Methods for discriminating hatchery fish and outcomes of stocking in the Murray-Darling Basin

Final Report, Murray-Darling Basin Authority, Native Fish Strategy Project MD741

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David A. Crook\textsuperscript{a}, Bronwyn M. Gillanders\textsuperscript{b}, Andrew C. Sanger\textsuperscript{c}, Andrew R. Munro\textsuperscript{b*}, Damien J. O’Mahony\textsuperscript{b*}, Skye H. Woodcock\textsuperscript{b}, Stephen Thurstan\textsuperscript{d}, Lee J. Baumgartner\textsuperscript{d}

\textsuperscript{a} Arthur Rylah Institute for Environmental Research
123 Brown Street, Heidelberg, Victoria 3084

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Executive summary

More than 60 million native fish produced by private and government hatcheries have been stocked into waterways of the Murray-Darling Basin (MDB). Despite the large scale of native fish stocking, little is known of the fate of stocked fish or their impact on wild populations in the MDB, at least in part due to a lack of methods for distinguishing between stocked and naturally recruited fish. Better information on the contribution of hatchery-produced fish to wild populations would be of great benefit to the management and conservation of fish in the MDB. For example, measures of abundance and species composition of native fish populations are often used as indicators in assessments of river health (e.g. the Sustainable Rivers Audit). At present, stocking has the potential to confound the results of studies that use fish assemblage data for such purposes.

This document is the final report for Native Fish Strategy (NFS) Project MD741, whose activities were undertaken between March 2007 and May 2010. Aspects of the work presented were also undertaken as part of previous projects funded by the then Murray-Darling Basin Commission, now Murray-Darling Basin Authority, and the Australian Research Council from 2002-2007.

The current project was initiated in response to Driving Action 6 (Managing Fish Translocation and Stocking) of the NFS. The primary objectives of the project were to:

- develop and evaluate practical mass marking techniques to allow for accurate discrimination of hatchery-produced and wild fish;
- facilitate and encourage broad-scale uptake of the marking techniques by government agencies and commercial hatcheries; and
- apply the marking techniques to quantify the contribution of stocked fish to populations in experimentally stocked rivers within the MDB.

A suite of chemical marking techniques for distinguishing between stocked and naturally recruited fish were developed during the project using Golden perch as the model species. Protocols for marking fingerlings with fluorescent dyes (calcein and alizarin red S) using "osmotic induction" were successfully trialled (Chapter 2). Calcein marking produced externally detectable marks that can be detected on live fish in the field. Osmotic induction marking with calcein had no detectable effects on fish health and is relatively quick and easy - it takes only 15 minutes to mark up to 20,000 fingerlings. A portable detection unit was tested and evaluated to allow for routine, non-lethal detection of calcein marked fish in the field. The simplicity and cost-effectiveness of osmotic induction marking make it feasible for widespread adoption in hatcheries. Large-scale calcein marking of hatchery fish commenced in 2009, and agencies from all State and Territory jurisdictions within the MDB have initiated processes to incorporate calcein marking into their stocking and/or research programs.

Methods for marking the otoliths (earstones) of fish with enriched stable isotopes were also developed as part of the project (Chapter 3). Female brood fish were injected with a solution of enriched stable isotope (e.g. $^{137}$Ba) to create an unambiguous chemical signature in the otoliths of the progeny. Alternatively, larvae and fingerlings were immersed in solutions of enriched stable isotopes to create chemical marks in the otoliths. The use of natural chemical signatures in otoliths for identifying stocked fish was also examined (Chapter 5). This was found to be a reliable technique in certain circumstances, although natural chemical markers were less definitive than artificially induced marks.

The use of chemical markers in otoliths to identify stocked fish, although requiring sacrifice of the fish, provides much more information about recaptured fish than calcein marking alone. For example, it may be possible to provide each hatchery with its own unique chemical marker: along with age information from annual otolith increments - this could tell us both the hatchery of origin and the year of release of recaptured stocked fish. Thus, a combination of otolith chemical marking and calcein marking has the potential to provide a very powerful tool for evaluating the outcomes of fish stocking.
A variety of statutory controls exist around the use of chemicals in veterinary medicine, agriculture, pest control, food production, and the environment. The use of chemicals for marking fish in hatcheries is arguably captured by some of the definitions contained in these statutory controls. Regulatory issues relating to the use of chemicals for marking fish were examined in detail and interpretation of chemical residue data and advice received from regulatory authorities was undertaken (Chapter 4). Based on this analysis, it was concluded that the chemical marking techniques developed during the project can be legally applied in hatcheries provided specific processes are undertaken.

As a first step towards understanding the impacts of native fish stocking on riverine fish populations in the MDB, chemically marked fish were stocked into three rivers of the southern MDB (Billabong Creek, Edward River, Murrumbidgee River) (Chapter 6). Post-stocking surveys were then conducted over a 5-year period and the proportion of marked fish determined. The results showed that at least a proportion of the stocked fish survived to reach the legal minimum size in all three rivers. However, the impacts of stocking on population structure were very different among rivers. In the Edward and Murrumbidgee Rivers, the age classes corresponding to the years of stocking were comprised of 18-38% experimentally stocked fish; and these fish made only a relatively minor contribution to the total catch of Golden perch. In contrast, stocked fish comprised up to 100% of age classes corresponding to stocking years in Billabong Creek, and stocking resulted in a four-fold increase in the catch rates of Golden perch. The results of the experimental stocking study demonstrate that stocking has the potential to affect population structure and abundance of the stocked species.

A range of extension and communication activities was undertaken to ensure efficient dissemination of the projects findings and outputs (Chapter 7). These activities included seminars at a variety of forums and scientific conferences (national and international), publication of articles in the peer reviewed scientific literature, production of a CD and pamphlet describing the projects activities, several radio interviews, visits to hatcheries to discuss the project and demonstrations of chemical marking techniques. In June 2009, a two-day stakeholder workshop was held in Melbourne to provide information and practical training in chemical marking techniques for hatchery fish. More than 40 stakeholders, including hatchery operators, recreational fishing representatives, scientists and fisheries and natural resource managers attended the workshop.

To further encourage uptake of the marking methods, 1 kg of calcein was distributed to relevant State/Territory agencies within the MDB for further trials of osmotic induction marking. Eight calcein field detection kits were also distributed to State/Territory agencies. These kits included a field fluorometer for quantitative measurement of calcein fluorescence and a specialised torch and glasses set for visual identification of marked fish. At the time of writing, more than 350,000 fish from eight species had been marked with calcein.

As we move from technique development to widespread implementation of chemical marking, an increased focus on developing coordinated strategies to facilitate uptake is required to build upon the momentum established during the project. Such a process needs to engage relevant stakeholders (e.g. via workshops, establishment of standing committees), and should consider a range of issues relating to the adoption of chemical marking, including: policy and management responses; resourcing and logistical issues; data acquisition and reporting requirements; and risk management. A coordinated and targeted implementation of methods for discriminating hatchery fish has the potential to greatly increase the quality of scientific information available for making decisions on native fish stocking and fisheries management in the MDB.
1 General introduction

In recent decades, the stocking of hatchery-produced fish into the wild has become a major tool for the management of depleted stocks of fish in freshwater, estuarine and marine environments, with many billions of fish from more than 300 species stocked world-wide (Brown and Day 2002). In the Murray-Darling Basin (MDB), more than 35 million native fish produced by private and government hatcheries have been stocked into waterways since 2001. Golden perch *Macquaria ambiguа* comprise ~65% of the stocked fish, with Silver perch *Bidyanus bidyanus* and Murray cod *Maccullochella peelii* making up most of the remainder (Gillanders et al. 2006). Despite the large number of fish stocked each year, little is known of the fate of stocked fish or their impact on wild populations in the MDB, in part because of a lack of suitably developed and tested methods for distinguishing between stocked and naturally recruited fish.

A variety of methods for identifying hatchery-produced fish have been developed to achieve this aim, including tagging, chemical marking and genetic analyses (see reviews by Nielsen 1992; Crook et al. 2005). However, the willingness of hatchery managers to adopt such methods is dependant on considerations including the direct costs of the equipment and consumables, logistical issues, any effects on the health, quality or quantity of fish produced, and any associated environmental or human health issues (particularly for potential food fish). It is important, therefore, that procedures for identifying hatchery fish accord with the requirements of hatchery operators and that the methods are optimised to minimise the impacts on hatchery operations.

The majority of native fish produced for stocking by hatcheries in the MDB are released as fingerlings of 20-50 mm total length (TL), and it is common for hatcheries to produce batches of 100,000 or more fish. Consequently, methodologies that require handling of individual fish (e.g. fin clipping, tagging) have generally been considered impractical to date. As a consequence, routine marking of hatchery-reared fish in the MDB has not been undertaken by commercial hatcheries, and marking of fish in government-owned hatcheries has been limited to a small number of research projects with specific objectives. More wide-ranging information on the contribution of hatchery-produced fish to wild populations would be of great benefit to the management of fish in the MDB, particularly if such information was routinely collected as part of large-scale survey programs such as the Sustainable Rivers Audit.

This project (MD741: Methods for discriminating hatchery fish and outcomes of stocking in the Murray-Darling Basin) was initiated in response to Driving Action 6 (Managing Fish Translocation and Stocking) of the Murray-Darling Basin Authority’s Native Fish Strategy (MDB 2004). The primary objectives of the project were to: 1) develop and evaluate practical mass marking techniques to allow for accurate discrimination of hatchery-produced and wild fish, 2) facilitate and encourage broad-scale uptake of the marking techniques by government agencies and commercial hatcheries, and 3) apply the marking techniques to quantify the contribution of stocked fish to populations in experimentally stocked rivers within the MDB.

