Stock Enhancement in the Murray-Darling Basin, Identification of Stocked Fish and Enhancing Post-stock Survival

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Abstract

Freshwater systems are experiencing a higher rate of decline in biodiversity compared with marine and terrestrial systems due primarily to water pollution, habitat degradation, species invasion, flow modification and over-exploitation. Stock enhancement programs to increase numbers of fish in these systems occur worldwide, however the success of these programs can be difficult to determine due to poor monitoring and the inability to distinguish between hatchery-stocked fish and wild recruitment. Severe declines in native fish distribution and abundance have occurred in the Murray-Darling Basin (MDB). Consequently, stock enhancement programs that stock millions of native fish annually have been established across the MDB to restore populations and provide recreational fishing opportunities. The success of these enhancement programs, however, is generally quantified solely in terms of the number of fish entering a system, not on the number surviving to reach reproductive age or enter the fishery. A method of marking stocked fish to determine post-release survival is required to assess the success of hatchery-reared fish after stocking into the MDB.

Native fish are not routinely marked before being stocked into the MDB, which makes it difficult to determine the percentage of fish in various populations comprised of stocked fish versus natural recruitment. A method that can be used to batch mark fish may be developed with the use of otolith (the ear bone of fish) chemistry, and an understanding of elemental incorporation. Using enriched stable isotopes, unique isotope signatures can be created into the calcifying structure of an otolith, leaving behind a permanent mark in stocked fish. I investigated whether larval otoliths of two native fish species, golden perch and Murray cod, could be marked with enriched stable isotopes. Each species has a different life history, and therefore the hatchery rearing of

larvae is different. Using a range of enriched stable isotopes from three elements, barium (Ba), strontium (Sr) and magnesium (Mg), singularly and in combination I explored the range of isotopic signatures that could be created. Unique signatures were created using stable isotopes of Ba and Sr; isotopes from these two elements had a high mark success rate and could be clearly distinguished from marks created with similar isotope combinations. Magnesium isotopes, however, were found to be poor markers with a low mark success compared to Ba and Sr stable isotopes.

Due to a lack of mark success with stable isotopes of Mg in comparison to stable isotopes of Ba and Sr, a trial was carried out on silver perch to determine the primary source of otolith Mg, to determine specifically if Mg in otoliths was sourced primarily from the water or diet. For this experiment, both the rearing water and diet were supplemented with increasing concentrations of Mg and the diet was spiked with an enriched Mg isotope. The experiment found that Mg was highly regulated in fish, although the majority of otolith Mg was sourced from the water.

Hatchery reared stocked fish are often predator naive, having never been exposed to a predator prior to release, this risk, combined with adjusting to a new environment results in low survival rates of stocked fish over the first few days after stocking. Exposing hatchery fish to chemical stimuli can increase predator awareness and possibly improve survival rates. To assess this behaviour, golden perch were exposed to a predator odour and alarm stimuli, and their post-stock behaviour in the presence of a potential predator was monitored. In addition, the presence and absence of habitat structure was also analysed to determine if it would influence the behaviour of golden perch. Habitat structure was found to increase the hiding time for all treatments. The exposure to predator odours had no influence on the behaviour of golden perch, and only influenced the time spent within a portion of the tank for one position. This

experiment indicated that choice of stocking site would contribute more to increasing post-stock survival of hatchery-reared golden perch rather than pre-stock predator training.

Enriched stable isotope marking was an effective method of mass marking different species of fish, with the methodology fitting within hatchery operating practices. This method of marking is not only applicable to fish stocking programs for the MDB, but can be applied to other stocking programs and hatcheries worldwide. Routine marking of stocked fish will enable fish monitoring programs to accurately assess how stocked fish are interacting within stocked systems. Research addressing the behaviour of stocked fish needs to identify and understand additional influential factors affecting survivorship of stocked fish. This research has developed a method for marking hatchery-produced fish, which can be used to evaluate fish stocking programs as well as providing some understanding of the differences in elemental incorporation into otoliths. Investigation into enhancing early survival of hatchery-stocked fish has found stocking density and appropriate habitat to be key in the early behavioural patterns of stocked fish.

Chapter 1 General Introduction

Photo: Murray cod pond at Narrandera Fisheries Centre

General Introduction

Attempts to re-establish species within their historical range through the release of wild or captive bred individuals have been occurring for at least 100 years (Seddon et al., 2007, Armstrong & Seddon, 2008) and include reintroductions of mammals, amphibians, fish and birds (Kleiman, 1989, Fischer & Lindenmayer, 2000, Lorenzen, 2005). The primary aims of reintroductions are to solve human animal conflicts (e.g. urbanisation influencing animal behaviours like home ranges), supplement populations for recreational or commercial exploitation and for conservation (Fischer & Lindenmayer, 2000). Methods for successful reintroductions begin with identification of the species at risk, understanding their behaviour and ecology, and what factors are contributing to their decline, such as hunting pressure, or the introduction of non-native species competing for resources (Kleiman, 1989). Reintroductions are generally considered successful once the released organisms have fully integrated into a local wild population and are able to survive without any further human interactions (Waples & Stagoll, 1997).

Saltwater covers 70% of the earth's surface with about 16,000 fish species inhabiting the marine environment, while freshwater systems such as rivers and lakes only cover 1% of the earth's surface yet contain 13,000 strictly freshwater fish species (Leveque et al., 2008). Freshwater systems are declining at a faster rate than other ecosystems. Between 1970 and 2007 the global Living Planet Index (LPI), a measure of the state of global biodiversity, of freshwater systems declined by 35%, more than either the global marine (24%) or terrestrial (25%) LPIs (WWF, 2010). With inland and coastal fisheries, productivity is often limited due to declining quality of the aquatic environment and overfishing. As a result, catches of wild fish are declining worldwide (Welcomme &

Bartley, 1998). In an effort to rebuild the worldwide loss in fish populations, at least 94 countries have programs to rear hatchery-propagated fish and release them into the wild (Hilborn, 1998, Welcomme & Bartley, 1998, Brown & Day, 2002).

Despite reintroduction programs, there has been little change in the abundance of target species contributing to fisheries, primarily due to early losses of fish after stocking (Fairchild et al., 2005, Brockmark et al., 2007). Furthermore, the success of many programs is difficult to determine due to lack of monitoring or the difficulty in distinguishing between stocked hatchery fish and wild recruitment (Brown & Day, 2002). Without methods to monitor the survival of stocked fish, and their contributions to natural populations, the success and impact of stocking programs cannot be fully determined. Two key areas for enhancing reintroduction success are (1) the preparation of fish prior to release and employment of optimal release strategies (see section 1.4 below) to ensure successful survival of hatchery fish in a wild environment, and (2) the ability to identify reintroduced species from wild counterparts.

1.1 Tagging and Marking Methodologies

Many methods are available for identifying fish using natural and artificial marks and tags (see reviews by Pawson & Jennings, 1996, Crook et al., 2005, Gillanders, 2009). Tagging and marking is common and can include both external and internal application. Although some tags and marks allow for recognition of individual fish, they generally require intensive handling and many methods are limited to large juveniles or adults. External tagging and marking include transbody, dart and anchor tags, fin clipping, branding and pigment marking, while internal tags include coded wire, passive integrated transponder (PIT) and visible implant tags (Gillanders, 2009). Of the various methods, not many are suitable or cost effective for large scale mass marking. In

addition, fish bred for stocking are generally reintroduced into systems as small fingerlings and their size can be limiting for many marking and tagging applications.

Natural marks eliminate the need for physical tagging (Thorrold et al., 2002). Identification can include differences in physical features such as colour, markings, differences in gill rakers or fin rays, the presence or absence of parasites, or differences in genetic or chemical composition (Gillanders, 2009). Differences in chemistry occur when there is a natural difference in the elemental or isotopic signatures between organisms collected from different areas because the physical and chemical environment in which the organisms are found differ (Thorrold et al., 2002, Elsdon & Gillanders, 2003a, Gillanders, 2009). Chemical signatures can be detected in the tissue of fish as well as calcified structures such as bones, scales and otoliths (Thorrold et al., 2002). Recent research has focused on otolith chemistry for identifying the origins and migratory patterns of fish. In addition, techniques have been developed to incorporate artificial marks into the otolith of fish to improve otolith chemistry use in fish identification.

1.2 Using Otoliths for Mark Identification in Fish

Otoliths are calcareous structures located in the inner ear of fish and have been used for marking and identifying different stocks of fish. There are three features of otoliths that make them useful for monitoring and assessing fish stock recovery programs (Campana et al., 2000). First, otoliths, unlike bone and scales, are metabolically inert and continue to grow throughout the life of the fish. Otoliths, therefore, provide a continuous growth record (Campana & Neilson, 1985). The second feature is that trace elements are incorporated into otoliths as they grow, often in proportion to dissolved concentrations in the ambient environment. Consequently, otoliths reflect the physical and chemical

environment that the fish inhabits. It is therefore possible to use the chemical signatures in fish otoliths to trace the environments inhabited (Campana et al., 2000, Kennedy et al., 2000). Finally, growth bands in the calcified structures of otoliths correspond to daily, seasonal or annual patterns (Campana & Thorrold, 2001). This feature provides the ability to relate chemical information to the age of the fish throughout its life.

1.2.1 Natural marks

Intrinsic marks are marks that are naturally present in the fish (e.g. otolith, scales, DNA and morphology) and can be induced through environmental variability or ontogenetic changes (Crook et al., 2005, Gillanders, 2009). Intrinsic marks eliminate the need to handle or physically mark fish (Thorrold et al., 2002). Conditions in hatcheries are often different from the wild, therefore fish from hatcheries often develop characteristics in their otoliths that are distinctive and can be used to distinguish hatchery fish from wild fish. Analysis of the variation in the microstructure of daily growth increments, as well as the shape and size of otoliths are commonly used to discriminate between hatchery and wild fish (Elsdon & Gillanders, 2003a, Gillanders & Joyce, 2005) or to identify fish from different geographical areas (Kennedy et al., 2000). As otoliths form a record of the chemical and physical environment to which a fish has been exposed throughout its life, it is possible to identify a fish's recruitment source. However in order for this to be achieved, hatchery signatures need to be distinct from the environment in which the fish are stocked (Crook et al., 2005).

1.2.2 Artificial marks

Another approach for identifying groups of fish based on otolith chemistry is to create distinctive artificial marks. Fish can be marked as early as the egg or embryo stage. There are three commonly used otolith marking techniques; (1) thermal marking, which involves changes to the temperature of rearing tanks, thereby altering the width of the

increments in the otolith (Volk et al., 1999, Courtney et al., 2000), (2) fluorescent dyes that bind to the calcified structures of fish (Crook et al., 2007, Crook et al., 2009), and (3) elemental (Ennevor & Beames, 1993, Brown & Harris, 1995) or enriched stable isotope marking (Thorrold et al., 2006, Munro et al., 2008, Munro et al., 2009), where artificial chemical changes are incorporated into otolith growth increments. All are easily applied, and can be used to mark large batches of fish with limited handling. In addition, marked fish usually display similar growth and mortality to unmarked fish (Thorrold et al., 2002, Crook et al., 2005).

Elements commonly used for marking usually occur at low levels in the natural environment, such as some lanthanide (rare earth) elements (Ennevor, 1994), or by enrichment of trace elements (Thorrold et al., 2002). Lanthanides occur naturally in the bones of many fish in extremely low concentrations, making them distinctive makers (Thorrold et al., 2002). Exposure to high concentrations of lanthanides, however may increase toxicity and accumulation, impair gill function and prevent further uptake of lanthanides, as well as essential ions and oxygen (Ennevor, 1994). Although they can be more ambiguous, the use of trace elements such as strontium (Sr) is more commonly used to mark fish otoliths (Pollard et al., 1999, Skov et al., 2001, Thorrold et al., 2002).

Elemental marks can be applied to a large numbers of fish with minimal handling (Secor and Houde, 1995). There are two main methods of using elements to mark the otolith of fish; these include transgenerational, via maternal transfer, or immersion. The influence of diet on otolith chemistry has also been investigated, however the incorporation of some elements from the diet is estimated to be variable and quite low (Walther & Thorrold, 2006).

Transgenerational marking of embryonic otoliths using artificial signatures involves injecting the abdominal cavity of the maternal fish with element or isotope enriched solutions, that are then transferred from adult to offspring, presumably via incorporation into the egg and yolk (Thorrold et al., 2006). Resulting progeny contain the mark that remains locked in the core of the otolith for the life of the tagged individual (Thorrold et al., 2006). Transgenerational marking techniques have been used on both freshwater (Munro et al., 2009) and marine species (Thorrold et al., 2006, Almany et al., 2007, Williamson et al., 2009a, Williamson et al., 2009b). To date, three stable isotopes of barium (Ba) have been successfully used to mark fish progeny using this technique (e.g. Munro et al., 2009, Williamson et al., 2009a).

Efforts to limit handling of fish, have led to the development of isotope immersion of fish, generally at the fingerling stage. They can be applied at any period of the fish's life cycle, as long as the fish can be held in a water source with a stable concentration of the chemical marker (e.g. recirculating or static). It has been suggested that an ideal time to use immersion techniques in hatchery settings is usually at the larval stage, where stable isotopes can be added to larval rearing tanks (Munro et al., 2008). Enriching the level of a stable isotope in the water creates a shift in the isotope ratio from the natural signature. For example, the natural abundance of ^{137}Ba and ^{138}Ba is 11.23 and 71.69 respectively (Rosman & Taylor, 1998), providing a natural ^{138}Ba/^{137}Ba ratio of 6.38, which is assumed to be invariant. By enriching ^{137}Ba in solution, the ratio is shifted downward, creating a mark that cannot be confused as a natural signal. By manually shifting the isotope ratio in a water source, an artificial mark can be created in the otolith of fish. These large shifts in isotope ratios can be detected using standard single collector laser ablation inductively coupled plasma-mass spectrometry (LA-ICP-MS), rather than less common multi-collector systems required to detect variation in natural

isotope ratios or isotope ratio mass spectrometers (Kennedy et al., 2000, Kennedy et al., 2002, Munro et al., 2008).

To date the stable isotopes that have been used to produce reliable marks are usually ^{137}Ba (Thorrold et al., 2006, Walther & Thorrold, 2006, Munro et al., 2008) and ^{86}Sr (Walther & Thorrold, 2006, Munro et al., 2008), and even fewer have investigated the combined use of multiple isotopes to create multi-isotopic signatures. The accuracy and precision with which marked fish can be identified, with different or a combination of isotopes, has yet to be established and in most cases it is unclear if different signatures created in otoliths will be easy to distinguish. If successful however, isotope/hatchery marks could be used to determine when a recaptured fish was released, its source hatchery, its age and the migratory patterns it displayed between release and re-capture.

1.3 Factors Affecting the Uptake of Elements in Otolith Marking

Mineralisation in otoliths, unlike other calcifying systems such as bones, shell, coral and enamel, takes place in a cellular medium known as the endolymph. The endolymph is a fluid that occurs within the inner ear sac and contains the ionic and organic precursors for the otolith formation (Romanek & Gauldie, 1996, Payan et al., 1999, Payan et al., 2004). From the environment to the otolith, the pathway of ionic uptake is a complex multi-step route involving several successive barriers (gills, intestine, skin, blood, inner ear epithelium, endolymph) (Ibsch et al., 2004, Payan et al., 2004). The otolith calcification process consists of $CaCO_3$ deposition within an organic matrix; whereby Ca^{2+} and HCO_3^- combine to form $CaCO_3$ and H^+ (Payan et al., 2004).

Substitution of elements for Ca into the otolith structure can occur and is dependent on the partitioning of elements across different barriers from the environment into the otolith. Partitioning between environment and otolith can be described by a partition

coefficient (D_{Me}), which is calculated by dividing the metal/calcium (Me/Ca) ratio measured in an otolith by the mean Me/Ca ratio measured in the surrounding environment or water. Values where $D_{Me} = 1$ indicate that there is no elemental discrimination, whereas values of $D_{Me} = 0$ specify that the element is discriminated against (Campana, 1999, Bath et al., 2000, Martin & Thorrold, 2005). Divalent metals, that have an ionic radii similar to Ca^{2+}, such as Sr^{2+}, Ba^{2+} and Mg^{2+}, are generally thought to substitute for Ca^{2+} ions in the otoliths structural lattice (Bath et al., 2000) but divalent metals may still be physiologically regulated.

1.3.1 Barium

Barium has seven naturally occurring stable isotopes, ^{130}Ba (0.11%), ^{132}Ba (0.10%), ^{134}Ba (2.42%), ^{135}Ba (6.60%), ^{136}Ba (7.85%), ^{137}Ba (11.23%) and ^{138}Ba (71.7%) (Rosman & Taylor, 1998). Barium is considered a minor element in otoliths, generally measured at <100 ppm (Campana, 1999). Elemental concentrations of Ba display a close relation with that of the water (Bath et al., 2000, Elsdon & Gillanders, 2003b, Dorval et al., 2007). To date, isotopic marking with Ba isotopes has been successful using three stable isotopes (^{135}Ba, ^{137}Ba and ^{138}Ba) (Munro et al., 2009, Williamson et al., 2009a).

1.3.2 Strontium

Strontium has three naturally occurring stable isotopes, ^{84}Sr (0.56%), ^{86}Sr (9.87%) and ^{88}Sr (82.53%), the other 7.04% is made up of ^{87}Sr which is radiogenic (Veizer, 1989, Rosman & Taylor, 1998). Strontium is a minor element in otoliths, occurring in concentrations >100 ppm (Campana, 1999). The chemical similarities between Ca^{2+} and Sr^{2+} ions allow strontium ions to replace calcium during the process of calcification (Pollard et al., 1999). Strontium is the most widely used element for marking larvae and juveniles (Thorrold et al., 2002). It can take up to 20 days for otoliths to reach Sr

saturation and reflect water concentrations (Elsdon & Gillanders, 2005b). As Sr varies naturally in the environment it needs to be used carefully to ensure elemental Sr marks in fish cannot be mistaken for natural levels (Thorrold et al., 2002). Only one stable isotope of Sr (^{86}Sr) has been used to mark both fish otolith and spines (Munro et al., 2008, Smith & Whitledge, 2011).

1.3.3 Magnesium

Magnesium (Mg) is the eighth most abundant element on earth, the fourth most abundant element in vertebrates and the most abundant divalent cation within cells. Magnesium occurs as three different isotopes in measurable natural abundances, ^{24}Mg (78.7%), ^{25}Mg (10.1%) and ^{26}Mg (11.2%) (Rosman & Taylor, 1998, Maguire & Cowan, 2002). The whole body content of Mg in most animals is around 0.4g·Kg^{-1}, in which, 60-65% resides in the bone, 35% in tissue compartments and 1-2% in extracellular fluids, including the plasma (Maguire & Cowan, 2002). Because many Mg^{2+} salts are highly soluble in water, it is very abundant in aquatic environments and is readily available to cells, unlike some transition metals or even calcium, that in general, precipitate from aqueous solutions at much lower concentrations (Maguire & Cowan, 2002). It has been suggested that Mg is physiologically regulated by fish (Dorval et al., 2007, Hamer & Jenkins, 2007); therefore, the utility of Mg as a marker is unclear at present. No previous studies have investigated enriched Mg isotopes for marking fish.

1.4 Preparing Fish for Stocking and Enhancing Post-Stock Survival

Several methods are now available for marking of hatchery-reared fish stocked into systems where the same species occurs naturally; however, the first few weeks after fingerlings are stocked into a system are critical to the success of enhancement

programs. Losses of over 70% have been observed within the first three days post-stocking (Secor et al., 1995). Survival skills of fish held in hatcheries can be reduced when captive animals do not acquire skills that they require in the wild (Waples & Stagoll, 1997). Differences in foraging behaviour, navigational skills, predator avoidance, social skills, aerobic fitness and wariness of human activities can all lead to behavioural differences between captive and wild counterparts (Waples & Stagoll, 1997).

1.4.1 Preparing fish for stocking

Many fish reared for stock enhancement are confined at high densities to a physical environment entirely lacking in structural complexity, exposure to predation and with feeding requirements met via feeding in a regular fashion with pellets (Brockmark et al., 2007). Fish held under such conditions are less likely to learn feeding and predator avoidance behaviours that are necessary for survival once released into the wild (Brown & Day, 2002). Some hatcheries, alternatively to these rearing methods, rear fish in large outdoor earthen ponds. Pond grow-out allows fish to experience a semi-natural environment, with natural light and temperature fluctuations, and avian predation (Brown & Day, 2002). Fish that experience environmental variability appear to have a more flexible behaviour, adapting to natural environments quicker than fish reared in plain hatchery tanks (Braithwaite & Salvanes, 2005, Brockmark et al., 2007). Suitable hiding structures may alter fish behaviour, reducing the movement of fish by providing a refuge from predators. Recently, Kawabata et al. (2011), found that acclimatising black-spot tuskfish *Choerodon schoenleinii* to shelters prior to exposure to white-streaked groupers *Epinephelus ongus* enhanced shelter utilization by the tuskfish, decreasing their post-release predation and mortality, in comparison to fish that were not acclimated to shelter.

The two most important behaviours an animal must develop to survive are the ability to eat and avoid being eaten (Olla et al., 1998, Brown & Day, 2002). To reduce cost, many stocked fish are reared on an artificial diet (Brown & Day, 2002) and therefore, these species lack prey recognition and foraging behaviours. For example, turbot *Scophthalmus maximus* which were naive to natural prey were more likely to attack pellets and stones than shrimp in comparison to wild turbot, which only attacked shrimp (Ellis et al., 2002). Similarly, hatchery reared Japanese flounder *Paralichthys olivaceus* juveniles rarely consumed natural prey initially after release (Tomiyama et al., 2011). In addition, having no opportunity to interact with predators prior to release, hatchery fish are often predator naive. Three key behaviours important to develop or reduce predator-induced mortality are (1) avoidance strategies that reduce the probability of encountering predators, (2) predator recognition and detection, and (3) anti-predator response (schooling, fleeing to refuge etc) (Brown & Day, 2002). If hatchery reared fish manage to survive their first few weeks after release, then their chances of long-term survival are greatly increased (Brown & Day, 2002).

Recent studies have examined ways of training fish before stocking as a means of enhancing post-stocking survival (Berejikian et al., 1999, Gazdewich & Chivers, 2002, Darwish et al., 2005). One of the key areas of research is ensuring stocked fish are predator aware. Many species of fish display a fear response when exposed to alarm substances released from the damaged skin of conspecifics. If the alarm substance is experimentally paired with the presentation of a neutral stimulus (e.g. an unknown potential predator), individuals acquire an alarm response (Griffin et al., 2000). Berejikian et al. (1999) predicted that if treating fish with paired alarm signals in the hatchery could achieve acquired predator recognition, then predator detection and survival could be improved after release into natural systems.

1.4.2 Optimising post-release survival

The choice of release site is an important factor for the survival of stocked fish. The stocked environment should be within the historic range of the species, contain suitable habitat with sufficient carrying capacity to sustain the growth of the introduced population, as well as offer protection and refuge from predators (Kleiman, 1989, Fairchild et al., 2005, Kawabata et al., 2011). Stocking experiments have found fish move around more after introduction into an environment where habitat is minimal compared to fish stocked directly within suitable habitat ranges (Taylor et al., 2006). When suitable habitat within stocking areas are not available, habitat restoration could provide suitable structure. By placing artificial habitat within brown trout *Salmo trutta* stocking locations, a greater number of favourable habitat locations were created not only for the brown trout, but for other fish and macro-invertebrates (Huusko & Yrjänä, 1997).

1.5 Fish Stocking in the Murray-Darling Basin

The Murray-Darling Basin (MDB), Australia, comprises approximately 5300 km of river, draining an area of 1.073 million km^2 (Walker, 1992, Humphries et al., 1999). The Basin is a semiarid river system and is subject to large fluctuations in discharge associated with floods and droughts (Walker, 1992). The MDB is home to approximately 50 fish species, but only 22 native species complete their entire life cycle within the freshwater river system. Of the others, 9 are introduced, 13 are primarily marine or estuarine and 5 are diadromous (Cadwallader, 1986). In addition to adapting to the irregularity of the system, increased pressure has been placed on the system since European settlement, that includes barriers to movement, river regulation, changes to water quality, vegetation and soil erosion resulting in a severe decline in the abundance and range of many freshwater species (Cadwallader, 1986, Koehn, 2004, Baumgartner

et al., 2006). Native fish distribution and abundances are currently estimated to be 10% of their pre-European levels (Barrett, 2004).

Stock enhancement programs for freshwater fish have been established across the MDB to restore populations and provide recreational opportunities (PIRSA, 1999). Through these programs, millions of juvenile Australian native freshwater fish are produced annually and stocked into public reservoirs and rivers throughout the basin (Brown & Harris, 1995, Gillanders et al., 2006). Some of the key species stocked in the MDB are golden perch *Macquaria ambigua*, silver perch *Bidyanus bidyanus* and Murray cod *Maccullochella peeli*. Golden perch occur mainly in the Murray-Darling system, Lake Eyre drainage system and some coastal streams of southern Queensland and northern New South Wales (NSW). Silver perch inhabit open waters throughout the MDB and within south-eastern Queensland (Lintermans, 2007), while Murray cod are distributed within the Murray-Darling region ranging from south-eastern Queensland throughout NSW and South Australia (SA) (Lintermans, 2007). The Murray cod is the largest freshwater fish in Australia and is the longest living percichthyid in Australia (Anderson et al., 1992a). Currently there is no way to evaluate the effectiveness of stocking programs within the MDB, as stocked fish are not routinely marked or tagged prior to release.

1.6 Thesis Outline

1.6.1 Aims and objectives

The primary aim of this study is to develop, test and evaluate stable isotope marking methods that can be easily incorporated into fish hatchery operations in both the MDB and elsewhere. I use detailed experiments to investigate the elemental incorporation into otoliths to increase our understanding of the processes that determine the success of

elemental marking. A final aim of the thesis is to explore ways to enhance post-stock survival by means of predator awareness training and optimising stocking conditions. Specific objectives of the study are:

- Determine concentrations and length of exposure required to produce a reliable isotope mark in otoliths of fish
- Develop hatchery marking protocols using enriched stable isotopes to batch mark the otoliths of hatchery fish
- Determine the ability to discriminate between different/similar isotope marks in the otoliths of fish, and evaluate the range of marks which can be used
- Investigate the incorporation of Mg into otoliths of freshwater fish; and whether otolith Mg concentrations are influenced by water or diet
- Determine if pre-stock exposure to predator odours and alarm substances influences the behaviour of stocked fish
- Investigate the influence that habitat structure and stocking density have on behavioural patterns of stocked fish

1.6.2 Chapter summary

*Chapter 2 Using enriched stable isotopes of barium and magnesium to batch mark otoliths of larval golden perch (*Macquaria ambigua*, Richardson)*

Golden perch are the most commonly stocked fish in the Murray-Darling Basin, yet methods for routinely marking these fish are only just being developed. Isotope techniques have previously been used to mark golden perch at the fingerling stage (Munro et al., 2008) and via embryo transfer (Munro et al., 2009), however a more cost-effective method may be to mark fish at the larval stage. This chapter investigates several isotopes of barium (^{136}Ba, ^{137}Ba and ^{138}Ba) and magnesium (^{24}Mg, ^{25}Mg and

^{26}Mg) for marking larval golden perch. First, optimal concentrations of isotopes and length of exposure were tested to determine the time it took otolith chemistry to reflect water chemistry. Marking trials were then carried out using a combination of enriched stable isotopes to create fifteen possibly distinctive signatures in larval otoliths.

Chapter 3 Determining mark success of fifteen combinations of enriched stable isotopes for batch marking larval otoliths

Murray cod, like golden perch, are also commonly stocked into the MDB, however they have a different reproductive and early life history to that of golden perch. In this chapter, I determine if Murray cod larvae can be held in static water over the endogenous feeding stage to allow for isotope immersion marking. In addition, I look at the creation of multiple signatures with four stable isotopes from three elements (^{137}Ba, ^{138}Ba, ^{88}Sr and ^{24}Mg), investigate the marking success of these isotopes, and how well different isotopic signatures can be distinguished from each other.

Chapter 4 Incorporation of magnesium into fish otoliths, determining contribution from water and diet

The first two chapters (2 & 3) showed that isotopes of Mg were poor markers of fish otoliths, as Mg isotopes were not incorporated into the otolith in proportion to Mg water concentrations. This chapter investigates the relation between Mg in the water and diet, and Mg in otoliths, to determine if fish regulate Mg incorporation into the otolith. Furthermore, by enriching the diet with ^{26}Mg I test the possibility to produce a signature shift in otoliths from the diet that can be used to determine the main contributor of otolith Mg (i.e. is otolith Mg sourced from water or diet).

Chapter 5 Effects of alarm stimuli, stocking density and habitat structure on the behaviour of hatchery-reared fish in the presence of an inter-specific predator

The most crucial time for the survival of stocked fish is the first few weeks after stocking, as fish adjust and become accustomed to their new environment. Hatchery reared fish are predator naive and can be easy prey for predators. Exposing fish to predator odours and alarm cues can provide a means of training fish to the presence of predicted predators. This chapter looks at the behaviour of stocked golden perch at different densities, with and without habitat structure, after being exposed to a predator odour or an alarm signal.

*Chapter 6 General discussio*n

Chapter 6 contains a general discussion of the four data chapters. A summary of the major findings is given with discussion of their implications not only for the Murray-Darling Basin, but also from a global perspective. Areas for further study are discussed.

1.6.3 Notes on structure and style

Each data chapter has been written as an independent manuscript for publication. Therefore, chapters 2-5 contain a list of co-authors, their associated affiliations along with their contribution to the manuscript and their permission to include the chapter in this thesis. Each chapter also has an independent abstract and acknowledgements. A comprehensive reference list for all chapters is included at the end of the thesis. Chapters 2 and 3 have been accepted for publication.

1.6.4 Appendices

Appendix A

This appendix provides an independent study of the difference in the incorporation of Ba and Sr into the otolith from diet and water in regards to temperature or salinity for

juvenile black bream. I assisted with the experimental design, data interpretation and writing of this study. The aim of this study was to determine if environmental factors such as temperature and salinity influence the elemental incorporation of Ba or Sr into otoliths, sourced from water or diet. Enriched stable isotopes were used to trace elemental sources from both diet and water.

Appendix B

Appendix B contains the full terms and conditions for the copyright agreement from John Wiley and Sons, to include Chapter 2 in this thesis.

Chapter 2 Using enriched stable isotopes of barium and magnesium to batch mark otoliths of larval golden perch (*Macquaria ambigua*, Richardson)

Photo: Set up of isotope immersion tanks for larval marking

Chapter 2 Preamble

This chapter is a co-authored paper published in the journal Ecology of Freshwater Fish. It is included with permission from John Wiley & Sons (see Appendix B), and can be cited as:

Woodcock SH, Gillanders BM, Munro AR, McGovern F, Crook DA & Sanger AC (2011) Using enriched stable isotopes of barium and magnesium to batch mark otoliths of larval golden perch (*Macquaria ambigua*, Richardson). Ecology of Freshwater Fish, 20: 157-165

In the paper Gillanders, Crook and Sanger contributed to the conception and design of the project, while Munro, McGovern and myself where involved with the acquisition and analysis of data. I was responsible for the drafting of the manuscript with all authors involved in revising of the manuscript for publication.

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Using enriched stable isotopes of barium and magnesium to batch mark otoliths of larval golden perch (*Macquaria ambigua*, Richardson)

2.1 Abstract

Enriched stable isotope immersion techniques were used to mark the otoliths of larval golden perch (*Macquaria ambigua*) immediately post-hatch. Two experiments were undertaken: the first involved rearing larvae in water enriched with three concentrations of ^{137}Ba for 1 to 5 days. Marks were produced in as little as 1 day; however, otolith isotope ratios reached equilibrium with the water in 5 days at 90 $\mu g \cdot L^{-1}$. The second experiment involved rearing larvae in isotope-enriched water with combinations of stable isotopes of Ba and Mg for 4 days after hatching. Seven significantly different isotopic signatures were produced using three Ba isotopes, which were reflective of the water. Only slight differences were found in otoliths of larvae that were reared in combinations of Mg isotopes, which did not reflect the water chemistry. The length of golden perch at 3 weeks of age showed that isotope immersion did not negatively affect early growth.

2.2 Introduction

Wild fisheries are under threat globally, with one fishery in four collapsing since 1950, due primarily to habitat degradation and overexploitation (Brown & Day, 2002, Mullon et al., 2005, Ward, 2006). One management response to the decline of fisheries is "stock enhancement" (Molony et al., 2003). The objective of stock enhancement programs is to increase the abundance of a particular stock by introducing artificially reared fish into the wild. However, the success of many enhancement programs is unclear (Brown & Day, 2002, Molony et al., 2003). An essential aspect in evaluating the success of an enhancement program is being able to identify and distinguish

between hatchery-produced fish and naturally occurring fish after stocking (Taylor et al., 2005b).

A range of methods are available to identify hatchery-produced fish, including dart, anchor and coded wire tags (Ingram, 1993, Booth & Weyl, 2008), fin clipping (Hansen, 1988), branding, tattooing and thermal marking (Refstie & Aulstad, 1975, Volk et al., 1999), as well as staining with fluorescent compounds such as calcein and alizarin (Secor et al., 1995, Crook et al., 2007, Crook et al., 2009). The artificial incorporation of identifiable isotopic signatures into the otoliths of fish has also recently been developed as a means of mass marking fish, both in the wild (Thorrold et al., 2006, Almany et al., 2007) and in hatcheries (Munro et al., 2008, Munro et al., 2009). Artificial chemical signatures have been introduced into fish otoliths via injection of enriched stable isotopes into brood fish to mark the progeny (Thorrold et al., 2006, Almany et al., 2007, Munro et al., 2009) or via direct immersion of fish into solutions of rare earth elements or enriched stable isotopes (Ennevor & Beames, 1993, Munro et al., 2008).

Munro et al. (2008) demonstrated that immersing juvenile golden perch, *Macquaria ambigua* (Richardson), in solutions of enriched stable isotopes of Ba and Sr for as few as 8 days resulted in a shift in the otolith isotope ratio, which could then be used to identify batches of fish. Among the benefits of this type of enriched stable isotope marking is that it requires no individual handling of fish and only very low concentrations of each isotope, thus making it a low cost marking method with negligible stress to the fish. However, a disadvantage of the technique described by Munro et al. (2008) is that standard hatchery protocols for the production of many species, including golden perch, do not include a hatchery holding period of 8 days or more in a static system at the juvenile (fingerling) stage. For instance, after harvesting

from earthen grow-out ponds, golden perch fingerlings are usually held in hatchery tanks for less than 2 days prior to being transported for stocking (S. Thurstan. pers. comm.). Because isotope marking of golden perch at the fingerling stage would require major alterations to existing hatchery protocols, it is unlikely to be adopted routinely for such species. An alternative approach is to mark hatchery fish at the larval stage; a period during which it is often routine, or at least feasible, to hold fish at very high densities in static water in which concentrations of enriched stable isotopes can be readily controlled. To our knowledge, mass marking of larvae via immersion in enriched stable isotopes has not been previously attempted.

