedure was performed for fertilised eggs collected from females that were not injected with either enriched isotope for both years. These progeny were then used further for immersion studies or as

experimental controls. Developing embryos were sorted daily and dead eggs or debris removed as needed, amount of dead eggs removed were recorded to determine fertilization and hatching success. Hatching began around 7-10 days post fertilization (dpf) in both years and further cleaning was conducted to remove egg shells and unfertilised or dead eggs. Live larvae were then transferred to small holding tanks for on-growing.

Larvae typically begin actively feeding approximately 17 dpf, and fish in all experimental and control tanks were fed a diet of *Artemia* sp. (San Francisco Bay Brand, Newark, CA, USA) *ad libitum* 3 times a day beginning at 17 dpf. During feeding water flow and lighting was turned off for 1 hour to optimise feeding opportunities for the larvae. Following each feeding event any remaining *Artemia* and larval mortalities were removed by siphon and recorded. At 46 days post-fertilization larvae were typically large enough to feed on a mix of *Artemia* and bloodworm (family Chronomidae) (Sally's Bloodworm). Throughout subsequent weeks the proportion of diet composed of bloodworm was increased then commercial trout chow (Silvercup Starter Crumble, Martin Mills, Elma, ON, Canada) was introduced as the larvae grow until the fish were on a trout chow only diet which was typically 8-9 months post fertilisation.

Determination of stable isotope persistence and the effects of timing on marking success

To examine the effect of immersion timing on marking success two random samples of 200 larvae that had not been previously exposed to enriched stable isotopes were transferred to 10 L re-circulating aquaria spiked with 100 $\mu g.L^{-1}$ of $^{86}SrCO_3$ or $^{137}BaCO_3$ at either twelve dpf or one hundred dpf. These fish were maintained in a similar light pattern as described, however, due to the static design 50% water changes were conducted on a daily basis in the fish immersed at 12 dpf and 90% daily water changes in the fish immersed at 100dpf. Tanks were monitored twice daily and any mortalities were removed when they were observed. At both time-points fish were

immersed for 8 days in the enriched isotopes and then transferred to flow through aquaria maintained at 12 ± 1°C and a 16-hour light: 8 hour dark schedule. Fin rays were sampled at 144, 511 and 650 dpf for the experimental and control fish. The initial sampling point was used to determine the validity of the stable isotope marking technique and second and third sampling points were used to determine the persistence of the mark.

Effect of immersion duration on mark retention and persistence

Initial analysis of the 2012 cohort indicated marking success was most successful in 100 dpf immersion groups. Thus an older age class was used in the immersion study for the 2013 cohort and fish were exposed at 185 dpf. In this experiment juvenile Lake Sturgeon were obtained from Grand Rapids Fish Hatchery that were the progeny of adults captured in Nelson River tributaries. Three groups of 30 fish were separated into three 60-gallon tanks at 185 dpf. Tanks were filled with either 100 $mg.L^{-1}$ of $^{137}BaCO_3$ or 100 mg L^{-1} $^{86}SrCO_3$ or dechlorinated city of Winnipeg tap water as controls. Fish were held under similar conditions as the 2012 cohort with the exception that following 3, 7 and 10 days immersion in the isotopes 10 fish from each treatment were removed and placed into a flow through aquaria held at 15°C with 16h:8h light:dark light regime. Fin rays were removed from all these experimental fish at 334 dpf, or 149 days post marking to determine marking success.

Fin Ray Sampling and Analysis:

Fish in all groups were first lightly anaesthetised through immersion in 50 $mg.L^{-1}$ tricaine methanesulfonate (MS-222). Once equilibrium was lost iris scissors were used to carefully dissect the leading fin ray from the pectoral fin on the right hand side of the fish. The fish were returned to the holding tank and allowed to on-grow. The fin ray was removed at the point of articulation (Currier 1951) then allowed to air dry for a period of at least one week. When the

second fin ray was sampled, fish were sacrificed by immersion in an overdose of anaesthetic and the pectoral fin ray from the left hand side of the fish was taken. After drying the fin rays were set in an epoxy (Epoxicure Resin and Hardener, Buehler, Lake Bluff, IL, USA) and left to dry for at least one week.

Fin rays were processed following protocols similar to those described by Smith and Whitledge (2011) in preparation for analysis on a laser ablation inductively coupled mass spectrometer (LA-ICP-MS). The laser settings used in this study are found in table 3.1. The laser used was an Nd:YAG laser (Merchantek LUV 213, New Wave Research/Merchantek, Fremont, California) coupled to an ICP-MS (Thermo Finnigan Element 2, Thermo Fisher Scientific Inc.).

Data analyses

The data was analysed by defining two simple classes, either marked or unmarked. Marked fin rays were defined as any profile of fin ray with a portion of the ratio that was at least five standard deviations from the mean of the control data. Unmarked fin ray profiles were defined as any profile that has no major deviations (5 or less than standard deviations away from the control mean). Although stringent all fin rays analysed fell into either category. Marking success was calculated as the number of marked fin rays per total fin rays analysed.