This document is the final report for Project MD741, whose activities were undertaken between March 2007 and June 2010. Aspects of the work presented here were also undertaken by the project team as part of previous projects funding by the Murray-Darling Basin Commission (R5003: Improved methodology for discrimination of stocked and wild fish; MD697: Chemical marker registration) and the Australian Research Council (LP0348611: Native fish stocking in rivers: discriminating between hatchery-reared and wild fish).
2 Osmotic induction marking with fluorescent dyes

2.1 Introduction

The ability to accurately and easily distinguish between wild and hatchery-produced fish is critically important for improving our capacity to evaluate the outcomes of fish stocking in the MDB. As part of this project, we developed and evaluated methods for "osmotic induction" marking of native fish. Osmotic induction is a chemical marking technique that involves immersing fish in a saline solution prior to immersion in a fluorescent dye (Mohler 2003; Crook et al. 2009). Immersion in the saline solution causes water to move out of the tissues of the fish via osmosis. When the fish is then placed in the less saline dye solution, the resulting osmotic gradient causes the dye to be taken up very rapidly. The osmotic induction marking system consists of inexpensive tubs and sieves, and it is feasible to mark 10's of thousands of fingerlings in less than 15 minutes (Figure 2.1). Trials found that osmotic indication marking of Golden perch fingerlings could be carried without any detectable effects on growth and mortality rates (Crook et al. 2009).

In addition to the permanent otolith mark produced by other chemical marking techniques, osmotic induction marking with calcein and alizarin red S (ARS) also produces externally detectable marks, thus allowing for non-lethal detection of marked fish. The simplicity and speed of the osmotic induction method makes it particularly amenable for use in large-scale hatcheries, where cost and logistics play a major role in determining the utility of marking methods.

2.2 Marking fish with calcein and alizarin red S

We used the most commonly stocked native fish in the MDB, Golden perch (Macquaria ambigua), as the main species for developing osmotic induction techniques for mass marking hatchery fingerlings. Successful trials have subsequently been conducted for Murray cod, Trout cod and Australian bass fingerlings. Osmotic induction marking involves two main steps. First, the fish are placed in a sieve and immersed for up to 5 minutes in a 5% (50% more salty than seawater) salt solution (Figure 2.1). The fish are then transferred to a solution of calcein or ARS for up to 10 minutes. Further details on the marking techniques are available in the references listed at the end of this chapter and in the CD that accompanies this report. Details on the regulatory aspects of chemically marking fish are dealt with in Chapter 4.

Figure 2.1. Osmotic induction marking with calcein

Approximately 8,000 Trout cod being marked with calcein at the Narrandera hatchery. The fish spent 3 minutes in a 5% salt solution before being transferred to a 0.5% calcein solution for 10 minutes.
2.3 Detecting fluorescent marks

Fluorescence microscopy

To detect calcein and ARS marked fish in the laboratory, fish were examined in a darkened room using a fluorescence dissecting stereomicroscope (Model MZ16 F, Leica, Switzerland) linked to a digital camera (Infinity Capture 3.5.1, Lumenera Corp., Canada) (Figure 2.2). A "GFP3" filter set was used for detecting the calcein marks (470 nm excitation filter with 40 nm half bandwidth, 525 nm band pass barrier filter with 50 nm half bandwidth). A "TXR" filter was used to detect the ARS marks (560 nm excitation filter with 40 nm half bandwidth, 610 nm long pass barrier filter). Photographs of the fish were taken using Image Pro Express 5.0.1.26 (Media Cybernetics, Bethesda, MD, USA) (Figure 2.2, 2.3).

Figure 2.2. Images of calcein and ARS marked fish under fluorescence microscope

(a) sectioned otolith of a calcein marked Golden perch, (b) sectioned otolith of an ARS marked Golden perch, (c) caudal fin of a calcein marked Golden perch, (d) anal fin and scales of an ARS marked Golden perch, (e) examining marked fish under fluorescence dissecting microscope.

Figure 2.3. Heads of calcein and ARS marked fish under fluorescence microscope

Top row - photographs taken under natural light; bottom row - non-marked control (left), ARS marked (middle), calcein marked (right).
Although fluorescence microscopy is the optimal method for detecting fluorescent marks on fish, it is generally not feasible to examine fish under a microscope in the field. This limits the utility of fluorescence microscopy primarily to the examination of euthanized fish or otolith samples.

**Field detection**

**Torch and glasses method**

Development of a system for non-lethal detection of marked fish in the field was one of the objectives of this project. To achieve this, we developed a technique for the visual detection of calcein marked fish that uses a bright torch fitted with an LED light source of the appropriate wavelength (470 nm blue light) as the excitation energy source. This light is shone onto the fish and causes any calcein marks to fluoresce. A pair of specialised sun glasses (Northwest Marine Technology, Shaw Island, WA, USA) is worn to act as the "barrier filter", thus excluding extraneous wavelengths of light and only allowing the calcein fluorescence wavelengths (560 nm green/yellow light) to pass. The torch and glasses method for detecting calcein marked fish is inexpensive (approx. AU$20 per set) and is easy to undertake in the field. Unfortunately, to date we have been unable to develop a similar system for detection of fish marked with alizarin red S. A unit for visually detecting calcein marked fish is also commercially available from Western Chemicals Inc., Ferndale, WA, USA (http://www.wchemical.com/Default.aspx).

![Figure 2.4. Detection of calcein fluorescence using torch and glasses method](image)

Measuring fluorescence on Golden perch juveniles using the torch and glasses method at the Chemical Marking of Fish Workshop (see Chapter 7).

**Field fluorometer**

Although visual detection of fluorescent marks using the torch and glasses method is effective, a disadvantage is that it relies upon a subjective judgement by the operator as to the presence of a mark. This can be potentially problematic for field staff without experience in identifying fluorescent marks. In addition, visual detection of fluorescent marks is best performed under darkened conditions to reduce incidental light that can obscure the marks - this can be difficult to achieve under field conditions.

As an alternative, we tested the application of a portable, hand-held fluorometer to measure fluorescence (GFP-Meter, Opti-Sciences, Inc., Hudson, NH, USA). These units are powered by four AA batteries, weigh 1 kg and are housed in a weather resistant plastic case. The units are configured with a flexible fiber-optic probe (1 m length, 4 mm diameter) to bring light energy to and from the sample. The end of this probe is held against the sample surface (e.g. the pre-operculum of the fish) and a reading is recorded after 3 sec (Figure 2.3). At the time of purchase (June 2009), the units cost approximately AU$5,000 each.
Results of tests on marked and unmarked fish demonstrated that calcein marked fish held under laboratory conditions could be unambiguously and objectively distinguished from non-marked fish more than 2 years after marking using the GFP unit (Figures 2.5 and 2.6). As part of our experimental stocking program (see Chapter 6), calcein marked fish were also stocked into Billabong Creek in February 2008. Fluorometer readings taken from recaptured fish have shown that accurate detection of marked fish is possible using both the fluorometer and the torch and glasses methods under field conditions. Although the ultimate longevity of detectable calcein marks in Golden perch requires longer term study, the results collected so far suggest that discrimination may be possible for significantly longer than 2 years. Gradual reduction in external calcein mark intensity is known to occur due to a number of factors, including photo-degradation of the calcein, growth of tissue over the marked bony material, and "dilution" of the mark due to tissue growth. Further sampling of Billabong Creek is planned to evaluate long term mark detectability of calcein marked fish. Similar to the torch and glasses method, we have so far been unable to successfully adapt a field fluorometer for detecting fish marked with ARS.

Figure 2.5. Detection of calcein fluorescence using field fluorometer
Measuring fluorescence from the pre-operculum of a live Golden perch fingerling (left), pre-operculum of a 104 mm TL Golden perch taken 26 months after calcein marking under normal light (middle) and using fluorescence microscopy (right).

Figure 2.6. Fluorometer readings of non-marked and marked fish
Readings from non-marked fish sampled from the Narrandera Fisheries Centre and Billabong Creek versus calcein marked fish reared in aquaria 26 months post-marking. All of the calcein marked fish recorded "signal overload" (>1.8 sample: reference fluorescence ratio). n= 12 for each sample group.
2.4 Costs and logistics of calcein and alizarin red S

A major advantage of osmotic induction marking with either calcein or ARS is that the marks can be easily detected on live fish. The cost of the chemicals is one of the most important considerations regarding the relative merits of the two dyes, as excessive cost could greatly limit the utility and uptake of the marking techniques. In this respect, ARS has clear advantages over calcein. The cost of preparing a 1 L solution of 0.025% ARS is ~AU$0.75, whereas the cost of a 1 L solution of 0.5% calcein is ~AU$140. However, we have been able to purchase bulk amounts of calcein (see Chapter 7 below), reducing the cost of calcein to approximately AU$100 per 1 L of 0.5% calcein solution. The relatively low concentrations of ARS used should also make disposal of surplus chemical easier and less expensive than calcein.

On the other hand, calcein has several advantages over ARS. Unlike ARS, the calcein treatment that produced the highest quality marks did not have any clear effects on mortality rates and fish treated with calcein had slightly increased rates of growth compared to unmarked control fish in our trials. Calcein has also been approved for skeletal marking of fish by the US Food and Drug Administration via the US Fisheries and Wildlife Service’s Investigative New Animal Drug (INAD) permitting process (US Fisheries and Wildlife Service 2006). As the potential health effects of calcein marking were thoroughly addressed during the INAD process, the regulatory issues relating to calcein for use in Australia should be more straightforward than for ARS, which has not yet been registered in the USA. In conclusion, osmotic induction marking with calcein or ARS has potential application for MDB hatcheries. The choice of either method will depend on the aims and limitations of the marking program and the permitting process required should marked fish be released into the wild.

2.5 Scaling up

The osmotic induction techniques for marking fish were developed and tested under laboratory conditions, with only small batches of <100 fish being marked at a time. One of the objectives of this project was to test the techniques at scales relevant to large hatcheries, where batches of 10,000-100,000 fish are typically produced. Large scale trials of the calcein marking method were conducted at the Narrandera Fisheries Centre. ARS was not examined further as field detection techniques are yet to be developed for fish marked with this dye.