There has also been very limited work on the range of isotopes that can be used to mark otoliths. To date, ^{137}Ba has been the most widely investigated isotope of barium and has been used to mark otoliths of different species of fish using various methods (Thorrold et al., 2006, Walther & Thorrold, 2006, Munro et al., 2008, Munro et al., 2009). Barium has seven isotopes (Rosman & Taylor, 1998) that could potentially be used to provide unique batch marks. No previous work has investigated using stable isotopes of magnesium (Mg) as a marker, although like Ba, it is thought to substitute for Ca, in the otolith matrix (Campana, 1999). Mg has three stable isotopes that exist in measurable natural quantities (Rosman & Taylor, 1998, Maguire & Cowan, 2002). A relationship exists between otoliths and water for Ba isotopes (Munro et al., 2008) where the ratios recorded in the otoliths reflect that of the water. Relationships between the environment and uptake of Mg isotopes into the otolith have not been previously investigated (Campana, 1999, Martin & Thorrold, 2005). Several researchers suggest that Mg might be physiologically regulated by fish (Shearer & Asgard, 1992, Martin & Thorrold, 2005, Dorval et al., 2007), and it is therefore unclear if shifts in the Mg isotope ratios in the water will be reflected in the otoliths.

The primary objective of the current study was to expand on the methods described by Munro et al. (2008) to evaluate practical techniques for mass marking golden perch otoliths via immersion in solutions of enriched stable isotopes at the larval stage, that limit disruption to standard hatchery protocols. First, we determine the concentrations and lengths of exposure required to produce unambiguous marks and investigate a range of stable isotope combinations to produce distinctive marks. Second, we examine relationships between otolith isotopic signatures and ambient water chemistry to explore the mechanisms that influence uptake of isotopes. Finally, we examine whether there are any effects of the marking procedure on mortality and growth rates.

2.3 Methods

2.3.1 Study species

Golden perch is native to waterways of inland Australia and is a popular angling species that grows to >5 kg. It is the most widely stocked native species within the Murray-Darling Basin (MDB), with stocked fish usually released as fingerlings of 30-40 mm total length (TL). During culturing, golden perch larvae are held in tanks in the hatchery for the first 4-6 days after hatching, while they feed endogenously on the yolk. Once the larvae begin exogenous feeding, they are transferred to grow-out ponds for 3-4 months. The fingerlings are then harvested and held in the hatchery for approximately 24 hours in flow-through tanks for assessment of condition prior to release. Therefore, the initial stage after hatching provides an ideal opportunity to enrich rearing tank water to mark larval otoliths, as it does not interrupt standard hatchery operating protocols and fish are held at a higher density than is typical at later life history stages.

2.3.2 Rearing conditions

For this study, golden perch were spawned at the Narrandera Fisheries Centre (NFC), Narrandera, New South Wales (NSW), using standard hormonal induction techniques. Eggs were incubated using methods similar to those described by Rowland (1996). Isotope enrichment took place during the first 4-5 days after hatching, in 1-L aquariums. For experimental purposes, a grow-out period of 2 weeks was achieved whereby treated fish were reared in 5 L of natural (un-enriched) water in dark blue plastic buckets. The buckets were cleaned daily to remove waste from the bottom and water changed every 4-5 days by siphoning through a PVC pipe fitted with a fine screen. All aquariums and buckets were pre-washed, rinsed and dried prior to use. Aquaria and buckets were all filled with bore water, the temperature was maintained throughout the experiment by placing aquariums and buckets into one of two flow-through water baths that were supplied with bore water. Temperature was recorded twice daily in each water bath.

2.3.3 Determining optimal concentration and length of exposure for isotope marking

Immediately after hatching golden perch larvae were transferred to 1-L treatment aquariums, at densities of 400-500 larvae·L^{-1} and were reared in isotope enriched water at different concentrations of ^{137}Ba (30, 60 or 90 μg·L^{-1}), for 1, 2, 3 or 5 days, resulting in 12 treatments. A control treatment in which fish were held in water with no enriched ^{137}Ba was also established. Two replicates of each treatment and control were used.

2.3.4 Creating multiple batch marks

Golden perch larvae were transferred to 1-L treatment aquariums at an approximate density of 200 larvae·L^{-1} immediately after hatching and were reared in isotope enriched water for 4 days using various combinations of enriched isotopes of ^{136}Ba, ^{137}Ba, ^{138}Ba, as well as ^{24}Mg, ^{25}Mg and ^{26}Mg (Table 2.1). Concentrations of 100 μg·L^{-1}

were used for each Ba isotope and 4000 $\mu g \cdot L^{-1}$ for Mg isotopes. Each treatment and control had a replicate tank, with 32 tanks in total.

Table 2.1: List of treatments used for isotope induced marks in the otoliths of larval golden perch in the multiple batch marks experiment.

Isotope Induced Signatures		
Ba Isotopes	Mg Isotopes	Ba and Mg Isotopes
^{136}Ba	^{24}Mg	^{137}Ba & ^{24}Mg
^{137}Ba	^{25}Mg	
^{138}Ba	^{26}Mg	Control
^{136}Ba & ^{137}Ba	^{24}Mg & ^{25}Mg	No enrichment
^{136}Ba & ^{138}Ba	^{24}Mg & ^{26}Mg	
^{137}Ba & ^{138}Ba	^{25}Mg & ^{26}Mg	
^{136}Ba, ^{137}Ba & ^{138}Ba	^{24}Mg, ^{25}Mg & ^{26}Mg	

2.3.5 Grow-out of larvae

At the end of the exposure time (1-5 days, dependent on treatment), each experimental tank was transferred into a larger grow-out bucket and enriched water was replaced with clean natural water until the yolk was absorbed, with final densities being reduced to 100-200 $larvae \cdot L^{-1}$, similar to grow-out pond densities. Upon switching to exogenous feeding, larvae were fed multiple times per day on zooplankton harvested from ponds or with *Artemia sp.* nauplii reared in the hatchery using standard techniques. All buckets were treated with 3 $g \cdot L^{-1}$ of sea salt to assist longevity of the live food source. Twenty larvae per treatment were collected 14-days after first feed, and stored in 70% ethanol until analysis. Some larval samples were collected before the 14 days if a large number of mortalities occurred within a tank. Length of larval golden perch was recorded before the dissection of each fish.

2.3.6 Water samples

For each experiment, water samples from each tank were collected twice: once after the initial spiking of the isotopes and then before fish were transferred into the grow-out buckets. Water samples were taken using a 20 mL syringe and filtered through a 0.45 μm filter into acid washed 30 mL plastic vials containing 0.5 mL of concentrated nitric acid. The water samples were stored frozen until analysis.

Water samples were analysed using ICP-MS (Inductively Coupled Plasma-Mass Spectrometry). Samples were tested against standards with known elemental concentrations and isotope ratios, to confirm that the treatments were spiked at the appropriate levels as well as to determine the contribution of isotopes in the water to the ratios in the otoliths of larval golden perch. Water samples were analysed for ^{138}Ba and ^{137}Ba for the optimal concentration and exposure experiment and for all three Ba and Mg isotopes used for the multiple batch marks in the second experiment (Table 2.1).

2.3.7 Otolith analyses

Otolith pairs from larval golden perch (a random sample of 10 fish per tank) were extracted and mounted whole onto microscope slides as per methods described by Munro et al. (2009). Otoliths from each experimental treatment were randomly analysed using Laser Ablation-ICP-MS (see Table 2.2 for laser and ICP-MS parameters). Vertical depth profiles were taken through the nucleus of the otolith using standard spot analysis (Munro et al., 2009). A reference standard (NIST 612, National Institute of Standards and Technology) was analysed periodically to determine correction factors. From the resulting data, three ratios were calculated for Ba (^{138}Ba/^{137}Ba, ^{138}Ba/^{136}Ba and ^{137}Ba/^{136}Ba) as well as for Mg (^{26}Mg/^{25}Mg, ^{26}Mg/^{24}Mg and ^{25}Mg/^{24}Mg). The maximum or minimum values in each depth profile for each

isotope ratio were recorded from the otolith data smoothed with a six point moving average (see Munro et al. 2008). Minimum values were recorded when there was a visible decrease from the natural ratio, resulting from enriching with the lighter of the isotopes, and maximum values used when there was a visible increase in the natural ratio, occurring from enrichment with the heavier isotope. Due to smaller isotopic shifts with the enrichment of lighter isotopes, compared to heavier isotopes, minimum values were taken for control treatment fish to determine mark success.

Table 2.2: Operating parameters for the New Wave Nd Yag 213 nm UV laser and Agilent 7500cs inductively coupled plasma-mass spectrometer (ICP-MS).

Laser		
Wavelength	213 nm	
Mode	Q-switched	
Frequency	5 Hz	
Spot Size	55 μm	
Laser Power	55%	
Carrier gas	Ar (0.92 $l.min^{-1}$)	
ICP-MS		
Optional gas	He (59.5%)	
Cone	Pt	
Dwell Times	^{136}Ba, ^{137}Ba	(400 ms)
	^{25}Mg, ^{26}Mg ^{138}Ba	(300 ms)
	^{24}Mg	(200 ms)

2.3.8 Statistical analysis

Univariate statistical analyses and linear discriminant function analysis were conducted using SPSS 15.0 or the updated software, PASW Statistics 17 for Windows (www.spss.com) and multivariate statistical analyses were conducted using PRIMER 6/PERMANOVA+ (www.primer-e.com). Data for determining optimal concentrations and exposure times for both the water and otoliths were analysed with a two-factor analysis of variance (ANOVA) with length of immersion and enriched Ba concentration as fixed factors. In addition, for the water analysis in the optimal concentrations and

exposure time experiment, replicate tanks were treated as a random factor (nested within the immersion × concentration term) whereas otolith were treated as a fixed factor and replicate tanks were pooled. Within the multiple batch marks experiment, one-factor ANOVAs were performed on each of the six isotope ratios to test for differences between the treatments for both the water and the otoliths. Tanks from the multiple batch mark experiment were pooled together, as preliminary analysis showed no tank effect present. If significant differences occurred within the six isotope ratio treatments, post-hoc Tukey's tests were used to identify which treatments differed, in particular whether experimental treatments differed from the controls, and whether isotope enriched treatments differed from each other. Where data did not meet assumptions of normality and homogeneity of variance, natural log transformations were applied for ANOVA analysis. In addition, a multivariate analysis of variance (PERMANOVA) was performed for the Ba and Mg isotope ratios to determine which treatments differed for the three combined isotope ratios (see Munro et al. 2008). Multidimensional scaling plots with stress coefficients were then produced. The stress coefficients provide an indication of the goodness of fit, and tend to zero when the data are perfectly represented (Clarke, 1993).

Mark success was determined from the percentage of otoliths that could be correctly classified into the treatment groups using linear discriminant function analysis (DFA; PASW). DFA takes a random sample of the data to establish a model; this model is then used to classify the remaining data and determine the percentage of fish that could be re-assigned to their correct enriched isotope treatment, as well as the percentage of fish misclassified and the treatment they were assigned. For the creating multiple batch marks experiment differences in the length of fish at the end of the 14-day grow-out

period and comparisons between the isotope ratios of the otoliths compared to the water samples were also analysed.

2.4 Results

2.4.1 Determining optimal concentration and length of exposure for isotope marking

The $^{138}Ba/^{137}Ba$ ratio in the water was significantly different for all three concentrations (Table 2.3). There was no detectable change in the $^{138}Ba/^{137}Ba$ ratio in the water over the course of the enriched isotope rearing period (Table 2.3). There was a significant tank effect that was a result of one or two pairs of the treatment tanks having slightly different Ba isotope ratios ($P < 0.001$). Given the difference in mean $^{138}Ba/^{137}Ba$ ratios in the water among the concentration levels, these small within-treatment variations were considered minor.

The $^{138}Ba/^{137}Ba$ ratios in the otolith varied for both the concentration of enriched ^{137}Ba and the length of exposure (Figure 2.1, Table 2.3). As the length of exposure to enriched isotopes was increased, the $^{138}Ba/^{137}Ba$ ratio decreased (Figure 2.1). The greatest change in $^{138}Ba/^{137}Ba$ ratios in the otoliths of larval golden perch occurred in the 5-day exposures where the Ba ratios decreased from the natural ratio of 6.38 to less than 1.0 for all concentrations (Figure 2.1). The same trend was seen with concentration of enriched ^{137}Ba, where the higher concentrations were associated with a more pronounced decrease in the $^{138}Ba/^{137}Ba$ ratio (Figure 2.1). All treatments were significantly different to the average minimum ratio in control fish (5.30 ± 0.48). Post-hoc tests indicated that the fish exposed to 30 $\mu g \cdot L^{-1}$ of ^{137}Ba for 1-day were significantly different to the 2, 3 and 5 day exposure times at the same concentration (Figure 1). One day exposures were also significantly different from the 3 day exposure time for the 60 $\mu g \cdot L^{-1}$ as well as the 3- and 5-days in the 90 $\mu g \cdot L^{-1}$ (Figure 2.1).

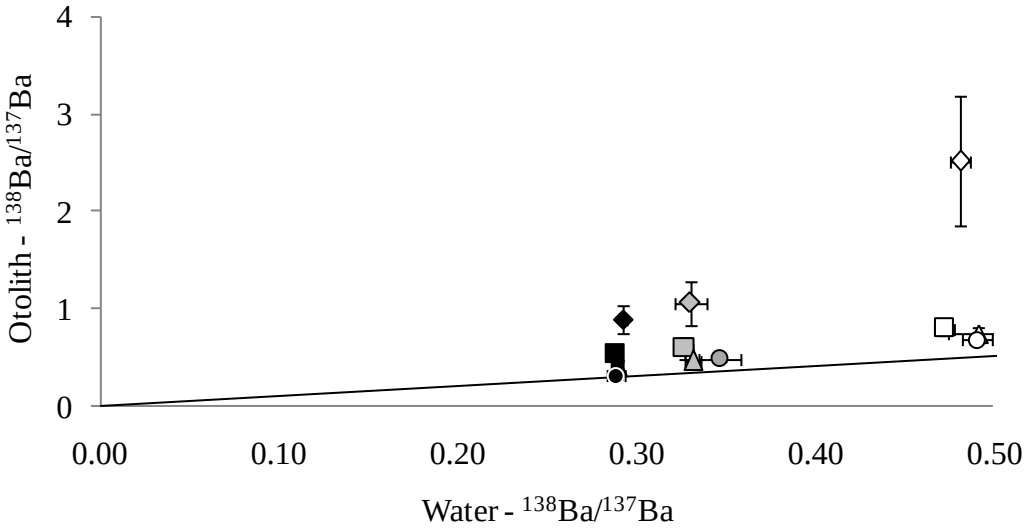

Figure 2.1: Relation between mean $^{138}Ba/^{137}Ba$ ratio, with standard error, in otoliths of golden perch and rearing water for optimal concentration and length of exposure experiment. The natural $^{138}Ba/^{137}Ba$ ratio is 6.38. Black = 90 µg·L^{-1}, grey = 60 µg·L^{-1} and white = 30 µg·L^{-1}, with ◇ 1, □ 2, △ 3 and ○ 5 days. Solid line is the 1:1 relation between the otolith and the water.

Table 2.3. ANOVA results for the difference in ^{137}Ba concentration (30, 60 or 90 µg·L^{-1}) and length of exposure (1, 2, 3 and 5 days) for $^{138}Ba/^{137}Ba$ ratio in the rearing water and larval golden perch otoliths.

	d.f.	MS	*F*	*P*
Water				
Concentration	2	0.134	317.920	<0.001
Exposure	3	≤0.001	0.630	>0.050
Concentration × Exposure	6	0.012	0.287	>0.050
Tank (Concentration × Exposure)	12	≤0.001	4.614	<0.001
Within Groups	24	≤0.001		
Otolith				
Concentration	2	3.505	17.725	<0.010
Exposure	3	4.851	24.528	<0.010
Concentration × Exposure	6	1.150	5.813	<0.001
Within Groups	53	0.198		

2.4.2 Creating multiple batch marks

Isotopic ratios were successfully altered with the addition of Ba and Mg isotopes into the rearing water of golden perch. Significant differences were found between the water for all Ba enriched treatments ($P < 0.001$) for each of the three Ba isotope ratios (Table 2.4). For the Mg enriched treatments, significant differences were found for the three Mg isotope ratios (Table 2.4, $P < 0.05$); however, post-hoc analysis found no difference between the control and the ^{24}Mg enriched treatment ($P > 0.05$).

Significant differences were found for marks in the otoliths of golden perch produced using combinations of enriched Ba isotopes (Table 2.5). Post-hoc analysis indicated all Ba isotope marks were significantly different from the average minimum ratio of the control treatment as well as from the natural ratio calculated from the natural abundance of isotopes ($P < 0.01$) (Figure 2.2a). There were also significant differences found between the seven treatments enriched with Ba isotopes ($P < 0.05$) (Figure 2.2a). Multivariate analysis of variance of the Ba enriched isotopes found significant differences among treatments (Table 2.5). Post-hoc tests showed that there were significant differences between the control ratios and all enriched treatments. There were also significant differences among all Ba enriched treatments, except for two combinations (^{136}Ba + ^{137}Ba was similar to the ^{136}Ba only treatment, as well as the ^{136}Ba + ^{137}Ba + ^{138}Ba treatment) (Figure 2.2a). As expected, there was no difference in the Ba isotope ratios for the ^{137}Ba and the ^{137}Ba + ^{24}Mg enriched treatments (Figure 2.2c). Using discriminant analysis, 93.8% of otoliths could be correctly assigned to their correct treatment and therefore considered successfully marked.

For the Mg isotope enriched treatments, significant differences were found for the marks created in the otoliths (Table 2.5). Post-hoc analysis revealed differences between marked otoliths and the control fish for all treatments (Figure 2.2b) except two

(the single ^{24}Mg and the combined ^{24}Mg + ^{25}Mg + ^{26}Mg enriched treatment) (P > 0.490). Multivariate analysis detected significant differences among the Mg enriched treatments (Table 2.5). Mark success in the Mg isotope treatments was lower than the Ba isotope treatments with only 62.5% of otoliths correctly classified to their isotope treatment using discriminant analysis.

Comparisons of the isotope signatures recorded in the otoliths with those of the corresponding spiked water showed a strong positive relationship (R^2 > 0.90) for the three Ba ratios (Figure 2.3). These relationships were similar to a 1:1 ratio between the otolith and the water for all Ba isotopes (Figure 2.3). In contrast, Mg isotopes showed a poor correlation (R^2 > 0.60) between the ratios in the otoliths and that in the water (Figure 2.4). The Mg isotope shifts in the otoliths were not a direct reflection of the water, and were significantly lower than that expected based on the water isotope ratios (Figure 2.4).

2.4.3 Effects of marking on growth

Due to the delicate nature of larvae, it was not possible to measure length at hatching. It was assumed that there were no significant differences in length of the fish at the time of marking as larvae were selected randomly from the same spawning event and from a single incubation tank. At the end of the two-week grow-out period for the multiple batch mark experiment, golden perch larvae had a mean length of 7.7 ± 0.07 mm TL. There were significant differences in lengths between some of the treatments (d.f. = 15, 203 F = 5.313, P < 0.001), however post-hoc analysis found none of the enriched isotope treatments had a significantly different length to the control treatment.

Table 2.4. ANOVA results for the difference in isotope ratios for the enriched water used to mark golden perch larvae in the multiple batch marks experiment.

Ratios		d.f.	MS	*F*	*P*
Log(^{138}Ba/^{137}Ba)	Between Groups	8	31.403	94.368	<0.001
	Within Groups	27	0.333		
Log(^{138}Ba/^{136}Ba)	Between Groups	8	19.463	1125.409	<0.001
	Within Groups	27	0.017		
Log(^{137}Ba/^{136}Ba)	Between Groups	8	11.004	54.969	<0.001
	Within Groups	27	0.200		
^{26}Mg/^{25}Mg	Between Groups	8	82.332	720.227	<0.001
	Within Groups	25	0.114		
^{26}Mg/^{24}Mg	Between Groups	8	2.637	42.417	<0.001
	Within Groups	25	0.062		
^{25}Mg/^{24}Mg	Between Groups	8	2.218	65.148	<0.001
	Within Groups	25	0.034		

Table 2.5. ANOVA results for the difference in isotope signatures of Ba and Mg and PERMANOVA analysis for the combined ratios for the otoliths of golden perch larvae marked in the creating multiple batch marks experiment.

Treatment		d.f.	MS	*F*	*P*
Log(^{138}Ba/^{137}Ba)	Between Groups	8	17.275	88.358	<0.001
	Within Groups	43	0.196		
Log(^{138}Ba/^{136}Ba)	Between Groups	8	30.443	770.739	<0.001
	Within Groups	43	0.039		
Log(^{137}Ba/^{136}Ba	Between Groups	8	49.318	94.354	<0.001
	Within Groups	43	0.523		
Combined Ba ratios	Between Groups	8	8432	85.106	0.001
	Within Groups	43	99.077		
^{26}Mg/^{25}Mg	Between Groups	8	1.029	38.026	<0.001
	Within Groups	43	0.270		
^{26}Mg/^{24}Mg	Between Groups	8	0.007	8.336	<0.001
	Within Groups	43	0.001		
^{25}Mg/^{24}Mg	Between Groups	8	0.006	15.841	<0.001
	Within Groups	43	<0.001		
Combined Mg ratios	Between Groups	8	1.040	36.659	0.001
	Within Groups	43	0.028		

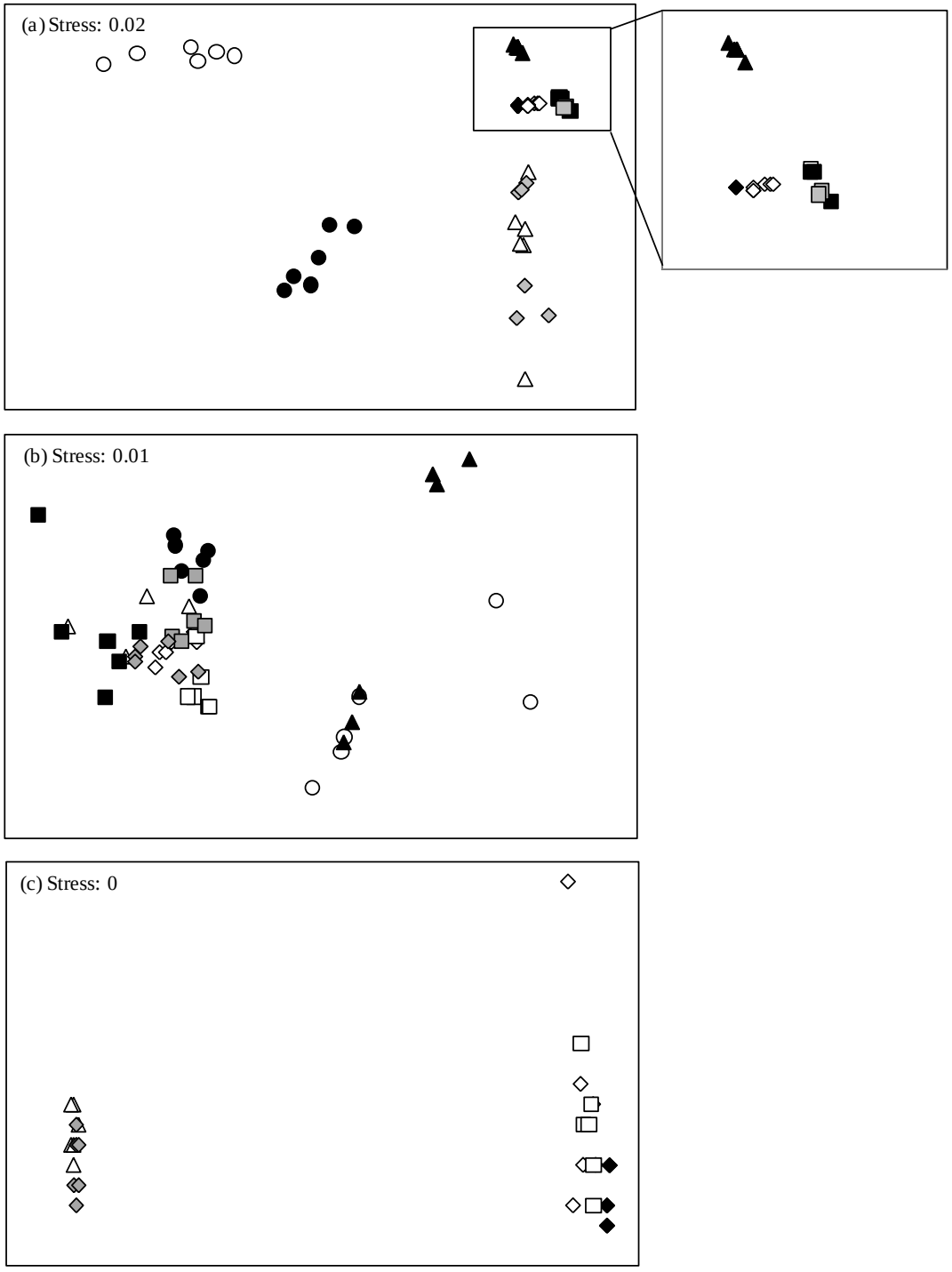

Figure 2.2: Multidimensional scaling plots, displaying multivariate analysis from the multiple batch mark experiment for (a) Ba enriched treatments ◆ Natural, ◇ Contro1, □ ^{136}Ba, △ ^{137}Ba, ○ ^{138}Ba, ■ ^{136}Ba & ^{137}Ba, ▲ ^{136}Ba & ^{138}Ba, ● ^{137}Ba & ^{138}Ba, ▣ ^{136}Ba, ^{137}Ba & ^{138}Ba, ◈ ^{137}Ba & ^{24}Mg, (b) Mg enriched treatments ◆ Natural, ◇ Contro1, □ ^{24}Mg, △ ^{25}Mg, ○ ^{26}Mg, ■ ^{24}Mg & ^{25}Mg, ▲ ^{24}Mg & ^{26}Mg, ● ^{25}Mg & ^{26}Mg, ▣ ^{24}Mg, ^{25}Mg & ^{26}Mg, ◈ ^{137}Ba & ^{24}Mg and (c) comparisons between the combined Ba and Mg enriched treatment ◆ Natural, ◇ Contro1, △ ^{137}Ba, □ ^{24}Mg, ◈ ^{137}Ba & ^{24}Mg.

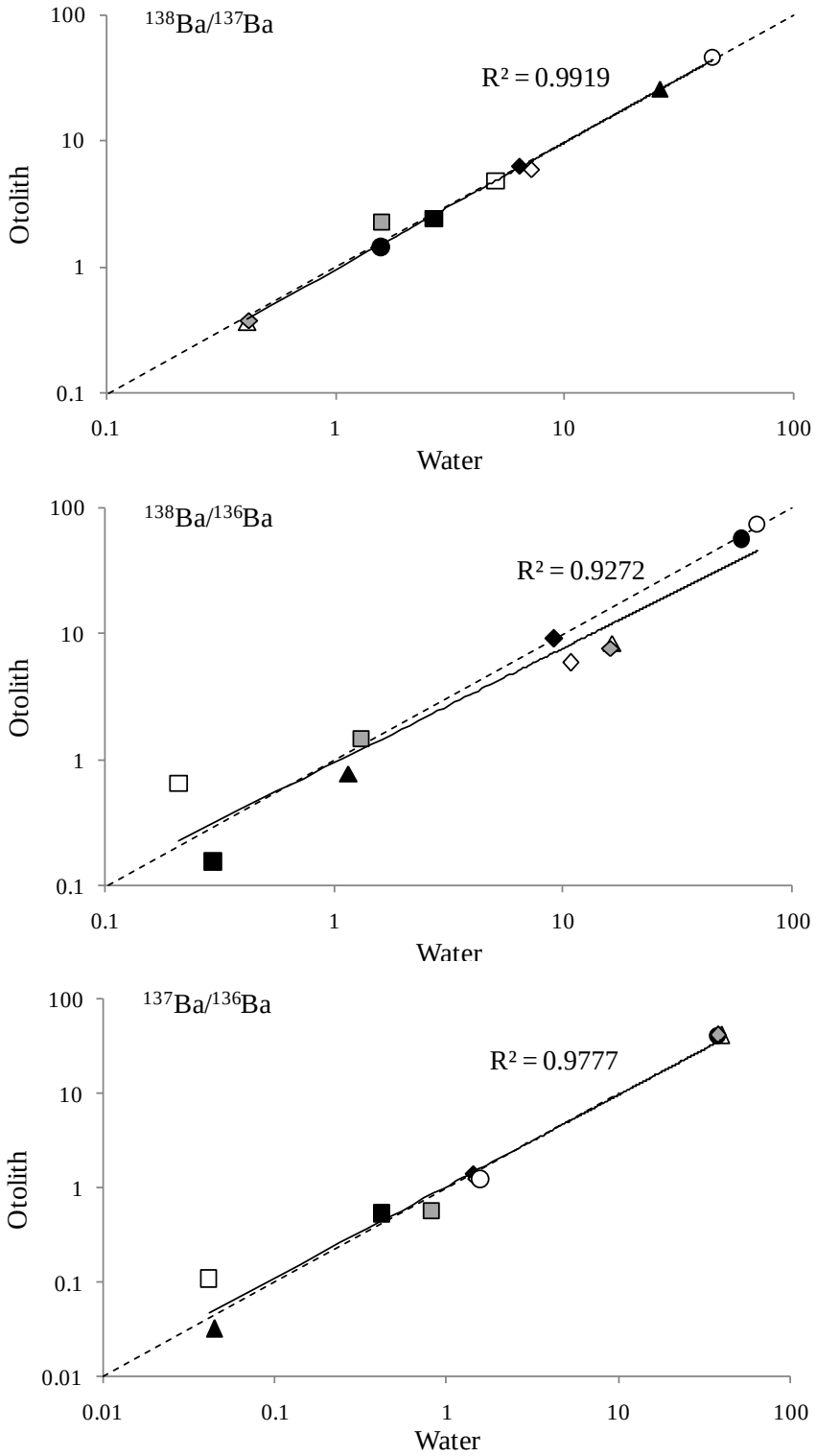

Figure 2.3: Relation between the mean otolith and the water isotope ratios from the multiple batch marks experiment, plotted on a log-scale, for (a) $^{138}Ba/^{137}Ba$, (b) $^{138}Ba/^{136}Ba$ and (c) $^{137}Ba/^{136}Ba$. Broken lines represent a 1:1 relation between otoliths and water, solid line is a line of best fit and symbols represent treatments, ◆ Natural, ◇ Contro1, □ ^{136}Ba, △ ^{137}Ba, ○ ^{138}Ba, ■ ^{136}Ba & ^{137}Ba, ▲ ^{136}Ba & ^{138}Ba, ● ^{137}Ba & ^{138}Ba, ▣ ^{136}Ba, ^{137}Ba & ^{138}Ba, ◈ ^{137}Ba & ^{24}Mg

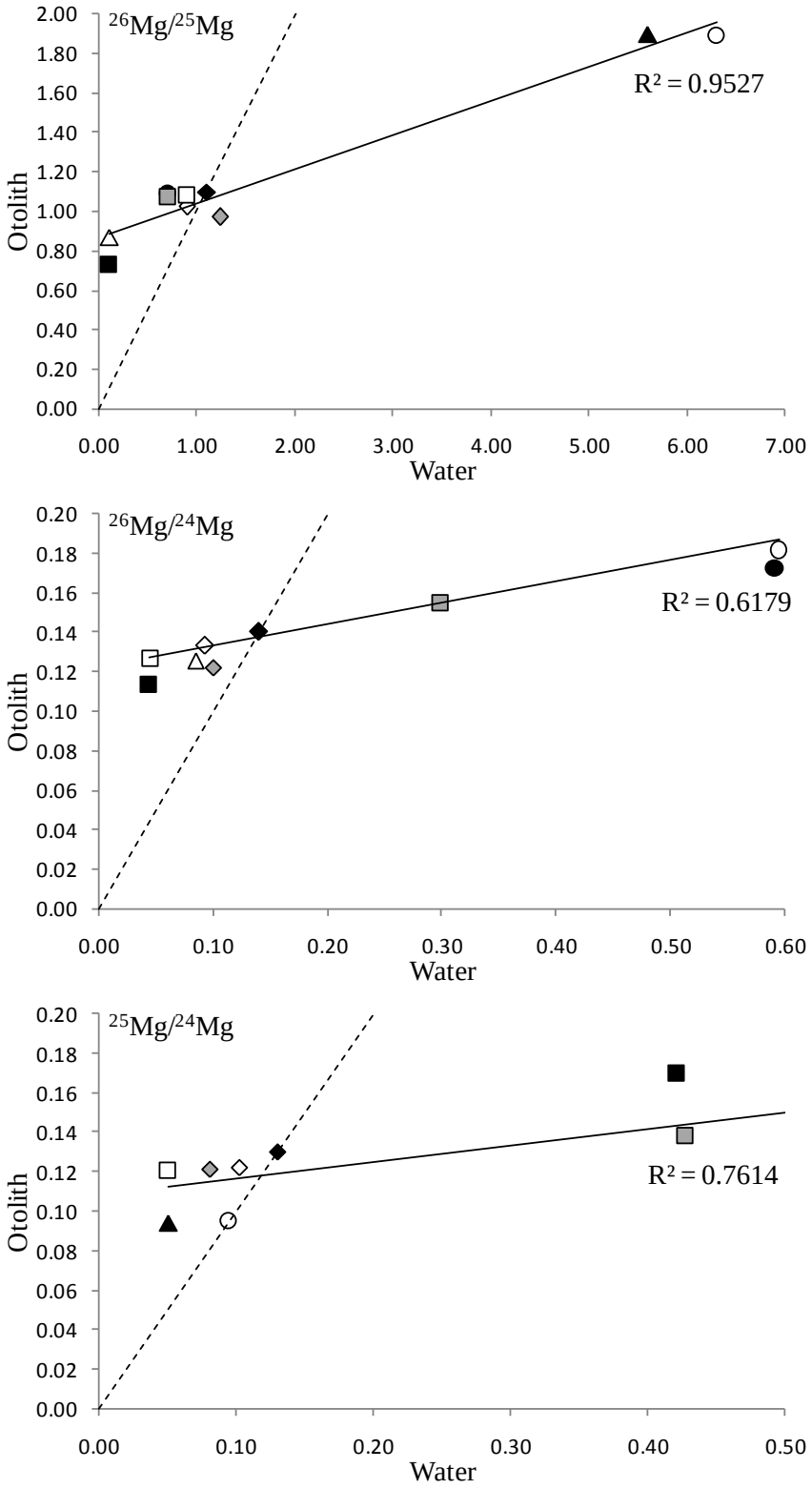

Figure 2.4: Relation between the mean otolith and the water isotope ratios for $^{26}Mg/^{25}Mg$, $^{26}Mg/^{24}Mg$ and $^{25}Mg/^{24}Mg$ from the multiple batch mark experiment. Broken lines represent a 1:1 relation between otoliths and water, solid line is a line of best fit and symbols represent treatments, ◆ Natural, ◇ Contro1, □ ^{24}Mg, △ ^{25}Mg, ○ ^{26}Mg, ■ ^{24}Mg & ^{25}Mg, ▲ ^{24}Mg & ^{26}Mg, ● ^{25}Mg & ^{26}Mg, ▩ ^{24}Mg, ^{25}Mg & ^{26}Mg, ◈ ^{137}Ba & ^{24}Mg. Note the x and y-axis vary among plots.