Table 3.1. Conditions used for LA-ICP-MS

Plasma power (W)	1385
Cool gas (L/min)	14
Auxiliary gas (L/min)	1.02
Sample gas (L/min)	1.05
ThO/Th (%)	7
Wash time (sec)	50
Sampling time (sec)	120
Nebulizer	Meinhard 200 µL/min
Spray Chamber	Cyclonic-Scott type combined
Analytical method	
Mass window (%)	5
Sample time (ms)	50
Sample/peak	10
Integration window (%)	5
Scanning type	EScan
Detection mode	Counting and analogue
Integration type	Average
Data reduction	
Standard reference material	NIST SRM 987 strontium carbonate
Mass-discrimination correction	Internally using 86Sr/88Sr ratio of 0.1194

Results

Scanning fin rays of fish that were not exposed to either strontium or barium enriched isotopes demonstrated a characteristic constant ratio between $^{88}Sr/^{86}Sr$ or $^{138}Ba/^{137}Ba$ (Fig. 3.1 & 3.2). The average ratio observed for control fish was 8.628 ± 0.006 for $^{88}Sr/^{86}Sr$ and 6.389 ± 0.007 for $^{138}Ba/^{137}Ba$. Both of these values fall very close to their theoretical values, obtained by dividing the natural abundances of the isotopes, of 8.375 and 6.383 respectively. Figures 3.3 and 3.4 are representative of laser ablation data obtained from scanned fin rays sampled from fish immersed and considered marked with ^{86}Sr and ^{137}Ba respectively. The ratio is significantly reduced either side of the central region of the fin ray, which is the middle region of the line

profile. The symmetry of the line profile is due to the scanning protocol which started on one side of the fin ray, scanned towards and through the core region and then outwards towards the opposite side. Figure 3.5 and 3.6 shows image of a fin ray that has been ablated showing the scanning path. To further understand the line profiles it is important to understand that distance 0 is the lateral edge of the fin ray where ablation began and the highest distance recorded on the line profile is the opposite lateral edge of the fin ray where ablation ended. An obvious marked pattern is seen when the control line profiles are compared to the line profiles from fish exposed to enriched isotopes (figs 3.1-3.4).

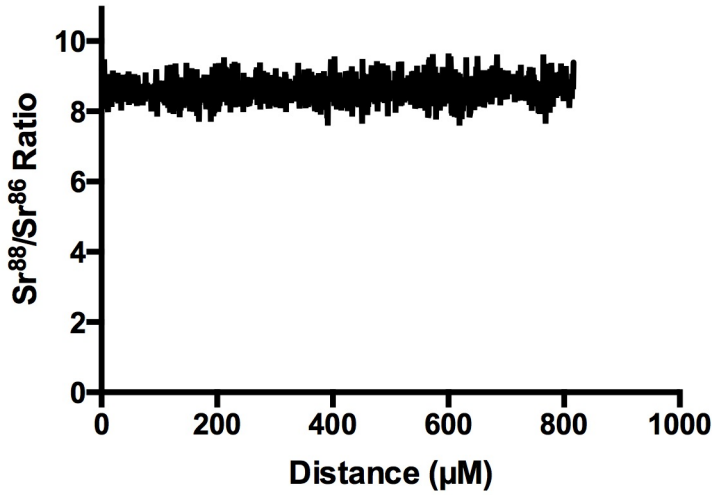

Figure 3.1. Line profile from Lake Sturgeon, *Acipenser fulvescens,* pectoral fin ray showing the $^{88}Sr/^{86}Sr$ ratio from a fish not exposed to an enriched ^{88}Sr environment (control). The ratio is determined from the respective counts per second of each isotope determined via laser ablation inductively coupled mass spectrometry (LA-ICPMS).

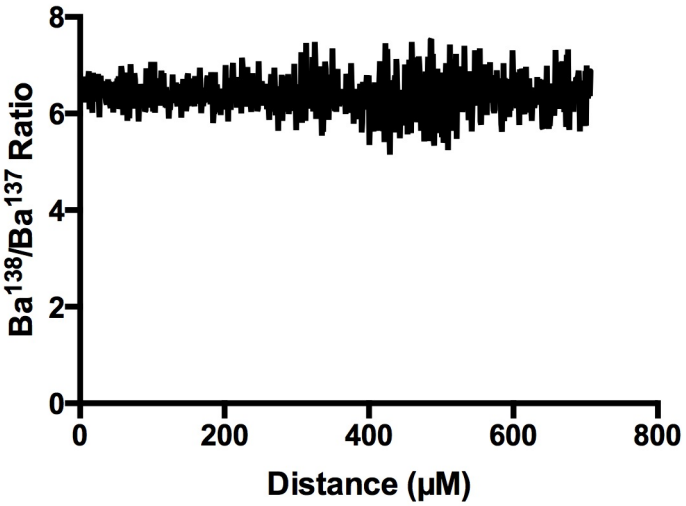

Figure 3.2. Line profile from Lake Sturgeon, *Acipenser fulvescens*, pectoral fin ray showing the $^{138}Ba/^{137}Ba$ ratio from a fish not exposed to an enriched ^{137}Ba environment (control). The ratio is determined from the respective counts per second of each isotope determined via laser ablation inductively coupled mass spectrometry (LA-ICPMS).