The first large scale trials were undertaken in January and March 2008 using 30 L plastic buckets with holes drilled into them as the holding vessels. Using this equipment, we marked 3,700 Murray cod fingerlings and 24,000 Golden perch fingerlings. The trials were very successful, with clearly detectable marks produced and no effects of marking on mortality rates recorded. Several minor issues emerged from the trials, including issues relating to oxygen stress, foaming and crowding due to the shape of buckets. Based on these experiences, we slightly adjusted the technique and developed a different marking system that included the use of high quality oxygen diffusers in the marking solutions, flatter holding vessels, and a purpose-built stainless steel sieve (see Figure 2.1). In 2009 and 2010, approximately 210,000 Golden perch, 144,000 Murray cod and 83,000 Trout cod were successfully marked at the I&I NSW hatchery at Narrandera using this system (for further details, see Chapter 7). Based on these trials, the estimated costs for marking fingerlings with calcein at the hatchery scale are approximately AU$20 per 1,000 fish.
Methods for discriminating hatchery fish and outcomes of stocking in the Murray-Darling Basin

For more detailed information on the material presented in this Chapter, see:


3 Marking fish otoliths with enriched stable isotopes

3.1 Introduction
Osmotic induction marking of fingerlings with fluorescent dyes is a practical means of marking fingerlings in hatcheries. However, marking fish with calcein does not provide any scope for identifying batches of fish or distinguishing fish from different hatcheries. Chemically marking otoliths, although requiring sacrifice of the fish, can provide much more information about recaptured fish than calcein marking. For example, it may be possible to provide each hatchery with its own unique chemical marker: along with age data from annual otolith increments, this could reveal both the hatchery of origin and the year of release of recaptured stocked fish. Thus, a combination of otolith chemical marking and calcein marking could provide a very powerful tool for evaluating the outcomes of fish stocking.

3.2 Isotope immersion marking
Isotope immersion otolith marking involves immersing fish in a solution of dissolved enriched isotope for sufficient time for a detectable chemical signature to be incorporated into the growing crystalline structure of the otoliths. The chemical mark can then be detected using a highly sensitive chemical analysis technique known as laser ablation inductively coupled plasma mass spectrometry (LA-ICPMS).

As for the osmotic induction marking technique, one of our primary goals in developing otolith chemical marking techniques was to ensure that the methods were compatible with hatchery practices. Our initial trial of isotope immersion marking was conducted on Golden perch fingerlings at the Narrandera Fisheries Centre (see Munro et al. 2008). Although the trial was successful, the project team concluded that, for Golden perch and Murray cod at least, isotope marking at the larval stage would be preferable - this fits more easily into normal hatchery protocols and results in marks that are easier to detect than when the fish are marked as fingerlings.

The newly hatched larvae of Golden perch (Figure 3.1) and Murray cod (and a number of other species) are usually held within the hatchery for several days before being released into grow-out ponds. Trials conducted at the Narrandera Fisheries Centre showed that it is possible to mark entire batches of Golden perch and Murray cod larvae by holding them in static, aerated water containing enriched isotopes of barium and strontium during the larval holding period (see Figure 3.2). Apart from adding the isotope solution and maintaining the fish in static water, this does not require any adjustment to normal hatchery protocols. Only a very tiny amount of an enriched isotope is required to shift the ratios in the hatchery water. For example, Munro et al. (2008) produced detectable shifts in the $^{138}\text{Ba}:^{137}\text{Ba}$ ratios of Golden perch otoliths by adding 0.001 g of $^{137}\text{Ba}$ to 200 L tanks at Narrandera.

![Figure 3.1. Golden perch larva](image)
Newly hatched Golden perch larva at the size of isotope immersion marking (~4 mm TL).
3.3 Transgenerational marking via brood fish injection

Recent studies of reef fish have described a method for marking fish embryos that involves injecting a chemical marking agent (enriched stable barium isotope) into the maternal parent prior to spawning (Thorrold et al. 2006; Almany et al. 2007; Williamson et al. 2009). Although this “transgenerational marking” method was developed for ecological studies of reef fish, the ability to mark all of the progeny of a maternal parent fish via a single injection has potential applications for marking hatchery produced fish.

We conducted a series of experiments on Golden perch at the Narrandera Fisheries Centre. Female brood fish were injected with different concentrations of enriched $^{137}$Ba at several different periods prior to hormone induced spawning (Figure 3.3). The progeny were then grown out and their otoliths analysed using LA-ICPMS. The results of the otolith chemistry analyses showed clear shifts in the otolith isotope ratios of the progeny of injected brood fish, thus producing an unambiguous chemical marker (Figure 3.4). Whilst the results of this pilot study were encouraging (see Munro et al. 2009), the project team decided not to pursue further development of this technique as part of the current project because it was felt that fluorescent dyes and isotope immersion techniques were more cost effective and had better potential for application in hatcheries.
Figure 3.3. Transgenerational marking of Golden perch
A female brood fish being injected with a solution of enriched stable barium isotope as part of the transgenerational marking trials conducted at the Narrandera hatchery.

Figure 3.4. Results of transgenerational marking trials on Golden perch
Laser ablation otolith profiles of $^{138}$Ba/$^{137}$Ba ratio in otoliths of juvenile Golden perch. (a) control fish with natural Ba isotope ratio, (b) progeny of brood fish injected with 40 µg $^{137}$Ba g$^{-1}$ at the same time as the hormones used to induce gonadal maturation. The transgenerational mark can be seen as a rapid decrease in the $^{138}$Ba/$^{137}$Ba ratio at around 50 µm along the transect in (b).
3.4 Costs and logistics of isotope marking

Enriched isotope marking of fish otoliths is easy to achieve and produces distinctive marks which cannot be mistaken with natural otolith chemical signatures. Stable isotope marking can be applied at different life stages, depending on the species or the operating protocols of the hatchery. The costs of isotope marking vary considerably depending on a range of factors. For example, the costs of different stable isotopes vary greatly, so the choice of isotope greatly influences the costs of artificially induced isotope markers.

Isotope immersion marking at the fingerling stage is estimated to cost AU$10.50 per 1000 fish with enriched $^{137}\text{Ba}$ (4 days with 50% water change at 15 µg·L$^{-1}$ and 10 fish·L$^{-1}$) and AU$45.00 to mark 1000 fish with enriched $^{86}\text{Sr}$ (4 days with 50% water change at 25 µg·L$^{-1}$ and 10 fish·L$^{-1}$). Immersion marking at the larval stage greatly reduces costs because larvae can be held at high densities in much smaller volumes of water during the marking period. It costs approximately AU$4.70 to mark 1000 larvae with enriched $^{137}\text{Ba}$ (5 days, no water exchange at 100 µg·L$^{-1}$ and 100 fish·L$^{-1}$) and $30.30 per 1000 larvae with enriched $^{86}\text{Sr}$ (5 days, no water exchange at 250 µg·L$^{-1}$ and 100 fish·L$^{-1}$). The selection of isotopes can further reduce cost, for instance it would cost AU$1.25 to mark with $^{138}\text{Ba}$ and $4.95 to mark with $^{88}\text{Sr}$, if the concentration of isotopes and marking conditions were the same as for $^{137}\text{Ba}$ and $^{86}\text{Sr}$ respectively. These four stable isotopes could be combined to further expand the number of mark signatures which can be created. Using just these four stable isotopes, 15 potential artificial isotopic signatures could be created.

In the case of transgenerational marking, it can be difficult to estimate exact costs due to variations in breeding success both within and between seasons - variations in fertilisation and hatch rates occur even within the highly controlled environment of a hatchery. For Golden perch, brood fish typically weigh about 1 kg. Using a $^{137}\text{Ba}$ solution at 20 µg·g$^{-1}$, it costs approximately AU$93 to inject one brood fish and mark success rates of up to 100% are achievable (see Munro et al. 2009). If progeny survival is high, it can cost less than AU$1 to mark 1000 fish, which is cheaper than isotope immersion marking of larvae or juveniles. However, if spawning fails or survival of progeny is low, the costs per marked fish can increase dramatically. Another disadvantage of transgenerational marking is that brood fish need to be handled in order to administer the isotopes. This can be problematic for species that are not routinely handled to induce spawning (e.g. Murray cod).

Analysis of otoliths to identify enriched isotope markers is usually carried out using laser ablation inductively coupled plasma mass spectrometry. Cost of LA-ICP-MS analysis is estimated as approximately AU$10 per otolith, although this costing is highly dependent on labour and instrument fees which can vary between laboratories. One of the major disadvantages of both isotope marking techniques is that fish must be killed for detection of the otolith marks. It may be possible in future to mark other bony structures (e.g. fin spines or scales). We conducted preliminary chemical analyses on Golden perch scales and fin spines, but were only able to reliably detect chemical markers using the otoliths. Further research is needed to determine whether reliable, non-lethal detection of enriched stable isotope markers is possible for hatchery fish.

3.5 Scaling up

The isotope marking methodologies trialled were specifically designed so that they could be easily incorporated into hatchery protocols. For transgenerational marking, brood fish are injected at or around the time that the fish are also injected with hormones extracted from carp pituitary glands to induce spawning (Rowland 1983). For species that are injected with such hormones, the additional injection of an isotope solution is relatively easy to incorporate into normal procedures. Our trials also suggest that injection with enriched isotopes does not significantly affect the fertilisation rate, number of fertilised eggs, or the hatching rate in comparison to control fish or
long-term hatchery records (Munro et al. 2008). However, as mentioned above, there are some issues with variability in spawning success of injected brood fish that may reduce the efficiency of this method. Although transgenerational marking has potential utility for a wide range of species and situations, in the case of hatcheries that produce native fish for stocking in the MDB, the project team concluded that immersion marking at the larval stage had several clear advantages, including cost, the simplicity of the method, and reduced risks to production.