2.5 Discussion

Recent research has found enriched stable isotopes to be useful for mass marking fish, with clear and distinguishable otolith marks (Thorrold et al., 2006, Munro et al., 2008, Munro et al., 2009). For a marking technique to be successful in large-scale hatchery settings, it needs to be cost effective and easy to administer, fitting within standard operating protocols. Applying isotope signatures at the larval stage of golden perch meets these criteria, as the larvae are routinely held in a static environment for 4-6 days before being stocked into rearing ponds. Enrichment of water can be done in standard larval rearing tanks, and once enriched stable isotopes are added to the water, no additional work is required. As many aquaculture operations require a hatchery for the production of larvae, the methodologies used here could be easily adapted for use with other species, for example silver perch (*Bidyanus bidyanus*), which has a similar rearing protocol to golden perch. Furthermore, the addition of enriched stable isotopes to the water had no detrimental effect on the growth of golden perch larvae when compared to larvae that were reared in water with no isotope enrichment. Similarly, Williamson et al. (2009b) found isotope marking resulted in no long-term health effects in the fish, and that the dose rate was unlikely to impact potential consumers of marked fish.

Marking of golden perch using 30 or 60 $\mu g \cdot L^{-1}$ of enriched ^{137}Ba and exposure time of one day was sufficient to achieve a mark on the otolith that was significantly different from non-marked fish. The Ba isotope ratios in golden perch otoliths reached equilibrium with the water at 90 $\mu g \cdot L^{-1}$ at five days, where otolith isotope ratios were a direct reflection of that spiked in the water. Larvae were held in a static environment for marking, which should be possible for many species, including salmonids, which absorb their yolk before feeding. Even though many larval rearing facilities use flow though water supplies rather than static tank methods, marking success at day one,

suggests that it may be possible to mark larvae of other species. The short duration required to induce detectable isotopic marks in otoliths in as little as one day may limit the physiological stress of the marking methods to acceptable levels for those species that, unlike golden perch, could not be held for four to five days.

The stable isotopes ^{136}Ba, ^{137}Ba and ^{138}Ba produced distinguishable marks when used singularly or in combination. One hundred percent of Ba marked fish could be effectively distinguished from non-marked fish and the different Ba treatments and could be distinguished with a high degree of accuracy (93.8% correctly classified). Shifts in Mg ratios were detected in the otolith despite the non-linearity between otoliths and water ratios. However, the shifts in Mg isotope ratios were not as distinct as with Ba isotope ratios. Only 77% of otoliths displayed an isotope signature distinguishable from non-marked (control) fish. There was little difference between the different Mg isotope enriched treatments due to large variation within a treatment and little difference among treatments, which resulted in only 62% of marked otoliths being correctly classified to their treatment.

As reported in previous research on elemental enrichment (Bath et al., 2000, Elsdon & Gillanders, 2003b, Martin & Thorrold, 2005, Dorval et al., 2007), Ba in the otoliths displayed a close relationship to Ba in the water. The Mg isotope ratios induced into the otoliths, however, were not in equilibrium with the water, despite the water being significantly altered by the addition of the enriched Mg isotopes. Past studies have found the main source of whole body Mg comes from the diet and secondarily from the water (Shearer & Asgard, 1992). As the larvae were feeding endogenously during the marking period, it appears likely that the yolk sac served as a major source of Mg for the larvae. Mg may be regulated by fish, whereby once the basic physiological Mg requirement has been meet, the fish can prevent further uptake (Shearer & Asgard,

1992). This could explain why the amount of Mg in the otoliths varied between and within different treatments, and shifts in the Mg isotope ratios in otoliths were lower than those in the water.

Furthermore, the combined $^{137}Ba + ^{24}Mg$ enriched treatment displayed a smaller shift in the Mg isotope ratio compared to singular ^{24}Mg enriched treatment. Otoliths are held in endolymph, and it is from this fluid that they incorporate the chemical constituents of their environment (Campana, 1999). Only 1 to 2% of Mg ions are taken into the extracellular fluid of fish, including the plasma from which the endolymph is derived (Maguire & Cowan, 2002). Therefore, as the level of Mg in the extracellular fluids is restricted, it would appear that the otolith is not being exposed to changes in the isotope ratios of Mg in the ambient water (i.e. physiological regulation predominates).

The current study suggests that there are a number of advantages to immersion marking of larvae versus marking at other life stages. Larvae are typically held at higher densities compared to fingerlings, which lowers the cost of immersion marking by reducing the volume of enriched water required. In addition, as mentioned above, larvae are often held within the hatchery for extended periods of time allowing for the growth of sufficient new otolith material to effectively incorporate the isotopic signatures. Mark recovery is also made simpler as marking at the larval stage locates the mark signature in the core of the otolith, whereas marking at a later stage, depending upon the size and age of the fish at the time of marking, the mark is located at some distance from the core of the otolith.

Multiple marks, as produced here with Ba isotopes, are relatively easy to create. Being able to produce multiple marks is potentially advantageous in both stock enhancement programs and commercial aquaculture. For example, each hatchery could be provided

with its own unique chemical "tag". The array of marks created could be used in combination with established otolith analysis to identify in which hatchery a fish was produced, the year of the stocking (via annual increment ageing) (Anderson et al., 1992b) or potentially the brood stock from which the fish came. Such detailed information has the potential to greatly increase our understanding of the survival, movements and impacts of hatchery-produced fish.

In summary, there is an increasing need to identify the origins of fish, whether hatchery-produced, naturally occurring, illegally stocked or escapees from aquaculture. Stable isotope marking techniques provide this capability and can be applied at the larval stage with negligible alterations to normal hatchery protocols. Increasing the number of stable isotopes, which can be used to effectively mark larval otoliths, will add to the number of unique signatures that can be created. Broad-scale uptake of methods for enriched stable isotope marking of hatchery fish has the potential to clarify our current uncertainties about the outcomes of stock enhancement programs and could provide an important tool for fisheries managers.

2.6 Acknowledgements

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Chapter 3 Determining mark success of fifteen combinations of enriched stable isotopes for batch marking larval otoliths

Photo: Larval Murray cod in grow-out tanks

Chapter 3 Preamble

This chapter is a co-authored paper in press in the *North American Journal of Fisheries Management*. As of thesis submission, copyright had not been handed over, but will likely be in the near future. This chapter can be cited as:

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In this paper, Gillanders, Crook, Munro, Sanger and Woodcock contributed to the conception and design of the project, while Munro and myself where involved with the acquisition and analysis of data. I was responsible for the drafting of the manuscript with all authors involved in revising of the manuscript for publication.

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Determining mark success of fifteen combinations of enriched stable isotopes for batch marking larval otoliths

3.1 Abstract

Chemical marking otoliths via immersion in solutions of enriched stable isotopes provides a means of distinctively marking large batches of hatchery-produced fish. This study investigated using enriched stable isotopes of barium, strontium and magnesium individually and in combination to determine mark success and the ability to correctly classify 15 unique batch marks in the otoliths of larval Murray cod *Maccullochella peelii peelii*. Marking with enriched stable isotopes ^{137}Ba, ^{138}Ba and ^{88}Sr individually or in combination produced clear and distinctive marks (98% mark success), with 93% of fish correctly classified to their respective isotope mark. Despite altering the Mg isotope ratio in the water, a corresponding shift in the otoliths was not observed (8% mark success), with miss-classification of many Mg enriched fish. Due to its low cost and minimal effects on hatchery protocols, we conclude that marking of hatchery-reared fish at the larval stage with isotopes of Sr and Ba has the potential to be a powerful tool in the production and management of a wide range of fish species.

3.2 Introduction

Otoliths, calcareous structures located in the inner ear of teleosts (Campana & Neilson, 1985), are considered biological archives reflecting age and growth of the fish as well as the chemistry of the water inhabited by a fish. As such, otoliths are routinely used for age and growth estimation, stock discrimination and characterization of life history events (Campana et al., 2000, Payan et al., 2004). Correlations between otolith and ambient water chemistry have been demonstrated for a range of fish species (Bath et al., 2000, Kennedy et al., 2000, Elsdon & Gillanders, 2003b, Dorval et al., 2007), and

several recent studies have shown that it is possible to manipulate elemental concentrations or stable isotope ratios in water to create artificial marks in otoliths (Ennevor & Beames, 1993, Walther & Thorrold, 2006, Munro et al., 2008).

Marking techniques using enriched stable isotopes have been developed using immersion techniques (Walther & Thorrold, 2006, Munro et al., 2008, Woodcock et al., 2011, Chapter 2) and maternal transfer to the embryo (Thorrold et al., 2006, Almany et al., 2007, Munro et al., 2009, Williamson et al., 2009a, Williamson et al., 2009b). Despite a range of elements and isotopes used for marking, few studies have investigated the use of multiple isotopes from different elements (but see Munro et al., 2008, Woodcock et al., 2011, Chapter 2). In addition, studies have generally indicated the effectiveness of the method, such as producing a shift in the isotope ratio of otoliths, but have not necessarily indicated the proportion of successfully marked fish for the element or isotope signature used. The most commonly used element is barium (Ba), with four of its seven isotopes having been used to mark fish, ^{135}Ba, ^{136}Ba, ^{137}Ba and ^{138}Ba (Thorrold et al., 2006, Munro et al., 2008, Munro et al., 2009, Williamson et al., 2009a, Williamson et al., 2009b, Woodcock et al., 2011, Chapter 2). Only one strontium (Sr) isotope has been trialed, ^{86}Sr, to mark both spines (Smith & Whitledge, 2011) and otoliths (Munro et al., 2008), and only one study has investigated the use of enriched magnesium (Mg) isotopes to mark otoliths (Woodcock et al., 2011, Chapter 2).

In this study, we describe a method for chemically marking the otoliths of Murray cod *Maccullochella peelii* with enriched stable isotopes. Murray cod is a large species that can grow to more than 100 kg and can live for more than 40 years. Occurring naturally in lowland rivers throughout the Murray-Darling Basin in southeastern Australia (Anderson et al., 1992a), Murray cod populations have decreased over the years due to increasing habitat loss, sedimentation, flow regulation, barriers to movement,

introduction of alien species, fishing and water quality deterioration (Koehn, 2004). Many areas of the Murray-Darling Basin are currently stocked with hatchery-produced fingerlings in attempts to increase Murray cod populations, but to date little is known of the outcomes of stocking programs, due, in part, to a lack of practical methods for marking large numbers of Murray cod in hatcheries.

Recent work on another native Australian species, golden perch *Macquaria ambigua*, demonstrated that successful marking could be achieved at three life stages, embryo (Munro et al., 2009), larvae (Woodcock et al., 2011, Chapter 2) and juveniles (Munro et al., 2008). Unlike golden perch, which are group spawners that release pelagic eggs that hatch into small (3.5–4.0 mm) poorly developed larvae (Lake, 1967, Rowland, 1996), Murray cod lay demersal, adhesive eggs in nests guarded by the male. Murray cod larvae range in size from 9.5–14.8 mm, are active and can begin feeding before complete consumption of the yolk (Koehn & Harrington, 2006). The differences in biology between golden perch and Murray cod require different hatchery production protocols (Rowland, 1983, Rowland, 1996) and it is therefore uncertain if an immersion marking technique will be a viable method for this species. The early life history characteristics of Murray cod, consisting of demersal eggs and benthic dwelling larvae, are common among freshwater and marine species; however the marking success of these larvae in comparison to pelagic larvae is unknown.

The objectives of this study were to first determine if Murray cod larvae could be marked post-hatch using enriched isotope immersion methods. We combined four stable isotopes from three elements, barium (^{137}Ba and ^{138}Ba) strontium (^{88}Sr) and magnesium (^{24}Mg) to produce 15 potentially unique isotope signatures. To our knowledge, this is the first study to trial the use of ^{88}Sr to mark fish. Second, we determined the extent to which the 15 isotope combinations used were successful in

marking otoliths and the percentage of correctly marked fish based on their otolith chemistry signatures.

3.3 Methods

3.3.1 Hatchery production of Murray cod

Adult Murray cod are maintained at the Narrandera Fisheries Centre (NFC), Narrandera, New South Wales, Australia, in semi-natural ponds where they spawn naturally in late spring (October-November). During spawning the adhesive eggs are deposited on mesh trays within large drums placed in the ponds. The naturally fertilized eggs are then brought into the hatchery and placed in flow-through tanks. Upon hatching, larvae are transferred to shallow flow-through troughs until they begin exogenous feeding (~6 days post-hatch) and are ready to be stocked into grow-out ponds. After growing to approximately 30-50 mm total length (TL), the fingerlings are harvested from the ponds and stocked into rivers. Methods for the production of Murray cod are generally similar between hatcheries (Rowland, 1983).

3.3.2 Isotope marking trials

Isotope immersion marking trials were conducted at the NFC hatchery during the first 6 days after hatching, when Murray cod are feeding endogenously on their yolk; this stage was selected for the trials as fish do not require an external food source, and therefore water changes were not required to maintain water quality. Larvae were transferred to 1 L treatment tanks at a density of 30-50 larvae·L^{-1}; treatment tanks were spiked once at the beginning of the immersion period using various combinations of enriched isotopes of ^{137}Ba, ^{138}Ba, ^{88}Sr and ^{24}Mg (Table 3.1). Enriched isotope solutions were made by dissolving isotopically enriched $BaCO_3$, $SrCO_3$ and MgO (Oak Ridge National Laboratories, Oak Ridge, Tennessee, USA) at concentrations of 100 μg·L^{-1} for

Ba isotopes, 250 $\mu g \cdot L^{-1}$ for Sr and 4000 $\mu g \cdot L^{-1}$ for Mg, with two replicate tanks per treatment (32 tanks in total). Spiking concentrations were based on previous studies marking golden perch larvae (Woodcock et al., 2011, Chapter 2) and from estimated elemental baseline water concentrations at NFC (Munro, unpublished data, approximate concentrations Ba = 0.015 $mg \cdot L^{-1}$, Sr = 0.0475 $mg \cdot L^{-1}$ and Mg = 3.10 $mg \cdot L^{1}$). During the isotope enrichment period, tanks were monitored to record any mortality that may have been attributed to the rearing conditions.

After 6 days of isotope immersion marking, fish from each tank were transferred to grow-out buckets containing 5 L of water that was not spiked with isotopes. Buckets were filled with bore water from the hatchery and randomly placed into one of two flow-through water baths that had a low flow rate to minimize temperature fluctuations. During the grow-out period, buckets were cleaned daily to remove waste from the bottom and water changed every 2-3 days by siphoning through a PVC pipe fitted with a fine screen. Upon commencement of exogenous feeding, Murray cod larvae were fed twice daily using *Artemia sp.* nauplii (3 *Artemia*$\cdot mL^{-1}$) reared in the hatchery using standard techniques and all buckets were treated with 3 $g \cdot L^{-1}$ of sea salt to assist longevity of the live food. Temperature was recorded twice daily at the water inlet and outlet for each water bath. Larvae were collected at the end of the 14 day grow-out period, placed into small sample vials and preserved with 70% ethanol. The total length of each larva was measured to the nearest 0.5 mm before dissection.

Table 3.1: Combinations of enriched stable isotopes used to mark the otoliths of Murray cod larvae.

Enriched Stable Isotope Treatments				
Control	*Single*	*Double*	*Triple*	*Quadruple*
No enrichment	^{137}Ba	$^{137}Ba + ^{138}Ba$	$^{137}Ba + ^{138}Ba + ^{88}Sr$	$^{137}Ba + ^{138}Ba + ^{88}Sr + ^{24}Mg$
	^{138}Ba	$^{137}Ba + ^{88}Sr$	$^{137}Ba + ^{138}Ba + ^{24}Mg$	
	^{88}Sr	$^{137}Ba + ^{24}Mg$	$^{137}Ba + ^{88}Sr + ^{24}Mg$	
	^{24}Mg	$^{138}Ba + ^{88}Sr$	$^{138}Ba + ^{88}Sr + ^{24}Mg$	
		$^{138}Ba + ^{24}Mg$		
		$^{88}Sr + ^{24}Mg$		

3.3.3 Water chemistry

One water sample per tank was collected on day 1, after the initial spiking of the water with the isotopes and a second sample on day 6, before fish were transferred into the grow-out buckets. Water was sampled from the treatment tanks using a 25-mL syringe, filtered through a 0.45-μm membrane filter into acid washed 30-mL plastic vials, and preserved with 0.5 mL of concentrated nitric acid. The samples were then stored frozen until analysis.

Water samples were analyzed using an Agilent 7500cs ICP-MS (Inductively Coupled Plasma-Mass Spectrometer) for all four isotopes used to mark the otoliths (Table 3.1), as well as two other isotopes necessary to calculate isotope ratios (^{86}Sr and ^{25}Mg). Isotope ratios calculated were $^{138}Ba/^{137}Ba$, $^{88}Sr/^{86}Sr$ and $^{25}Mg/^{24}Mg$. Isotope ratios were calibrated against known isotope standard ratios and concentrations, in order to confirm that the treatments were spiked at the appropriate levels as well as to compare the isotope ratios in the water to the ratios in the larval Murray cod otoliths. Elemental standards were made by diluting elemental natural stock solutions to desired concentrations using a 2% nitric acid solution. Standard concentration ranges were based on water samples previously taken from NFC. Isotope standards were made by

dissolving the isotopic enriched compounds in 0.05 mL hydrochloric acid and then diluting to the required concentrations with a 2% nitric acid solution. All water standards were stored in a cold room until analysis.

3.3.4 Otolith analysis

Sagittal otoliths from larval Murray cod were extracted and mounted whole onto microscope slides as described by Munro et al. (2009). Otoliths from each tank (n = 6 otoliths) were analyzed in random order using a New Wave Nd Yag 213nm UV laser ablation unit attached to an Agilent 7500cs ICP-MS (see Table 3.2 for operating parameters). Depth profile analyses were taken through the centre of each otolith. The isotopes of interest were recorded, as well as manganese (^{55}Mn) and indium (^{115}In). ^{55}Mn was analyzed to ensure that the laser had hit the nucleus and ^{115}In as an indicator for when the laser went through the otolith and penetrated the indium spiked thermal plastic glue on the slide (see Munro et al., 2009). A reference standard (NIST 612, National Institute of Standards and Technology) was analyzed to adjust for drift in the ICP-MS, which was then fitted to the otolith ratio data, on the assumption that the isotope ratios in the NIST standard were the natural ratios (see Munro et al., 2008 for details). Shifts in the isotope ratios were determined from a six-point moving average of the depth profile. Where a heavier isotope was used to enrich holding water (e.g. ^{138}Ba or ^{88}Sr) the maximum value of the profile was recorded, and for enrichment with the lighter isotopes (e.g. ^{137}Ba or ^{24}Mg and the combined ^{137}Ba + ^{138}Ba) the minimum values were recorded (Woodcock et al., 2011, Chapter 2).

Table 3.2: Operating parameters for analysis of otoliths for the New Wave Nd Yag 213nm UV laser and Agilent 7500cs inductively coupled plasma-mass spectrometer (ICP-MS).

Laser		
Wavelength	213 nm	
Mode	Q-switched	
Frequency	5Hz	
Spot size	55μm	
Laser power	60%	
Carrier gas	Ar (0.92 L·min-1)	
ICP-MS		
Optional gas	He (59.5%)	
Cone	Pt	
Dwell times	^{136}Ba, ^{137}Ba	(400 ms)
	^{138}Ba, ^{25}Mg, ^{26}Mg	(300 ms)
	^{24}Mg, ^{88}Sr, ^{86}Sr	(200 ms)
	^{55}Mn	(100 ms)
	^{115}In	(50 ms)

3.3.5 Analysis of the difference in fish length and isotope ratios among treatments

Univariate and multivariate analysis of variance were carried out using permutational ANOVA/MANOVA - a dissimilarity/distance-based alternative to traditional analysis of variance (PRIMER 6/PERMANOVA+; http://www.primer-e.com). A two-factor nested model was used for all analyses with treatment (isotope combinations) as a fixed factor and replicate tanks as a random factor nested within treatment. Univariate analyses were used to test for differences in mean length of fish at the end of the marking period among treatments. It was not possible to measure fish at the start of the experiment due to their small size, although it was assumed that all fish were of similar length because they were from a single spawning event and were randomly divided among treatment tanks. Differences in the three isotope ratios (^{138}Ba/^{137}Ba, ^{88}Sr/^{86}Sr and ^{25}Mg/^{24}Mg) in both rearing water and otoliths were also tested using univariate

analyses. Multivariate analyses that included all three isotope ratios as response variables were done for both rearing water and otoliths for all treatments. For the analyses involving the rearing water, samples taken at the beginning and end of the enrichment period were treated as replicate samples with the assumption that isotope ratios had remained constant during the marking process and represented the average ratio the fish were exposed to during the marking process.

Post hoc pair-wise tests were used to test for significant differences between pairs of treatments, if there was a significant treatment effect. Of particular interest were differences between the control and enriched isotope treatments, between the enriched isotope treatments, as well as differences between treatments enriched with the same stable isotope. Although statistically significant tank effects were detected, the variation within tanks was minor compared to the treatment effect (see Results); therefore, for ease of interpretation of *post hoc* tests, analyses of the water and otolith data were reanalyzed using a simplified one-factor model with only the fixed treatment factor.

3.3.6 Determination of mark success and correct classification

The percentage of otoliths successfully marked was estimated by comparing the otolith ratios from the enriched isotope treatments with the control for each of the three isotope ratios ($^{138}Ba/^{137}Ba$, $^{88}Sr/^{86}Sr$ and $^{25}Mg/^{24}Mg$). If the otolith isotope ratio of an individual differed by more than ±2 standard deviations (+2 for fish marked with the heavier isotopes, ^{138}Ba or ^{88}Sr, and –2 for fish marked with the lighter isotopes, ^{137}Ba or ^{24}Mg and the combined $^{137}Ba + {}^{138}Ba$) of the mean ratio of the control, the fish was considered marked for that particular isotope ratio. To determine mark classification success, linear discriminant function analysis (DFA) was carried out using PASW Statistics 17 (http://www.spss.com). Otoliths were randomly divided into training

(n = 128) and testing (n = 64) subsets. Discriminant functions were developed using the training data and the test data were used to determine the percentage of otoliths that could be correctly assigned to their respective enriched isotope marking treatment (see White & Ruttenberg, 2007 for further details).

3.3.7 Correlation between water and otoliths

The isotope signatures produced in the otoliths were plotted against the isotope ratios from the water. Linear models were fitted to the data to determine the relationship between the isotope ratios in the otolith and the water.

3.4 Results

3.4.1 Effects on larval growth

The mean length of Murray cod larvae recorded at the end of the experiment was 13.10 ± 0.03 mm (standard error) with no significant difference among treatments (d.f. = 15, 280, MS = 0.439, $P > 0.050$). There was however a significant tank effect within treatments for length (d.f. = 15, 16, MS = 0.697, $P = 0.001$), with four treatments showing a statistically significant tank effect (*post hoc* pair-wise tests: Control; ^{88}Sr; ^{137}Ba+^{138}Ba and ^{138}Ba+^{88}Sr). The difference in mean length between pairs of tanks for the Control and ^{137}Ba + ^{138}Ba treatments was <0.5 mm. The difference in mean length for the other two treatments was <1.0 mm. These differences, while statistically significant, were very minor and are not considered to be biologically important given the range and variation in length of fish among all treatment tanks. In addition, no mortalities were observed over the isotope enrichment period in any of the experimental tanks. The mean temperature over the experimental period was 21.8 ± 0.32 °C.

3.4.2 Water chemistry

A tank effect was detected within each of the isotope ratios and for the combined ratio analysis (Table 3.3). *Post-hoc* analysis revealed that differences were a result of small variations between enriched tanks; however these variations were minor when compared to the shift in the isotope ratios from the control (see Figure 3.1). As expected, significant differences in the isotope ratios of the water in the various treatments were found (Table 3.3). Enrichment of water with Ba isotopes produced three significantly different $^{138}Ba/^{137}Ba$ ratios, namely ^{137}Ba enrichment (0.47 ± 0.03; mean of all treatments enriched with ^{137}Ba), ^{138}Ba enrichment (39.09 ± 2.56) and enrichment of both Ba isotopes (1.96 ± 0.32). These ratios differed significantly from the control treatment and other non-Ba enriched treatments (7.02 ± 0.19). Treatments enriched with ^{88}Sr resulted in an increase in the $^{88}Sr/^{86}Sr$ ratio (11.95 ± 0.45), which was significantly different from the control treatment and all other non-Sr enriched treatments (8.99 ± 0.17). Treatments enriched with ^{24}Mg displayed a significant decrease in the $^{25}Mg/^{24}Mg$ ratio (0.19 ± 0.004), with similar $^{25}Mg/^{24}Mg$ ratio between treatments, with the exception of the combined ^{137}Ba + ^{24}Mg treatment, which displayed a significant difference to six out of the seven other ^{24}Mg enriched treatments. All ^{24}Mg enriched treatments were significantly different from the control treatment and non-Mg enriched treatments (0.22 ± 0.002). PERMANOVA indicated eight treatments had similar isotope ratios. These were mainly treatments where Ba or Sr isotopes were enriched in combination with ^{24}Mg. Enrichment of water with the various combinations of the four isotopes (^{137}Ba, ^{138}Ba, ^{88}Sr and ^{24}Mg) resulted in 14 different isotope signatures that were significantly different from the control treatment or non-enriched water samples.

Table 3.3: ANOVA results testing for differences in water chemistry between the treatment and tanks nested within treatments. Results are shown for individual isotope ratios and combined ratios.

Water Ratios	df	MS	*F*	*P*
$^{138}Ba/^{137}Ba$				
Treatment	15	1059.8	244.05	0.001
Tank(Treatment)	16	3.755	6.205	<0.010
Residual	32	0.605		
$^{88}Sr/^{86}Sr$				
Treatment	15	9.451	30.369	0.001
Tank(Treatment)	16	0.273	7.442	0.001
Residual	32	0.037		
$^{25}Mg/^{24}Mg$				
Treatment	15	0.0007	67.617	0.001
Tank(Treatment)	16	0.0000	3.273	0.010
Residual	32	0.0000		
Combined Ratios				
Treatment	15	1069.3	229.72	0.001
Tank(Treatment)	16	4.029	6.276	<0.010
Residual	32	0.642		

3.4.3 Otolith analysis

There were significant differences between replicate tanks for the $^{88}Sr/^{86}Sr$ ratio and the $^{25}Mg/^{24}Mg$ ratio, but not for the $^{138}Ba/^{137}Ba$ ratio and the combined three ratios (Table 3.4). Four treatments differed between tanks for the $^{88}Sr/^{86}Sr$ ratio, whereas eight treatments differed for the $^{25}Mg/^{24}Mg$ ratio. Similar to the water analysis, differences in otolith chemistry between tanks were minor compared to treatment isotope ratio shifts (Figures 3.1 and 3.2). All Ba enriched treatments were significantly different from the control treatment (Figure 3.2, Table 3.4). The three different Ba enriched combinations (^{137}Ba, ^{138}Ba and a combination of the two) were all significantly different from each other. In addition, treatments enriched with the same Ba isotopes showed similar isotope ratios, with the exception of the ^{137}Ba enriched treatment compared to the combined ^{137}Ba + ^{88}Sr + ^{24}Mg treatment (Figure 3.2). All ^{88}Sr enriched treatments were

significantly different from the control and non-enriched ^{88}Sr treatments (Figure 3.2, Table 3.4). ^{88}Sr enriched treatments differed, such that the combined $^{138}Ba + ^{88}Sr + ^{24}Mg$ treatment was different from four of the seven other ^{88}Sr enriched treatments, and the combination treatment of all four isotopes was different from the $^{88}Sr + ^{24}Mg$ enriched treatment (Figure 3.2). The addition of ^{24}Mg to the water did not alter the $^{25}Mg/^{24}Mg$ ratio of otoliths (Figure 3.2, Table 3.4). Multivariate analysis of the otolith chemistry signatures indicated significant differences between the control treatment and all enriched stable isotope treatments except the enriched ^{24}Mg (Table 3.4). Differences were also found among the majority of the enriched isotope treatments.

Table 3.4: ANOVA results testing for differences in otolith chemistry between the treatment and tanks nested within treatment. Results are shown for individual isotope ratios and the combined ratios.

Otolith Ratios	df	MS	*F*	*P*
$^{138}Ba/^{137}Ba$				
Treatment	15	1717.7	339.14	0.001
Tank (Treatment)	16	5.065	0.561	>0.050
Residual	160	9.025		
$^{88}Sr/^{86}Sr$				
Treatment	15	3.900	12.616	0.001
Tank (Treatment)	16	0.309	2.347	<0.010
Residual	160	0.132		
$^{25}Mg/^{24}Mg$				
Treatment	15	0.00003	0.273	>0.050
Tank (Treatment)	16	0.00010	4.085	0.001
Residual	160	0.00002		
Combined Ratios				
Treatment	15	1721.6	320.4	0.001
Tank (Treatment)	16	5.374	0.587	>0.050
Residual	160	9.157		

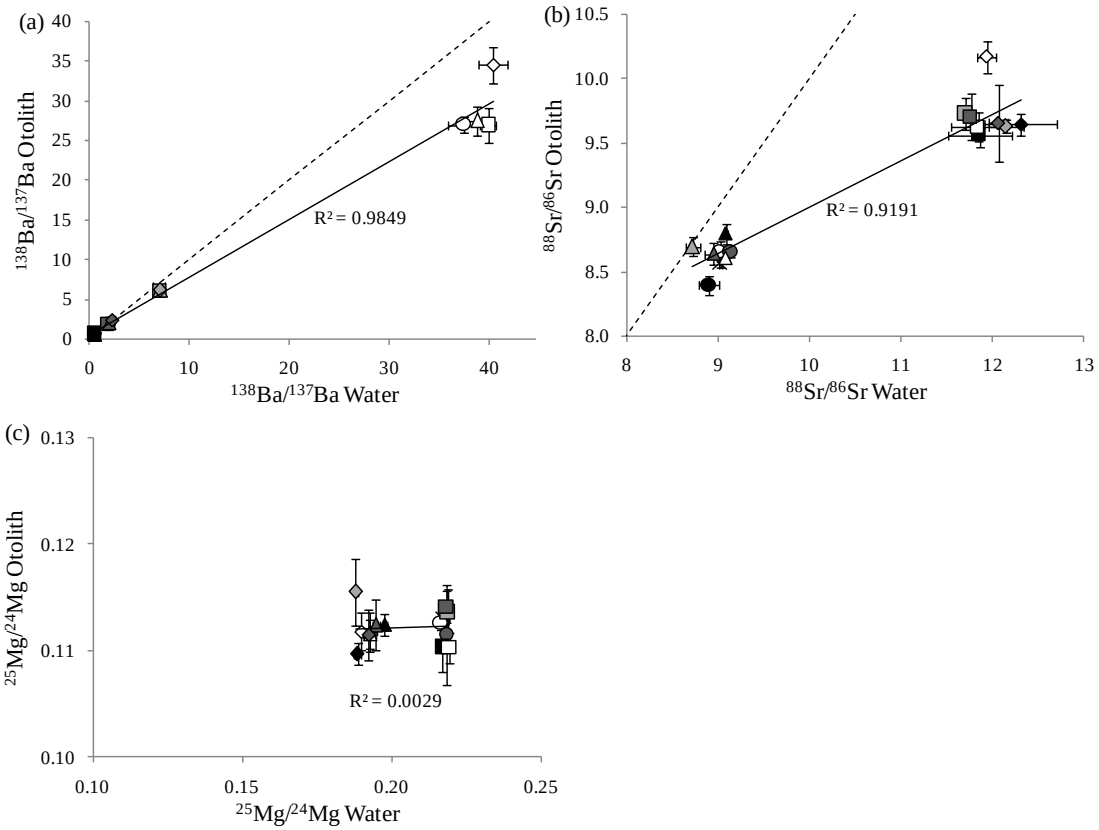

Figure 3.1: Relationship between the isotope ratios in otoliths of larval Murray cod compared to the water samples (a) $^{138}Ba/^{137}Ba$, (b) $^{88}Sr/^{86}Sr$ and (c) $^{25}Mg/^{24}Mg$. Symbols: × Control, ● ^{137}Ba, ○ ^{138}Ba, □ ^{88}Sr, △ ^{24}Mg, ● ^{137}Ba and ^{138}Ba, ■ ^{137}Ba and ^{88}Sr, ▲ ^{137}Ba and ^{24}Mg, □ ^{138}Ba and ^{88}Sr, △ ^{138}Ba and ^{24}Mg, ◇ ^{88}Sr and ^{24}Mg, ■ ^{137}Ba, ^{138}Ba and ^{88}Sr, ▲ ^{137}Ba, ^{138}Ba and ^{24}Mg, ◆ ^{137}Ba, ^{88}Sr and ^{24}Mg, ◇ ^{138}Ba, ^{88}Sr and ^{24}Mg, ◆ ^{137}Ba, ^{138}Ba, ^{88}Sr and ^{24}Mg. Dashed lines represent the 1:1 relation between otolith and water for each isotope ratio and the solid line is a line of best fit.