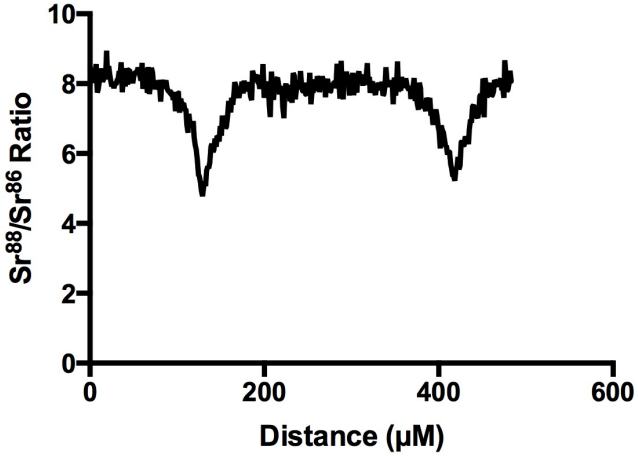

Figure 3.3. Line profile from Lake Sturgeon, *Acipenser fulvescens*, pectoral fin ray showing the $^{88}Sr/^{86}Sr$ ratio from a fish exposed to an enriched ^{86}Sr environment (treatment). The ratio is determined from the respective counts per second of each isotope determined via laser ablation inductively coupled mass spectrometry (LA-ICPMS). The x-axis for these line profiles represent the distance ablated with 0 being one edge of the fin ray and the highest value recorded being the opposite edge, because of this the line profile is symmetrical and the middle of the line profile is where the core of the fin ray is located and ages outward to distance 0 and the highest distance.

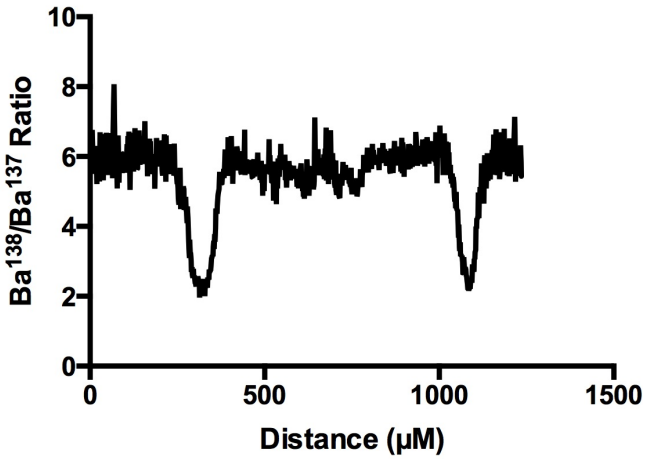

Figure 3.4. Line profile from Lake Sturgeon, *Acipenser fulvescens*, pectoral fin ray showing the $^{138}Ba/^{137}Ba$ ratio from a fish exposed to an enriched ^{138}Ba environment (treatment). The ratio is determined from the respective counts per second of each isotope determined via laser ablation inductively coupled mass spectrometry (LA-ICPMS). The x-axis for these line profiles represent the distance ablated with 0 being one edge of the fin ray and the highest value recorded being the opposite edge, because of this the line profile is symmetrical and the middle of the line profile is where the core of the fin ray is located and ages outward to distance 0 and the highest distance.

Marking success varied with both the technique used (immersion or transgenerational), with the isotope used (barium or strontium), the timing of when fish were immersed (12 dpf or 100 dpf), and the duration of immersion in the stable isotope (3, 7 or 10 days) (Table 3.2). The highest marking success observed from the 2012 cohort when 100dpf fish were immersed in ^{137}Ba for seven days at 100 dpf. The second highest marking success was in the same cohort of fish but following immersion in ^{86}Sr. Comparison between the 12 dpf and 100 dpf immersion in ^{137}Ba is unfortunately not available due to excessive mortality in the 12 dpf ^{137}Ba immersed group.