A disadvantage of immersion marking at the fingerling stage is that standard hatchery protocols for the production of most species, including Golden perch and Murray cod, do not include a hatchery holding period of 8 days or more 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. Thus, isotope marking of fingerlings is unlikely to be adopted routinely for Golden perch, Murray cod and other similar species. In contrast, larval immersion requires almost no adjustment to the normal hatchery protocols - the isotope solution is simply poured into the hatchery containers and the fish held in static conditions during the holding period. As the larvae feed endogenously at this stage, holding fish in static, aerated conditions is not problematic. A large scale marking trial of the isotope immersion method using Golden perch larvae was successfully undertaken at the Narrandera Hatchery in 2009.

For more detailed information on the material presented in this chapter, see:


4 Regulatory issues relating to the use of chemicals to mark hatchery fish

4.1 Introduction

Broad scale adoption by government and private hatcheries of the marking techniques developed as part of this project has the potential to greatly enhance our ability to effectively manage and monitor fish populations of stocked species in the Murray-Darling Basin. However, a variety of statutory controls exist around the use of chemicals in veterinary medicine, agriculture, pest control, food production, and the environment. The use of chemical marking compounds on fish produced at hatcheries is arguably captured by some of the definitions contained in these statutory controls.

A project objective, therefore, was to establish whether the chemical marking techniques developed were captured by relevant legislation at national and state levels, and whether any of the chemicals needed to be registered, could be registered or were registered as veterinary chemical products for their intended use as chemical markers. The project also aimed to identify any other steps that would be necessary to clear statutory hurdles that may inhibit the extension of the experimental marking techniques to broad scale use by hatcheries throughout the Murray-Darling Basin.

4.2 Fluorescent dyes

The use of calcein for skeletal marking of fish has been approved for use by the US Food and Drug Administration via an investigative new animal drug exemption (INAD 10-987; for details visit www.fws.gov/fisheries/aadap/home.htm). To establish the regulatory requirements for the use of calcein and alizarin red S in Australia, a consultant (AgAware Consulting Pty Ltd) was engaged by the Arthur Rylah Institute for Environmental Research to act on behalf of the project team in correspondence with the relevant authorities. The consultant and a representative of the project team had discussions with the Australian Pesticides and Veterinary Medicines Authority (APVMA) and requested a ruling on whether calcein and ARS, when used for the purpose of marking fish, constituted the use of a veterinary chemical product. The ruling received from the APVMA stated that the use of these chemicals for marking fish as described in a summary report to the authority did not require registration under the Agriculture and Veterinary Chemical Code Act 1994 (see Sanger and Crook 2007).

The Biological and Chemical Risk Management Unit of the NSW DPI Division of Biosecurity Compliance and Mine Safety and the Chemical Standards Branch of the Victorian DPI Biosecurity Victoria division also provided further written advice that the use of calcein and ARS for the purpose of marking fish in hatcheries would not constitute use of an unregistered stock medicine under the NSW Stock Medicines Act 1989 and the Victorian Agricultural and Veterinary Chemicals (Control of Use) Act 1992 (see Sanger and Crook 2007).

The project team’s interpretation of the advice from the APVMA and the State agencies responsible for the use of veterinary and agricultural chemicals is that the techniques and the two fluorescent compounds have effectively been cleared for use provided that they are used solely for the purposes of marking fish (i.e. not for any type of veterinary purpose).

Following the advice received on the use of fluorescent dyes as veterinary chemicals, there remained some doubt about the legality of the methods for fish that may eventually be consumed as food. The project team, via AgAware Consulting, corresponded with Food Standards Australia New Zealand (FSANZ), the statutory authority charged with overseeing the registration of chemical additives to foods in Australia and New Zealand. FSANZ advised that since calcein and alizarin red S are not registered chemical additives in Australia, their presence as quantifiable residues in food sold in Australia may not be permitted.
Based on further consultations with FSANZ, the project team concluded it was necessary to develop a quantitative analytical technique for determining whether quantifiable traces of the two chemicals were present in marked fish. A NATA registered analytical laboratory (Leeder Consulting) was engaged to develop techniques for quantifying calcein and alizarin red S residue in fish. Although it is clear that very small amounts of calcein and ARS will remain present in the bony parts of marked fish (i.e. the chemical marks), the results of the residue tests have been used to determine the amount of residue in the edible parts of the fish alone (fillets) and in cleaned whole fish on a mg/kg basis. Using a similar approach to that employed by Leeder Consulting in the development of a test for malachite green in seafood, a result below the limits of detection can be used as evidence of no quantifiable residue of calcein or ARS in marked fish.

The project team commissioned residue tests for on-grown Golden perch and Murray cod marked with calcein as fingerlings of 0.2-1.5 g. No detectable traces were present in the fillets of either species and no traces were found in cleaned whole Golden perch. Only one of six cleaned whole Murray cod recorded levels at the detection limits of the test, with the other fish below detection limits. This evidence was provided to FSANZ and advice was subsequently provided that calcein did not need to be registered as a chemical food additive for fish marked according to the methods outlined in Crook et al. (2006; 2009).

The project team’s interpretation of the advice from FSANZ is that registration of calcein and ARS as food additives is not required provided it can be demonstrated that the marking procedures employed do not produce quantifiable residues in fish fillets or cleaned whole fish at the legal minimum size (i.e. when they become legally available as food). In the case of Murray cod and Golden perch, we marked individuals with calcein as fingerlings (0.2-1.5 g) using the osmotic induction technique, and subsequently grew the fish out until they had increased at least ten-fold in mass. The residue tests conducted on these fish are considered conservative because the fish were still well below the minimum legal size when tested (all fish tested were less than 160 mm in length).

To determine whether quantifiable residues are present when marking other species of fish, or if making alterations to the marking techniques, we would recommend following a similar protocol to that used for the residue testing conducted on Golden perch and Murray cod:

1. On-grow marked fish until they have increased at least ten times in mass prior to conducting residue tests.
2. Employ a conservative approach by testing fish when they are still well below the legal minimum size and by conducting tests on gilled and gutted whole fish, as well as fillets.

Under the US Fish and Wildlife Service’s INAD 10-987, calcein marking of fish greater than 2 g is not permitted to ensure a long withdrawal period prior to potential consumption by humans. Whilst this condition is not legally binding in Australia, we would recommend that marking of fish with fluorescent dyes be restricted to fish of less than 2 g unless further research establishes that it is appropriate to mark fish at larger sizes.

A method for determining residues of ARS in fish was developed; however, we have not conducted residue tests because there is no method currently available for field detection of ARS marked fish. The project team would not recommend marking of potential food fish with ARS until residue testing has been conducted and appropriate advice from FSANZ received.

4.3 Stable isotopes

4.3.1 Isotope immersion
For the use of stable isotopes of barium and strontium to mark fish otoliths via immersion, consideration of the potential requirements for registration of the enriched stable isotopes $^{137}$Ba
and $^{86}\text{Sr}$ was undertaken by the project team and the consultant (AgAware Consulting Pty Ltd). A written interpretation by the Consultant of the advice received from the Australian Pesticides and Veterinary Medicines Authority (APVMA) stated that the use of enriched isotopes for the sole purpose of marking fish does not require registration under the Commonwealth Agriculture and Veterinary Chemical Code Act 1994 (see Sanger and Crook 2007).

With regards to the requirement for Food Standards Australia New Zealand (FSANZ) approval as a chemical food additive, the total concentrations of Ba used for the immersion method of marking fish are well below the maximum concentrations of 2 mg L$^{-1}$ outlined in the Australian Drinking Water Guidelines. The concentrations of both Ba and Sr are also within the typical range of concentrations found in water in the natural environment (Kraus and Secor 2004; Crook et al. 2006). Consequently, there is no potential for treated fish to assimilate residues of either element above the levels commonly found in fish inhabiting natural freshwater and marine environments. On this basis, the project team concluded that there is no potential requirement for FSANZ registration of enriched stable isotopes of Ba or Sr for the immersion marking of fish as described by Crook et al. (2006) and Munro et al. (2008).

**Transgenerational marking**

For the transgenerational method of marking fish, the maximum total mass of barium (Ba) enrichment was determined to be <0.1 µg per marked fish. The average daily dietary intake of Ba is ~800 µg per day and levels of up to 10 mg L$^{-1}$ in drinking water have been shown to have no adverse effects on humans after 8 weeks (NHMRC and NMMERC 2004). The maximum concentration of the Ba solution injected into the maternal broodfish (1 g L$^{-1}$) exceeds the maximum concentration of Ba for drinking water in Australia of 2 mg L$^{-1}$. Research on the assimilation of Ba suggests that it is unlikely that broodfish would retain high levels of Ba in their edible tissues (Williamson et al. 2009). However, as a conservative approach, broodfish injected with Ba should not be released into the wild or otherwise made available as food until research on their suitability for consumption is conducted. Given the minimal Ba enrichment of marked progeny fish, and provided that Ba injected brood fish will not be made available as food, the project team concluded that there is no potential requirement for FSANZ registration or approval of the use of the enriched stable isotopes Ba for transgenerational marking of fish as described by Munro et al. (2009). Similarly, Williamson et al. (2009) found that transgenerational marking with Ba presented no risk to humans based on analyses of marine fish.

### 4.4 Disposal of chemicals

**Calcein and alizarin red S**

Calcein and ARS dye solutions must be disposed of properly after use - they should not be poured down the drain or otherwise disposed of into sewers or waterways. In the USA, where calcein marking has been undertaken on a large scale for a number of years, no discharge of calcein solution is allowed under the Investigative New Animal Drug (INAD 10-987) approval for SE Mark® (calcein). According to the INAD, all used calcein solution must be stored in sealed containers on station prior to disposal by a registered chemical disposal company. Mohler and Bradley (2008) also recently described a method for reducing the volume of waste from calcein marking using activated carbon. Such methods have potential to minimise the costs of calcein solution disposal.