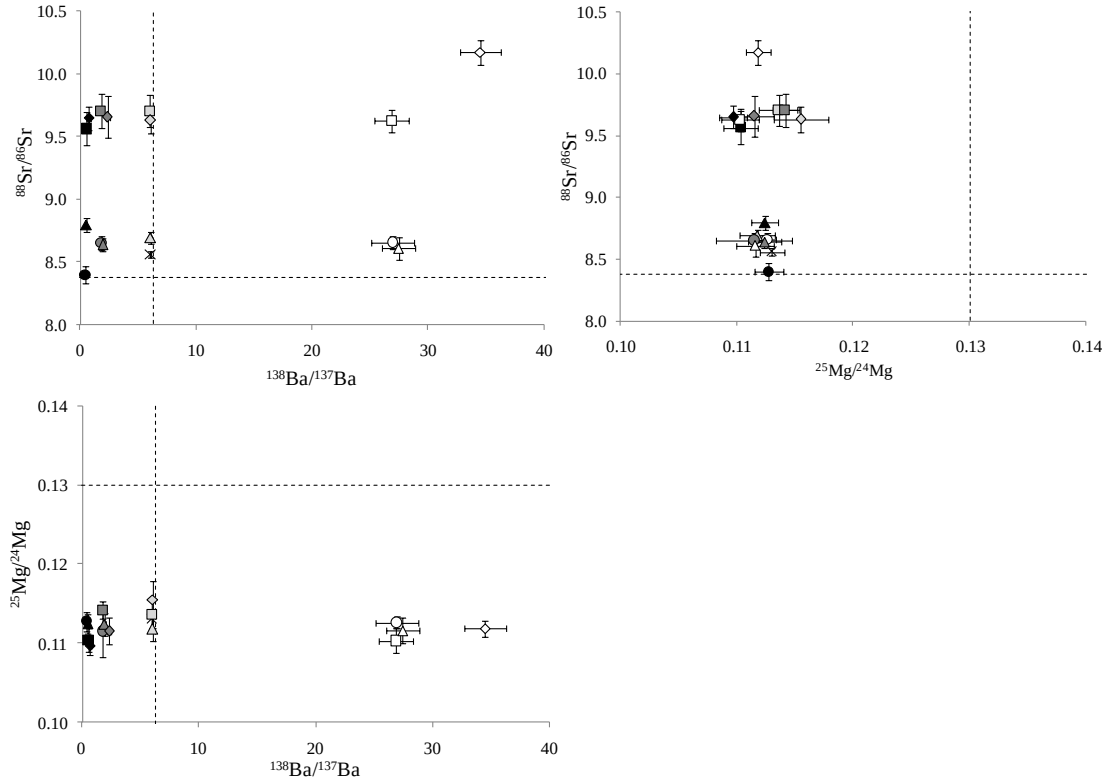

Figure 3.2: Isotope ratios in the otoliths of larval Murray cod for all enriched isotope treatments. Symbols: × Control, ● ^{137}Ba, ○ ^{138}Ba, □^{88}Sr, △ ^{24}Mg, ● ^{137}Ba and ^{138}Ba, ■ ^{137}Ba and ^{88}Sr, ▲ ^{137}Ba and ^{24}Mg, □ ^{138}Ba and ^{88}Sr, △^{138}Ba and ^{24}Mg, ◇^{88}Sr and ^{24}Mg, ■^{137}Ba, ^{138}Ba and ^{88}Sr, ▲^{137}Ba, ^{138}Ba and ^{24}Mg, ◆ ^{137}Ba, ^{88}Sr and ^{24}Mg, ◇^{138}Ba, ^{88}Sr and ^{24}Mg, ◆ ^{137}Ba, ^{138}Ba, ^{88}Sr and ^{24}Mg. Dashed lines represent the natural isotope ratios.

3.4.4 Determination of mark success and correct classification

Otolith mark and classification success varied and was dependent on the enriched isotope treatment. Comparisons of the isotope signature of each fish to the control for each of the three isotope ratios using the ±2 SD criteria found treatments enriched with Ba stable isotopes had a 100% mark success and ^{88}Sr enriched treatments had mark success of 99%. Treatments enriched with ^{24}Mg had the lowest mark success rate where only 8.3% of otoliths were classified as being marked.

Discriminant function analysis showed that only 57.8% of otoliths from the 15 treatments could be correctly classified to their respective treatment due to misclassification of treatments enriched with ^{24}Mg (Table 3.5). When the ^{25}Mg/^{24}Mg ratio was removed from the analysis along with all treatments enriched with ^{24}Mg, 93.3% of otoliths were correctly classified to their respective treatment. Only two of the eight treatments had misclassified fish (Table 3.6), with the other six treatments having 100% correct classification (Table 3.6). Seven significantly different isotope signatures in the otoliths were found using three stable isotopes (^{137}Ba, ^{138}Ba and ^{88}Sr) either individually or in combination.

3.4.5 Correlation between water and otolith

The isotope ratios in the otoliths were strongly correlated with the isotope ratios of the water for ^{138}Ba/^{137}Ba (R^2 = 0.98, Figure 3.1a) and ^{88}Sr/^{86}Sr (R^2 = 0.92, Figure 3.1b). However, the enriched treatments for both ratios displayed a lower otolith isotope ratio shift than was achieved in the water. This effect was more marked for Sr than for Ba. Interestingly, there was no evidence of a relationship between the water and the otoliths for Mg enriched treatments (R^2 = 0.0028, Figure 3.1c).

Table 3.5: Linear discriminant function analysis displaying the percentage of fish that are correctly, or miss-classified to the respective isotope mark using the three isotope ratios measured ($^{138}Ba/^{137}Ba$, $^{88}Sr/^{86}Sr$ and $^{25}Mg/^{24}Mg$) for each treatment. Sample size were n=12 for each treatment.

	Treatment															
Percentage of cases classified to respective treatment	Control	^{137}Ba	^{138}Ba	^{88}Sr	^{24}Mg	^{137}Ba ^{138}Ba	^{137}Ba ^{88}Sr	^{137}Ba ^{24}Mg	^{138}Ba ^{88}Sr	^{138}Ba ^{24}Mg	^{88}Sr ^{24}Mg	^{137}Ba ^{138}Ba ^{88}Sr	^{137}Ba ^{138}Ba ^{24}Mg	^{137}Ba ^{88}Sr ^{24}Mg	^{138}Ba ^{88}Sr ^{24}Mg	^{137}Ba ^{138}Ba ^{88}Sr ^{24}Mg
Control	42.9				57.1											
^{137}Ba		100														
^{138}Ba			50							50						
^{88}Sr											100					
^{24}Mg	33.3				66.7											
^{137}Ba + ^{138}Ba						66.7							33.3			
^{137}Ba + ^{88}Sr							75							25		
^{137}Ba + ^{24}Mg							25	75								
^{138}Ba + ^{88}Sr			20						60						20	
^{138}Ba +^{24}Mg			50							50						
^{88}Sr + ^{24}Mg				50							50					
^{137}Ba, ^{138}Ba + ^{88}Sr												100				
^{137}Ba, ^{138}Ba + ^{24}Mg						25							75			
^{137}Ba, ^{88}Sr + ^{24}Mg							40					20			40	
^{138}Ba, ^{88}Sr + ^{24}Mg									25						75	
^{137}Ba, ^{138}Ba, ^{88}Sr + ^{24}Mg												57.1				42.9

Table 3.6: Linear discriminant function analysis displaying the percentage of fish that are correctly, or miss-classified to the respective isotope mark using only Ba and Sr isotopes and ratios measured ($^{138}Ba/^{137}Ba$ and $^{88}Sr/^{86}Sr$) for each treatment. Sample size were n=12 for each treatment.

		Treatments								
		Control	^{137}Ba	^{138}Ba	^{88}Sr	^{137}Ba ^{138}Ba	^{137}Ba ^{88}Sr	^{138}Ba ^{88}Sr	^{137}Ba, ^{138}Ba ^{88}Sr	Total %
Percent of cases classified to respective treatment	Control	66.7			33.3					100
	^{137}Ba		100							100
	^{138}Ba			100						100
	^{88}Sr				100					100
	^{137}Ba + ^{138}Ba					100				100
	^{137}Ba + ^{88}Sr						100			100
	^{138}Ba + ^{88}Sr			33.3				66.7		100
	^{137}Ba, ^{138}Ba + ^{88}Sr								100	100

3.5 Discussion

Enriching the rearing water with combinations of stable isotopes of Ba and Sr resulted in successful marking of Murray cod otoliths. Murray cod are usually reared in flow-through troughs before being moved out into rearing ponds. However, holding the larvae in a closed system over the 6 day isotope immersion period, similar to that used by Woodcock et al. (2011, Chapter 2) to mark golden perch, had a negligible impact on normal hatchery protocols, and comparisons of length between the enriched stable isotope treatments indicated that the marking procedure had no detrimental effects on growth of larvae compared to controls over the 21 day grow-out period.

As the density of fish that can be held together during the endogenous feeding stage is higher compared to later life stages, immersion marking using isotope enriched solutions at the endogenous feeding stage offers a range of benefits compared to the application of this or similar techniques at later life history stages. These include

minimizing costs of water enrichment, removing the requirement to feed the fish, maintaining water quality that is reduced as a result of waste excretion, and allowing for fish to be held in the same body of water for the entire marking period. Our findings suggest that it should be possible to mark most species of fish by immersion in enriched isotopes if rearing conditions can include a static holding period of several days (see also Munro et al., 2008). This is generally possible for any species relying on an endogenous yolk supply, whether pelagic or demersal spawners.

Isotopes of Ba have now been used to successfully mark several species of marine fish (Thorrold et al., 2006, Walther & Thorrold, 2006, Almany et al., 2007, Williamson et al., 2009a, Williamson et al., 2009b) and freshwater fish (Munro et al., 2009, Woodcock et al., 2011, Chapter 2 and this study). By enriching water with ^{137}Ba, ^{138}Ba and a combination of the two, ^{137}Ba + ^{138}Ba, in this experiment, three distinct signatures were produced in the otoliths of larval Murray cod. These three ratio shifts were significantly different from non-marked fish and were distinguishable from each other. Ba signatures in the otoliths closely reflected those of the water (i.e. the slope of the relationship was close to one), which was similar to findings of Woodcock et al. (2011, Chapter 2) for golden perch.

To our knowledge, this is the first study to mark fish using ^{88}Sr, with previous studies focusing on the use of ^{86}Sr to mark mummichogs *Fundulus heteroclitus* (Walther & Thorrold, 2006), golden perch (Munro et al., 2008) and lake sturgeon *Acipenser fulvescens* (Smith & Whitledge, 2011). These two Sr isotopes have been shown to create distinctive signature shifts. Although the ratios of enriched ^{88}Sr values in the otolith were lower than that measured in the water, a positive relationship was still detected. These two Sr isotopes (^{86}Sr and ^{88}Sr) are both useful markers. The lower isotopic shift in the otoliths compared to the water for Sr ratios could be due to a lag

effect in the incorporation of this element. A previous study found that it takes 20 days for Sr concentrations in the otolith to reflect that of the ambient water (Elsdon & Gillanders, 2005a). Although a significant isotope shift was created in Murray cod otoliths over the 6 day immersion period, it is likely that a longer marking period may be required for Sr isotopes. Further work examining the concentration and length of exposure required to produce isotopic signatures reflective of the water may be required for Sr.

Marking with ^{24}Mg did not shift the isotope ratios in the otoliths of Murray cod, despite having successfully altered the Mg isotope ratio in the water. A similar result was also found when marking golden perch larvae with ^{24}Mg; however enrichment with ^{25}Mg and ^{26}Mg in golden perch resulted in isotope shifts that could be detected in otoliths (Woodcock et al., 2011, Chapter 2). There are a number of reasons that could explain the difficulty in shifting Mg isotope ratios in otoliths through altering the isotope ratios in the rearing water. The chemical structure of the aragonite matrix is distorted when Mg is included or substituted for Ca, because of the smaller size of the Mg ion and bond distance, whereas Sr substitutes for Ca with minimal distortion (Crick, 1989, Finch & Allison, 2007). Also, Mg is a physiologically necessary element and once physiological Mg requirements have been met, fish may regulate Mg to prevent further uptake from either water-borne or dietary sources (Shearer & Asgard, 1992). The physiological regulation of Mg has been suggested in previous studies (Dorval et al., 2007, Hamer & Jenkins, 2007, Melancon et al., 2009), and the regulation of Mg may therefore, explain why Mg isotopes do not produce signatures in the otoliths that are reflective of the water.

Ba and Sr enriched treatments had ≥ 98% mark success, with all combinations of Ba and Sr enriched isotope treatments being distinguishable from each other, as well as

from unmarked fish. A high proportion of fish marked with Ba and Sr enriched stable isotopes were also correctly classified to their respective treatments (93.3%). Using combinations of ^{137}Ba, ^{138}Ba and ^{88}Sr isotopes produced seven distinct signatures. A recent study using similar methods to mark golden perch larvae reported an additional four signatures which could be created using three Ba isotopes (^{136}Ba, ^{137}Ba and ^{138}Ba; Woodcock et al., 2011, Chapter 2). Using the six isotopes that have successfully been used to mark fish otoliths; ^{135}Ba, ^{136}Ba, ^{137}Ba, ^{138}Ba, ^{86}Sr and ^{88}Sr (Thorrold et al., 2006, Walther & Thorrold, 2006, Munro et al., 2008, Munro et al., 2009, Williamson et al., 2009a, Woodcock et al., 2011, Chapter 2) then a total of sixty-three possible signatures could conceivably be created.

The ability to create unique batch-marks in otoliths with combinations of enriched isotopes has a variety of potential uses. For example, it is possible to assess the success of stock enhancement programs or different stocking strategies where batches of fish from different hatcheries, brood stocks, or stocking events are uniquely marked in order to evaluate growth and survival among groups. Artificial isotope signatures could also assist in ecological studies of fish movements and population dynamics, or by commercial aquaculture and aquarium fish producers for quality assurance and stock management purposes. Production of unique markers, as demonstrated here, is a low cost and practical method for discriminating batches of fish and has the potential to be a powerful tool in the production and management of a wide range of fish species. Such techniques increase our ability to accurately identify hatchery-produced fish and develop a better understanding of the outcomes of fish stocking.

3.6 Acknowledgements

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Chapter 4 Incorporation of magnesium into fish otoliths: determining contribution from water and diet

Photo: Holding of juvenile silver perch after calcein marking

Chapter 4 Preamble

This chapter is a co-authored paper, with intention to publish in a peer-reviewed scientific journal.

All authors contributed to the conception and design of the project, I was responsible for the acquisition and analysis of data and for the drafting of the manuscript with all authors involved in revising the manuscript for publication

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Incorporation of magnesium into fish otoliths: determining contribution from water and diet

4.1 Abstract

Magnesium is a commonly measured element in otolith chemistry analysis and is often included in the suite of elements used to discriminate fish from different environments. Poor relations between Mg in water and otolith chemistry are however often found. We examined the uptake of Mg into the otoliths of a fresh water fish (silver perch *Bidyanus bidyanus)*, to determine the extent to which this element can be used to record previous environmental conditions. Silver perch fingerlings were reared for 30 days in water with four concentrations of Mg (14.5, 36.6, 52.1 and 69.6 $mg \cdot L^{-1}$) and fed on a diet supplemented with a combination of natural Mg and enriched ^{26}Mg at five concentrations (1496, 1626, 1902, 2005 and 2036 $\mu g \cdot g^{-1}$). Enriched ^{26}Mg was added to the diet to achieve a $^{26}Mg/^{25}Mg$ ratio that was different from the natural ratio, such that the relative contribution of water and diet to Mg incorporated into otoliths could be determined. Enriching the diet with ^{26}Mg resulted in an isotope shift in the otolith of silver perch from the natural $^{26}Mg/^{25}Mg$ ratio of 1.10 to 1.42; however, this was not as high as the ratio in the diet (> 3.7) suggesting that the fish did not fully incorporate Mg from the diet. Water was the primary source of otolith Mg, contributing on average > 80% to otolith Mg (range 74-95%). The fact that Mg concentrations in the otolith did not change in response to Mg concentrations in the water or diet, indicates that Mg is likely physiologically regulated and therefore is not a reliable environmental indicator.

4.2 Introduction

Over recent decades, otolith chemistry has been used to address issues relating to the ecology and management of fish and fisheries, for example identifying different fish

stocks (De Pontual et al., 2000, Bastow et al., 2002), tracing the environmental histories of fish (Limburg, 1995, Campana et al., 2000, Elsdon & Gillanders, 2005a) and chemically tagging fish at various life history stages (Thorrold et al., 2006, Munro et al., 2008, Munro et al., 2009, Woodcock et al., 2011, Chapters 2 & 3). Despite the widespread use of otolith chemistry analysis, relations between elemental concentrations in otoliths and the surrounding water and diets of fish remain unresolved for many elements. In freshwater systems, water passing over the gills is thought to be the primary source of most elements, whilst water absorbed via the intestines is the main source of water borne elements in marine fish (Campana, 1999). Elements then pass into blood plasma through to the endolymph and are incorporated into the crystallizing otolith (Campana, 1999). An unknown proportion of elements are sourced from the diet, and it is suggested that the variation in otolith chemistry may be due to differences in elemental concentrations between diets (Campana, 1999, Buckel et al., 2004). The relative influence of water chemistry as opposed to diet chemistry on otolith composition is largely unknown for many elements.

The four most commonly used elements in otolith chemistry applications are barium (Ba), strontium (Sr), magnesium (Mg) and manganese (Mn) (e.g. Elsdon & Gillanders, 2002, Wells et al., 2003, Dorval et al., 2007). Of these elements, correlations between elements in the water and otoliths have been well documented for Sr and Ba (Bath et al., 2000, Elsdon & Gillanders, 2003b, de Vries et al., 2005); but no correlation is generally found for Mn (Elsdon & Gillanders, 2003b, Martin & Thorrold, 2005, Hamer & Jenkins, 2007). The results for Mg are more variable and a poor relationship between water and otoliths is sometimes found for Mg (see Table 4.1, Wells et al., 2003, Dorval et al., 2007, Hamer & Jenkins, 2007). Magnesium in otoliths has, however, been found to be useful in distinguishing fish from different sites when used in combination with

other elements, although no water chemistry data were provided for these studies (for example Swan et al., 2003, Sarimin et al., 2009). The concentrations of some elements are highly regulated in fish including Na, K, S, P and Cl (Thresher et al., 1994, Proctor et al., 1995) and it has been suggested that Mg is also physiologically regulated in fish (Dorval et al., 2007, Hamer & Jenkins, 2007). The regulation of Mg could help to explain the variations found in previous research.

Magnesium is the eighth most abundant element on earth, the fourth most abundant element in vertebrates and the most abundant divalent cation within cells (Maguire & Cowan, 2002). Magnesium exists as three different isotopes in measurable natural abundances, ^{24}Mg (78.7%), ^{25}Mg (10.1%) and ^{26}Mg (11.2%) (Rosman & Taylor, 1998, Maguire & Cowan, 2002). Magnesium is enriched in the blood compared to the endolymph fluid which surrounds otoliths (Melancon et al., 2009), indicating physiological fractionation of this element into the endolymph. Elemental discrimination of elements into the otolith can be calculated using a partition coefficient equation ($D_{element}$ = (element:Ca)$_{otolith}$/ (element:Ca)$_{water}$)); values where D = 1 indicate that no elemental discrimination occurs, while values of D = 0 indicate that none of the element is incorporated into the otolith from the water (Campana, 1999). This equation can be used to determine the discrimination of elements from different sources into fish (Campana, 1999, Melancon et al., 2009). In comparison to Ba and Sr, which show high partition coefficients from the water (Table 4.1), Mg has low partition coefficients, indicating a higher level of discrimination against Mg into the otolith from the water compared to Ba and Sr (See Table 4.1 for a comparison in the partition coefficients for Mg, Ba, Sr and Mn).

In support of high regulation of Mg into otoliths, enriched isotope studies involving Mg isotopes, suggest that Mg is not readily incorporated into the otolith from the water

compared to Sr and Ba (Chapters 2 & 3), as only 8% of fish were successfully marked using ^{24}Mg (Chapter 3). This contrasts with studies using enriched isotopes of Ba and Sr, which found that the majority of Ba and Sr was sourced from the water (Walther & Thorrold, 2006, Chapter 2 & 3). The major contributor of otolith Mg (i.e. water or diet) is unknown. Rainbow trout *Oncorhynchus mykiss* obtain their Mg requirements from a combination of dietary and water-borne sources (Shearer & Asgard, 1992). At low Mg concentrations there was a linear relation between whole body tissue Mg concentrations and water-borne Mg concentrations. However, once Mg requirements were met, the relation remained stable as the fish apparently regulate Mg uptake from both the water-borne and dietary source (Shearer & Asgard, 1992). Whether a similar relation occurs for otolith Mg is unknown.

This study investigates the relation between Mg in the water and diet, and Mg in otoliths. Firstly, we aim to determine the extent to which Mg concentrations in the otolith reflects those of the water and diet. By using a diet enriched with ^{26}Mg, we then aimed to determine whether the primary source of otolith Mg is from the diet or water. Finally, we determine if Mg is physiologically regulated from water or diet into the otolith. We test the hypothesis that fish regulate Mg incorporation into the otolith and predict that as the concentration of Mg in the water and diet increases, the concentration of otolith Mg will increase to a point, after which the concentration in the otolith will plateau despite increases in water or dietary Mg.

Table 4.1: Review of the studies that have examined (a) the influence of Mg in the water on Mg otolith chemistry, with partition coefficient comparisons for Ba, Sr and Mn, and (b) the effect of diet on otolith Mg chemistry. Magnesium concentrations are reported as total Mg (ppm) or as Mg:Ca (mol·mol^{-1}, mmol·mol^{-1}, µmol·mol^{-1}).

Effects of Water on Otolith Mg		Mg Concentration		Relation	Partition Coefficients				Influence of Water on	
Species		Water	Otolith	(R^2)	D_{Mg}	D_{Ba}	D_{Sr}	D_{Mn}	Otolith Mg	Reference
Temperature and Salinity										
Controlled Manipulations										
Acanthopagrus butcheri		1.70 mol·mol^{-1}	>80µmol·mol^{-1}	-	-	-	-	-	None	Elsdon and Gillanders, 2002
Leiostomus xanthurus		4.75 mol·mol^{-1}	1.8 mmol·mol^{-1}	-	3.8×10^{-4}	0.37	-	0.196	None	Martin and Thorrold, 2005
Field Study										
Cynoscion nebulosus		4.56 mol·mol^{-1}	0.33 mmol·mol^{-1}	0.442	7.3×10^{-2}	0.31	0.23	14.80	None	Dorval et al., 2007
Water Chemisrty										
Oncorhynchus keta		0.29 mol·mol^{-1}	2.29 mmol·mol^{-1}	-	-	-	-	-	Useful in discriminant analysis	Sohn et al., 2005
Pagrus auratus		4.97 mol·mol^{-1}	20 ppm	0.01	1.18×10^{-5}	0.43	0.08	0.92	None	Hamer and Jenkins, 2007
Platycephalus bassensis		4.97 mol·mol^{-1}	10 ppm	0.36	0.60×10^{-5}	0.09	0.10	0.11	None	Hamer and Jenkins, 2007
Oncorhynchus clarki		0.74 mol·mol^{-1}	0.13 mmol·mol^{-1}	0.39	2.0×10^{-4}	0.04	0.40	-	Negative	Wells et al., 2003
Salvelinus namaycush	*Aragonite*	6.52ppm	11.7 ppm	-	1.19×10^{-4}	0.034	0.35	0.021		Melancon et al., 2009
Lota lota	*Aragonite*		13.4 ppm	-	1.36×10^{-4}	0.0037	0.28	0.054		Melancon et al., 2009
	Veterite		331 ppm	-	3.37×10^{-3}	0.00024	0.024	0.43		
Bidyanus bidyanus		4-18 mol·mol^{-1}	23.9 mmol·mol^{-1}	0.002	1.10×10^{-5}	-	-	-	None	This Study

Effects of Diet on Otolith Mg			Mg Concentration		Influence of Diet	
Species	Diet Type / Supplement	Diet	Water	Otolith	on Otolith Mg	Reference
Oncorhynchus mykiss	$MgS0_4$ and MgO	78–725 ppm	1.4-1000 ppm	-	Parabolic in flesh	Shearer and Asgard (1992)
Pomatomus saltrix	Shrimp vs. fish	1768 vs. 2338 ppm	-	20.4-24.3 ppm	None	Buckel et al. (2004)
Anguilla anguilla	fish, amphipods, insects shrimp, crustacea, pellet	1600 - 4800 ppm	0.38-0.18 mol.mol^{-1}	37.24-69.2 µmol.mol^{-1}	None	Marohn et al. (2009)
Sciaenops ocellatus	Test diet (high, med, low element levels)	1790, 812, 308 ppm	410-1453ppm	34-38 ppm	None	Hoff and Fuiman (1995)
Bidyanus bidyanus	Gelatin-$MgCl_2\cdot H_20$ and ^{26}Mg (MgO)	1496-2036 ppm	14.5-69.6 ppm	23.9 mmol·mol^{-1}	None	This Study

4.3 Methods

4.3.1 Study species

Silver perch *Bidyanus bidyanus* is a native freshwater fish, inhabiting the Murray-Darling Basin, Australia. Juvenile silver perch (43.23 ± 0.53 mm, total length) were sourced from Aquablue Seafoods, Pindimar, New South Wales (NSW) and acclimatised for 48 hours after arrival at The University of Adelaide, South Australia, in one of three 40 L tanks, fitted with filtration and aeration. The otoliths of the fish were then marked with calcein using osmotic induction techniques (Crook et al., 2009). The calcein mark was used to distinguish between pre- and post-experimental growth within the otolith. Fish were held for an additional 24 hours after marking to ensure successful recovery from the marking procedure before the start of the experiment. Silver perch were randomly distributed among experimental tanks with seven fish stocked into 10 L of water. Fish were exposed to one of 20 treatments combinations (crossed design of four water treatments × five diet treatments, each treatment was replicated with 40 experimental tanks in total). A small submerged filter was placed in each tank (JT Filtration Pump, JHJ-411B, 300 $L \cdot h^{-1}$) along with an air stone to aerate and circulate the water. Experimental tanks were randomly assigned to one of five water baths that were maintained at 20^{o}C throughout the experiment to maintain water temperature. Fish were reared in experimental tanks for 30-days.

4.3.2 Water enrichment

$MgCl_2 \cdot 6H_20$ was used as the chemical form of Mg for the water because it is very soluble and a previous study showed that the chemical form of Mg in diets did not appear to be a significant factor contributing to reported differences in dietary Mg requirements in rainbow trout (Shearer & Asgard, 1990). Silver perch were reared in four increasing concentrations of water (14.5 $mg \cdot L^{-1}$, 36.6 $mg \cdot L^{-1}$, 52.1 $mg \cdot L^{-1}$ and

69.6 mg·L^{-1}) by supplementing water at concentrations of 0 mg·L^{-1} 50 mg·L^{-1}, 100 mg·L^{-1} and 150 mg·L^{-1}. Weekly 50% water changes were conducted using aged water (to ensure dechlorination of water). Once water changes had been completed, appropriate amounts of Mg were added to bring the Mg concentrations up to treatment levels for each tank. Water samples were taken on the day after initial set up and approximately 2 h after each water change using a 25 mL syringe. Samples were filtered through 0.45 µm membrane filters into acid-washed plastic vials containing 0.5 mL of concentrated nitric acid. Water samples were analysed (see section 4.3.4 below) to ensure that consistent Mg concentrations were maintained throughout the duration of the experiment and to check for possible leaching of Mg from the feed. All water samples were frozen until analysis.

4.3.3 Diet enrichment

Silver perch were fed on a gelatine based diet using a modified recipe described by Royes and Chapman (2003). Ingredients included prawns in brine (drained), frozen spinach, grated carrot, rolled oats, wheat germ and cod liver oil. Silver perch were fed one of five diets, each supplemented with increasing Mg to create five treatment levels, with a total Mg concentration in the diets of 1496 µg·g^{-1}, 1626 µg·g^{-1}, 1902 µg·g^{-1}, 2036 µg·g^{-1} and 2005 µg·g^{-1}. Mg was added to the diet in two forms: as Mg with natural isotopic abundances (in the form $MgCl_2 \cdot 6H_20$), at a rate of 0 µg of Mg·g^{-1} (baseline) 50 µg·g^{-1}, 100 µg·g^{-1}, 150 µg·g^{-1} and 200 µg·g^{-1}. MgO enriched with ^{26}Mg was also added at a corresponding rate of 50 µg·g^{-1}, 60.5 µg·g^{-1}, 71.0 µg·g^{-1}, 81.0 µg·g^{-1} and 91.5 µg·g^{-1} to produce an altered $^{26}Mg/^{25}Mg$ isotope ratios that was similar across all treatment levels (although this was not critical to achieve – the only requirement was that the ratio was significantly different from the natural). Like $MgCl_2$, it was assumed the Mg in the MgO would be assimilated by the fish in the same manner as other

chemical forms of Mg (Shearer & Asgard, 1990). The fish were fed twice daily and any uneaten food was removed one hour after feeding to prevent leaching of Mg from the diet into the water. For chemical analysis of diet a sample of each diet (n=3 lots of 5 g) was oven dried at 60^{o}C for 24 hours, ground using a mortar and pestle and dissolved in 0.025 g·mL^{-1} nitric acid. Dilution was based on a pilot study done to determine the ability to measure the concentration of Mg in the diet. Samples were left to dissolve for one week. Each sample was then diluted to achieve a 2% nitric acid concentration using ultrapure water, before being filtered through 0.45 μm membrane filter for analysis.

4.3.4 Water and diet analysis

Water and diet samples were analysed for Mg concentrations and isotope ratios using an Agilent 7500cs inductively coupled plasma-mass spectrometer (ICP-MS); see Table 4.2 for operating parameters. Elemental standards were made from natural elemental stock solutions at 0.05, 0.10, 0.50 and 1.0 mg·L^{-1}. Isotope standard concentrations were made using a 4 μg·L^{-1} Mg natural stock, which was enriched at two concentrations 200 and 600 μg·L^{-1} for the three Mg stable isotopes. Agilent Mass Hunter software was used to collect raw data, which was calibrated against the elemental standards. Isotope counts per second were further corrected for machine drift against both the elemental and isotope standards. ^{26}Mg/^{25}Mg isotope ratio and the concentration of Mg were calculated from the corrected data. Due to the relative proportions of the three Mg isotopes being different among samples, the total Mg concentrations were based on the sum of the counts of the three Mg isotopes (^{24}Mg, ^{25}Mg and ^{26}Mg). Mg:Ca ratios were calculated by dividing the molar concentration of Mg by the molar concentration of Ca (based on the ^{43}Ca counts).

4.3.5 Otolith analysis

After 30-days, fish were euthanized using an ice slurry and immediately frozen. The total length of each fish was recorded and the sagittal otoliths removed, cleaned and dried in a fume hood for 24 hours. One otolith from each fish was embedded in a two-part epoxy (Struers, EpoFix resin and hardener) spiked with 40 ppm indium, and 0.35 mm sections were cut using a low speed saw (Buehler, Isomet low speed saw). Sections were polished using lapping film (3 µm grit size) and fixed onto microscope slides using indium spiked CrystalBond 509 thermoplastic glue (see Munro et al., 2008 for additional details). Otoliths were analysed on a New Wave Nd Yag 213nm UV laser operated in Q-switch mode connected to an Agilent 7500cs ICP-MS; see Table 4.2 for operating parameters. Spot analyses were taken outside the calcein mark on the outer edge of the otolith, to analyse the otolith material laid down during the experiment. Indium counts were monitored to determine penetration through the otolith, or if laser was positioned too close to the otolith edge, thus ablating the resin. A reference standard (NIST 612, www.nist.gov/) was analysed periodically throughout each session, including at the beginning and end. Mg concentrations were determined using GLITTER 5.3 (www.glitter-gemoc.com) using similar methods for calculating the Mg concentrations in the water. For isotope ratios, the same data reduction methods were used as described by Munro et al. (2008, 2009), with the exception that the average value from the smoothed data was used for the $^{26}Mg/^{25}Mg$ isotopic ratio for the otolith.

Table 4.2: Operating parameters on the Agilent 7500cs inductively coupled plasma mass spectrometer (ICP-MS) used to analyse water and diet samples and the operating parameters for the New Wave Nd Yag 213 UV laser with ICP-MS used to analyse otoliths.

Solution ICP-MS	Collision Cell	He (5 mL·min^{-1})
	Cone	Pt
	Integration time	0.10 sec with 3 replicates for each isotope (^{43}Ca, ^{24}Mg, ^{25}Mg and ^{26}Mg)
Laser	Wavelength	213 nm
	Mode	Q-switch
	Frequency	5 Hz
	Spot size	30 µm
	Laser power	65%
	Carrier	Ar (0.92 L·min^{-1})
Laser ICP-MS	Optional gas	He (58%)
	Cone	Pt
	Dwell times	^{25}Mg and ^{26}Mg (30 ms)
		^{24}Mg (20 ms)
		^{43}Ca (10 ms)

4.3.6 Data analysis

All statistical analyses were carried out using PRIMER 6/PERMANOVA+ (www.primer-e.com). Water and otolith samples were tested for differences in the ^{26}Mg/^{25}Mg ratio and Mg concentrations, for both total Mg and Mg:Ca, between treatment tanks, and among water enrichment and diet enrichment treatments using three-way ANOVA. The ^{26}Mg/^{25}Mg ratios in the water samples were examined to determine if Mg from the diet had leached into the water. Tanks nested within treatment (water × diet) were analysed to determine if there was any tank effect. Differences in Mg concentrations in diet samples were tested using one-way ANOVA. If differences were found, pair-wise tests were used to determine which treatment levels were significantly different from each other. Differences in the ^{26}Mg/^{25}Mg isotope ratio among diet levels and otoliths were also tested using one-way ANOVA, although the

assumption was that the $^{26}Mg/^{25}Mg$ ratios in the diets should be the same across all levels.

Regression analyses were done to determine the relation between the concentrations of Mg in the otoliths and either water or diet. The Mg concentrations of the otoliths were plotted against the Mg concentrations of the water or diet; linear models were then fitted to the data to determine the relationship. The partition coefficients for otoliths of silver perch were calculated using the following equations:

$$D_{Mg(water)} = \frac{Mg{:}Ca_{otolith}}{Mg{:}Ca_{water}} \quad or \quad D_{Mg(diet)} = \frac{Mg{:}Ca_{otolith}}{Mg{:}Ca_{diet}}$$

Percent contribution of otolith Mg from diet or water was determined using the following equations (Kennedy et al., 2000), where the measured $^{26}Mg/^{25}Mg$ ratio in each source was used. Differences in the percent contribution among the different treatments were determined using similar ANOVA to those described above for Mg concentrations in water and otoliths.

$$\%Element_{(water)} = \left[1 - \left(\frac{Isotope\ Ratio_{(water)} - Isotope\ Ratio_{(otolith)}}{Isotope\ Ratio_{(water)} - Isotope\ Ratio_{(diet)}}\right)\right] \times 100$$

$$\%Element_{(diet)} = \left[1 - \left(\frac{Isotope\ Ratio_{(otolith)} - Isotope\ Ratio_{(diet)}}{Isotope\ Ratio_{(water)} - Isotope\ Ratio_{(diet)}}\right)\right] \times 100$$

4.4 Results

4.4.1 Water enrichment

The Mg concentration in the water increased with increasing amounts of $MgCl_2$ added, demonstrating that water Mg concentrations and Mg:Ca ratios were successfully altered (Tables 4.3 and 4.4). There was a significant difference in the total Mg:Ca concentrations for the water treatment levels (Table 4.4, Figure 4.1) with *post-hoc* tests

demonstrating a significant difference between all Mg water treatments. A significant interaction was detected between water and diet treatments for the Mg:Ca ratio (Table 4.4), where diet treatments resulted in variation within the lower concentration water treatments 14.5 mg·L^{-1} and 36.6 mg·L^{-1}, but not for other levels of diet (Fig 4.1). This variation is minor and could possibly be due to small differences between replicate tanks. As predicted, no significant difference in the ^{26}Mg/^{25}Mg in the water was found (Table 4.4). The mean ratio in the water was 1.23 ± 0.01 (standard error), slightly higher than the natural ^{26}Mg/^{25}Mg ratio of 1.10, suggesting that if leaching from the diet did occur, it was minimal.