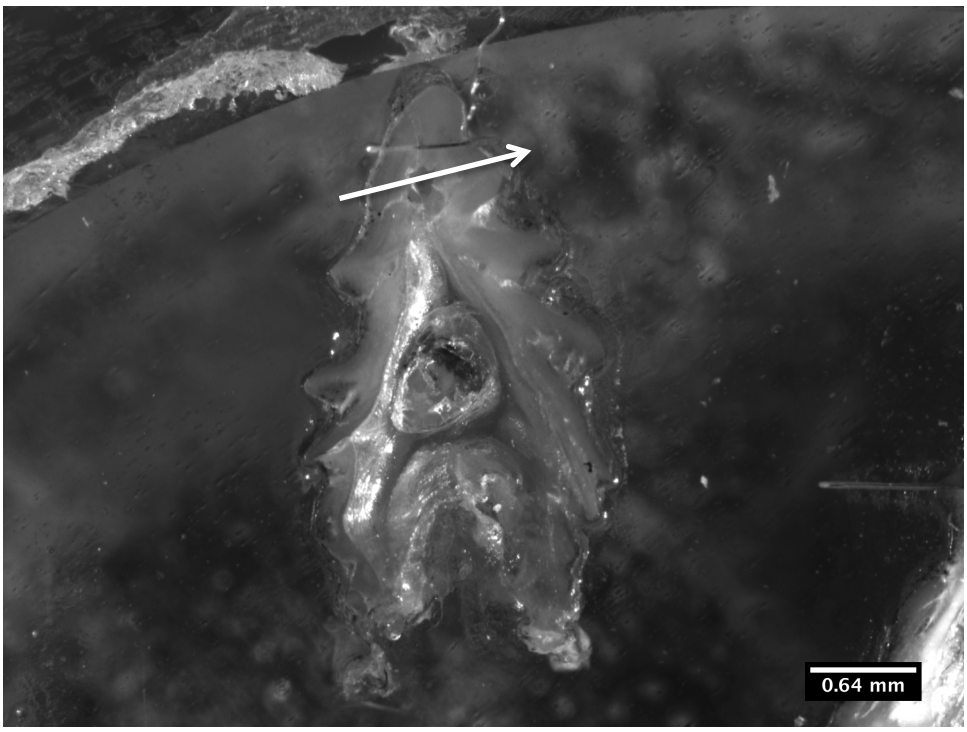

Figure 3.5. Photograph of a Lake Sturgeon, *Acipenser fulvescens*, fin ray after laser ablation. The arrow indicates the direction and path of ablation.

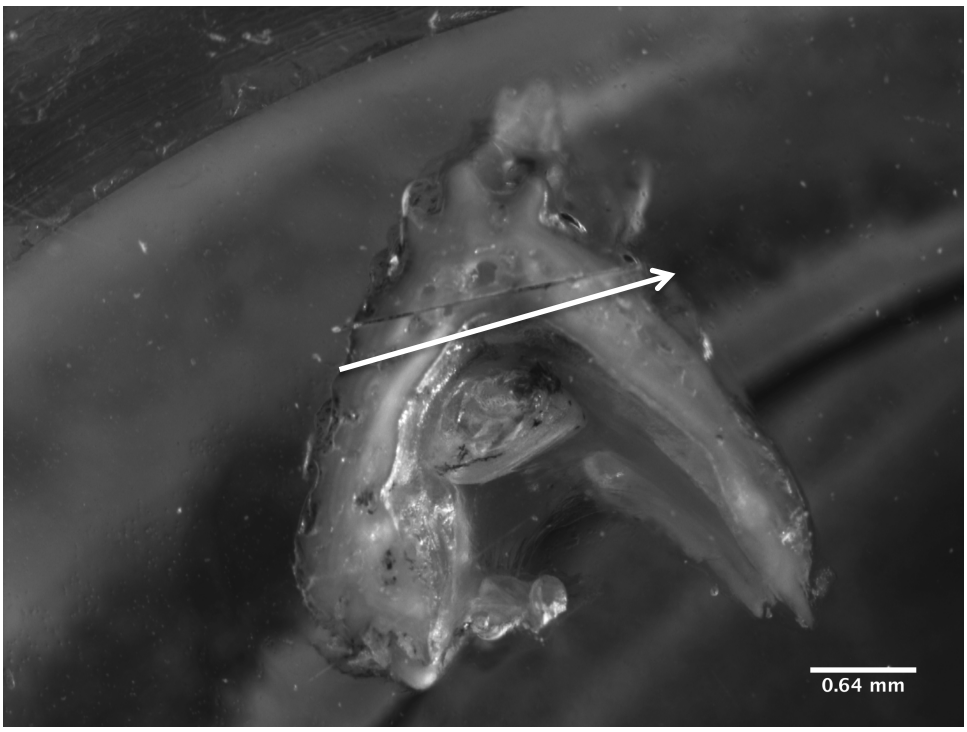

Figure 3.6. Photograph of a Lake Sturgeon, *Acipenser fulvescens*, fin ray after laser ablation. The arrow indicates the direction and path of ablation.