If the marking of hatchery fish with fluorescent dyes is to be undertaken on a large scale in Australia, protocols for the appropriate disposal of remaining dye solutions, as well as suitable record keeping, will need to be established. The information contained in INAD 10-987 could be used as a basis for establishing such protocols by the relevant agencies, such as State Environment Protection Authorities, etc. As no standard protocol currently exists, it is the responsibility of each hatchery (whether government or private) to develop and implement site specific protocols for the disposal of waste dye solutions that are compliant with relevant State or Territory regulations on
chemical use and disposal. Assistance with developing these protocols may be sought from registered chemical disposal companies.

**Isotope immersion and trans-generational marking**

The concentrations of stable barium isotopes used for the immersion method of marking fish are well below the maximum concentrations outlined in the Australian Drinking Water Guidelines (there is no reference to stable isotopes of strontium in the guidelines). The concentrations of both barium and strontium are also within the typical range of concentrations found in water in the natural environment (e.g. Kraus and Secor 2004; Crook et al. 2006). Consequently, the project team did not identify any regulatory issues relating to the disposal of the water used to treat the fish for isotope immersion.

No left-over solution needs to be disposed of when using the trans-generational method, although as previously mentioned, broodfish injected with barium should not be released into the wild or otherwise made available as food until research on their suitability for consumption is conducted.

**4.5 Other considerations**

In addition to the “nuts and bolts” of the techniques used to mark fish with chemicals, practitioners need to consider a number of related issues prior to marking fish, including the following:

**Hatchery practice and animal welfare**

Good hatchery practice is critical to the success of all hatchery operations. It is made all the more important when marking fish with chemicals because the fish may experience short term stress during and after the marking procedures. The use of chemicals in hatcheries also requires accurate record keeping and the use of appropriate handling and disposal protocols. Staff undertaking chemical marking activities also need to be fully aware of their obligations with regards to animal welfare and, in the case of fish used for research purposes, animal care and ethics approvals.

**Responsible fish stocking**

Although the stocking of fish produced in hatcheries is an important tool for fisheries management, it carries environmental, social and economic risks (see Gillanders et al. 2006). These risks need to be addressed for stocking activities to proceed in a sustainable manner, and the necessary approvals from the responsible authorities need to be granted prior to stocking.

**Occupational Health and Safety**

The importance of ensuring the safety and welfare of staff whilst undertaking hatchery operations cannot be overstated. Relevant Occupational Health and Safety legislation that applies in each State or Territory must be complied with. Activities associated with the chemical marking of fish carry risks and it is the responsibility of the hatchery operator to ensure that appropriate safeguards are in place to minimise these risks. Material Safety Data Sheets (MSDS’s) are available for all of the chemicals used in the marking procedures described in this report. These should be understood and followed when using these chemicals to mark fish. Gloves, safety glasses and protective clothing should be worn at all times when marking fish.

For more detailed information on the material presented in this Chapter, see:


5 Natural otolith chemical signatures

5.1 Introduction

The otoliths of fish are composed of a calcium carbonate matrix that grows continuously throughout the life of the fish. As otoliths grow, trace elements present in the water are accreted into the otolith surface and, as there is no turnover of the deposited material, the otolith forms a permanent record (“otolith chemical signature”) of the chemical environment to which a fish has been exposed throughout its life (Campana 1999). By measuring the relative amounts of trace elements present in various regions of the otolith (e.g. the embryonic/larval growth region in the core), it is possible to identify the natal chemical signatures of individual fish (Crook and Gillanders 2006). As described in Chapter 3, the chemical composition of otoliths can be precisely measured using methods such as LA-ICPMS.

In the case of stocked fish, the embryonic, larval and early juvenile portions of otoliths contain the chemical signature of the hatchery in which the fish was produced. If the water chemistry conditions within the hatchery are sufficiently different to the natural environment (e.g. due to the use of fertilisers or other chemicals), it should be feasible to distinguish the core chemical signatures of hatchery and wild fish without the need for artificial markers. Furthermore, if different hatcheries have distinctive water chemistries, it may be possible to discriminate between fish produced at different hatcheries. In this chapter, we undertake a series of analyses to determine the feasibility of using natural otolith chemical marks for identifying hatchery fish.

5.2 Hatchery versus wild chemical signatures

We collected Golden perch otoliths from wild populations across of the MDB and the majority of major hatcheries in order to determine whether the otolith chemical signatures of hatchery fish were distinguishable from wild fish. Samples from hatcheries were collected over several years to examine the temporal stability of otolith signatures. Trace element concentrations in the core regions of the otoliths were analysed using LA-ICPMS. Discriminant function analyses were used to statistically analyse the data.

Analysis of wild fish collected in 2004 revealed a high degree of overlap among sites within regions and a classification accuracy of only 44% (Figure 5.1). However, when sites within a region were combined, there was clear separation at the regional scale and 93% of fish were correctly classified to their region of origin based on their otolith chemical signatures (Figure 5.1).

![Figure 5.1. Otolith chemical signatures of fish collected from the wild.](image)

Discriminant function plots showing otolith chemical signatures of fish collected from 14 sites within three river basins in the MDB. Sites shown separately on left (44% classification accuracy) and grouped into river basins on the right (93% classification accuracy).
Methods for discriminating hatchery fish and outcomes of stocking in the Murray-Darling Basin

Analysis of hatchery fish collected in 2004 and 2005 revealed reasonably strong separation among hatcheries, with classification accuracy of 76% among eight hatcheries in 2004 and 90% accuracy among four hatcheries in 2005 (Figure 5.2). Despite the relatively high classification rates within years, the chemical signatures of individual hatcheries were not stable between years: only 6% classification accuracy achieved when the 2004 baseline data were used to classify the 2005 fish.

Figure 5.2. Otolith chemical signatures of fish collected from MDB hatcheries. Discriminant function plots showing otolith chemical signatures of fish collected from eight hatcheries in 2004 and four hatcheries in 2005.

Comparisons between the otolith chemical signatures of fish collected from hatcheries and the wild in 2004 showed that it was possible to identify hatchery fish with a high degree of accuracy for at least some of the hatcheries (Figure 5.3). However, this was not always the case. For example, the chemical signature of the Uarah hatchery at Grong Grong, NSW, overlapped extensively with the Darling River basin chemical signature. Thus, it would not be possible to discriminate fish produced at the Uarah hatchery in 2004 if they were stocked into the Darling River.

Figure 5.3. Otolith chemical signatures of fish collected from river basins and hatcheries. Discriminant function plots showing otolith chemical signatures of fish collected in 2004 from three MDB hatcheries and from the wild in the lower Murray, mid-Murray and Darling regions.
5.3 Conclusions

Our analyses suggest that natural otolith chemical signatures have potential utility for discriminating between stocked and wild fish in certain circumstances. However, there are a number of limitations that need to be considered when attempting to adopt such an approach. First, the multi-elemental otolith signatures of both wild and hatchery fish are not always stable across years (Figure 5.2; A. Munro unpubl. data). An exception to this was the Narrandera hatchery whose chemical signature remained stable across all years sampled (2004-2008). The existence of inter-annual variation in hatchery and wild chemical signatures means that the accuracy of discrimination between hatchery and wild fish is likely to change between years (possibly even within years). Thus, a “library” of otolith samples would need to be collected from hatcheries at least annually to allow otolith chemical signatures of fish collected from the wild to be matched back to candidate hatcheries. This is a relatively complex analytical process and may be difficult to achieve in practice.

Despite the relatively high levels of classification accuracy that were achieved in some instances, natural chemical signatures are not as definitive as the artificially induced otolith chemical marks described in Chapter 3. There are high levels of variability in otolith chemical signatures amongst regions and, as mentioned, signatures may change over time. Furthermore, it is often impossible to sample fish from all possible natal sources - classifications of fish based on natural otolith signatures can only consider the sampled populations, and thus, there can be some doubt as to the origin of fish even when classification accuracy is high. In contrast, enriched stable isotope markers cannot be confused with natural signatures because the isotope ratios achieved do not occur naturally.

The analyses used in the current study examined only trace element concentrations (e.g. strontium, barium, manganese). It is possible that the inclusion of additional trace elements and more temporally stable chemical parameters, such as $\text{Sr}^{87}/\text{Sr}^{86}$ ratios (Kennedy et al. 2000), would lead to more discriminatory power in this type of analysis. Whilst our research suggests that analysis of natural otolith chemical signatures has some utility for discriminating hatchery and wild fish, further research is required to fully explore the potential of this technique.
6 Outcomes of stocking in Murray-Darling Basin rivers

6.1 Introduction
The current project has tested, developed and implemented a series of techniques for discriminating hatchery and wild fish. Ultimately, however, the aim of this research is to develop an understanding of the impacts and outcomes of native fish stocking in the MDB. In this chapter, we describe an experimental stocking study conducted in four rivers of the southern MDB. This work began in 2002 as part of a previous project funded by the Australian Research Council's Linkage Program (2003-2006) and has continued with funding under the NFS since 2004.

The aim of the field study was to experimentally stock rivers with chemically marked fish and to conduct follow-up surveys to determine the contribution of stocked fish to resident populations. Golden perch were chosen as the test species, as this species was used for the development of the marking techniques and is the most commonly stocked in the MDB. Relatively large numbers of fish were stocked over a broad spatial extent to mimic the types of large-scale stockings that are currently undertaken in the MDB, thus ensuring that the findings of the study have relevance to real world situations.

6.2 Experimental stocking
We conducted experimental stocking and fish assemblage surveys in the Murrumbidgee River, Edward River and Billabong Creek in the south-eastern region of the MDB (Table 6.1). We also surveyed the Murray River in the region between Yarrawonga and Tocumwal as an unstocked reference - fish stocking in this region of the Murray River has not been permitted since the early 1990’s. Golden perch fingerlings (~30-40 mm TL) for the study were produced at the Narrandera Fisheries Centre, Narrandera, New South Wales, Australia.