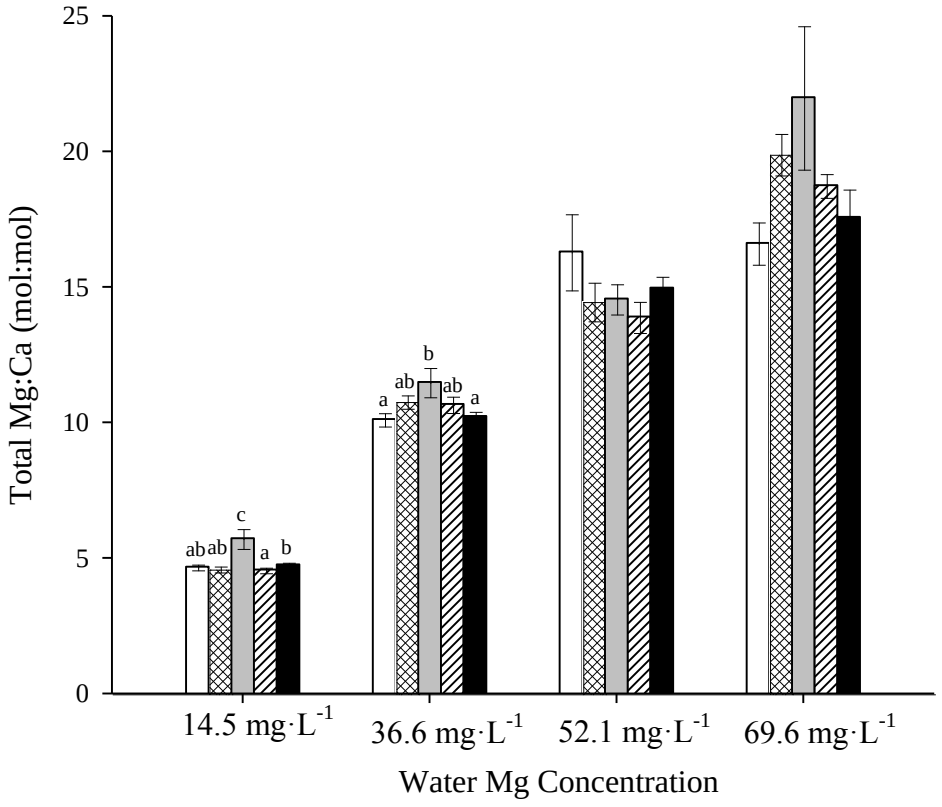

Figure 4.1: Mean (SE) concentration of Mg:Ca (mol:mol) in the rearing water of silver perch. Shaded bars represent diet; □ 1496 µg·g^{-1}, ⊡ 1626 µg·g^{-1}, ▩ 1902 µg·g^{-1}, ▨ 2036 µg·g^{-1} and ■ 2005 µg·g^{-1}. Different letters represent significant difference found for diet within each water treatment. Within water concentrations 52.1 mg·L^{-1} and 69.6 mg·L^{-1}, no significant differences were found.

4.4.2 Diet enrichment

The gelatine based diet was successfully supplemented with Mg (Table 4.3) with significant differences among diets found for the total Mg concentration ($F_{4,10}$ = 179.9, $P \leq 0.001$). *Post-hoc* tests found differences between all diets with the exception of the 150 $\mu g \cdot g^{-1}$ and 200 $\mu g \cdot g^{-1}$ supplemented diets, which had similar total Mg concentrations of 2036 $\mu g \cdot g^{-1}$ and 2005 $\mu g \cdot g^{-1}$ respectively. Significant differences were also found for the Mg:Ca ratio ($F_{4,10}$ = 13.44, $P < 0.010$). *Post-hoc* tests revealed that there were some similarities between diets (Figure 4.2). Enrichment with ^{26}Mg also successfully shifted the $^{26}Mg/^{25}Mg$ ratio from the natural ratio of 1.10 to greater than 3.70. Differences in the isotope ratio between diets were found ($F_{4,10}$ = 176.17, $P \leq 0.001$), where a larger shift from the natural ratio was found with the increasing Mg enrichment (Figure 4.3). The larger ratio shift is likely due to a slightly lower overall Mg concentration in these diets than was expected (Table 4.3, Figure 4.3).

Table 4.3: Mean (SE) otolith Mg concentrations ($\mu g.g^{-1}$) of silver perch for different magnesium concentrations in rearing water and diet. Water and diet were supplemented with increasing concentrations of $MgCl_2 \cdot 6H_20$, and diet was also enriched with the stable isotope ^{26}Mg (in the form MgO).

Water Mg	Diet Mg Concentration ($\mu g \cdot g^{-1}$)				
Concentration ($mg \cdot L^{-1}$)	1496 ± 23	1626 ± 11	1902 ± 6	2036 ± 15	2005 ± 24
14.49 ± 0.44	9.31 ± 0.62	10.53 ± 1.57	7.82 ± 0.60	9.31 ± 0.51	8.69 ± 1.28
36.58 ± 0.87	9.61 ± 0.96	8.74 ± 1.05	10.07 ± 0.89	10.30 ± 0.71	11.53 ± 1.04
52.10 ± 1.83	9.75 ± 0.85	10.73 ± 1.34	11.66 ± 1.18	8.72 ± 0.47	9.14 ± 0.63
69.57 ± 2.14	9.02 ± 0.64	8.63 ± 0.75	9.34 ± 1.07	7.33 ± 0.41	12.14 ± 1.14

Table 4.4: ANOVA results for the total Mg concentration, Mg:Ca concentration and the $^{26}Mg/^{25}Mg$ ratio for the rearing water of silver perch enriched with Mg at four concentrations (14.5, 36.6, 52.1 and 69.6 mg·L^{-1}). Note that the water was not enriched with ^{26}Mg; therefore, significant differences would not be expected.

Water	d.f.	MS	*F*	*P*
Mg Concentration (ppm)				
Water	3	32845.00	295.30	0.001
Diet	4	402.63	3.62	<0.050
Water×Diet	12	156.70	1.41	>0.050
Tank(Water×Diet)	20	111.23	0.86	>0.050
Residual	200	128.81		
Mg:Ca (mol:mol)				
Water	3	2178.50	305.83	0.001
Diet	4	20.90	2.93	<0.050
Water×Diet	12	15.94	2.24	<0.050
Tank(Water×Diet)	20	7.12	0.87	>0.050
Residual	200	8.19		
$^{26}Mg/^{25}Mg$				
Water	3	0.002	1.456	>0.050
Diet	4	0.001	0.383	>0.050
Water×Diet	12	0.002	1.447	>0.050
Tank(Water×Diet)	20	0.001	0.181	>0.050
Residual	160	0.007		

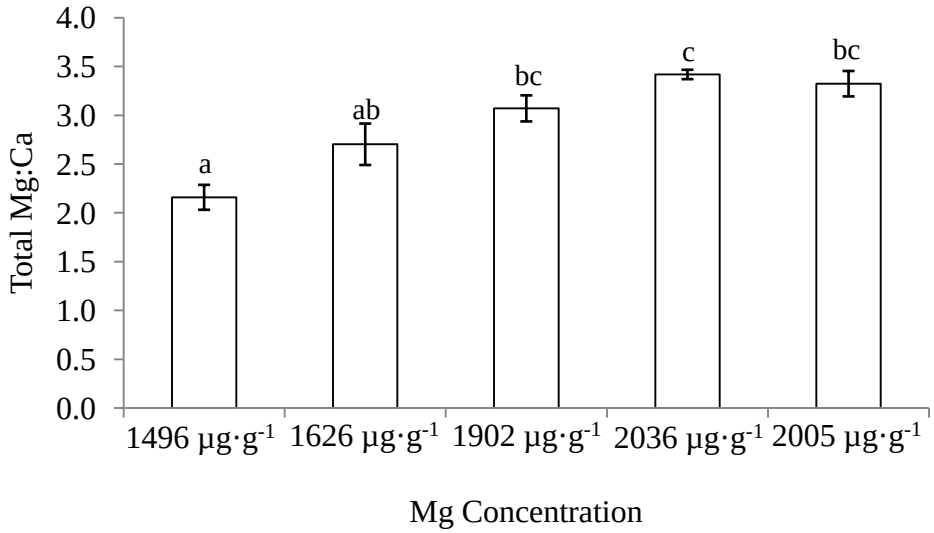

Figure 4.2: Mg:Ca (mol:mol) (mean ± SE) in diet treatments used to feed silver perch. Different letters represent significant differences ($P < 0.05$) between diets using pair-wise tests.

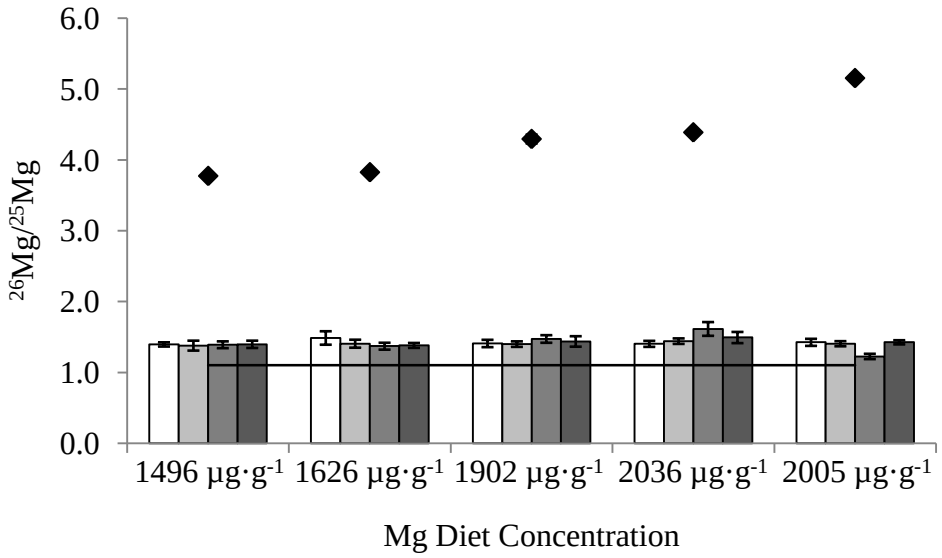

Figure 4.3: $^{26}Mg/^{25}Mg$ isotope ratio for diet (represented by diamonds) and otoliths of silver perch (represented by bars, with shading representing water Mg supplement treatments, □ 14.5 mg·L^{-1}, ▒ 36.6 mg·L^{-1}, ▓ 52.1 mg·L^{-1} and ■ 96.6 mg·L^{-1}). Solid line is the natural $^{26}Mg/^{25}Mg$ ratio, 1.10.

4.4.3 Otoliths

The concentration of Mg, and the Mg:Ca ratio in the otoliths of silver perch did not differ for fish reared at different concentrations of Mg in the water or for different concentrations of Mg in the diet (Table 4.3, 4.5). Similarly, there was no difference in the $^{26}Mg/^{25}Mg$ isotope ratios in otoliths of any treatment (Table 4.5, Figure 4.3). The mean otolith $^{26}Mg/^{25}Mg$ across all treatments was 1.42 ± 0.01, a slight increase from the natural ratio of 1.10 indicating that some of the ^{26}Mg from the diet may have been incorporated into the otolith (Figure 4.3).

Water was the major contributor of Mg into silver perch otoliths (Figure 4.4), with diet only contributing approximately 6% of otolith Mg (Figure 4.4). The concentration of Mg in the water had no significant effect on the amount of Mg incorporated into the otolith ($F_{3,215} = 7.929$, $P > 0.05$), however, a significant difference was found for diet ($F_{4,215} = 128.74$, $P < 0.050$). Fish fed the highest Mg supplement diet (200 $\mu g \cdot g^{-1}$, concentration 2005 $\mu g \cdot g^{-1}$) obtained significantly less Mg from their diet (3.5%) compared to an average of 7.7% for fish fed all other diet treatments (Figure 4.4).

Despite creating significant shifts in the rearing water and the diet of silver perch, no relation was found between the Mg concentration of silver perch otoliths and either the water ($R^2 = 0.0002$, Figure 4.5a) or the diet ($R^2 = 0.006$, Figure 4.5b). The concentration of otolith Mg remained relatively constant despite the increasing concentrations in the water or diet (Figure 4.4). There was a significant difference in the partition coefficient in the otoliths coming from the water ($F_{3,187} = 157.04$, $P < 0.050$), with significant differences found between Mg water supplement treatments. The overall mean was $D_{Mg(water)} = 0.002 \pm 0.0003$. There was also a significant difference in the partition coefficient of otoliths for diet treatments ($F_{4,187} = 18.97$, $P < 0.050$), with difference between all diet levels except for comparison of diet with the Mg

concentration of 2005 $\mu g \cdot g^{-1}$ with the 1626 $\mu g \cdot g^{-1}$ and the 1902 $\mu g \cdot g^{-1}$ Mg concentration diets. A non-linear relationship was found for both the D_{Mg} compared to the water concentration and to diet (Figure 4.6). In both comparisons, the partitioning of Mg increased with increasing concentrations in both sources (Figure 4.6).

Table 4.5: ANOVA results for total Mg concentration, Mg:Ca concentration and the $^{26}Mg/^{25}Mg$ ratio of the otoliths of silver perch reared in increasing concentrations of Mg and fed a ^{26}Mg enriched diet.

Otoliths	d.f.	MS	*F*	*P*
Mg Concentration (pmm)				
Water	3	12.46	0.459	>0.050
Diet	4	13.94	0.513	>0.050
Water×Diet	12	19.90	0.723	>0.050
Tank(Water×Diet)	20	27.75	3.677	<0.050
Residual	167	7.55		
Mg:Ca (mmol:mol)				
Water	3	29.30	0.84	>0.050
Diet	4	23.85	0.68	>0.050
Water×Diet	12	26.58	0.76	>0.050
Tank(Water×Diet)	20	35.35	2.04	<0.010
Residual	167	17.37		
$^{26}Mg/^{25}Mg$				
Water	3	0.006	0.14	>0.050
Diet	4	0.095	2.15	>0.050
Water×Diet	12	0.063	1.42	>0.050
Tank(Water×Diet)	20	0.045	1.32	>0.050
Residual	176	0.034		

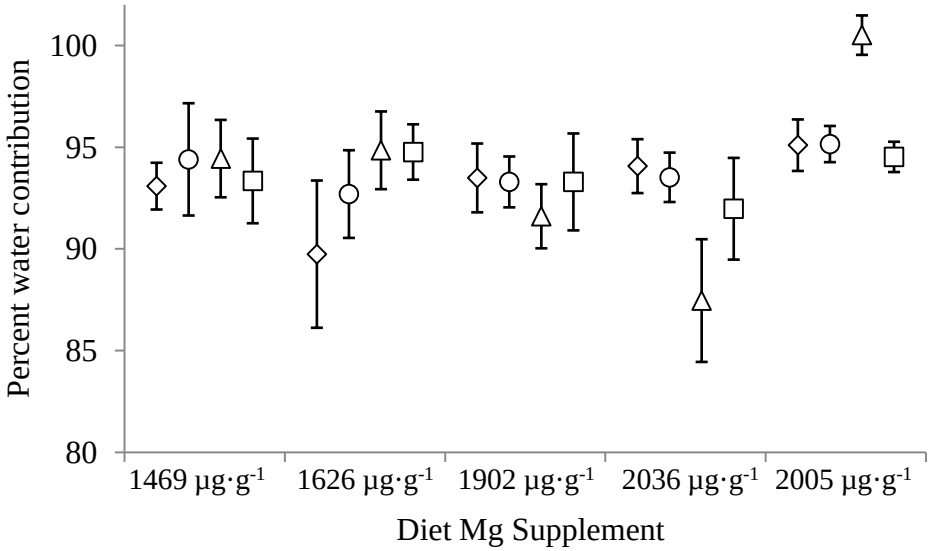

Figure 4.4: Mean percent magnesium contribution (±SE) from the water into the otoliths of silver perch reared in water with supplemented Mg and fed an isotope enriched diet (^{26}Mg) with supplemented Mg. Treatments were: ◇ 14.5 mg·L^{-1}, ○ 36.6 mg·L^{-1}, △ 52.1 mg·L^{-1}, □ 69.6 mg·L^{-1} of Mg concentration of the water.

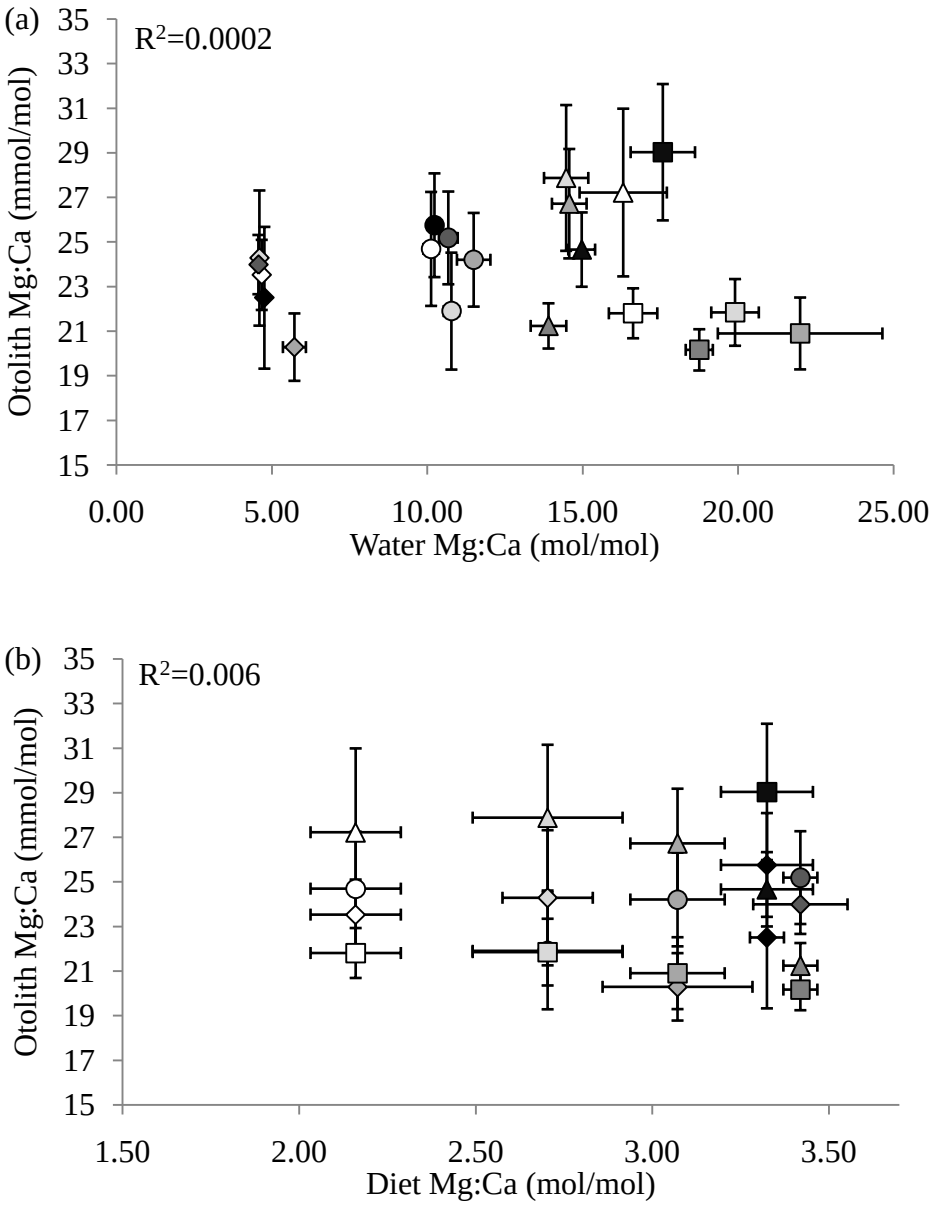

Figure 4.5: Mean (±SE) magnesium concentration (Mg:Ca, mmol:mol) for (a) otolith and water and (b) otolith and diet. Water concentrations are represented by symbol, ◇ 14.5 mg·L^{-1}, ○ 36.6 mg·L^{-1}, △ 52.1 mg·L^{-1}, □ 69.6 mg·L^{-1}, while shading represents diet, ◇ 1496 µg·g^{-1}, ◈ 1626 µg·g^{-1}, ◆ 1902 µg·g^{-1}, ◆ 2036 µg·g^{-1} and ◆ 2005 µg·g^{-1}.

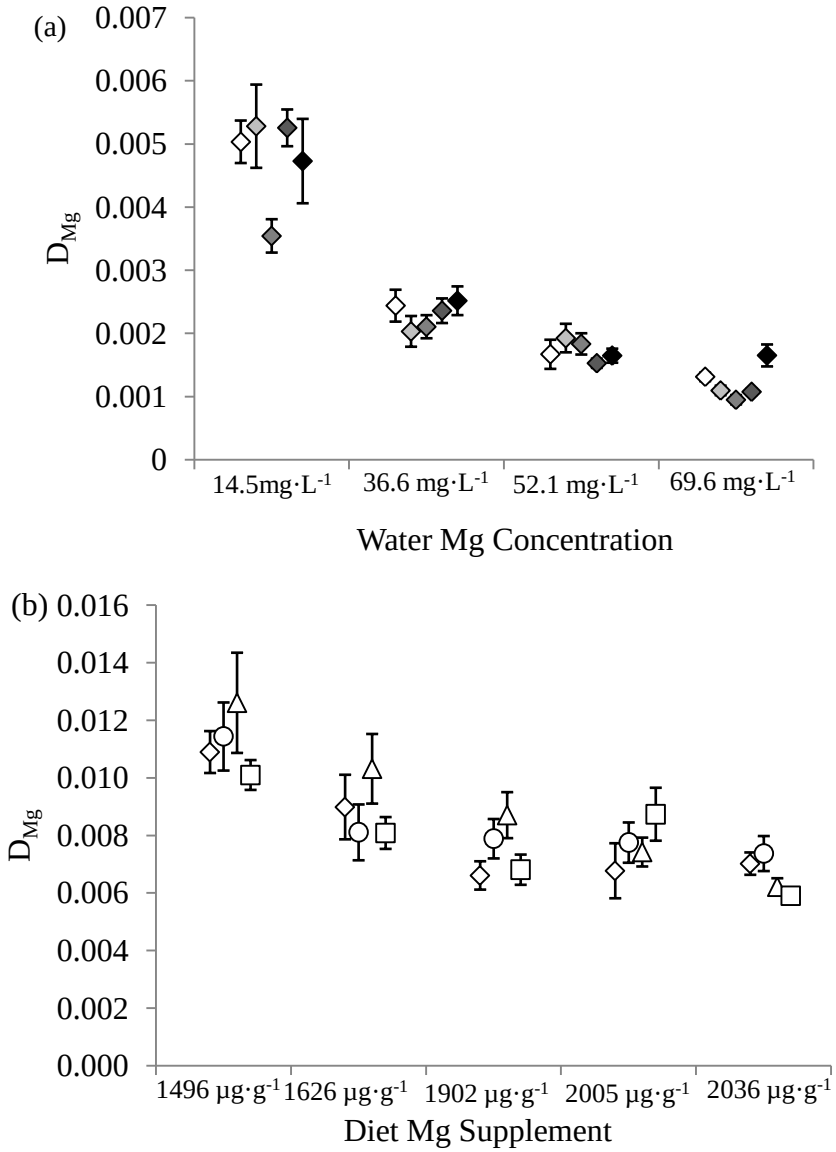

Figure 4.6: Mean (±SE) partition coefficients for silver perch versus (a) Mg concentration of the water and (b) Mg concentration of the diet. Shading represents diet treatment levels, ◇ 1496 µg·g^{-1}, ◈ 1626 µg·g^{-1}, ◆ 1902 µg·g^{-1}, ◆ 2036 µg·g^{-1} and ◆ 2005 µg·g^{-1}, while water treatment levels are represented by symbol, ◇ 14.5 mg·L^{-1}, ○ 36.6 mg·L^{-1}, △ 52.1 mg·L^{-1}, □ 69.6 mg·L^{-1}.

4.5 Discussion

Concentrations of Mg have been used in numerous studies investigating the links between otolith and environmental history, with varying results (Table 4.1). Our study found that despite increased Mg concentration available to silver perch from the water and diet, the Mg concentrations in the otoliths did not change. There was no relation between Mg in the otoliths and that of either water or diet across the concentrations examined. This indicates that Mg is not taken up into the otolith in proportion to its availability and therefore is unlikely a reliable environmental indicator.

4.5.1 Effect of Mg water concentration on otolith chemistry

Correlations between otolith and water chemistry have found little to no relation (Table 4.1), with Mg contributing little to discrimination of groups of fish (Wells et al., 2003, Crook & Gillanders, 2006, Hamer & Jenkins, 2007) and showing no significant interaction with salinity or temperature (Elsdon & Gillanders, 2002, Martin & Thorrold, 2005, Dorval et al., 2007). Increasing the concentration of Mg in the rearing environment of silver perch had no significant effect on the concentration of Mg in their otoliths. The lowest Mg:Ca concentration in the water, 4.8 $mol \cdot mol^{-1}$, was similar to natural concentrations found in water chemistry studies (see Table 4.1, Martin & Thorrold, 2005, Dorval et al., 2007, Hamer & Jenkins, 2007). In wild fish, a negative correlation between otolith and the water has been found for Mg:Ca ratios (Wells et al., 2003). Similarly, a controlled laboratory study by Shearer and Asgard (1992), found that whole body Mg concentrations decreased with increasing Mg concentrations in the water. Although Mg has the potential to allow discrimination between fish from different sources, the lack of a reliable pattern between water and otolith Mg concentrations suggests that Mg cannot be relied upon to reflect ambient water conditions.

The lack of a consistent relation between water and otolith Mg concentrations is highlighted by the partitioning of Mg between the water and otolith. Partition coefficient values calculated here were similar to other otolith and water studies (see Table 4.1, Dorval et al., 2007, Melancon et al., 2009). The discrimination of Mg from the water to the otoliths is a strong indicator that Mg is likely physiologically regulated by fish. Melancon et al. (2009) found that metal concentrations in the endolymph and blood were similar, with the exceptions of Mg (and Fe), which were enriched in the blood relative to the endolymph suggesting partitioning of Mg into the endolymph before it can be incorporated into the otolith. Further research is required to determine the amount of Mg that is found in the flesh, blood and other compartments of fish, to determine where in fish the majority of elemental discrimination occurs.

Our study provides further evidence that immersing fish in water enriched with Mg to produce a chemical tag in the otolith is not as effective as the use of Ba and Sr (Munro et al., 2008, Munro et al., 2009, Williamson et al., 2009a, Smith & Whitledge, 2011, Chapter 2 & 3). Of the two previous studies that have examined the use of enriched stable isotopes of Mg (Woodcock et al., 2011, Chapters 2 & 3), both found ^{24}Mg to be a poor marker, and limited success was found with isotopes of ^{25}Mg and ^{26}Mg (Woodcock et al., 2011, Chapter 2).

4.5.2 Effect of Mg in diet on otolith chemistry

Feeding silver perch a diet supplemented with Mg also had no detectable effect on the concentration of Mg in the otolith, which supports previous research where Mg was found to have no influence on overall otolith composition (see Table 4.1, Hoff & Fuiman, 1995, Buckel et al., 2004, Marohn et al., 2009). Studies on the effect of diet on whole body Mg have found up to 57% retention of dietary Mg in tissue (Shearer & Asgard, 1990). Although increasing dietary levels of Mg did increase whole-body Mg

concentrations, this effect was reduced with high concentrations of dietary Mg (Shearer & Asgard, 1992). Partition of Mg from the diet into the otolith was similar to water, but was lower for fish fed on the diet with the highest Mg concentration.

The concentration of the diets used in this study were high in comparison to other studies that used Mg enriched diets (Shearer & Asgard, 1992), or natural diets (Marohn et al., 2009) (Table 4.1). However, they were similar to the concentration of Mg in shrimp and fish reported by Buckel et al. (2004). The high concentrations of Mg in the diet used to feed silver perch in this experiment may have been beyond the Mg threshold requirement of the fish. The majority of the Mg may have been physiologically regulated form all diets by the fish. Running an experiment using diets low in Mg but which still contain diet requirements to promote healthy eating and growth in fish may help to determine at what concentration a Mg threshold exists in otoliths.

In this study we were able to alter the $^{26}Mg/^{25}Mg$ in otoliths to 1.42, by enriching the diet with ^{26}Mg to ratio levels of >3.70. The lower $^{26}Mg/^{25}Mg$ ratio in the otoliths compared to that of the diet indicated that not all of the Mg in the diet was incorporated into the otoliths of fish, despite feeding for 30 days. This is the first study, to our knowledge, that has spiked a diet with enriched stable isotopes. Interestingly, the Mg isotope ratio shift created in the silver perch otoliths was very similar to the shift created in the larval golden perch otoliths, whereby water was enriched with ^{26}Mg (Woodcock et al., 2011, Chapter 2). It could therefore be theoretically possible to alter otolith chemistry using an isotope-enriched diet.

4.5.3 Percent contribution of water and diet

Water was the primary source of otolith Mg, contributing 74 to 95% of Mg. Walther and Thorrold (2006) also found that water was the main source of Ba and Sr. These studies highlight the influence of water on otolith chemistry. In comparison, Woodcock et al. (2011) found enriching water with stable isotopes of Mg was a poor method for altering the Mg isotope ratio in golden perch otoliths. Although similar isotope ratios were created in silver perch and golden perch, 80% of the Mg in otoliths of silver perch came from the water, whereas only 11% came from the water in golden perch (Woodcock et al., 2011, Chapter 2). The differences in contribution are likely due to the difference between the otolith and water isotope ratios for each study. The percent contribution equation estimates the difference between the two contributing sources (water and diet) to the otolith, and determines the source with the closest isotope ratio (Figure 4.7). Because only a small isotope shift was created in the otoliths of both species compared to the shifts created in the diet fed to the silver perch, or the rearing water of the golden perch (Figure 4.7), the equation has calculated Mg coming primarily from the diet for golden perch, whereas for the silver perch, the Mg is from the water (Figure 4.7). In these experiments, the high discrimination of Mg from both the water and diet resulting in a similar Mg isotope ratios in the otoliths of these fish, may be an explanation to why there is a difference in the percent contribution between the two sources for these studies. Further work with the use of mixing models, which can include more factors, like the partitioning of elements, may help to resolve the differences found in this and the Woodcock et al. (2011, Chapter 2) study.

To our knowledge, this is the first study to directly test the incorporation of Mg into the otolith from both water and diet. Coupled with the results of previous research, this study demonstrates the importance of using caution when utilising Mg as an

environmental indicator. Whilst Mg concentrations may still have use for discrimination of fish from different regions, it cannot be assumed that such differences reflect environmental conditions or diet. Our study has shown that otolith Mg isotope ratios can be artificially altered and it may be possible to chemically batch mark the otoliths of fish by enriching the diet with specific Mg isotopes. This study shows further support for the high physiological regulation of Mg in fish indicating that otolith Mg composition is not a reliable environmental indicator.

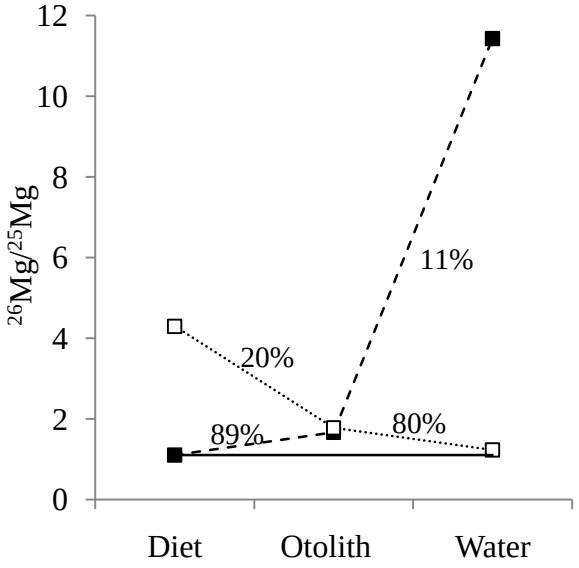

Figure 4.7: Comparison of the $^{26}Mg/^{25}Mg$ ratios for diet, otoliths and water for silver perch (□) and golden perch (■) (Woodcock et al., 2011). Percent values represent the relative contribution of water and diet to otoliths for each species. Solid line is the natural $^{26}Mg/^{25}Mg$ ratio (1.10).

4.6 Acknowledgements

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Chapter 5 Effects of alarm stimuli, stocking density and habitat structure on the behaviour of hatchery-reared fish in the presence of an inter-specific predator

Photo: Murray cod

Chapter 5 Preamble

This chapter is a co-authored paper, with intention to publish in a peer-reviewed scientific journal.

All authors contributed to the conception and design of the project, I was responsible for the acquisition and analysis of data and for the drafting of the manuscript with all authors involved in revision of the manuscript for publication

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Effects of alarm stimuli, stocking density and habitat structure on the behaviour of hatchery-reared fish in the presence of an inter-specific predator

5.1 Abstract

Hatchery-reared fish are released into a range of environments to rebuild populations, but the hatchery release of organisms remains a controversial technique in terms of its ability to increase abundances. Hatchery-reared fish often show differences in behaviour to wild fish and may suffer high mortality rates. Golden perch fingerlings were exposed to different alarm stimuli (predator odours and alarm cues) to determine if pre-stock training would influence their post-stock behaviour. After exposure to stimuli golden perch were stocked with a predator, Murray cod, at high and low densities with habitat structure present or absent, and their behaviour and the area used monitored. Pre-stock predator training had no influence on the behaviour and movement of golden perch fingerings when stocked with Murray cod compared to naive fish. Stocking at a higher density reduced the total time fish remained hiding, compared to stocking fish singularly and the addition of artificial habitat structure increased the hiding time of stocked fish for all treatments. This study indicates that choice of stocking site would contribute more to increasing post-stock survival of hatchery-produced golden perch than pre-stock predator training.

5.2 Introduction

Globally, freshwater ecosystems and the fish assemblages they support are in a state of rapid decline (Ricciardi & Rasmussen, 1999, Sala et al., 2000) due to water pollution, habitat degradation, the impacts of invasive species, flow modification and over-exploitation (Dudgeon et al., 2006). A commonly used approach for restoring depleted stocks of fish is the stocking of hatchery-reared fish into the natural environment (Olla

et al., 1998). However inadequate knowledge of the complex interactions between fish and the physical and biological environment into which they are to be released means that there can be high rates of movement from the area in which fish are stocked and or high rates of mortality (Olla *et al.*, 1998).