Table 3.2. Percent marking success in fin rays sampled from the 2012 cohort of Lake Sturgeon, *Acipenser fulvescens,* for each treatment at different times post-transgenerational or immersion marking. (--) represents no sampling, number in parentheses represents the number of available samples that were analysed. (R) represents fin rays sampled from the right side of the fish and (L) represents fin rays sampled from the left side of the fish. Percent success was calculated as the number of individuals marked divided by the total number of individuals analysed for either the ^{86}Sr or ^{137}Ba isotope. None of the control fish exhibited a change in the isotopic ratio of either ^{86}Sr or ^{137}Ba hence there are no percentages assigned to these fish. Sample size for ^{86}Sr transgenerational and 12 dpf is low due to a mortality event.

Days post marking	44 (L)	132 (L)	144 (L)	411 (R)	499 (R)	550 (R)	638 (R)
Transgenerational							
^{86}Sr	--	--	33% (3)	--	--	--	--
^{137}Ba	--	--	--	--	--	--	--
Immersion							
12 dpf ^{86}Sr	--	25% (4)	--	--	0% (4)	--	0% (4)
100 dpf ^{86}Sr	91% (11)	--	--	88% (8)	--	75% (8)	--
12 dpf ^{137}Ba	--	--	--	--	--	--	--
100 dpf ^{137}Ba	93% (14)	--	--	10% (5)	--	0% (5)	--
Control							
	--	--	(8)	--	(8)	--	(8)

In the 2012 cohort, 100 dpf fish immersed in ^{86}Sr showed a 91% marking success compared to a 25% marking success for 12 dpf immersed in ^{86}Sr and a 33% marking success for the transgenerational ^{86}Sr treatment group. No viable eggs were obtained from the ^{137}Ba transgenerational marked female (Table 3.2).

Both the 12 dpf strontium immersion and 100 dpf barium immersion treatment groups from the 2012 cohort showed a sharp decrease in marking success within the first year and a half post marking (Table 3.2). When the 100 dpf barium immersion cohort were sampled at 411 days post mark the retention of mark decreased from 93% to 10% when compared to fin rays sampled 44 days post mark. The 12 dpf strontium immersion also experienced a decrease in marking

success during this time period decreasing from 25% to 0%. The 100 dpf strontium immersion also experienced a decrease in marking success during this period from 91% to 88%. When the fish were sampled again at 550 days post mark, marking success was found to have decreased further in both the 100 dpf ^{137}Ba immersion cohort to 0% detection of a mark and 75% detection in the ^{86}Sr immersed fish. The 12 dpf strontium immersion cohort had no increase or decrease in marking success and remained at 0% marking success at 638 days post marking sampling time point.

Table 3.3. Effect of immersion time on fin ray marking success in Lake Sturgeon, *Acipenser fulvescens*, 185 dpf immersed in either ^{86}Sr or ^{137}Ba for 3, 6 or 10 days Samples were taken from these fish at 149 days post immersion and data were calculated from the number of marked individuals divided by the total number of individuals analysed for each isotope and are expressed as a percent success (%). Number in parentheses represents the number of samples available that were analysed.

	Number of days immersed in isotope		
	3	6	10
^{86}Sr	90% (10)	90% (10)	100% (10)
^{137}Ba	75% (8)	100% (10)	100% (10)

Marking success was also observed to vary with the length of immersion to the enriched isotope environment in the 2013 cohort. In both the ^{137}Ba and ^{86}Sr immersion cohort there appeared to be a positive relationship between duration of immersion and marking success where the longest immersion time (10 days) lead to 100% marking success regardless of isotope used (Table 3.3).

Discussion

Marking validation

This study confirmed the potential of using the rare stable isotope of strontium ^{86}Sr as an isotopic mark in the fin ray of age 0 Lake Sturgeon (Smith and Whitledge, 2011). Analysis of fin rays from fish that were exposed to 100 $\mu g.L^{-1}$ $^{86}SrCO_3$ demonstrated a distinct pattern of a lower

$^{88}Sr/^{86}Sr$ ratio when analysed using LA-ICPMS. The results are also supported by similar research conducted on the otoliths of a variety of teleost species including Golden Perch, Murray Cod, Mummichogs, *Fundulus heteroclitus,* and Atlantic Salmon, *Salmo salar* (Munro et al. 2008; Woodcock et al. 2011; Walther and Thorrold 2006; de Braux et al. 2014). The present study also confirmed the validity of using ^{137}Ba as a stable isotope to mark the fin rays of age 0 Lake Sturgeon. As with ^{86}Sr; immersion of fish in 100 $\mu g.L^{-1}$ of $^{137}BaCO_3$ resulted in a characteristic drop in the pectoral fin ray $^{138}Ba/^{137}Ba$ ratios. This is the first report of the use of ^{137}Ba as a stable isotope in marking sturgeon but is supported by similar studies examining the validity of using ^{137}Ba as an isotopic mark in the otolith of Golden Perch, Murray Cod, Mummichog and Atlantic Salmon (Munro et al. 2008; Woodcock et al. 2011; Walther and Thorrold 2006; de Braux et al. 2014).