The fish stocked into the Murrumbidgee River and Edward River from 2002-2006 were marked with alizarin complexone using the methodology described by van der Walt and Faragher (2003). The fish released into Billabong Creek in 2006 and 2007 were not chemically marked due to uncertainty regarding the regulation of chemicals for marking fish. This issue was resolved (see Chapter 4; Sanger and Crook 2006) and most of the fish released into Billabong Creek in 2008 were marked with calcein using the methodology described in Chapter 2. The non-marked fish stocked into Billabong Creek were identified by conducting otolith chemistry analyses similar to those described in Chapter 5. Stocked fish could be classified with an overall accuracy of 92-97% (depending on the year) in the samples collected from Billabong Creek (Gillanders unpubl. data).

In addition to the stockings undertaken as part of our study, stockings of Golden perch in our study reaches were undertaken by fishing clubs as part of the Industry and Investment NSW “dollar for dollar” stocking program (Table 6.1). In Billabong Creek, we were able to treat these fish as part of our experimental stocking program because the source of the additional stocked fish was known and movement by fish within the study reach was restricted by upstream and downstream weirs throughout the study. The additional stocked fish in Billabong Creek were identified using analysis of the natural otolith chemical signatures as described above. Such an approach was not attempted in the Edward and Murrumbidgee rivers, as movement by fish in these systems is less restricted and the potential sources of fish were much less certain. Thus, we were only able to identify the sources of the marked fish stocked specifically as part of this project in these rivers.

Standardised surveys of the fish assemblages in the study rivers were conducted using boat-mounted electrofishing units (Figure 6.1). The time that electricity was applied to the water was recorded and used to quantify sampling effort. Catch per unit effort (CPUE) was measured as the number of individual fish collected per hour of time that electricity was applied. Sampling effort increased during the study as further funding for the project was obtained (see Table 6.2).

Collected fish were identified, counted and measured for length (mm, FL or TL) (Figure 6.2). The weight (g) of a sub-set of fish was also measured. A sample of Golden perch from each survey was
euthanised for otolith analysis. Otoliths were embedded in resin, sectioned using a slow speed saw and examined under a fluorescence microscope (see Chapter 2) to identify alizarin complexone or calcein marks. As mentioned, LA-ICPMS was used to identify the unmarked stocked fish in Billabong Creek. Fish were aged by counting the annual growth increments and the year of birth was back-calculated (Anderson et al. 1992). The year classes of fish were calculated as the year of spawning, rather than the year of stocking. For example, a fish spawned in October 2007 and released as a fingerling in January 2008 would be considered a member of the 2007 year class.

Table 6.1: Experimental Golden perch stockings.
The number of Golden perch fingerlings experimentally stocked into the three study rivers and the chemical markers used for identification. All fish were sourced from the Narrandera Fisheries Centre with the exception of those marked (*). * includes 20,000 unmarked fish from the Murray-Darling hatchery stocked in the study reach in Billabong Creek in 2007, and 2,000 unmarked fish from the Uarah hatchery stocked in the study reach in Billabong Creek in 2008.

<table>
<thead>
<tr>
<th>Year of Stocking</th>
<th>Billabong Creek (Wanganella - Moulamein)</th>
<th>Edward River (Stevens Weir - Moulamein)</th>
<th>Murrumbidgee (Berembed - Yanco)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Marker</td>
<td>Number</td>
</tr>
<tr>
<td>2002</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2004</td>
<td>-</td>
<td>-</td>
<td>50,000</td>
</tr>
<tr>
<td>2005</td>
<td>-</td>
<td>-</td>
<td>49,000</td>
</tr>
<tr>
<td>2006</td>
<td>60,000</td>
<td>Otolith chemistry</td>
<td>-</td>
</tr>
<tr>
<td>2007</td>
<td>68,000*</td>
<td>Otolith chemistry</td>
<td>-</td>
</tr>
<tr>
<td>2008</td>
<td>22,000*</td>
<td>Calcein/otolith chem.</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 6.2: Details of standardised electrofishing surveys conducted during the study.
Effort was measured as the electrofishing time (h) that power was applied to the water.

<table>
<thead>
<tr>
<th></th>
<th>Billabong</th>
<th>Edward</th>
<th>Murrumbidgee</th>
<th>Murray</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trips</td>
<td>Effort (h)</td>
<td>Trips</td>
<td>Effort (h)</td>
</tr>
<tr>
<td>2005</td>
<td>1</td>
<td>1.07</td>
<td>1</td>
<td>1.27</td>
</tr>
<tr>
<td>2006</td>
<td>2</td>
<td>5.77</td>
<td>2</td>
<td>6.88</td>
</tr>
<tr>
<td>2007</td>
<td>2</td>
<td>5.44</td>
<td>2</td>
<td>3.96</td>
</tr>
<tr>
<td>2008</td>
<td>3</td>
<td>8.94</td>
<td>3</td>
<td>6.59</td>
</tr>
<tr>
<td>2009</td>
<td>3</td>
<td>6.15</td>
<td>3</td>
<td>6.45</td>
</tr>
</tbody>
</table>
6.3 Contribution of stocked fish

In Billabong Creek, the CPUE of Golden perch increased dramatically following stocking (Table 6.3, Figure 6.3). This increase in CPUE is likely to be an underestimate of the true effect, as the Golden perch sampled during each trip were removed for otolith analysis. Rather than becoming depleted over time, however, the catch rates of stocked Golden perch continued to rise throughout the study. This was most likely due to a gradual increase in collection efficiency as the stocked fish grew larger and more susceptible to electrofishing. There was little evidence of any natural recruitment in Billabong Creek in the years following stocking, with stocked fish from the Narrandera hatchery estimated to comprise 100%, 79% and 92% of the 2005, 2006 and 2007 year classes respectively (i.e. year classes corresponding to experimental stockings) (Figure 6.4). Although a small number of fish was stocked in the study reach in Billabong Creek in 2009, none of these fish were collected in subsequent surveys.

In the Edward River, the experimentally stocked fish comprised 18 and 38% of all Golden perch collected in the 2003 and 2004 year classes respectively (Figure 6.3, 6.4). The 2003 year class was particularly strong, with a large number of non-marked individuals collected in addition to the experimentally stocked fish. These fish were either natural recruits or were unmarked hatchery fish from other stockings. The 2004 year class was much weaker than 2003, and although our stocked fish made up a higher proportion of the age class than in 2003, fewer stocked individuals from the 2004 year class were collected. According to stocking records, approximately 25,000 and 35,000 unmarked Golden perch from a number of different hatcheries were stocked into other regions of the Edward River in 2004 and 2005 respectively. Stockings of Golden perch were also conducted in the connected Murray River in these years. As previously mentioned, we were not able to positively identify the origins of the unmarked fish collected from the Edward River. The age frequency distributions suggest a lack of recruitment of Golden perch since 2005 in the Edward River, despite continued stocking of unmarked fingerlings in regions of the Edward and Murray rivers between 2006 and 2009. CPUE was variable among years, but unlike Billabong Creek, there was no obvious upward or downward trend over the study.

In the Murrumbidgee River, experimentally stocked fish comprised 37, 29 and 33% of all Golden perch collected in the 2001, 2003 and 2004 year classes respectively (Figure 6.3, 6.4). Similar to the Edward River, the 2003 year class was the most abundant year class at most sites. However, there was more variability in the relative contributions of the different year classes amongst sites within the Murrumbidgee River. The contribution of stocked fish from the different stocking years was also highly variable amongst sites. For example, fish from the 2002 stocking (ie. 2001 year
Methods for discriminating hatchery fish and outcomes of stocking in the Murray-Darling Basin

class) comprised 42, 14 and 57% at Berembed, Narrandera and Buckingbong, but did not
contribute at all to the samples collected from Yanco/Euroly. Fish from the 2004 stocking (ie.
2003 year class) comprised 23, 86 and 21% at Berembed, Buckingbong and Yanco/Euroly, but did
not contribute at all to the samples collected from Narrandera.

Similar to the Edward River, stocking of un-marked Golden perch was also undertaken by fishing
clubs in the Murrumbidgee River during the study. According to stocking records, approx.156,000
unmarked Golden perch fingerlings were released in the region between Berembed Weir and
Yanco Weir between 2002 and 2009 (Ben Doolan, I&I NSW pers. comm.). As we were not able to
distinguish between these unmarked fish and natural recruits, it is not possible to quantify the total
contribution of stocked fish from our data. However, it is likely that these other stocking also
contributed significantly to populations in the Murrumbidgee River. Unlike Billabong Creek and
the Edward River, there was evidence of limited recruitment of juvenile fish after 2005 in the
Murrumbidgee River, with small numbers of fish from the 2006 and 2007 year classes present in
the samples. Similar to the Edward River, there was no obvious trend in CPUE over the study.

The samples collected from the Murray River were dominated by large fish more than 5 years old
and 350 mm TL (Figure 6.4). There was little evidence of recruitment into the population after
2005, with only one fish from each of the 2005 and 2006 year class collected at a single site
(Brears Road). Given that this reach of the Murray River is not stocked, the dominance of older
fish and the apparent lack of recruitment post-2005 suggest that natural recruitment in this reach
has been at low levels in recent years and that immigration of adult fish may be sustaining the
current population of Golden perch.

Figure 6.2: Two year classes of stocked Golden perch.
Fish from the 2005 (left) and 2006 (right) year classes collected from Billabong Creek in May 2007.
Figure 6.3. Contribution of stocked fish to Golden perch populations.