Fish raised in captivity often have difficulties adapting to wild conditions, with low initial survival rates of newly stocked fish commonly reported (Fairchild et al., 2005, Brockmark et al., 2007). For example, losses of over 70% within the first three days post-stocking, have been observed (Secor et al., 1995). However if hatchery-reared fish manage to survive their first few weeks after release, then their chances of survival are greatly enhanced (Brown & Day, 2002). Reduced survival of hatchery-produced fish often occurs because captive animals are reared in such a way that they do not develop behaviours required for survival in the wild (Waples & Stagoll, 1997). Differences in foraging behaviour, navigation, predator avoidance, social interactions, aerobic fitness and wariness of humans can all lead to differences in survival rates between captive and wild counterparts (Waples & Stagoll, 1997).

The two most important behaviours an animal must develop are the ability to find food and avoid being eaten (Brown & Day, 2002). Often hatchery-produced fish are fed on an artificial diet and are not exposed to predators prior to stocking. Such fish may not have learnt prey recognition and foraging behaviours required to find food or develop predator recognition (Olla et al., 1998, Brown & Day, 2002). Recent studies have evaluated methods for training fish before stocking as a way of enhancing post-stocking survival (Berejikian et al., 1999, Darwish et al., 2005). One of the key aims of such training is to ensure that stocked fish respond appropriately to the presence of predators. Many species of fish display a fear response when exposed to alarm substances released from the damaged skin of conspecifics. If the alarm substance is experimentally paired

with the presentation of a neutral stimulus (an unknown odour, such as, an odour of an unknown potential predator), it is possible to induce an acquired alarm response in fish (Griffin et al., 2000). If acquired predator recognition can be achieved by training fish with paired alarm signals in the hatchery, it is possible to improve predator detection and survival following release into natural systems (Berejikian et al., 1999).

Native fish populations in the Murray-Darling Basin (MDB), Australia, have been in decline since early European settlement (Rowland, 1996, Barrett, 2004). The reduction in native fish populations has been attributed to a range of factors, including fishing pressure, interactions with exotic species, pollution, habitat loss and flow regulations (Barrett, 2004). The decline of native fish has had a detrimental effect on important recreational fisheries. One of the responses of government agencies has been the development of stocking programs that aim to restore populations of recreationally important native fish. Since 2001, more than 30 million juvenile golden perch *Macquaria ambigua*, Murray cod *Maccullochella peelii peelii* and silver perch *Bidyanus bidyanus* have been stocked into the MDB (Gillanders et al., 2006). Generally, these fish are reared in semi-natural earthen ponds, where the fish feed upon live zooplankton and are exposed to limited avian predation (Rowland, 1996). They are not normally exposed to piscivorous predation or complex habitats present in the natural environment, although some cannibalism can occur. There is little information on the early survival of golden perch, Murray cod or silver perch fingerlings following stocking, although variability in stocking success has been widely reported. Predation in the immediate post-stocking period has been suggested as a potentially important factor in determining stocking success.

The objective of this study was to determine whether hatchery-reared golden perch fingerlings could be trained via exposure to alarm cues to alter their responses to the

presence of an inter-specific predator. We also examined the effects that stocking density and habitat complexity has on the behaviour of golden perch fingerlings in the presence of a predator. We predicted that pre-exposing golden perch to predator odours will result in changes to fish behaviour, as would increase habitat structure and lower stocking densities.

5.3 Methods

Golden perch were exposed to one of three different training conditions prior to being exposed to a predator; (1) no chemical exposure (naive fish), (2) exposure to the predator odour, or (3) exposure to an alarm cue paired with the predator odour. Golden perch were then stocked into experimental tanks at two different stocking densities (low, n = 1 and high, n = 10) and with habitat structure present or absent. Each trial was replicated six times, whereby each training trial was run at each stocking density with habitat structure present or absent (total trials, n = 72).

Laboratory experiments were undertaken using four 20 L training tanks and two 250 L experimental tanks (1400 mm × 540 mm × 350 mm). A 25 mm^2 mesh screen was fixed in place across the experimental tanks 1000 mm from the stocking end of the tank using aquarium grade silicon adhesive (Figure 5.1). Golden perch fingerlings, approximately 20 mm total length (TL), were obtained from the Narrandera Fisheries Centre, New South Wales (NSW), in late March, 2010. Golden perch were housed in a recirculating tank, at The University of Adelaide, South Australia, fitted with a bio-filter, and fed a frozen commercial diet consisting of brine shrimp, bloodworms and a beef-heart mix. Golden perch were held for 27-days before the experimental trails began. Murray cod was selected as the predator for the experiment as it is a dominant predator in the Murray-Darling Basin. Six Murray cod, approximately 300 mm TL each, were sourced

from Aquablue Seafoods, Pindimar, NSW. Murray cod were held in 250 L tanks (similar to experimental tanks) fitted with a recirculating bio-filter, and were fed a pelletised diet provided by the hatchery. All holding and experimental tanks were kept at 20°C.

5.3.1 Training conditions

Alarm cue and predator odour was prepared using modified methods of Berejikian et al. (1999) and Gazdewich and Chivers (2002). For the alarm cue, skin extract was collected from juvenile golden perch, sourced from Narrandera Fisheries Centre. A total of 9 cm^2 of skin and muscle was removed from multiple juvenile golden perch and added to 200 mL of distilled water and homogenized. This was then filtered through aquarium filter wool to remove any visible particles and the resulting solution was frozen in 5 mL lots. Predator odour was prepared by holding two Murray cod in an aerated, but unfiltered 42 L aquarium for 18 hours. After this time, Murray cod were removed and the water frozen in 30 mL lots. Each training trial used 60 mL of this water (Berejikian et al., 1999, Gazdewich & Chivers, 2002).

Golden perch to be used for a trial were removed from a holding tank, and placed into one of two pre-stocking training tanks. Desired stimuli were slowly introduced into the training tanks using a syringe at a rate of approximately 1 $mL \cdot sec^{-1}$. De-chlorinated water was added to the naive fish tank at similar rates. Fish were left exposed to the treatments for 1 day before being removed and stocked into experimental tanks. After the stocking trial, fish were removed from experimental tanks; golden perch were placed into a second holding tank. Each golden perch was used in one trial only.

5.3.2 Stocking trials

For each experiment, Murray cod were placed behind the predator screen 2-hours before the start of the experiment, to allow fish to acclimatise. Following acclimatisation, golden perch fingerings were then stocked into the opposite end of the tank (within position 1-3, Figure 5.1) in two different stocking densities (low, n = 1 and high, n = 10) and with habitat structure present or absent. Habitat structure consisted of PVC pipe placed at different distances from the predator screen (Figure 5.1). The mesh size of the predator screen was selected to allow juvenile golden perch to move freely between the two sections, but prohibit the movement of Murray cod into the stocking area. Experimental stocking trials were randomly sequenced, with four trials occurring per-day, two in the morning and two in the afternoon. Each Murray cod was used once per day and randomly allocated to experimental tanks.

The behaviour and movement of stocked fish was recorded using a camera mounted above each tank looking down that recorded the entire experimental arena for 1 hour after stocking. Video was recorded via a video capture card (GC-650, Geovision, Taiwan) and displayed on a computer monitor using EziViewLog500 software (Geovision, Taiwan). Video footage was examined, and three behaviour patterns were quantified across the 1-hour period for each trial. The amount of time spent exhibiting specific behaviour (to nearest second) was recorded using three categories. These were "moving", where fish were more than a body length away from structure or corners of tanks, "hiding" total time spent within the PVC pipes, close to the dividing wall or in the corners of the tank, and "motionless", fish in the open but not moving continuously for longer than 3-seconds. For high stocking density tanks, movement was defined as periods when one or more fish were moving; whilst time spent hiding was defined as when all fish were out of view. The initial 10 minutes after stocking was analysed in

further detail to calculate the time fish spent at different positions along the tank (Figure 5.1) to determine if initial distribution was influenced by the predator. For high stocking density treatments, each position was watched and the number of fish and the time spent within the position was recorded; this was then converted into a percentage of time fish spent within a position. Due to time constraints, it was not possible to trace the movement of fish for the full hour.

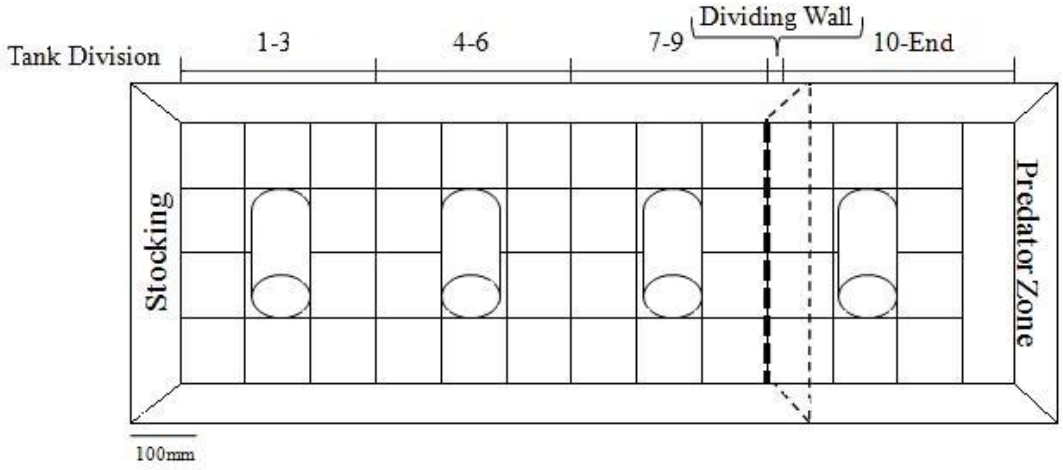

Figure 5.1: Experimental stocking tank 1400 mm × 540 mm × 350 mm. Dashed square indicates dividing wall separating the Murray cod from the stocking area.

5.3.3 Statistical analysis

PRIMER 6/PERMANOVA+ (http://www.primer-e.com) was used to conduct all statistical analyses. As fish in the high stocking density could not be individually tracked, analysis of the three behaviours (hiding, moving and motionless) was conducted for the low and high stocking density treatments separately using two-way ANOVA, with the different training treatments as fixed factors. Three-way ANOVA was used to determine if training, stocking density or habitat had an effect on the percentage of time golden perch spent within each position of the tank for the initial 10 minutes post-stocking. Where significant differences were found, *post-hoc* pair-wise tests were performed to identify which training conditions differed. Chi-square tests were performed using PASW Statistics 17 (http://www.spss.com) to determine the

effect of stocking density and habitat on the distribution of golden perch within a tank. This analysis used pooled data from the three training treatments as training was found to have little to no influence on the distribution of golden perch in the experimental stocking trials (see results). Multivariate analysis was not possible on the distribution of golden perch as individual positions were not independent of one another.

5.4 Results

5.4.1 Behaviour

There was no significant effect of pre-stock stimuli training on the behaviour of golden perch stocked at low or high stocking densities (Table 5.1, Figure 5.2). Habitat structures had a significant effect for all three observed behaviours for the low stocking density (Table 5.1). Additional habitat increased the amount of time fish spent hiding in the low stocking density. Habitat structure had no influence on the behaviour of fish stocked at the higher density.

5.4.2 Initial distribution

When looking at the time golden perch remained within a position, several significant effects were found (Table 5.2). An interactive training and stocking effect was found for position 4-6, where at low stocking, alarm cue paired with predator odour exposed fish remained within this position longer than naive or predator odour trained fish (Figure 5.3). Stocking density was the most influential single factor, with stocking density influencing the time golden perch remained in positions 4-6, 7-9 and near the dividing wall (Figure 5.3). The presence of habitat did not influence the time spent within any areas of the tank (Table 5.2). Across the three training treatments, significant differences in the distribution of golden perch where found using chi-square analysis for all habitat structure × stocking density combinations (Table 5.3). Within the low

stocking density treatment without habitat structure, fish spent more time at the dividing wall followed by position 1-3 (furthest from the predator, Figure 5.3). For all other stocking and habitat structure combinations, fish spent more time within position 1-3 (Figure 5.3). For low stocking treatments, less time was spent within position 4-6 for both habitat structure present or absent (Figure 5.3). For high stocking treatments, fish spent less time within position 10-End when habitat was absent, and less time within the dividing wall with habitat present. While golden perch had access to the predator zone, no predation occurred during experimental trials.

Table 5.1: Analysis of variance for the influence of pre-stock training and habitat structure on the behaviour (hiding, moving and remaining motionless) of golden perch stocked at low (n=1) and high (n=10) density over 1-hour after being stocked with a Murray cod.

Source	df	MS	*F*	*P*(perm)
Low Stocking Density				
Hiding				
Training	2	118	0.14	>0.05
Habitat structure	1	10553	12.67	<0.01
Training × Habitat structure	2	259	0.31	>0.05
Residual	30	833		
Moving				
Training	2	279	0.72	>0.05
Habitat structure	1	3598	9.24	≤0.001
Training × Habitat structure	2	797	2.05	>0.05
Residual	30	389		
Motionless				
Training	2	562	1.40	>0.05
Habitat structure	1	1828	4.55	<0.05
Training × Habitat structure	2	201	0.50	>0.05
Residual	30	402		
High Stocking Density				
Hiding				
Training	2	65	0.19	>0.05
Habitat structure	1	719	2.11	>0.05
Training × Habitat structure	2	209	0.61	>0.05
Residual	30	340		
Moving				
Training	2	67	0.20	>0.05
Habitat structure	1	709	2.08	>0.05
Training × Habitat structure	2	211	0.62	>0.05
Residual	30	341		
Motionless				*P*(MC)
Training	2	0.03	1.00	>0.05
Habitat structure	1	0.03	1.00	>0.05
Training × Habitat structure	2	0.03	1.00	>0.05
Residual	30	0.03		

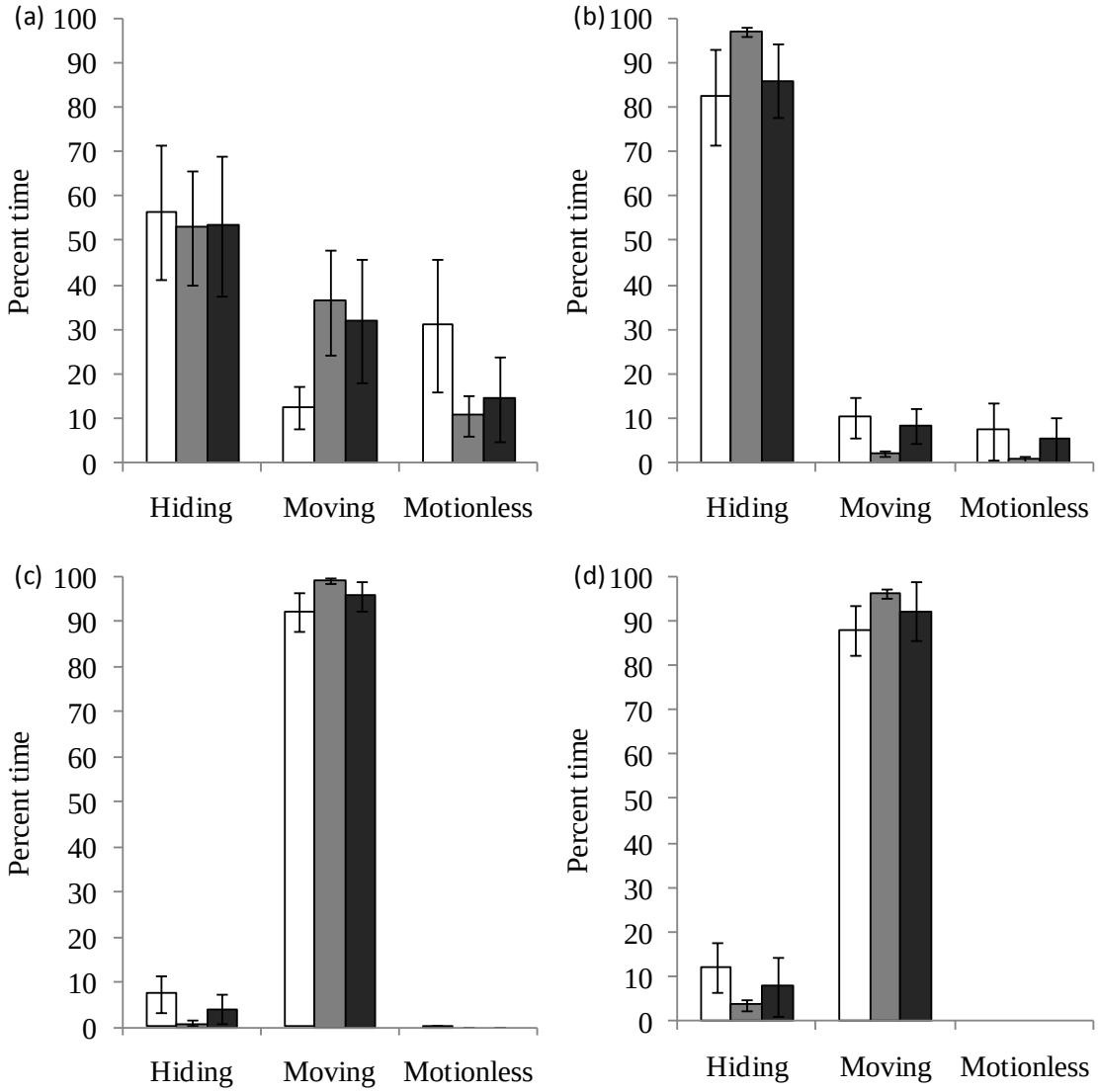

Figure 5.2: Percentage of time golden perch spent displaying different behaviour under different training, □ naive, ■ predator odour and ■ predator odour and alarm cue, and treatment conditions (a) Low stocking density without habitat, (b) low stocking density with habitat, (c) high stocking density without habitat and (d) high stocking density with habitat.

Table 5.2: Analysis of variance testing the influence that training, stocking density and additional habitat structure had on the position of golden perch fingerlings within the initial 10-minutes post-stocking

Source	df	MS	*F*	*P*
Position 1-3				
Training	2	185	0.115	>0.050
Stocking density	1	682	0.847	>0.050
Habitat structure	1	71	0.088	>0.050
Training × Stocking density	2	645	0.400	>0.050
Training × Habitat structure	2	3579	2.221	>0.050
Stocking density × Habitat structure	1	42	0.052	>0.050
Training × Stocking density× Habitat structure	2	3930	2.439	>0.050
Residual	60	48349		
Position 4-6				
Training	2	1561	4.080	<0.050
Stocking density	1	1059	5.538	<0.050
Habitat structure	1	342	1.789	>0.050
Training × Stocking density	2	1741	4.552	<0.010
Training × Habitat structure	2	606	1.585	>0.050
Stocking density × Habitat structure	1	71	0.373	>0.050
Training × Stocking density× Habitat structure	2	1054	2.756	>0.050
Residual	60	11474		
Position 7-9				
Training	2	57	0.076	>0.050
Stocking density	1	1424	3.801	<0.050
Habitat structure	1	38	0.102	>0.050
Training × Stocking density	2	126	0.168	>0.050
Training × Habitat structure	2	2121	2.832	>0.050
Stocking density × Habitat structure	1	108	0.288	>0.050
Training × Stocking density× Habitat structure	2	65	0.086	>0.050
Residual	60	22473		
Position Wall				
Training	2	2220	1.286	>0.050
Stocking density	1	3738	4.330	<0.050
Habitat structure	1	2156	2.497	>0.050
Training × Stocking density	2	5318	3.080	>0.050
Training × Habitat structure	2	3247	1.881	>0.050
Stocking density × Habitat structure	1	28	0.033	>0.050
Training × Stocking density× Habitat structure	2	1232	0.714	>0.050
Residual	60	51795		
Position 10-End				
Training	2	833	1.189	>0.050
Stocking density	1	289	0.823	>0.050
Habitat structure	1	177	0.505	>0.050
Training × Stocking density	2	1346	1.920	>0.050
Training × Habitat structure	2	369	0.526	>0.050
Stocking density × Habitat structure	1	9	0.027	>0.050
Training × Stocking density× Habitat structure	2	98	0.139	>0.050
Residual	60	21033		

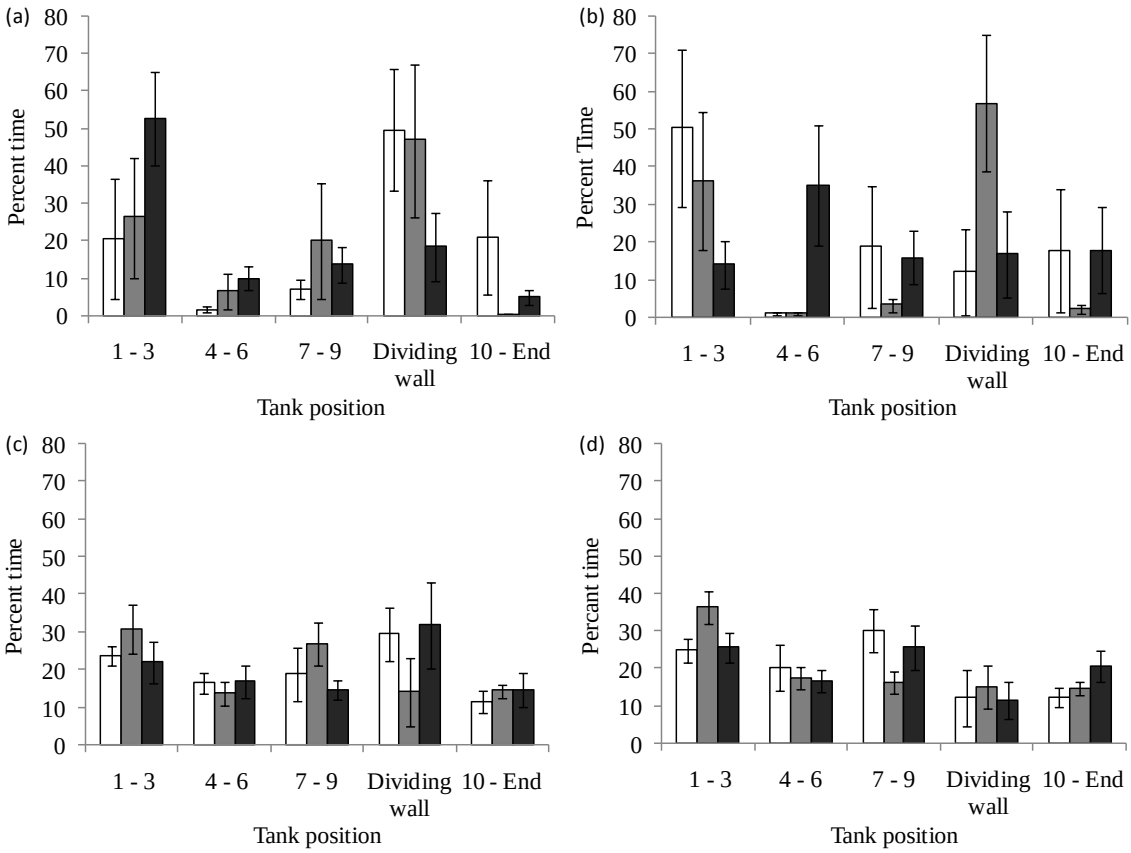

Figure 5.3: Mean percentage of time (± SE) fish spent within each division of the tank during the first 10 minutes after stocking, □ control, ▒ predator odour and ■ predator odour and alarm cue (a) low stocking density without habitat, (b) low stocking density with habitat, (c) high stocking density without habitat and (d) high stocking density with habitat.

Table 5.3: Chi-square test for the distribution of golden perch across each division of the stocking experimental tank for the initial 10mins post-stocking. This analysis uses pooled data from the three training treatments.

Treatment	df	Chi-Square	*P*
(a) Low stocking without habitat structure	4	130.88	≤0.001
(b) Low stocking with habitat structure	4	63.09	≤0.001
(c) High stocking without habitat structure	4	18.03	≤0.001
(d) High stocking with habitat structure	4	24.63	≤0.001

5.5 Discussion

Under both stocking densities, the presence of habitat structure was the most important factor influencing the behaviour and distribution of golden perch fingerlings post-stocking. Exposing golden perch to predator odour and alarm stimuli had no influence on their behaviour when stocked with a potential predator, and had a minimal effect on the distribution of golden perch within the experimental tank. Golden perch did not appear to respond to the presence of the Murray cod, spending similar time within the predator zone to areas where they could not be preyed upon. These results suggest that ensuring availability of suitable habitat structure when stocking into a region has a greater chance of improving post-release survival of this species than pre-release training with chemical stimuli. It should also be noted that, whilst the two density treatments were not directly comparable, a much greater total amount of time was spent by fish in the high density treatment exhibiting non-hiding behaviour. This observation suggests that stocking density may have a strong effect on survival rates as at higher densities, more time was spent in open water where fish have an increased vulnerability to predation.

Although exposing golden perch to training stimuli before stocking had no influence on their post-stock behaviour, exposing stocked fish to similar stimuli has been successful with some species, such as chinook salmon *Oncorhynchus tshawytscha* (Berejikian et al., 1999) and glowlight tetras *Hemigrammus erythrozonus* (Darwish et al., 2005). Both of these species displayed reduced foraging activity and area use following exposure to predator odours (Berejikian et al., 1999, Darwish et al., 2005). Arctic charr *Salvelinus alpines,* when exposed to a predator odour or predator odour paired with a disturbance signal, displayed no behavioural difference to naive control fish (Vilhunen et al., 2005). However, directly conditioning with a live predator resulted in less activity and

increased freezing time, with fish being completely motionless, compared to the other training treatments (Vilhunen et al., 2005). Visual training may be a more suitable method for golden perch.

The presence of structure in which to hide (i.e. habitat) had an influence on the behaviour of golden perch, and a small influence on the position of golden perch. The time spent at the dividing wall, compared to other positions, could be explained by fish being able to hide within the mesh screen of the wall and the structures holding it in place. Although silicone was used to fill most of the joining gaps, the small size of the golden perch fingerlings enabled them to take advantage of the small spaces. Suitable habitat structure has been shown to be important in post-stock survival for a number of species, including brown trout *Salmo trutta*, whereby enhancement structures provided a greater number of favourable habitat locations for brown trout, as well as other fish and macro-invertebrates (Huusko & Yrjänä, 1997). Furthermore small hatchery-reared mulloway *Argyrosomus japonicas* released into shallow waters, displayed significantly higher movement than mulloway released directly into deep habitats over the first two days post-release (Taylor et al., 2006). This difference in movement was reduced after three days as fish relocated from shallow to deeper habitats. Identifying optimal release sites and stocking densities are key criteria in developing effective stocking strategies and are likely to be important for optimising the survival of stocked golden perch fingerlings. Release sites should have high prey availability and necessary habitat to provide protection and shelter from predators, whist stocking densities should be chosen to reflect the availability of suitable prey and habitat (Fairchild et al., 2005).

Golden perch did not show behavioural differences when pre-exposed to predator stimuli, indicating that this method of pre-stock training may not be suitable for this species. Understanding the movement and behaviour of fish post-stocking, however,

may help to provide information to assist efforts to increase survival after release. Identifying suitable stocking locations and stocking densities in regards to the availability of habitat that provides shelter from predators and foraging opportunities is essential for the early survival of stocked fish while they acclimatise to a new environment. Enhancing post-stock survival will help to increase the probability of stocked fish reaching sexual maturity and contributing to fisheries. Further research is needed to determine the differences in behaviour between hatchery reared and wild recruits to compare factors including predatory impacts, competitive abilities, social interactions and dispersal potential.

5.6 Acknowledgements

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Chapter 6 General Discussion

Photo: Murrumbidgee River

General Discussion

6.1 Background

The reintroduction of hatchery-reared fish to boost natural stocks and enhance fisheries is being undertaken by an increasing number of organisations across the world (Hilborn, 1998, Welcomme & Bartley, 1998). Despite the large financial investment represented by these activities, fish raised in captivity often have difficulty adapting to wild conditions, displaying poor post-release performance. The proportion of hatchery fish that survive to adulthood is rarely assessed as part of stocking programs, but has been found to be as low as 5% (Brown & Day, 2002, Brockmark et al., 2007). In systems where natural recruitment occurs, distinguishing between reintroduced and wild fish is essential to determine the success of stocking programs, and while there are some methods available to identify stocked fish, many are not suitable for the scale of stocking that can occur. Therefore, a technique to identify hatchery-stocked fish in systems is required. Knowledge of the proportion of stocked fish in a river system would enable assessment of the success of stock enhancement programs and river health.

The objective of this thesis was to develop and evaluate isotope immersion marking techniques, to provide a method of marking that could not only be used on different species, but could be easily incorporated into hatchery operations. I sought to develop a method that would minimise the handling of fish and would not require fish to be held for longer periods than normal hatchery operations. In addition, post-stock behaviour of golden perch *Macquaria ambigua* was assessed to determine the influence of pre-stock predator training and habitat structure. In the following sections I summarise the major findings of the thesis and discuss their implications in terms of the technical factors

influencing marking success and the potential utility and implications of the techniques for fisheries management and research.

6.2 Enriched Stable Isotope Marking

Enriched stable isotope marking of fish otoliths has been used in a number of studies over the last five years to mark both marine and freshwater species, including hatchery-produced and wild spawned fish. The range of stable isotopes used to produce successful marks has varied along with the methods of application (transgenerational and immersion marking) and the life stages marked (embryo, larvae and fingerling). In addition, both otoliths and fin spines have been marked (see Thorrold et al., 2006, Almany et al., 2007, Munro et al., 2008, Munro et al., 2009, Williamson et al., 2009a, Williamson et al., 2009b, Smith & Whitledge, 2010, Woodcock et al., 2011 and Chapter 2 & 3 for examples).

For a mark to be successful it should meet certain criteria. These criteria include: (1) the mark does not influence mortality rates and behaviour of the tagged individual; (2) the tagging methods are cost-effective and can be easily applied at a large scale; (3) marked fish can be unambiguously identified, for extended periods after application; and (4) the mark is quick and inexpensive to detect (Thorrold et al., 2002). For successful uptake of new tag and marking methodologies, these four criteria need to be determined if the application is to be incorporated into stock enhancement/re-introduction programs. While I could not successfully test all criteria, such as marking at a large scale and determining mark identification after an extended period, I have been able to show that stable isotope larval immersion techniques fit within standard hatchery operating protocols and marking has no impact on the mortality or growth of larval fish. Multiple

isotopes can be used to create unique signatures, and these signatures can be discriminated, with high accuracy.

6.2.1 Suitability as a hatchery marking technique

Holding fish in intensive hatchery settings can be costly in food and maintenance, and high stocking densities and overcrowding can increase physiological stress to fish, diminishing their quality and therefore survival once stocked. Golden perch and Murray cod *Maccullochella peelii* are only held in the hatchery at two life stages prior to stocking. The first is after initial hatching, when fish feed endogenously on the yolk, which can range from 4-6 days. They are then stocked out into grow-out ponds, which have an approximate surface area of 3500 m^2, and have a natural zooplankton population upon which fish feed. The second time the fish are held in the hatchery is prior to release, when fish are held for 1-2 days to assess fish condition, before being released into rivers or reservoirs.

Fish were successfully marked just after hatching in this study (Chapters 2 & 3), although fingerlings have previously been marked just prior to release (Munro et al., 2008). The two species marked at the larval stage have different life histories. Golden perch are group spawners that lay pelagic eggs, which hatch into small (3.5–4.0 mm) poorly developed larvae (Lake, 1967, Rowland, 1996), whereas Murray cod lay demersal, adhesive eggs in nests guarded by the male. Murray cod larvae are larger (9.5–14.8 mm) and more developed larvae than golden perch larvae at hatching; they are active and can begin feeding before complete absorption of the yolk (Koehn & Harrington, 2006). The difference in biology between golden perch and Murray cod require different hatchery production protocols (Rowland, 1983, Rowland, 1996) and approaches to applying otolith chemical marks. Golden perch eggs are held in flow-through cylindrical tanks with light aeration to help suspended eggs, upon hatching the

water flow is turned off while aeration is continued to help maintain pelagic larvae throughout the water column. Murray cod eggs that have been laid on mesh plates are hung vertically in large flow-through tanks, when close to hatching they are placed in long shallow flow through troughs. Upon hatching, water flow is maintained, as larvae tend to sit on the bottom of the tank. However, for the purpose of immersion marking Murray cod larvae, these rearing protocols were successfully adjusted to include a static holding period to allow for successful marking of fish without compromising the health of the larvae.

Both golden perch and Murray cod were successfully held in enriched isotope holding tanks with various combinations and concentrations of enriched isotopes. Larval fish can be maintained at higher densities than fingerlings, which reduces the cost of immersion marking because less chemical is required to mark fish. In addition, larvae are generally held in a closed, aerated system (i.e. non flow-through), which again minimises costs relative to a flow-through system, as constant supplement of enriched isotopes is not required. Using various concentrations of ^{137}Ba, a shift in the $^{138}Ba/^{137}Ba$ isotope ratio could be created in as little as one day, however it took 5-days for the otolith isotope ratio to equilibrate with the ratio of the water (Woodcock et al., 2011, Chapter 2). Longer enrichment periods provided at the larval stage, allow for distinctive signatures, as otolith increment growth can incorporate more of the enriched isotope signatures. In addition, marking during the larval period ensures the mark is positioned close to the core of the otolith. A mark near the core that covers a few days of otolith growth potentially allows for easier identification, compared to marking at a later stage or using transgenerational-marking techniques. The success of marking both species at the larval stage suggests that other species could be marked in a similar

manner in hatcheries if water quality can be maintained long enough to allow for successful incorporation of marking chemicals.

6.2.2 Correctly identifying isotope signatures in otoliths

In Chapters 2 and 3, I used seven stable isotopes from three elements, Ba (^{136}Ba, ^{137}Ba and ^{138}Ba), Mg (^{24}Mg, ^{25}Mg and ^{26}Mg) and Sr (^{88}Sr) to create unique marks in the otoliths of larval golden perch and Murray cod (Table 6.1). I tested twenty-five isotope signatures of which eleven could be clearly distinguished. Successful marking was only accomplished using isotopes of Ba and Sr. Magnesium isotopes were not successful in creating distinctive marks. Isotope ^{24}Mg was tested on both species and failed to create a shift in isotope ratios in the otoliths of larval golden perch after 4-days immersion or in Murray cod after 6-days of immersion at the concentrations used. Although ^{25}Mg and ^{26}Mg isotopes created an isotope shift in the otoliths of golden perch, considerable variation in the otolith Mg ratios was found among fish, and these isotopes were considered poor markers. Isotopes ^{25}Mg and ^{26}Mg were not tested on Murray cod. Further discussion on Mg incorporation is provided under Factors Influencing the Success of Otolith Chemical Marking (section 6.3).