In addition to confirming the use of stable isotopes to mark fin rays by immersing age 0 Lake Sturgeon in either $^{86}SrCO_3$ or $^{137}BaCO_3$ this study also examined the potential for transgenerational marking by injecting gravid females with either 2 $mg.kg^{-1}$ $^{86}SrCO_3$ or $^{137}BaCO_3$ to mark the resulting progeny. Although marking success was low a characteristic drop in $^{88}Sr/^{86}Sr$ ratios in fin rays was observed in a proportion (33%) of fish from those that were on-grown from gravid females injected with $^{86}SrCO_3$. This is the first report of successful use of this technique in sturgeon and results are supported by previous studies employing transgenerational marking and isotopic signatures in the otoliths of a number of teleost fish including; Common Carp and Eastern Rainbowfish, *Melanotaenia splendida* (Zitek et al. 2014; Starrs et al. 2013). Transgenerational marking using $^{137}BaCO_3$ in Lake Sturgeon was unsuccessful, despite reported success in teleost fish including the Cinnamon Clownfish, Black Sea Bass, and Coral Reef Grouper (Thorrold et al. 2006; Williamson et al. 2009).

Marking success

Following validation of the marking technique in age 0 Lake Sturgeon the marking success of each technique was examined. Fin ray marking success observed in the present study following immersion of age 100 dpf Lake Sturgeon in ^{86}Sr was comparable to otolith marking success following immersion in similar concentrations of ^{86}Sr for a number of teleost fish. Immersion of Golden Perch in 15 $\mu g.L^{-1}$ ^{86}Sr for 8 days resulted in 83% otolith marking success when fish were sacrificed 24 days following immersion (Munro et al. 2008). Further, immersion of Murray Cod in 250 $\mu g.L^{-1}$ ^{86}Sr led to 50-100% otolith marking success (Woodcock et al. 2011); 100% otolith marking success was observed following immersion of Mummichogs in 100 $\mu g.L^{-1}$ ^{86}Sr (Walter and Thorold 2006) and finally immersion of Atlantic Salmon in 75 $\mu g.L^{-1}$ ^{86}Sr for 1 hour resulted in 71% success when using osmotic induction (de Braux et al. 2014). Fin ray marking success in Lake Sturgeon that were immersed at an age of 12 dpf and Lake Sturgeon from the transgenerational marking technique in the present study was appreciably lower at 25% and 33% respectively. These stark differences may be due to development of the pectoral fin ray in larval Lake Sturgeon. At hatch in studied Acipenserids there is rarely any origins of the pectoral fin present, when there is only a fleshy thickening (Dettlaff et al.1993). However, it was informative to examine how persistent a isotopic mark would be in these early development fish. After 23 hours post hatch the origins of the pectoral fins are present in the majority of the prelarvae. After 9 days in examined Acipenserids the fin rays were visible to the naked eye (Detlaff et al. 1993). The smaller size of the fin rays in the post-hatch larvae may explain the lower detection or lower marking success. With smaller fin rays there is a smaller band of "marked" fin ray matrix, which may be missed in the analysis process. Conversely at 100 dpf the fin ray has substantially more material present in the structure. It is possible therefore

that increased ^{86}Sr may be incorporated thus creating broader bands providing greater resolution in measurement of the scanned fin ray. It is possible that a mark may have been present in the fin ray of fish from both the transgenerational and 12 dpf immersion experimental groups; however, the scale may have been too small for the instrumentation to discern the presence or absence of a mark on a consistent basis.

Interestingly fin ray marking success of the 12 dpf ^{86}Sr immersed fish in the present study was much lower than the 83-92% marking success previously observed in Lake Sturgeon immersed in 100 $\mu g.L^{-1}$ ^{86}Sr for either 10 or 24 days (Smith and Whitledge 2011). However, fish used by Smith and Whitledge (2011) were 162.5 ± 0.89 mm total length (TL), substantially larger than the 12 dpf larvae used in the present study but were more similar in size range to the 100 dpf immersed fish 202.8 mm ± 2.70 mm TL in the present study, suggesting that differences in size or developmental stage will influence marking success. Indeed at 12 dpf organs are not fully developed and growth trajectory is substantial (Dettlaff et al. 1993). It is possible that significant remodelling is occurring in hard structures such as fin rays and therefore the initial ^{86}Sr that is deposited in the fin ray maybe resorbed over the course of the following few months as the fish continues to develop and grow. At 100 and 185 dpf while growth trajectory remains significant the majority of organ development is complete and the fish would be investing primarily in somatic growth in preparation for over wintering. At this stage there may be less remodelling of hard structures such as the fin ray and therefore less resorption of an isotopic signature like ^{86}Sr once it is incorporated in the fin ray. Analysis of otolith isotopic ratios would help provide insight into this hypothesis as otoliths are thought not to undergo remodelling such that once an isotope is incorporated in the otolith it is not removed (Campana 1999).