Length frequency plot (adjusted to catch per unit effort (CPUE), fish per hour of electrofishing time) from the standardised electrofishing surveys showing contribution of experimentally stocked and non-marked fish to populations in each year of the study. The Murray River was not experimentally stocked and is presented as a reference. Experimentally stocked fish (grey bars), non-marked fish (black bars). The minimum legal size of 300 mm for Golden perch is shown as a dashed line. Note the different scales on the y-axes between rivers.
Figure 6.4. Contribution of stocked fish to year classes of Golden perch.
The contribution (% of total catch) of experimentally stocked and non-marked fish within each year class at each site. The Murray River was not experimentally stocked and is presented as a reference. Fish from year classes 2000 and earlier (≤2000) have been combined. Experimentally stocked fish (grey bars), non-marked fish (black bars).
6.4 Resident fish assemblages

In addition to monitoring golden perch populations, we examined the species composition and relative abundance of the fish assemblages in the four rivers. The experimental stockings and subsequent surveys were not designed to directly assess the impacts of stocking on resident fish assemblages. However, information on fish assemblages provides contextual information about the factors that may have influenced the outcomes of the experimental stockings (e.g. abundance of predatory species). Details of all of the species collected during the study are shown in Table 6.3.

As mentioned previously, the CPUE of Golden perch in Billabong Creek increased dramatically following stocking, whilst there were no clear trends in the other rivers. It should be noted that we did not conduct pre-stocking surveys in the Edward and Murrumbidgee rivers, so it is not possible to examine any effects of stocking on CPUE in these two rivers. Of the other species collected, Murray cod and common carp were the most common large bodied species. Considering that common carp were found to comprise the vast majority of fish biomass in previous surveys of MDB rivers (Harris and Gehrke 1997), it was interesting that Murray cod vastly outnumbered carp in Billabong Creek and the Edward River. In fact, the CPUE of Murray cod recorded in these rivers during the current study is amongst the highest ever recorded for the species (DSE, I&I NSW unpubl. data). Murray cod were much less abundant in the Murrumbidgee and Murray rivers than in the Edward River and Billabong Creek, with carp greatly outnumbering Murray cod. It is also interesting to note that stocking in Billabong Creek successfully increased Golden perch abundance despite the presence of extremely high numbers of Murray cod: a species well known as a predator of fish (Ebner 2006).

Most of the other large bodied fish species collected were patchy in time and space. The threatened Trout cod was only collected in the Murray and Murrumbidgee rivers. However, within these systems they were in consistently present in relatively high abundances. Silver perch were recorded in all four rivers, but were extremely patchy both spatially and temporally. Bony herring were recorded only occasionally in the Edward River and Billabong Creek, although when present they were often highly abundant. River blackfish were recorded in low abundances in the Murrumbidgee and Murray rivers, as were introduced redfin perch. The introduced goldfish was recorded in all four rivers in low to moderate abundances.

The distributions and abundances of small bodied species revealed some interesting patterns. First, several usually widespread and abundant species (Australian smelt, Murray River rainbowfish, unspecked hardyhead) were extremely rare in Billabong Creek, with only a handful of individuals of these species collected over the five years of surveys. Murray River rainbowfish were not recorded at all in Billabong Creek. In contrast, these species were abundant in the other three rivers, including the Edward River into which Billabong Creek flows. Reasons for the low numbers of small bodied native species in Billabong Creek are unclear at present.

Another interesting finding was that the abundance of Australian smelt declined markedly during the study in the Edward and Murrumbidgee rivers. This was most noticeable in the Edward River where CPUE dropped each year from 828 fish/h in 2005 to 5 fish/h in 2009. Again, the reasons for this decline are currently unclear. Carp gudgeon and the introduced Gambusia were widespread and abundant in all four rivers. However, we were not able to estimate CPUE for these species because catchability was very low using boat mounted electrofishing.
Methods for discriminating hatchery fish and outcomes of stocking in the Murray-Darling Basin

Figure 6.3: Murray cod from Billabong Creek.
Typical sized Murray cod from Billabong Creek (approx. 400 mm TL). More than 50 Murray cod were collected from Billabong Creek per hour of electrofishing time over the study.

Figure 6.4: Freshwater catfish from Billabong Creek.
This was the only Freshwater catfish collected during the study. The fish was 460 mm TL and was collected in March 2006 at Windouran.

Figure 6.4: Bony herring from Edward River.
Bony herring were recorded only occasionally in the Edward River and Billabong Creek, although when present they were often highly abundant.
Table 6.3: Catch per unit effort (CPUE) for all species.

Numbers of each species in each year per hour of electrofishing time. Carp gudgeon (*Hypseleotris* sp.) and the introduced Gambusia (*Gambusia holbrooki*) were also recorded in all rivers but CPUE was not calculated due to poor susceptibility of these species to boat mounted electrofishing.

<table>
<thead>
<tr>
<th>Species</th>
<th>Billabong</th>
<th>Edward</th>
<th>Murrumbidgee</th>
<th>Murray</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>2005</td>
<td>2006</td>
<td>2007</td>
<td>2008</td>
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<tr>
<td>Golden perch</td>
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<tr>
<td><em>Macquaria ambigua</em></td>
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<td>Murray cod</td>
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<td>12.9</td>
<td>47.9</td>
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<td>Trout cod</td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>Maccullochella macquariensis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silver perch</td>
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<td>0.1</td>
<td>2.9</td>
<td>1.6</td>
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<td><em>Bidyanus bidyanus</em></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Freshwater catfish</td>
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<td></td>
</tr>
<tr>
<td><em>Tandanus tandanus</em></td>
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<td></td>
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<tr>
<td>River blackfish</td>
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<tr>
<td><em>Gadopsis marmoratus</em></td>
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<tr>
<td>Bony herring</td>
<td>1.9</td>
<td>1.3</td>
<td>0.3</td>
<td>0.3</td>
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<tr>
<td><em>Nematalosa erebi</em></td>
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<td>Australian smelt</td>
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<td></td>
</tr>
<tr>
<td><em>Retropinna semoni</em></td>
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<td>0.7</td>
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<tr>
<td>Un-specked hardyhead</td>
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<td></td>
</tr>
<tr>
<td><em>Craterocephalus stercusmuscarum fulvus</em></td>
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<td>0.7</td>
<td>8.4</td>
<td>21.7</td>
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<td>Murray-Darling rainbowfish</td>
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<td>248.7</td>
<td>1.5</td>
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<td><em>Melanotaenia fluviatilis</em></td>
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<td>2.0</td>
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<td>1.0</td>
<td>0.9</td>
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<td>Redfin perch</td>
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<td></td>
</tr>
<tr>
<td><em>Percula fluviatilis</em></td>
<td>1.5</td>
<td>0.6</td>
<td>1.7</td>
<td>3.2</td>
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</tbody>
</table>

Numbers of each species in each year per hour of electrofishing time. Carp gudgeon (*Hypseleotris* sp.) and the introduced Gambusia (*Gambusia holbrooki*) were also recorded in all rivers but CPUE was not calculated due to poor susceptibility of these species to boat mounted electrofishing.
6.5 Conclusions

The results of the field surveys showed that at least a proportion of the experimentally stocked fish survived to reach the legal minimum size of 300 mm TL in all three rivers. However, the impacts of stocking on population structure were very different among the rivers. In Billabong Creek, the stockings had a dramatic impact upon Golden perch population structure, with massive increases in CPUE and the proportion of smaller size classes.

In the Edward and Murrumbidgee rivers, stocked fish contributed to all of the year classes in which stocking was conducted. Although we were unable to determine whether unmarked fish in the Edward and Murrumbidgee rivers were wild recruits or had been released as part of other stocking programs, it is clear that stocking contributes considerably to Golden perch populations in all three of the stocked rivers surveyed. In contrast, the Golden perch population in the non-stocked reference reach (mid-Murray River) was dominated by older fish, with evidence of only very limited recruitment of juveniles in recent years.

These findings suggest that stocking of hatchery reared fish is having a major impact on the structure and abundance of Golden perch populations in rivers of the southern MDB. This is encouraging from conservation and fisheries management perspectives, as it demonstrates the effectiveness of stocking as a means of increasing the abundance of Golden perch to augment riverine populations and contribute to recreational fisheries. However, the findings also raise some issues worth consideration.

First, the year class composition of the four rivers suggests low levels of natural recruitment between 2002 and 2008. It is possible that environmental conditions (e.g. low river flows due to drought conditions – see Mallen-Cooper and Stuart 2003; King et al. 2009) in the southern MDB have not been suitable for successful spawning or recruitment for Golden perch in recent years. The fact that stocking of fingerlings was successful in increasing Golden perch densities, at least in Billabong Creek, suggests that limits to population size are likely related to "bottlenecks" in spawning success and/or survival of the early life history stages. If this is the case, stocking of a small number of recreationally important species, such as Golden perch and Murray cod, may be masking underlying environmental issues that could be affecting a much broader range of fish and other aquatic species whose populations are not artificially augmented via stocking.

The two species most commonly stocked into rivers (Golden perch and Murray cod) are top order predators. Changes to fish assemblage composition caused by stocking have the potential to affect the trophic structure of aquatic ecosystems via alteration of carbon processing pathways in the food chain (see Gawne et al. 2007; Hunt et al. 2003). Furthermore, it is possible that stocking effects local genetic diversity within populations, with potential impacts on the ability of populations to adapt to changes in environmental conditions (Bearlin and Tikel 2003; Rowland and Tully 2004). Brood stock management requirements outlined in the NSW Hatchery Quality Assurance Guidelines (Rowland and Tully 2004) are designed to minimise any such genetic impacts: our study further demonstrates the importance of adherence by hatcheries to brood stock management guidelines.