With the four enriched isotopes that could be used successfully to create marks (^{136}Ba, ^{137}Ba, ^{138}Ba and ^{88}Sr), fifteen unique marks are possible. I only marked fish with eleven of the fifteen possible signatures (Table 6.1). Fish marked with these eleven marks could be clearly discriminated and classified to their respective mark with high accuracy (> 90%). Isotopes of Ba and Sr have also been used to mark other species using transgenerational techniques (Thorrold et al., 2006, Almany et al., 2007, Munro et al., 2009, Williamson et al., 2009a, Williamson et al., 2009b), or immersion at the fingerling stage (Munro et al., 2008). These studies have used two additional isotopes to those I investigated including ^{135}Ba (Williamson et al., 2009a) and ^{86}Sr (Munro et al.,

2008). Using the seven isotopes that have been successful in altering otolith isotope ratios (^{135}Ba, ^{136}Ba, ^{137}Ba, ^{138}Ba, ^{86}Sr and ^{88}Sr), sixty-three isotope marks could potentially be produced. When using more than one isotope, the number of comparable natural ratios required to be used for comparison increases. For example, marking with ^{137}Ba and ^{88}Sr, the natural ^{137}Ba/^{138}Ba and ^{88}Sr/^{86}Sr ratios can be used for comparison. However, if combinations of isotopes from one element are used, a more sophisticated approach will be required to distinguish treatments. For example combining ^{137}Ba with ^{138}Ba, the combined ^{137}Ba+^{138}Ba ratio signature created may not be that dissimilar from the natural ^{137}Ba/^{138}Ba ratio. Therefore comparison against an unaltered isotope, for example ^{136}Ba, to give comparable ratios ^{137}Ba/^{136}Ba and ^{138}Ba/^{136}Ba, may be required to distinguish between marks made up of these multiple isotopes.

Table 6.1: Summary of isotope signatures used to mark larvae using immersion methods in Chapters 2 & 3. *Asterisks indicate treatments with high mark success and correct classification (> 90%)

Golden perch	Both species	Murray cod
Ba isotope signatures	Ba isotope signatures	Sr isotope signatures
*^{136}Ba	*^{137}Ba	*^{88}Sr
*^{136}Ba + ^{137}Ba	*^{138}Ba	
*^{136}Ba + ^{138}Ba	*^{137}Ba + ^{138}Ba	Combined isotope signatures
*^{136}Ba + ^{137}Ba + ^{138}Ba		*^{137}Ba + ^{88}Sr
	Mg isotopes signatures	*^{138}Ba + ^{88}Sr
Mg isotopes signatures	^{24}Mg	^{138}Ba + ^{24}Mg
^{25}Mg		^{24}Mg + ^{88}Sr
^{26}Mg	Combined isotope signatures	^{137}Ba + ^{138}Ba + ^{24}Mg
^{24}Mg + ^{25}Mg	^{137}Ba + ^{24}Mg	*^{137}Ba + ^{138}Ba + ^{88}Sr
^{24}Mg + ^{26}Mg		^{137}Ba + ^{24}Mg + ^{88}Sr
^{25}Mg + ^{26}Mg		^{138}Ba + ^{24}Mg + ^{88}Sr
^{24}Mg + ^{25}Mg + ^{26}Mg		^{137}Ba + ^{138}Ba + ^{24}Mg + ^{88}Sr

Multiple marks allows for identification of stocking several cohorts of fish in a system, in addition, these marks could be used to determine the success of different stocking parameters, as discussed in Chapter 5, to determine the influences of stocking density and location. The use of multiple isotope marks in fish stocked into the one system, however, may result in complications of mark identification, if testing for an unknown isotope signature. Records of isotopes used to mark fish, the species marked and the year stocked need to be established and maintained by hatcheries that take up this method of marking. The North Pacific Anadromous Fish Commission (NPAFC), keep records of otolith mark patterns to improve the mark recognition and determine hatchery origin of stocked salmon in the North Pacific (NPAFC, 2006). Detailed records of marks used for hatchery fish stocked into river systems will assist in the later identification of marked fish to allow the focus on the isotope ratios used to easily detect and identify marked fish.

6.2.3 Influence on mortality and early growth

In Chapters 2 & 3, I demonstrated that enriched isotopes of Ba and Sr could be successfully used to mark larval golden perch and Murray cod. No differences in mortality or growth of marked individuals compared to control treatments were detected for either species or for any of the isotope combinations used to mark fish. This is similar to findings from other immersion marking studies which used elements (Ennevor & Beames, 1993) or fluorescent compounds (Taylor et al., 2005a, Crook et al., 2007, Crook et al., 2009). Mortality and growth is generally only investigated for days or weeks post-marking and therefore further investigations to test for potential long-term impacts are required.

6.2.4 Future directions

Isotope marking studies have focused on the ability to mark, and identify marks with clarity. Further research is required to determine the ease of scaling up marking techniques, looking at the success of enriching standard larval rearing tanks and detecting isotope or elemental signatures after a significant time when there has been reasonable otolith growth. Larval and embryo marking should be detectable by focusing analysis close to the core of the otolith (Macdonald et al., 2008). Marking at a later life history stage may require knowledge of the average otolith diameter at the time of marking to pin-point the region of the otolith where the mark is likely to be located, or marking with enriched isotopes could be paired with fluorescent banding with calcein (Crook et al., 2009) to highlight the marked area.

The other issue is the width of the isotope mark within the otolith. By marking at the larval stage while fish feed endogenously, the otolith can be exposed to isotope- or element- supplemented water for 4-6 days of growth, increasing the width of the mark in the otolith and therefore the ability to subsequently detect a mark. Similarly, marking at a later stage where immersion time may be limited, reduces not only the width of the marked increment, but the marked section will likely not be a direct reflection of the water chemistry due to the time it takes for otolith and water chemistry to match (for examples see Chapters 2 & 3 and Munro et al., 2008). Using small laser beams, detecting the exact isotope mark may be complicated and likely require transects across the core. Likewise, if too large a laser beam is used, the mark signature quality may be reduced due to dilution of the isotope signal by ablation of non-marked regions of the otolith (Ruttenberg & Warner, 2006, Macdonald et al., 2008). Otolith-sampling techniques, may therefore, need to focus on identifying the best methods for identifying the mark; for example, using solution techniques, by dissolving the core and

surrounding growth, compared to laser ablation may be a possible solution. For laser techniques, different approaches such as drilling through the otolith or analysis of thin transects across the core may be required.

6.3 Factors Influencing the Success of Otolith Chemical Marking

Many studies have investigated factors that influence otolith chemistry. These can include environmental factors such as temperature and salinity or trace elements in the water (Bath et al., 2000, Elsdon & Gillanders, 2002, Elsdon & Gillanders, 2003b, Martin & Thorrold, 2005) as well as physiological factors such as regulation of elemental intake (Thresher et al., 1994, Proctor et al., 1995, Melancon et al., 2009). In addition, the source of elements into fish otoliths, namely diet vs. water contributions may also influence otolith chemistry (Buckel et al., 2004, Walther & Thorrold, 2006).

6.3.1 Elemental source: water vs. diet

Elements incorporated into otoliths are derived from either the environment in which a fish inhabits or its diet (Campana, 1999, Elsdon & Gillanders, 2003a, Elsdon et al., 2008). The use of otolith elemental composition to understand past environmental histories has been based on the assumption that elemental incorporation in otoliths is proportional to the ambient concentration of a particular element in the water (Martin & Thorrold, 2005). Similarly, immersion-marking techniques also rely on uptake from the water. Linear relations between water and otoliths have been shown for elements such as Ba and Sr (Bath et al., 2000, Elsdon & Gillanders, 2002) with over 80% of otolith Sr and more than 95% of otolith Ba originating from the rearing water (Walther & Thorrold, 2006, Appendix A). This relationship for Ba and Sr between the water and otolith was the basis for successful immersion marking techniques using enriched isotopes from these elements (Chapters 2 & 3). Water was the major contributor of Mg

in the otolith of silver perch *Bidyanus bidyanus* (Chapter 4). Despite water being the major contributor, there was a poor relation between otolith and water Mg with little change in the overall Mg concentration or shift in the Mg isotope ratio in otoliths of fish. This is comparable to the lack of a successful mark using enriched stable isotopes of Mg in golden perch and Murray cod (Chapters 2 & 3 respectively).

When otolith chemistry does not reflect the water chemistry, diet is assumed the main contributor. Studies investigating the influence of diet on otolith chemistry have shown contradictory results. Fish fed a diet of either shrimp or fish prey for 60-days had different otolith chemical compositions (Buckel et al., 2004) suggesting an influence of diet. However, diet has also been found to have little to no effect on otolith chemistry for a range of elements (Hoff & Fuiman, 1995, Milton & Chenery, 2001). My research on Mg, where contributions of water and diet were assessed, found no relation between Mg concentrations in fish otoliths and their diet or water. The poor relation between fish otoliths and both diet and water suggests that Mg is physiologically regulated in fish.

6.3.2 Physiological regulation of elements

A number of elements (e.g. Na, K, S, P and Cl) are physiologically regulated in fish (Thresher et al., 1994, Proctor et al., 1995). Many studies have investigated the partitioning of elements from the water into the otolith, however it is important to consider that regulation of elements by the gills in freshwater fish, or the intestine wall in marine fish, and other membranes, occurs before the elements are transported by the blood to the endolymph (Melancon et al., 2009). Recent research investigating the difference in the concentration of elements between the water, blood, endolymph and otoliths of two species of freshwater fish, *Salvelinus namaycush* and *Lota lota*, found elements were more enriched in the blood compared to the water. Furthermore, while

blood and endolymph shared similar concentrations of metals, elements were depleted in the otolith compared to the endolymph (Melancon et al., 2009). Unfortunately, fish otolith and endolymph samples were taken from different fish to which blood samples were taken, and water samples were collected a year after fish sampling, therefore direct comparisons were not possible (Melancon et al., 2009). Further studies may be required to determine the fractionation of elements between fish and the environment.

The similarities in the endolymph chemistry between *Salvelinus namaycush* and *Lota lota* which occupy similar bottom-dwelling niches and display similar feeding behaviours, was not reflected in the otoliths of these two species in which the chemical composition of the otoliths were significantly different (Melancon et al., 2009). Differences in the elemental composition of otoliths of different species of fish have been found, despite being collected from within the same site (Gillanders & Kingsford, 2003, Hamer & Jenkins, 2007, Melancon et al., 2009). Although these differences are often small and do not prevent the use of otolith elemental composition to distinguish differences between collection sites (Hamer & Jenkins, 2007), further work looking at differences in otolith structure, difference in fish physiology and variations in diet or habitat use may be required to identify the differences in elemental incorporation between species.

6.3.4 Future directions

A better understanding of the transport of elements within fish and inter-species differences is needed to determine where elements are partitioned and to achieve a better understanding of how otolith chemistry reflects the environment for a range of elements. The partitioning of elements does not necessarily invalidate the use of otolith chemistry for reconstructing environmental conditions experienced by individual fish, providing that the degree of regulation for various elements remains constant, or

changes in a predictable manner for the variable of interest, for example, temperature or salinity (Martin & Thorrold, 2005).

Otoliths are generally composed of one of three polymorphs of calcium carbonate (aragonite, calcite and vaterite) each with a unique crystal shape (Tomás & Geffen, 2003). The difference in the structure of the crystal allows different elements to be incorporated into the growing lattice structure, dependent on the size of the element. Aragonite has high concentrations of Ba and Sr compared to vaterite that characteristically has high concentrations of Mg and Mn (Tomás & Geffen, 2003, Melancon et al., 2005, Melancon et al., 2009). The difference in the chemical composition between the different CaCO3 crystal structures has implications for otolith chemistry in regards to stock discrimination and chemical marking. Further research looking at the differences in elemental incorporation into different otolith structures is required to clarify variation in otolith chemistry studies.

6.4 Enhancing Post-Stock Survival

With stock enhancement programs occurring worldwide (Hilborn, 1998, Welcomme & Bartley, 1998), information on the factors contributing to the success or failure of stocking techniques is needed. Simply rearing and stocking fish into water systems is no longer sufficient for successful stock enhancement. Many factors influence the success of stock enhancement programs with the major one being the survival and acclimation of fish after stocking.

Despite large scale stocking of native species into river systems (Brown & Harris, 1995, Gillanders et al., 2006), the behaviour and survival of newly released fish is difficult to determine. In addition, research on other stocked systems has found high mortality of hatchery-reared fish while acclimating to a new environment due to translocation,

stocking stress and predation (Armstrong & Seddon, 2008). Therefore, efforts to better prepare fish for release while within a hatchery are being trialled and include habitat selection, predator awareness and prey recognition (Berejikian et al., 1999, Ellis et al., 2002, Gazdewich & Chivers, 2002, Kawabata et al., 2011).

In Chapter 5, pre-stock predator training was conducted on golden perch, and their behaviour when stocked with a potential predator was observed. This method has been trialled on a number of species, with fish displaying reduced foraging activity and area of occupancy following exposure to predator odours (Berejikian et al., 1999, Darwish et al., 2005). Golden perch (Chapter 5) showed no behavioural differences in fish pre-exposed to predator and alarm stimuli compared to naive fish. Other methods, including visual training (Vilhunen et al., 2005), have been successful for other species, and similar methods should be trialled on native Australian fish to determine if they induce a behavioural response. The effectiveness of pre-stock training and the practicality of training methods, may limit methods that can be incorporated into hatcheries. Further research into the behaviour and survival of fish once stocked into natural systems needs to be conducted to validate pre-stock training to justify potential changes to fish stocking programs.

In addition to testing the influence of pre-stock training, Chapter 5 also investigated the influence habitat structure had on the behaviour of stocked fish. Increased habitat reduced the movement of fish at both high and low stocking densities. Efforts to better prepare fish, especially those reared in intensive aquaculture systems to natural prey, daily light cycles, complex environments and predation, paired with the identification of suitable stocking habitats for individual stocking species are required. Selection of suitable stocking locations to enhance the post-release survival of stocked fish needs to include assessment of appropriate habitat, prey abundance and reduced predation

pressure to increase the likelihood of stocked fish contributing to fisheries and later population recruitment. This will lead to better success of fish stock enhancement programs.

6.4.1 Future directions

The success of stocking programs needs to be evaluated. Mortality is generally higher at early life history stages; therefore, it is important to determine survival of stocked fish immediately after stocking, as well as later in life. The stocking sites may also require monitoring to determine if the available food supply and habitat is adequate for survival of stocked fish. Depending on the availability of prey, stocking locations may need to be re-evaluated or fish stocked at lower densities. If predation is responsible for low survival rates, further investigation is required to make stocked fish predator aware or fish could be stocked into areas of reduced predator abundances. This might include visual training to predators, or a better understanding of the mechanisms involved in predator recognition for particular species of fish that do not respond to odour cues.

6.5 Conclusion

For successful evaluation of stock enhancement programs, stocked fish need to be distinguishable from natural recruits. Enriched stable isotope marking provides a method to mass mark fish during early life history stages, allowing all hatcheries that produce and stock marked fish to be distinguished. Marking with stable isotopes of Ba and Sr displayed not only high mark success rates, but these marks could be distinguished from not only unmarked fish, but from fish marked with similar isotope signatures (e.g. marking with ^{137}Ba + ^{138}Ba compared to only ^{137}Ba). Continued research into the mechanisms controlling the uptake of elements and the fractionation of elements from the water to the otolith will help not only to clarify elements or isotopes

which will create successful mark signatures, but as with Mg, will help to clarify elements which are not suitable for marking due to high physiological regulation. Further techniques to maximise survival of stocked fish needs to occur. By selecting appropriate sites, and stocking fish at densities that will reduce crowding, will help fish to adapt to a new environment, reducing risk of early predation. Techniques developed in this thesis will help to improve stocking programs by providing a way of assessing the contribution that stocked fish have on natural systems.

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Photo: Golden perch fingerlings in holding tank

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Appendices

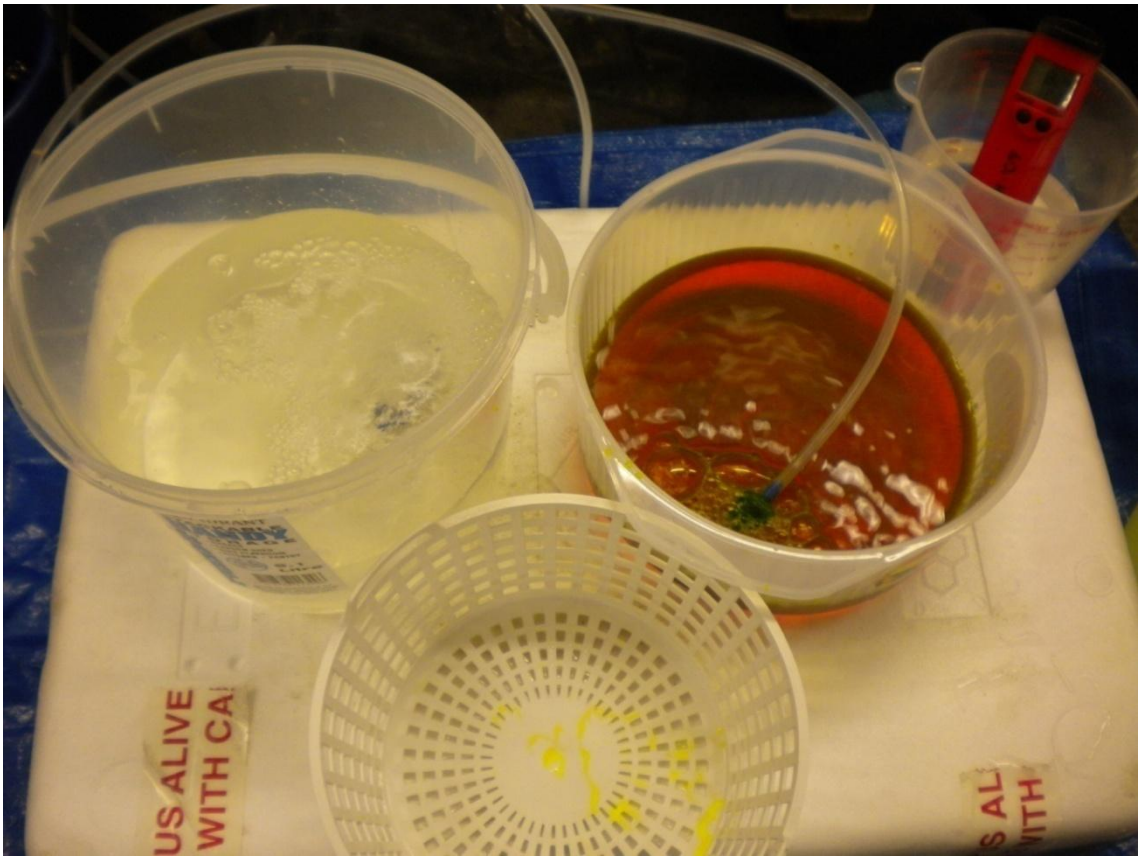

Photo: Osmotic induction marking chemical bath set up

Appendix A

Preamble

This appendix is a co-authored paper, with intention to publish in a peer-reviewed scientific journal. The paper is an adaption from the honours work conducted by Simon Webb 2010, citation of work conducted in this Appendix should be to Simon Webb's honours thesis, "Sources of otolith barium (Ba) and strontium (Sr) in black bream (*Acanthopagrus butcheri*) and the potential influences of salinity and temperature" until publication of the work.

Gillanders and Woodcock conceived and designed the project. Webb was responsible for the acquisition and analysis of data and for the initial draft of the manuscript. Woodcock and Gillanders extensively revised the manuscript for publication. I contributed to the design and assisted with experimental set up and analysis on samples. I was also largely involved in data interpretation and revision of the text.

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Sources of otolith barium and strontium in estuarine fish and the influence of salinity and temperature

Abstract

Otoliths are commonly used to reconstruct migratory patterns and to determine stock structure of fish, owing to the chemical relations between water and otolith chemistry. Water is only one source of variation in otolith chemistry and the contribution that diet plays to the chemical composition of otoliths under different environmental influences, temperature and salinity, has been widely unexplored. To determine the percentage contributions from water and diet to otolith Ba and Sr, juvenile *Acanthopagrus butcheri* (Munro 1949) (Family Sparidae) were reared in three different salinities under three different water temperatures. Rearing water and diet were enriched with stable isotopes of Ba (^{137}Ba and ^{136}Ba) and Sr (^{88}Sr and ^{86}Sr) thus enabling contributions of water versus diet to the otolith to be determined. Ambient water was the primary contributor to otolith Ba (between 54 and 95 %) and Sr (between 49 and 87 %). Water contributions to otolith Ba were not significantly affected by temperature or salinity. A significant interactive effect of temperature and salinity on water contributions to otolith Sr was detected, which was most evident at high temperatures where contributions decreased with increasing salinity. This study supports water as the primary contributor of otolith chemistry and suggests that the contribution of water can be influenced by environmental factors such as temperature and salinity.

Introduction

Elemental and isotopic concentrations of calcified structures have been used to make inferences of past diets and/or environmental history of terrestrial and aquatic organisms. Such inferences are made through comparisons of element concentrations

and isotopic ratios present in calcified structures with those in the organism's surrounding environment. The most commonly used calcified structure in fish are otoliths, paired calcium carbonate ($CaCO_3$) structures present in all teleost fish which assist in balance and hearing (Campana, 1999); they are found within the inner ear, suspended in endolymphatic fluid and isolated by a semi-permeable membrane, the endolymph (Campana & Thorrold, 2001). The chemical composition of otoliths reflects that in which the fish are exposed to throughout their life (Campana, 1999). In addition, otoliths are metabolically inert (Campana & Thorrold, 2001), which means that they are not subject to re-absorption and therefore their structure and subsequent elemental concentrations remain unaltered throughout the fish's life. These characteristics of otoliths make them an ideal candidate for reconstructing the past history of fish.

Elements are crystallised out of the surrounding endolymph fluid onto the otolith edge of fish in concentric rings (Campana & Neilson, 1985). Increments and their subsequent element concentrations can thereby provide a chemical 'journal' of a fish's life, which can then be used to determine past history (Campana, 1999). Elements such as strontium (Sr) and barium (Ba) which are known to substitute for calcium (Ca) in the $CaCO_3$ structure (Campana, 1999, Bath et al., 2000), display high correlations between the environment and otoliths (Bath et al., 2000, Elsdon & Gillanders, 2003, de Vries et al., 2005). Ambient water and diet represent the two known sources of otolith chemistry (Campana, 1999) and incorporation of trace elements from these sources maybe controlled by environmental (Elsdon & Gillanders, 2003, Arai et al., 2004, Kraus & Secor, 2004) and physiological variables (Kalish, 1989, Sadovy & Severin, 1994, Arai et al., 2003), which may affect biomineralisation of elements into the otolith.

Few studies have investigated the relative contributions of diet or water to otolith composition (but see Farrell & Campana, 1996, Kennedy et al., 2000, Walther & Thorrold, 2006, Gibson-Reinemer et al., 2009). Most conclude that water is the major contributor to otolith chemistry and that diet has little or no effect on otolith chemistry (Farrell & Campana, 1996, Walther & Thorrold, 2006, Gibson-Reinemer et al., 2009). However, Kennedy et al. (2000) suggested that 70 % of Sr in *Salmo salar* otoliths was derived from dietary sources. Past contribution studies, have examined the contribution from either water or diet only at a single salinity and temperature, and therefore were unable to report any salinity, temperature or interactive effects on source contributions. Despite this, salinity or temperature may influence contributions from each source, through impacts on osmoregulation as well as water and/or food intake.

Osmoregulation is the process of altering water potential to maintain the fluid and electrolyte balance within the fish despite changes in ambient water conditions (Lignot et al., 2000). Osmoregulation is important for both marine and freshwater fish. Marine fish require a large water intake as water-loss is high due to osmosis and the passive movement of water out of the fish into ambient water (Campana, 1999, Boeuf & Payan, 2001). Freshwater fish, however, do not require a large water intake, but they need to actively uptake ions from ambient water as concentrations are lower in water compared to the fish (Perry, 1997). Since marine and freshwater fish have different adaptations for osmoregulation, different amounts of elements may be available from ambient water for otolith incorporation. Therefore, estuarine fish, such as *Acanthopagrus butcheri* (Munro 1949) (Family Sparidae), which moves between fresh and marine waters, may have different amounts of elements available for uptake due to the water they reside in. This may lead to either an increase or a decrease in the relative contribution from water and the opposite trend in dietary contribution.

Dietary intake in fish is influenced by water temperature and salinity (Swanson, 1998, Boeuf & Payan, 2001, Arjona et al., 2009). Food intake tends to be greater at higher temperatures (Swanson, 1998, Boeuf & Payan, 2001, Imsland et al., 2001, Arjona et al., 2009) and higher salinities (Rubio et al., 2005). There may however, be an upper temperature and salinity limit for food intake, where intake declines with further increases in temperature and salinity (Imsland et al., 2001, Handeland et al., 2008, Luz et al., 2008). A change in environment may lead to a change in food intake thereby affecting relative contributions to otolith chemistry since varying amounts of elements will be available for incorporation from the diet.

Relative contributions of diet and ambient water to otolith chemistry from past studies have reported varying results and therefore the contributions of each are still relatively unknown. In addition, no studies have addressed the potential influence of changing salinity and temperature on diet versus water contributions to otolith chemistry. Thus, we test the hypotheses that (1) ambient water is the main contributor to barium and strontium otolith chemistry, and (2) salinity and temperature will influence the percent contribution of elements into the otolith, due to changes in diet and water requirements

Methods

Experimental design

Juvenile *Acanthopagrus butcheri* (Munro 1949) (Family Sparidae), approximately 20-30 mm total length (TL), were obtained from Challenger TAFE hatchery, Western Australia, and held in three 250 l fibreglass tanks at a salinity of 35 ‰ and a water temperature of 16 °C. Each tank was adjusted to one of three salinities (10, 20 and 30) through 5 ‰ changes per day. After an acclimatisation period of at least 24 hrs at set salinities, fish were marked with 0.5 % calcein using the osmotic induction technique

described by Crook et al. (2009). Following calcein marking, fish were assigned to treatment tanks at their respective salinities, which were then adjusted to one of three different water temperatures (16, 20 and 24 °C) through 2 °C changes per day.

Experimental treatments consisted of two replicate 15 l acid- and bleach-washed plastic tanks containing 10 l of water. A small internal filter was placed in each tank (JT Filtration Pump, JHJ-411B, 300 l h^{-1}) along with an air stone. Tanks were held in water baths to maintain desired water temperature during the experimental period, and were covered with clear plexiglass lids to minimise evaporation. All saltwater used during the experiment was delivered from South Australian Research and Development Institute (SARDI) Aquatic Sciences division at West Beach, South Australia, and held in an outdoor storage tank, and diluted to the desired salinity using aged freshwater. Water was spiked with ^{137}Ba or ^{88}Sr by dissolving isotopically enriched $BaCO_3$ or $SrCO_3$ (Oak Ridge National Laboratories, Oak Ridge, Tennessee, USA) at a concentration of 0.1 mg l^{-1} for ^{137}Ba or 0.25 mg l^{-1} for ^{88}Sr; enriched concentrations were based on elemental baseline water concentrations. Enriched Ba and Sr treatments consisted of all possible combinations of water temperature (16, 20 and 24 °C) and salinity (10, 20 and 30), referred to hereafter as low, ambient and high, respectively. Water quality was maintained through weekly 50 % water changes.

Fish were fed a gelatin-based diet during the acclimatisation period based on the recipe by Royes and Chapman (2003). Ingredients included prawns in brine (drained), frozen spinach, grated carrot, rolled oats, wheat germ and cod liver oil. During the experiment, fish were fed the same gelatin-based diet spiked with either ^{136}Ba at 0.1 µg g^{-1} for Ba treatments or ^{86}Sr at 0.25 µg g^{-1} for Sr treatments. Isotopes were in a similar carbonate form as those used to enrich water. Fish were fed twice daily throughout the experiment

and any detritus remaining after 30 min was siphoned away. Fish were exposed to experimental conditions for 32 d, after which fish were euthanised through immersion in an ice slurry and immediately frozen until otolith extraction.

Water and diet sampling and analysis

Water samples were collected after each water change throughout the experimental period (n = 7). Each sample was collected from each tank using a 25 ml syringe, filtered through a 0.45 µm filter into acid-washed 30 ml plastic vials, and acidified with 0.5 ml of concentrated nitric acid. Water samples were frozen until analysis, where they were diluted 1:10 with 2 % nitric acid. To determine the isotope concentrations of Ba and Sr in the diets, a sample of each diet (n = 3 lots of 5 g) was oven dried at 60 $^{\circ}$C for 24 hrs, ground using a mortar and pestle and dissolved in 0.025 g mL^{-1} nitric acid. Dilution was based on a pilot study done to determine the ability to measure the concentration of Sr and Ba in the diet. Samples were left to dissolve for 1-week. Each sample was then diluted to achieve a 2 % nitric acid concentration using ultrapure water, before being filtered through 0.45 µm filter for analysis.

Water and diet samples were analysed using an Agilent 7500 cs (www.agilent.com) Inductively Coupled-Plasma Mass Spectrometer (ICP-MS); see Table I for operating parameters. Sr and Ba water samples were analysed separately using individual sets of standards. A natural multi-element stock standard was run for both Ba and Sr samples at 1, 50, 100 and 500 µg l^{-1}. Ba standards included two additional standards for each isotope, ^{136}Ba and ^{137}Ba at 50 and 200 µg l^{-1}. The Sr standards included four additional isotope-enriched solutions, two for both ^{86}Sr and ^{88}Sr at 150 and 350 µg l^{-1}. Standards and blanks were analysed periodically throughout the session. Agilent Mass Hunter was used to collect raw data, which was calibrated against the elemental standards. Isotope

counts per second were further corrected against both the elemental and isotope standards, before being used to calculate the isotope ratios of interest. The water $^{137}Ba/^{43}Ca$ and $^{88}Sr/^{43}Ca$ ratios were used as the general water Ba:Ca and Sr:Ca ratios.

Table I: Operating parameters on the Agilent 7500cs inductively coupled-plasma mass spectrometer (ICP-MS) used to analyse water and diet samples and the operating parameters for the New Wave Nd Yag 213 UV laser with ICP-MS used to analyse otoliths.

Solution ICP-MS	
Collision Cell	He (5 ml/min)
Cone	Pt
Integration time	0.10 sec with 3 replicates for each isotope (^{43}Ca, ^{88}Sr, ^{86}Sr, ^{136}Ba, ^{137}Ba)
Laser	
Wavelength	213 nm
Mode	Q-switch
Frequency	5 Hz
Spot Size	30 μm
Laser Power	65%
Carrier Gas	Ar (0.95 L/min)
ICP-MS	
Optional Gas	He (58%)
Cone	Pt
Dwell Times	^{115}In (50 ms)
	^{43}Ca (100 ms)
	^{88}Sr, ^{86}Sr (200 ms)
	^{136}Ba, ^{137}Ba (400 ms)

Otolith preparation and analysis

One otolith from each fish was embedded in a two-part epoxy (Struers, EpoFix resin and hardener) spiked with 40 ppm indium, and 0.35 mm sections were cut using a low speed saw (Buehler, Isomet low speed saw, model no. 11-1280-250). Sections were then polished using lapping film (3 μm grit size) before being fixed onto microscope slides using indium spiked CrystalBond 509 thermoplastic glue (see Munro et al., 2008 for additional details). Otoliths were analysed on a New Wave Nd Yag 213nm UV laser

operated in Q-switch mode connected to an Agilent 7500cs ICP-MS; see Table 1 for laser and ICP-MS operating parameters. The edges of otoliths were sampled using spot analyses to ensure otolith material laid down during experimental conditions was analysed. Ba and Sr isotopes (^{137}Ba, ^{136}Ba, ^{88}Sr and ^{86}Sr) used to enrich holding water and diet were measured along with ^{43}Ca for element:Ca ratios and ^{115}In, to determine when otolith material was no longer analysed. A reference standard, NIST 612, (National Institute of Standards and Technology; www.nist.gov) was analysed throughout each session and used to correct for mass bias and machine drift. The average value of the smoothed ^{137}Ba/^{136}Ba isotope ratio data and the ^{88}Sr/^{86}Sr isotope ratio data were used as the isotopic value for each sample. The otolith ^{137}Ba/^{43}Ca and ^{88}Sr/^{43}Ca ratios were used as the overall otolith Ba:Ca and Sr:Ca ratios (henceforth referred to as Ba:Ca and Sr:Ca).

Statistical analysis

Statistical analyses were conducted using PRIMER 6/PERMANOVA (www.primer-e.com). Differences in the Ba and Sr ratio of water samples and otoliths were analysed individually using three-way permutational univariate analysis of variance (ANOVA) with unrestricted permutations for both Ba:Ca and Sr:Ca and for isotopic ratios ^{137}Ba/^{136}Ba and ^{88}Sr/^{86}Sr. Temperature and salinity were treated as fixed factors with replicate tanks treated as a random factor (nested in both temperature and salinity). Differences in the isotope ratios of the diets were tested using one-way ANOVAs. If significant differences were detected within single or multi-factor ANOVAs, *post-hoc* pair-wise tests were used to determine which treatments or tanks differed.

Percentage contributions

The percent contributions from ambient water or diet to otolith Sr and Ba were calculated using the following equations (Kennedy et al., 2000). Calculations used log values of the isotope ratios ($^{137}Ba/^{136}Ba$ or $^{88}Sr/^{86}Sr$) for each factor (otolith, water and diet). To determine if there were significant differences in the percent contribution among the different treatments similar ANOVAs to those described above were used.

$$\%Element_{(water)} = \left[1 - \left(\frac{Isotope\ Ratio_{(water)} - Isotope\ Ratio_{(otolith)}}{Isotope\ Ratio_{(water)} - Isotope\ Ratio_{(diet)}}\right)\right] \times 100$$

$$\%Element_{(diet)} = \left[1 - \left(\frac{Isotope\ Ratio_{(otolith)} - Isotope\ Ratio_{(diet)}}{Isotope\ Ratio_{(water)} - Isotope\ Ratio_{(diet)}}\right)\right] \times 100$$

Results

Rearing conditions

The temperature and salinity of the tanks were similar to the required experimental parameters (Table II). A significant difference was detected among temperature and salinity treatments for both Ba and Sr enriched treatments (Table III), which corresponded to differences between the set treatment parameters (Table II). Slight variation in temperature and salinity was detected between replicate tanks for both Ba and Sr (Table III), likely due to interactive effects of salinity and temperature and evaporation at high temperatures.

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Table II: Summary of rearing conditions of *Acanthopagrus butcheri* for (a) Ba enriched treatments and (b) Sr enriched treatments at temperature (Low (L) 16°C, Ambient (A) 20 °C and High (H) 24°C) and salinity (Low (L) 10, Ambient (A) 20 and High (H) 30).