At the time of writing there was no data for comparison of our strontium transgenerational fin ray marking experiments with other sturgeon species. In the present study Lake Sturgeon fin ray marking success was quite low using this technique (33%) compared to the literature values observed for Murray Cod (100%) (Munro et al. 2009). The difference between these two values may be due to a variety of factors such as size of adult, timing, egg maturation and environment. Timing of injection for one species may not be optimal for another species. For example, Munro et al. (2009) observed that marking success in Murray Cod was 81% when injected with 20 $\mu g.g^{-1}$ or 40 $\mu g.g^{-1}$ at 21 days prior to hormonal induction of spawning, but increased to 100% when the same concentration of isotopes were administered 24h prior to induction of spawning. Furthermore, injection of enriched ^{137}Ba delivered concurrently with induction of spawning resulted in 25% marking success of progeny when the dose was 20 $\mu g.g^{-1}$ and 100% when the dose was 40 $\mu g.g^{-1}$ (Munro et al. 2009). Finally environmental factors such as salinity may influence the deposition of injected ^{86}Sr into the eggs as it is well recognized that increased strontium in the otolith characterizes a marine-type life history phase (Campana 1999). Clearly further research is warranted to understand the efficacy and validity of transgenerational marking in Lake Sturgeon.

Marking success data for age 0 Lake Sturgeon pectoral fin rays from sturgeon immersed in ^{137}Ba is novel and there are no comparators from the chondrosteans. The data can be compared to teleost marking otolith data from similar marking conditions. Munro et al. (2008) reported otolith marking success of between 82-93% in Golden Perch, which is lower than the otolith marking success reported by Woodcock et al. (2011), where greater than 98% of the otoliths from 6 days post hatch Murray Cod immersed in 100 $\mu g.L^{-1}$ ^{137}Ba after 14 days post immersion. The timing of this immersion is closer to that of the 12 dpf trials that were conducted

in this study but are comparable to the marking success of the fish immersed at 100 dpf. At present, species differences and environmental parameters likely best explain the observed differences in marking success between the Lake Sturgeon and Murray cod.

Immersion time

The present study examined the differences in marking success from 185 dpf fish exposed to ^{86}Sr and ^{137}Ba from the 2013 cohort for different lengths of time and found that length of immersion influenced fin ray marking success for both isotopes. Perhaps unsurprisingly there was a positive relationship between marking success and length of immersion for both isotopes, which is most likely due to increased residence time and therefore opportunity for the isotopes to be taken up and incorporated in the fin ray. The more of the rare isotope present the greater the likelihood of a reduced isotopic ratio and thus an expanded "marked" area in the fin ray. These two factors will increase the marking success for longer immersed sturgeon.

This data is not consistent with the findings of Smith and Whitledge (2011) who found in age 0 Lake Sturgeon that there was no significant trend in length of immersion compared to marking success. However, the minimum immersion time used by Smith and Whitledge (2011) was 10 days, which corresponds to the maximum immersion time used in the present study. Thus based on the data from the present study and Smith and Whitledge (2011) immersion of fish beyond 10 days may not improve the immediate marking success. In support of the positive relationship between immersion times and marking success observed in the present study research on length of immersion on marking success in teleost otoliths has also demonstrated an effect of length of immersion on marking success. In Golden Perch marking success increased with immersion time when perch were immersed for 1, 4 or 8 days in either 5 $\mu g.L^{-1}$ or 15 $\mu g.L^{-1}$ reaching 100% when fish were immersed for 8 days in the higher concentration (Munro et al.

2008). The differences between the findings of Munro et al. (2008) and Smith and Whitledge (2011) may be due to the difference in concentrations. Golden Perch were immersed in a lower concentration, 5 or 15 $\mu g.L^{-1}$, (Munro et al. 2008) than that of this study and Smith and Whitledge (2011), 100 $\mu g.L^{-1}$, and this will play a role as both Munro et al. (2008) and Smith and Whitledge (2011) showed a significant effect of concentration on isotope on marking success.

Persistence of mark

The presence of the ^{86}Sr isotopic mark in the fin ray of fish immersed in $^{86}SrCO_3$ at 100 dpf fell from 91% at 44 days post mark to 88% at 411 days post mark and then to 75% at 550 days post mark. Interestingly, despite having a higher initial marking success of 93% at 100 days post immersion the presence of a ^{137}Ba signature in fish immersed in ^{137}Ba at 100 dpf fell precipitously to 10% at 411 days post-mark and 0% at 550 days post-mark. At present it is unknown as to reasons for the differences between ^{86}Sr and ^{137}Ba mark persistence

The presence of ^{86}Sr isotopic signature remained in the fin ray of Lake Sturgeon for up to 120 days following immersion of age 0+ fish (162.5 ± 0.89 mm TL) for 10 days in 25, 50 or 100 $\mu g.L^{-1}$ (Smith and Whitledge 2011). However, it is worth noting that comparison of the persistence of a stable isotopic signature between the fin ray and otolith should be made with caution as resorption of the otolith is considered to be limited and thus isotopic signatures are thought to be retained for the lifetime of the organism (Campana 1999), whereas the fin ray in chondrosteans may undergo resorption as seen in white sturgeon (Veinott and Evans 1999). However Veinott and Evans (1999) demonstrated that strontium concentrations in similar age regions of the fin ray remain relatively constant in the wild over the span of 6 years.