Treatment										
Temperature	Salinity	Tank	Ba: Ca	$^{137}Ba/^{136}Ba$	Temperature (°C) n = 32	Salinity (‰) *n* = 32	Sr: Ca	$^{88}Sr/^{86}Sr$	Temperature (°C) *n* = 32	Salinity (‰) *n* = 32
(a)							(b)			
L	L	1	10.97 ± 0.68	34.57 ± 1.33	15.51 ± 0.19	10.2 ± 0.03	45.19 ± 0.55	10.43 ± 0.11	16.16 ± 0.20	10.1 ± 0.03
	L	2	10.29 ± 0.56	33.17 ± 0.69	15.50 ± 0.20	10.3 ± 0.04	45.13 ± 0.55	10.47 ± 0.09	16.22 ± 0.20	10.1 ± 0.03
	A	1	5.02 ± 0.28	40.18 ± 1.91	15.54 ± 0.20	20.4 ± 0.07	42.09 ± 0.52	9.42 ± 0.06	16.19 ± 0.20	20.1 ± 0.04
	A	2	5.08 ± 0.34	39.41 ± 1.08	15.50 ± 0.20	20.7 ± 0.10	42.47 ± 0.47	9.42 ± 0.05	15.83 ± 0.20	20.2 ± 0.05
	H	1	3.13 ± 0.20	51.27 ± 1.75	15.59 ± 0.20	30.3 ± 0.13	41.10 ± 0.49	9.10 ± 0.03	15.85 ± 0.20	30.0 ± 0.06
	H	2	3.19 ± 0.21	49.14 ± 1.50	15.66 ± 0.20	30.4 ± 0.11	40.35 ± 0.30	9.09 ± 0.03	16.03 ± 0.20	30.2 ± 0.06
A	L	1	10.50 ± 0.55	33.27 ± 0.62	19.58 ± 0.26	10.4 ± 0.05	44.97 ± 0.69	10.49 ± 0.13	19.95 ± 0.13	10.2 ± 0.04
	L	2	11.41 ± 0.83	34.49 ± 0.96	19.68 ± 0.26	10.4 ± 0.05	45.30 ± 0.87	10.44 ± 0.11	20.15 ± 0.13	10.1 ± 0.03
	A	1	5.34 ± 0.20	38.98 ± 0.68	19.71 ± 0.27	21.0 ± 0.13	42.37 ± 0.48	9.44 ± 0.05	20.02 ± 0.13	20.9 ± 0.10
	A	2	5.54 ± 0.43	39.08 ± 0.43	19.64 ± 0.26	21.2 ± 0.15	42.36 ± 0.41	9.41 ± 0.07	20.23 ± 0.13	20.6 ± 0.07
	H	1	3.16 ± 0.17	49.17 ± 1.85	19.80 ± 0.26	30.5 ± 0.19	40.85 ± 0.51	9.15 ± 0.04	20.01 ± 0.11	31.1 ± 0.17
	H	2	3.01 ± 0.20	47.69 ± 2.05	19.65 ± 0.26	30.9 ± 0.19	40.79 ± 0.25	9.13 ± 0.03	19.73 ± 0.12	30.9 ± 0.16
H	L	1	10.49 ± 0.61	33.73 ± 0.80	23.89 ± 0.16	10.5 ± 0.05	45.72 ± 0.63	10.64 ± 0.16	23.78 ± 0.15	10.9 ± 0.12
	L	2	8.58 ± 0.81	29.73 ± 1.90	24.04 ± 0.17	10.9 ± 0.11	43.94 ± 0.60	10.29 ± 0.20	23.59 ± 0.14	11.2 ± 0.15
	A	1	5.06 ± 0.31	38.71 ± 0.66	24.19 ± 0.15	20.9 ± 0.13	42.48 ± 0.60	9.51 ± 0.07	23.79 ± 0.14	21.8 ± 0.24
	A	2	5.39 ± 0.40	39.27 ± 0.96	24.03 ± 0.16	21.2 ± 0.17	42.38 ± 0.48	9.49 ± 0.04	23.67 ± 0.16	22.3 ± 0.30
	H	1	3.33 ± 0.24	47.30 ± 0.90	23.76 ± 0.16	31.6 ± 0.29	40.76 ± 0.50	9.12 ± 0.04	23.57 ± 0.14	31.7 ± 0.28
	H	2	3.61 ± 0.35	46.46 ± 1.99	23.42 ± 0.15	33.3 ± 0.43	40.70 ± 0.51	9.13 ± 0.06	23.67 ± 0.14	32.4 ± 0.34

Data are displayed as means ± SE.

Table III: Analysis of variance examining differences in rearing conditions (temperature and salinity) among treatment tanks for Ba enriched tanks ((a) temperature and (b) salinity) and Sr enriched tanks ((c) temperature and (d) salinity).

	Source of Variation	df	MS	*F*	*P*
Ba					
	a) Temperature				
	Temperature	2	3337.10	9115.80	≤0.001
	Salinity	2	0.71	1.94	>0.050
	Temperature × Salinity	4	2.13	5.83	<0.050
	Tank(Temperature × Salinity)	9	0.37	0.26	>0.050
	Residual	558	1.41		
	b) Salinity				
	Temperature	2	123.72	8.40	<0.050
	Salinity	2	50325.00	3416.70	≤0.001
	Temperature × Salinity	4	46.73	3.17	>0.050
	Tank(Temperature × Salinity)	9	14.73	6.98	≤0.001
	Residual	558	2.11		
Sr					
	c) Temperature				
	Temperature	2	2795.30	4051.80	≤0.001
	Salinity	2	1.54	2.23	>0.050
	Temperature × Salinity	4	0.40	0.58	>0.050
	Tank(Temperature × Salinity)	9	0.69	0.84	>0.050
	Residual	558	0.82		
	d) Salinity				
	Temperature	2	319	73.39	≤0.001
	Salinity	2	49771	11457	≤0.001
	Temperature × Salinity	4	15	3.41	>0.050
	Tank(Temperature × Salinity)	9	4	2.19	<0.050
	Residual	558	2		

Water and diet chemistry

Temperature had no significant effect on the Ba:Ca ratio of the water (Table IVa, Figure 1a). A significant difference, however, was detected for salinity (Table IVa, Figure 1a), where *post hoc* tests indicated that Ba:Ca ratios differed across all three salinity treatments. Water was successfully altered using enriched ^{137}Ba, water had a mean $^{137}Ba/^{136}Ba$ ratio of 40.31 ± 1.56 (mean ± S.E.), compared to the natural ratio of 1.43. Temperature and salinity both had a significant impact on the $^{137}Ba/^{136}Ba$ ratios of the water (Tables IIa, IVb). *Post-hoc* tests indicated that the $^{137}Ba/^{136}Ba$ ratios differed between low and high temperature treatments, and differed across all three salinity treatments.

Salinity was the only factor which influenced both the Sr:Ca and $^{88}Sr/^{86}Sr$ ratios, with differences among all three salinities (Table V, Figure 1b). Strontium isotope ratios in the treatment tanks were successfully altered with ^{88}Sr, and displayed a mean $^{88}Sr/^{86}Sr$ ratio of 9.86 ± 0.14, which differed from the natural ratio of 8.38.

The diet fed to *Acanthopagrus butcheri* was successfully altered using enriched stable isotopes. The ^{136}Ba enriched diet had a significantly reduced $^{137}Ba/^{136}Ba$ ratio compared to the non-isotope enriched and Sr enriched diets ($F_{2,12} = 132.42$ $P \leq 0.001$) displaying an isotope shift from the non-enriched diet of 1.33 ± 0.05 to 0.66 ± 0.02. The ^{86}Sr enriched diet also displayed a significant difference in the $^{88}Sr/^{86}Sr$ ratio compared to the non-isotope enriched and Ba enriched diet ($F_{2,12} = 86.21$, $P \leq 0.001$) with a decrease in the $^{88}Sr/^{86}Sr$ ratio from the non-enriched diets of 9.03 ± 0.03 to 7.99 ± 0.09.

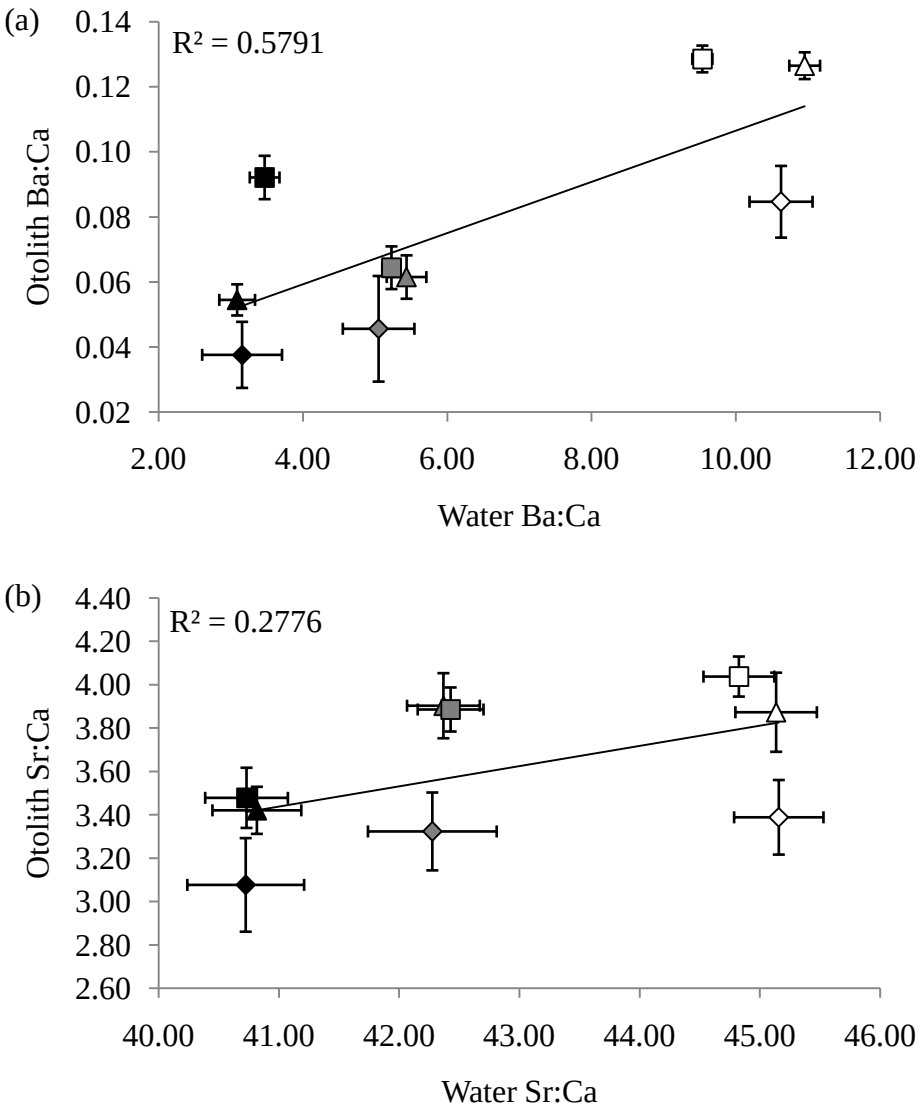

Figure 1: Mean (± S.E.) for (a) Ba:Ca and (b) Sr:Ca ratios in the otoliths and rearing water. Symbols represent temperature (◇ low, △ ambient and □ high), shading represents salinity (◇ low, ◆ambient and ◆ high).

Otolith chemistry

A significant interactive temperature × salinity influence was found for the Ba:Ca ratios in otoliths (Table IVc, Figure 1a). *Post-hoc* tests indicated that at ambient and high temperature, Ba:Ca ratios differed between low salinity treatments and the ambient and high salinity treatments. *Post-hoc* tests also indicated that at high salinity, Ba:Ca differed between the high temperature treatment and both the low and ambient temperatures. Temperature and salinity had no influence on the $^{137}Ba/^{136}Ba$ ratios measured in the otoliths of *Acanthopagrus butcheri* (Table IVd, Figure 2a). A significant tank effect was detected for otolith $^{137}Ba/^{136}Ba$ ratios (Table IVd). *Post-hoc* tests indicated variation between tanks at high temperature and either ambient salinity or high salinity (Figure 2).

A significant difference was detected in otolith Sr:Ca ratios among temperature and salinity treatments (Table Vc, Figure 1b). *Post-hoc* tests indicated that Sr:Ca differed between low temperature and both the ambient and high temperature treatments. Sr:Ca differed between the highest salinity and both the low and ambient salinity treatments. A significant difference in the otolith $^{88}Sr/^{86}Sr$ ratios was detected among tanks (Table Vd). *Post-hoc* tests found that differences occurred between tanks for three treatments (Figure 2b). A significant interactive temperature × salinity influence was also detected (Table Vd, Figure 2b). *Post-hoc* tests indicated that at high salinity, both low and ambient temperatures differed from high temperatures. There was also a significant difference in otolith $^{88}Sr/^{86}Sr$ between low and high salinity treatments at high temperature, where otolith $^{88}Sr/^{86}Sr$ ratios decreased with increasing salinity at high temperatures.

Table IV: ANOVA for the influence that temperature and salinity had on Ba:Ca and $^{137}Ba/^{136}Ba$ ratios in the rearing water and otoliths of *Acanthopagrus butcheri*.

Source of Variation	df	MS	*F*	*P*
Water				
a) Ba:Ca				
Temperature	2	0.004	1.180	>0.050
Salinity	2	3.194	856.820	≤0.001
Temperature × Salinity	4	0.007	1.810	>0.050
Tank(Temperature × Salinity)	9	0.004	0.900	>0.050
Residual	108	0.004		
b) $^{137}Ba/^{136}Ba$				
Temperature	2	47	4.000	<0.050
Salinity	2	2507	233.000	≤0.001
Temperature × Salinity	4	8	1.000	>0.050
Tank(Temperature × Salinity)	9	11	1.000	>0.050
Residual	108	13		
Otolith				
c) Ba: Ca				
Temperature	2	0.015	35.700	≤0.001
Salinity	2	0.040	91.730	≤0.001
Temperature × Salinity	4	0.002	4.890	<0.050
Tank(Temperature × Salinity)	9	0.000	0.380	>0.050
Residual	102	0.001		
d) $^{137}Ba/^{136}Ba$				
Temperature	2	10	0.240	>0.050
Salinity	2	61	1.480	>0.050
Temperature × Salinity	4	97	2.326	>0.050
Tank(Temperature × Salinity)	9	42	3.565	≤0.001
Residual	102	11		

Table V: ANOVA for the influence that temperature and salinity had on Sr:Ca and $^{88}Sr/^{86}Sr$ ratios in the rearing water and otoliths of *Acanthopagrus butcheri*.

Source of Variation	df	MS	*F*	*P*
Water				
a) Sr: Ca				
Temperature	2	0.13	0.08	>0.050
Salinity	2	196.60	126.33	≤0.001
Temperature × Salinity	4	0.24	0.15	>0.050
Tank(Temperature × Salinity)	9	1.56	0.77	>0.050
Residual	108	2.03		
b) $^{88}Sr/^{86}Sr$				
Temperature	2	0.02	0.38	>0.050
Salinity	2	20.38	396.78	≤0.001
Temperature × Salinity	4	0.01	0.18	>0.050
Tank(Temperature × Salinity)	9	0.05	0.93	>0.050
Residual	108	0.06		
Otolith				
c) Sr: Ca				
Temperature	2	3.18	10.42	≤0.010
Salinity	2	2.31	7.57	<0.050
Temperature × Salinity	4	0.08	0.26	>0.050
Tank(Temperature × Salinity)	9	0.31	0.95	>0.050
Residual	104	0.32		
d) $^{88}Sr/^{86}Sr$				
Temperature	2	0.27	0.62	>0.050
Salinity	2	3.96	8.98	<0.050
Temperature × Salinity	4	2.08	4.71	<0.050
Tank(Temperature × Salinity)	9	0.45	4.28	≤0.001
Residual	104	0.10		

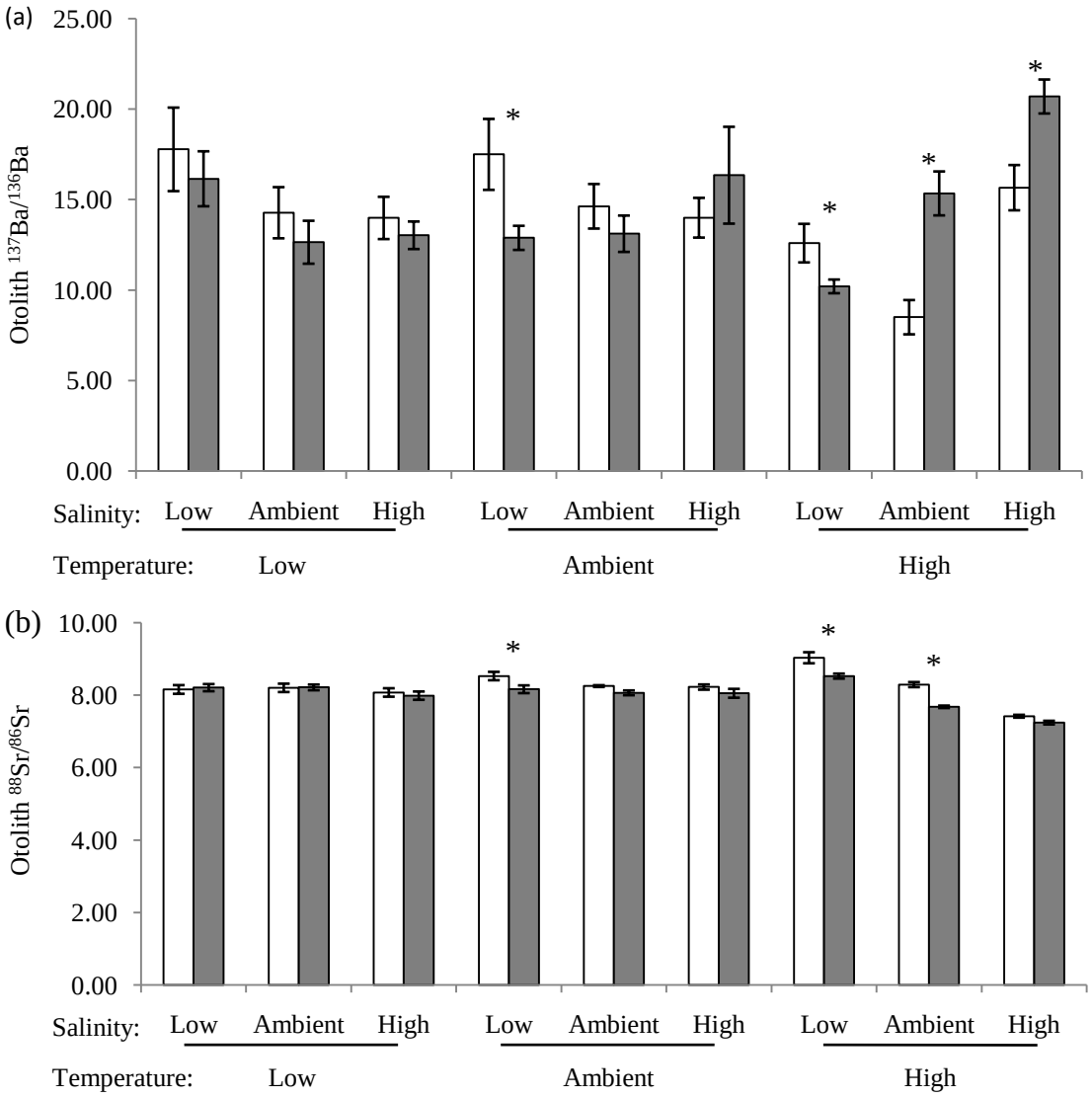

Figure 2: Mean (± S.E) isotope ratios in the otoliths of (a) $^{137}Ba/^{136}Ba$ and (b) $^{88}Sr/^{86}Sr$ for salinity (low, ambient and high) and temperature (low, ambient and high) treatments. Shading represents replicate tanks (□ Tank 1, ■ Tank 2), significant differences between replicate tanks indicated by asterisk (*).

Percent contribution of water and diet

Water contributed from 54 to 95 % of otolith Ba. No significant differences in water contributions were detected among salinity or temperature treatments for Ba (Table VIa, Figure 3a). Significant differences were detected among replicate tanks (Table VIa), with *post-hoc* tests indicating three treatment levels differed (Figure 3a).Water contributed between 49 and 87 % of otolith Sr. A significant interaction between

temperature and salinity was detected for percentage contributions from water into otoliths for Sr (Table VIb, Figure 3b). *Post-hoc* tests indicated that at low salinity, water contributions differed between the low temperature treatment and both the ambient and high temperature treatments. *Post-hoc* tests also indicated that at low temperature, water contributions differed between the low and high salinity, and at the high temperature, differences in salinity were found between the high salinity treatment with both the low and ambient salinity treatments. Water contributions appeared to increase with increasing salinity except at the high temperature treatment where contributions appeared to decrease with increasing salinity. A significant tank effect was also detected (Table VIb) with *post-hoc* tests indicating that there were differences between tanks for two treatments (Figure 3b).

Table VI:ANOVA comparing the percent contributions from ambient water to otolith Ba and Sr among treatments of water temperature, salinity and replicate tanks.

Source of Variation	df	MS	*F*	*P*
Contribution from water				
a) Ba				
Temperature	2	35	0.268	>0.050
Salinity	2	374	2.853	>0.050
Temperature × Salinity	4	259	1.973	>0.050
Tank(Temperature × Salinity)	9	132	4.233	≤0.010
Residual	102	31		
b) Sr				
Temperature	2	408	3.304	>0.050
Salinity	2	291	2.354	>0.050
Temperature × Salinity	4	940	7.586	≤0.010
Tank(Temperature × Salinity)	9	125	4.212	≤0.001
Residual	104	30		

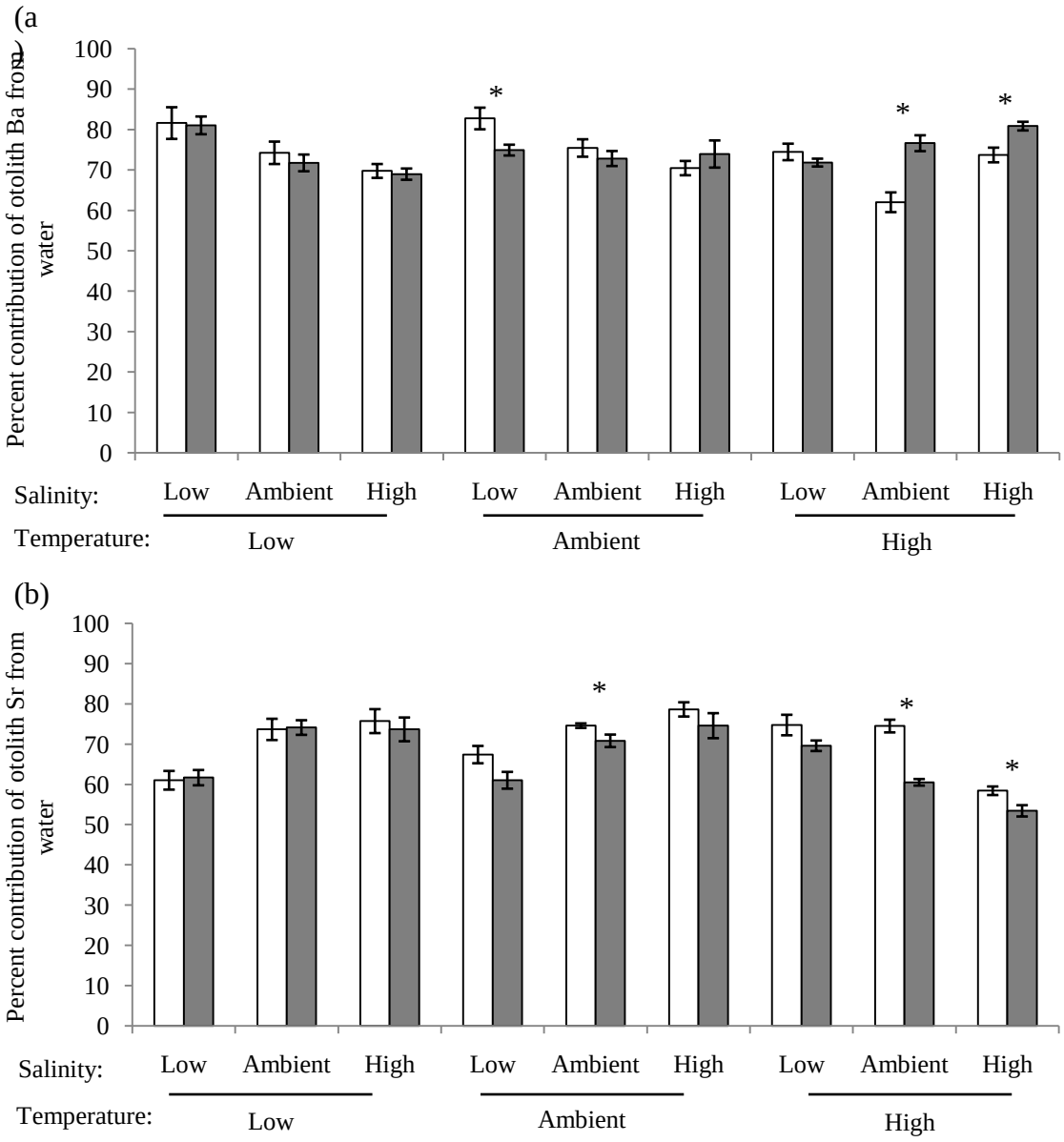

Figure 3: Mean (± S.E) percent contribution from the water into the otoliths for (a) Ba and (b) Sr for salinity (low, ambient and high) and temperature (low, ambient and high) treatments. Shading represents replicate tanks (□ Tank 1, ■ Tank 2), significant differences between replicate tanks indicated by asterisk (*).

Discussion

Otolith chemistry has been used for a number of applications, including reconstructions of fish migratory pathways (for example Gillanders, 2005, Elsdon & Gillanders, 2006), and the determination of fish stock structure (for example Bergenius et al., 2005, Jónsdóttir et al., 2006). The majority of this research relies on the assumption that otolith chemistry is reflective of the environment in which the fish lives (Bergenius et al., 2005, Jónsdóttir et al., 2006, Elsdon et al., 2008), although it is not necessary to know why otolith chemistry varies. There is not always a linear relation between otolith and water chemistry, suggesting that for some elements a close relationship between otoliths and water does not exist. In this study, water was the main contributor of otolith Ba and Sr, with environmental factors, salinity and temperatures influencing the incorporation of these elements into the otoliths.

Barium chemistry

Water Ba:Ca ratios were influenced by salinity, but not for temperature, with Ba:Ca decreasing with increasing salinity, as is often found in studies looking at elemental concentrations in water (Coffey et al., 1997, Elsdon & Gillanders, 2006). Otolith Ba:Ca ratios were affected by temperature and salinity, but there was not a simple relation. Otolith Ba:Ca ratios generally increased with increasing temperature and, similar to the water, decreased with increasing salinity. However, at the high temperature this was not the case. This may be explained by biological effects such as kinetics (Kalish, 1989). Changes in proteins surrounding the otolith are thought to be due to kinetic effects (Kalish, 1989, Elsdon & Gillanders, 2002). The activity of these proteins is likely to be affected by temperature and may affect the morphology of the otolith crystal. Changes in the morphology of the otolith have been shown to affect the uptake of elements

(Brown & Severin, 1999). The results suggest that the otolith crystal may have been compromised at high temperature and thus Ba may have been more readily incorporated into the otolith at the high temperature and high salinity treatment. Elsdon and Gillanders (2002) found a temperature salinity interaction for otolith Ba:Ca ratios whereby the interactive effect was significant only at the high temperature treatment, supporting the results shown here. Other studies that investigated temperature and salinity effects on otolith Ba:Ca ratios found no significant temperature-salinity interactions (Martin & Thorrold, 2005, Martin & Wuenschel, 2006).

Temperature and salinity both influenced water $^{137}Ba/^{136}Ba$ ratios. In general, water $^{137}Ba/^{136}Ba$ ratios displayed a slight decrease with increasing temperature. This pattern may be explained by loss of water through increased evaporation at higher temperatures. Water $^{137}Ba/^{136}Ba$ ratios increased with increasing salinity. This may be explained by the general relationship between Ba:Ca in the water and salinity. High salinity treatments had a lower total Ba:Ca content than low salinity treatments and thus were more affected by the ^{137}Ba spiking concentration. No other studies have investigated the relation between Ba isotopes and either temperature or salinity. Otolith $^{137}Ba/^{136}Ba$ ratios were not affected by temperature or salinity. The lack of a significant effect may be due to similar amounts of Ba derived from water and diet despite different temperatures and salinities (see below). The only studies that have previously investigated otolith $^{137}Ba/^{136}Ba$ ratios focused on a single temperature and/or salinity.

Water contributed between 54 and 95 % of otolith Ba in this study. No detectable temperature or salinity effects were found for water contributions to otolith Ba. These results suggest that water is the primary contributor of otolith Ba, and contributions from water and diet to otolith Ba are similar regardless of water temperature and

salinity. This supports previous research that concluded ambient water was the primary contributor to otolith Ba either through studies of Ba:Ca ratios (Buckel et al., 2004, Martin & Thorrold, 2005, Martin & Wuenschel, 2006) or relative contributions of diet and water based on isotope ratios (Walther & Thorrold, 2006). Our estimates are lower than those calculated in Walther and Thorrold (2006) for a single temperature and salinity, as they reported 98% of Ba in juvenile mummichog *Fundulus heteroclitus* otoliths was derived from ambient water. This may be due to species-dependent differences and/or the different treatments used.

Strontium chemistry

Water Sr:Ca ratios did not change with temperature, but decreased with increasing salinity. This suggests that either Sr concentration decreased with increasing salinity or Ca concentration increased at a greater rate than Sr with increasing salinity. The latter is more likely given the general relationship between Sr concentration and salinity. Martin et al. (2004) also found the same patterns in water Sr:Ca ratios with temperature and salinity. They suggested that Sr:Ca ratios decreased with increasing salinity due to higher Sr:Ca concentrations being present in the deionised water used to make the lower salinity treatments. Otolith Sr:Ca ratios were affected by both temperature and salinity. Otolith Sr:Ca ratios increased with increasing temperature, which again may have been due to kinetics, similar to Ba incorporation at higher temperatures. The results suggest that changes in otolith structure due to increasing temperature may have led to Sr being selected more often than Ca in the otoliths. Otolith Sr:Ca ratios decreased with increasing salinity, which reflected that of water.

Temperature and salinity influenced the $^{88}Sr/^{86}Sr$ ratios in the water in a similar manner to water Sr:Ca. Water $^{88}Sr/^{86}Sr$ ratios decreased with increasing salinity, similar to Ba,

and increased concentrations of Sr at high salinities lead to a reduced effect of the spiking concentration of ^{88}Sr on the water $^{88}Sr/^{86}Sr$ ratios. No other studies have experimentally manipulated Sr isotopes in water at various temperature and salinities, however an inverse relation was found for $^{86}Sr/^{88}Sr$ versus salinity in the rearing water of *Brevoortia patronus* (Chesney et al., 1998). Strontium isotope ratios have been shown to typically change over a salinity gradient, with higher ratios occurring at lower salinities, which was found in the rearing water of *Acanthopagrus butcherui* in this study and for Chesney et al. (1998).

Otolith $^{88}Sr/^{86}Sr$ ratios were affected by an interaction between temperature and salinity, where the pattern was most evident at high temperatures and ratios decreased with increasing salinity. This pattern is most likely due to an increased food intake at higher temperatures. Increased food intake would permit a greater amount of diet-derived Sr to be incorporated into otoliths. As diet was spiked with ^{86}Sr, a greater contribution of ^{86}Sr from diet would have lowered the $^{88}Sr/^{86}Sr$ ratio in the otoliths below the natural ratio (8.38). However, as water was spiked with ^{88}Sr, the $^{88}Sr/^{86}Sr$ ratio in otoliths would have been raised above the natural level if ambient water was the primary contributor of Sr to otoliths. Therefore, the increased food intake would have limited the shift in $^{88}Sr/^{86}Sr$ ratios caused by the water. Food intake in fish has been shown to increase with both increasing salinity (Rubio et al., 2005) and temperature (e.g. Boeuf & Payan, 2001, Handeland et al., 2008, Arjona et al., 2009). The decrease in otolith Sr ratios across salinity treatments only occurred at high temperatures. As high temperatures may enhance salinity effects (Elsdon & Gillanders, 2002), food intake may have been further increased through the cumulative effects of salinity and temperature, thus leading to the pattern seen among otolith $^{88}Sr/^{86}Sr$ ratios at high temperature treatments.

Sr water contributions were affected by an interaction between temperature and salinity where contributions from water decreased with increasing salinity at the high temperature treatment. Given that contribution from diet is 100 % minus the percent contribution from water, the decrease in water contribution suggests that the contribution from diet increased with increasing salinity at high temperature. Food intake was likely enhanced through the cumulative effects of salinity and high temperature and therefore led to a greater percentage of diet-derived otolith Sr at these higher treatment levels. As water contributed between 49% and 87% of otolith Sr for all treatments, the results imply that water is the primary contributor of Sr to otoliths. Water has also been reported as the primary contributor of Sr isotopes to otoliths in other studies (Farrell & Campana, 1996, Walther & Thorrold, 2006, Gibson-Reinemer et al., 2009). This study's estimates are consistent with those reported in previous studies. Farrell and Campana (1996) suggested 88% otolith Sr was water-derived in Nile tilapia *Oreochromis niloticus*, Walther and Thorrold (2006) suggested 83% in mummichogs *Fundulus heteroclitus* and Gibson-Reinemer et al. (2009) suggested 66% in rainbow trout *Onchorynchus mykiss*. The differences in water estimates may be due to species-specific differences or the different treatments used.

Conclusion

Reliable reconstructions of fish environmental history using otolith chemistry can only be achieved if general patterns of elemental uptake in otoliths are known. The present study provides two important contributions to reconstructions of fish life history. First, it provides support for past studies which conclude that ambient water is the primary contributor to otolith Sr and Ba, and that diet has a minor influence on otolith chemistry. It has been documented that ambient water was the primary contributor to otolith Sr in Nile tilapia *Oreochromis niloticus* (Farrell & Campana, 1996) and in

rainbow trout *Onchorynchus mykiss* (Gibson-Reinemer et al., 2009) and to otolith Sr and Ba in mummichogs *Fundulus heteroclitu* (Walther & Thorrold, 2006). Second, this study provides the first piece of evidence to show that percentage contributions from water to otolith Sr and Ba may vary according to salinity and temperature. All contribution studies to date have tested only one temperature and one salinity treatment and therefore have been unable to report the effects of both factors. The contributions of diet and water to otolith chemistry could impact migratory reconstructions, especially in regions where high temperatures and high salinities coincide, which may result in greater contributions from diet. Such areas are likely in shallow estuarine areas where freshwater input is low, e.g. estuaries of southern Africa and southern Australia (Potter et al., 1990). Greater contributions from diet could be problematic for environmental history reconstructions if food sources contain isotope ratios that differ from the natural isotope ratios.

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