Based on the experiments conducted the most persistent mark was achieved when at least 100 dpf fish were immersed in ^{86}Sr for at least ten days. This marking technique could be utilized

to identify hatchery-reared sturgeon that were released into the wild and subsequently caught and the fin ray was sampled for analysis of the presence of an isotopic signature. The more persistent mark of the otolith is not possible due to the destructive nature of otolith sampling in this endangered species. Further the ability to immerse an entire cohort and produce a mark that will successfully mark 91%-100% of fish exposed without handling makes the stable isotope marking technique suitable for use in stock enhancement identification and assessment of the successes, or failures, of stock enhancement programs.

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General Discussion

The previous chapters showed that strontium enters the organism and is accumulated into the tissue long term. The results show the biological half-life of strontium and barium span from several weeks to a time long after the organism's natural lifespan. These results confirm the use of the enriched stable isotope marking technique using barium and strontium is well suited for the application in a long-lived species such as the Lake Sturgeon. Further the research confirmed the presence of the mark predicted by the flux and accumulation studies and previous work in other species.

However there was a discrepancy found between results of chapters two and three. In chapter two the biological half-life of barium was found to be very high compared to the half-life of strontium in all tissues, this finding disagrees with the findings in chapter three that showed the persistence of the strontium mark was much greater than that of the barium mark in the fin ray. A possible explanation for this discrepancy is not that the mark is not there but we are unable to detect it as well. As mentioned previously use of a single detector LA-ICP-MS causes a temporal lag and thus spatial lag between readings of different isotopes. This coupled with the previously mentioned lower concentrations of barium compared to strontium in marked tissue, could cause barium marked areas to be spatially smaller and thus harder to detect with a single detector apparatus. Another possible hypothesis to explain this discrepancy may be due to the fact there is more strontium in the fin ray than barium (Campana 1999). Figure 2.5 and 2.9 show a relatively greater effect of barium treatment on its ratio than the strontium treatment has on its ratio. This could be due to the fact that the entire fin ray was digested. With larger amounts of strontium in the fin ray tissue there is possibility the common ^{88}Sr released from these areas may

add noise to the data. Future research into the half-life of barium and strontium in these tissues may focus specifically on marked regions and the change in ratio there, to limit the effect that non-marked areas may have on this. This could be done by monitoring the change in ratio between the two isotopes in the tissue in marked areas via spot analysis with LA-ICPMS of the tissue over time.

The results from this thesis can be furthered by additional research on the physiology and application of the stable isotope marking technique. Physiologically a greater understanding on the uptake mechanisms of strontium and barium is needed to understand uptake kinetics and the relationship between uptake rates and environmental parameters such as pH, calcium concentration and temperature. Such studies would lead to optimizing immersion times and concentrations to maximise environment and species specific marking success for improved reliance on stable isotope marking as a technique for fisheries management and conservation. Additional research on other possible elements in the alkaline earth metal group, in particular magnesium, which has been used to mark otoliths in Golden Perch (Woodcock et al. 2011) are warranted. Indeed, Woodcock et al. (2011) tested the use of ^{138}Ba, ^{137}Ba, ^{88}Sr and ^{24}Mg in isolation and combination as potential isotopic markers in the otolith of the Golden Perch. All produced marks in the otolith of the Golden Perch but success was variable depending on the isotopes used. Such combinations would be a useful avenue of research in Lake Sturgeon as this could lead to genetic population specific marking and subsequently improved management practice within a given river system.

Other chemicals may be used in addition to isotopes to further increase the marking variability that managers and researchers may use. Including, but not limited to, oxytetracycline and calcein. Immersion of eggs and larvae in oxytetracycline has been shown to produce unique

marks under UV light (Secor et al. 1991; Tsukamoto 1988; Reinert et al. 1998; Jones et al. 1999). Calcein is a chemical that binds to Ca and is also detectable under a UV light source and has successfully been used to mark Rainbow Trout. (Elle et al. 2010). Use of additional chemicals and isotopes could potentially provide an infinite number of combinations to chemically mark and therefore identify fish. While it is not the intent at this stage of the present study to examine multiple marks it is evident that multiple marking for population assessment of fisheries where stock enhancement has been/is implemented could provide very pertinent and useful information from a management perspective.

In addition future work in this field may also include field studies testing the efficiency of the stable isotope marking technique. Long-term studies are needed to determine the length in which the mark in the fin ray will remain in the natural environment. In addition studies determining the fitness of fish marked with the stable isotope marking technique versus unmarked hatchery-reared fish and wild progeny will be necessary to ensure marking with the stable isotope marking technique does not damage stock enhancement efforts.

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