In conclusion, the results of this study demonstrate that stocking of native fish is a powerful tool for improving inland fisheries in the MDB. Whilst there are many potential benefits of stocking for both recreational fishers and the environment, our study supports previous documents and policies (e.g. NSW Freshwater Fish Stocking Environmental Impact Statement; NSW Hatchery Quality Assurance Guidelines, Victorian Guidelines for Assessing Translocations of Live Aquatic Organisms) in re-iterating the need for careful management of stocking programs in the MDB to avoid adverse outcomes to natural populations of the stocked species, as well as riverine ecosystems more generally.
Methods for discriminating hatchery fish and outcomes of stocking in the Murray-Darling Basin

7 Extension activities

7.1 Communications

A range of extension and communication activities was conducted to ensure efficient dissemination of the projects findings and outputs. With the assistance of the Victorian Native Fish Strategy Co-ordinator (Fern Hames), a communication plan was developed by the project team to identify the project’s target audience and develop a strategy for undertaking appropriate communication activities. As the outputs of the project were considered relatively specialised and technical, direct engagement with potential end users of the outputs (i.e. hatchery managers, fisheries managers, recreational fishers, fisheries researchers) was identified as the primary aim of the communication plan for the project.

To engage with the target audience, communication activities undertaken during the project included seminars at a variety of forums and scientific conferences (national and international), publication of articles in the peer reviewed scientific literature, production of a CD and 4 page pamphlet describing the projects activities, several radio interviews, visits to hatcheries to discuss the project and demonstrations of chemical marking techniques. In addition to these activities, a 2-day “Chemical Marking Workshop” was held to communicate the outputs of the project directly to the target audience.

7.2 Chemical Marking Workshop

The Chemical Marking Workshop was held on 18-19th June 2009 at the Metropole Hotel in Melbourne (Figure 7.1). A specifically targeted audience of 40 stakeholders, including hatchery managers, fisheries managers, recreational fishers and fisheries researchers was invited to attend. The full list of attendees is shown in Table 7.1. A CD containing detailed information about the chemical marking techniques developed as part of the project was prepared and handed out to all participants at the beginning of the workshop. The contents of the CD provide detailed technical information on fish marking techniques for potential end users. Copies of the CD are available from the authors.

The topics covered and activities undertaken at the workshop were:

**Day 1**
Introduction to workshop and Native Fish Strategy (Dos O’Sullivan)
Fish marking techniques/marking with fluorescent dyes (Dave Crook)
Otolith chemistry techniques/broodfish injection (Bronwyn Gillanders)
Isotope immersion marking (Skye Woodcock)
Regulatory issues, animal welfare, OHS (Andrew Sanger)
Workshop CD content (Dave Crook).
General discussion/question session (Dos O’Sullivan)

**Day 2**
Practical demonstrations:
- Osmotic induction marking (Stephen Thurstan)
- Field detection of calcein marks (Dave Crook)
- Otolith analysis and fluorescence microscopy (Bronwyn Gillanders).

Group discussions
Methods for discriminating hatchery fish and outcomes of stocking in the Murray-Darling Basin

Figure 7.1: Stephen Thurstan demonstrating osmotic induction marking at the Chemical Marking Workshop.

Table 7.1: List of workshop participants.

| Dos O'Sullivan (facilitator) (Dosaqua Pty Ltd) | Brenton Zampatti (SARDI) |
| David Crook (Vic DSE) | Jonathon McPhail (PIRSA) |
| Fiona Warry (Vic DSE) | Matt Beitzel (ACT Parks, Conservation & Lands) |
| John Koehn (Vic DSE) | Mark Lintermans (University of Canberra) |
| Jason Lieschke (Vic DSE) | Janet Pritchard (MDBA-NFS) |
| Fern Hames (Vic DSE) | Frederick Bouckaert (MDBA-SRA) |
| Karen Weaver (Vic DSE) | Steven Brooks (Qld DPI) |
| Neil Hyatt (Vic DPI) | Adam Butcher (Qld DPI) |
| Cameron McGregor (Vic DPI) | Andrew Norris (Qld DPI) |
| Hui King Ho (Vic DPI) | Malcolm Pearce (Qld DPI) |
| Andrew Sanger (I&I NSW) | John Russell (Qld DPI) |
| Lee Baumgartner (I&I NSW) | Bronwyn Gillanders (Adelaide University) |
| Prue McGuffie (I&I NSW) | Skye Woodcock (Adelaide University) |
| Gavin Butler (I&I NSW) | Damien O'Mahony (River to Sea Research) |
| Cameron Westaway (I&I NSW) | Bruce Malcolm (Uarah Hatcheries) |
| Stephen Thurstan (I&I NSW) | Noel Penfold (Murray Darling Fisheries) |
| Ian Boutell (I&I NSW) | Bruce Sambell (Ausyfish) |
| Ben Doolan (I&I NSW) | Christopher Collins (VR Fish) |
| Les Kowitz (Freshwater Fishing and Stocking Association of Qld) | David Kramer (Future Fish Foundation) |

During the workshop, open discussions of issues relating to the use of chemical marking techniques were held. Some of the main points raised during the discussions are summarised below.

Applications

- Marking methods have been successfully developed and tested primarily using Golden perch. There is a need to develop and test protocols on other species, including freshwater, estuarine and marine fishes. This includes undertaking appropriate residue tests for different species.
In addition to their use in distinguishing stocked and wild fish, the chemical marking techniques have a variety of other potential applications that should be explored. For example, chemical marks could be used for compliance purposes to identify the sources of fish sold in the aquarium trade or fish markets. Chemical markers may also have application for threatened species management and fish ecology research.

Chemical markers could serve as an indicator or metric in assessments of river health (e.g. the Sustainable Rivers Audit) by providing information on the proportion of wild recruits in populations of stocked species. At present, stocking has the potential to confound the results of studies that use fish assemblage data as an indicator of river health.

Further testing of combinations of chemical markers (e.g. calcein and isotope marking) is an important future direction to maximise the information available from chemical markers.

Research on the development of a field detection system for ARS marked fish is needed.

Communication/extension

Effective communication is vital to the successful implementation of chemical marking at large scales. Accurate and scientifically defensible information relating to food safety and regulatory issues must be presented to avoid misconceptions about the use of chemicals to mark fish.

Adoption of the techniques is reliant upon effective and sustained communication between researchers, fisheries managers, hatchery managers and recreational fishers. It was agreed that the Chemical Marking Workshop was an excellent format for achieving this type of interaction.

It was suggested that a second Chemical Marking Workshop and/or further hatchery demonstrations should be conducted for interested stakeholders who did not attend the June 2009 workshop. The potential to produce more sophisticated training material (e.g. instructional DVD) was also raised.

Implementation and regulation

Due to the wide range of potential uses and users of chemical marking methods, there is a considerable risk that implementation of chemical marking protocols will be fragmented and piecemeal. The release of chemically marked fish without careful consideration of other releases has the potential to undermine the integrity of research/management activities using marked fish.

It is essential that implementation of chemical marking techniques proceeds in an organised and well documented manner across the MDB. Although a range of stakeholders are interested in chemically marking fish, responsibility for implementation ultimately rests with the relevant State/Territory agencies.

A number of participants suggested that it would be ideal for all stocked fish to be marked with calcein prior to release so that the contribution of stocked fish to populations could be assessed regardless of the location and timing. However, concerns regarding the feasibility of marking all stocked fish were also raised. It was agreed that further thought needs to be invested in resolving the best ways of implementing chemical marking techniques in hatcheries in terms of costs, logistics and record keeping. Although this issue was considered beyond the scope of the current study, several mechanisms for facilitating implementation were discussed (e.g. formation of a high level, multi-agency steering committee).
• It was recognised that it is the hatchery operator’s responsibility to ensure that relevant legislative requirements associated with chemical marking are met, including occupational health and safety, use and disposal of chemicals, and animal welfare.

• It was suggested that standardised methods for chemically marking fish could be included in the NSW Hatchery Quality Assurance Program and in other hatchery quality assurance programs as they are introduced. This could include standard protocols for all uses (research, recreational stocking, aquaculture, compliance).

7.3 Facilitating uptake

To further encourage uptake of the marking methods, 1000 g of calcein was distributed to the relevant State and Territory agencies within the MDB for further trials of osmotic induction marking. Eight calcein field detection kits were also distributed to the State and Territory agencies at the Chemical Marking Workshop (Table 7.2). These kits included a field fluorometer for quantitative measurement of calcein fluorescence and a specialised torch and glasses set for visual identification of marked fish (Figure 7.2). Training in the use of the kits was provided at the Chemical Marking Workshop. In addition, eight osmotic induction marking kits (consisting of three 40 L plastic tubs and a stainless steel sieve [see Figure 2.3]) were constructed and freighted to the relevant State and Territory agencies (Vic. DPI; I&I NSW; QDPI; ACT Parks, Conservation & Lands; SARDI).

At the time of writing, more than 350,000 fish from 10 species had been marked with calcein provided as part of this project (Table 7.3). A large number of Golden perch, Murray cod and Trout cod were marked with calcein in hatcheries in New South Wales and Queensland during the 2009/10 season. Further trials are also being conducted on brown trout, Australian bass and Macquarie perch by researchers in New South Wales, Victoria and the ACT. Although development of calcein marking techniques was originally targeted towards use in hatcheries, the techniques have already found wider applicability. For example, researchers at SARDI and the Arthur Rylah Institute in Victoria are trialling calcein marking for assessments of the status of threatened populations of Yarra pygmy perch (Nannoperca obscura) and dwarf galaxias (Galaxiella pusilla) respectively. Calcein marking is also being used by researchers at the Arthur Rylah Institute to mark the introduced Gambusia (Gambusia holbrooki) in a project examining control options for this pest species.

Table 7.2: Details of recipients of GFP fluorometer units.

<table>
<thead>
<tr>
<th>State</th>
<th>Organisation</th>
<th>Contact person</th>
<th>GFP unit number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vic</td>
<td>DSE (Arthur Rylah Institute)</td>
<td>Jason Lieschke</td>
<td>923Z07</td>
</tr>
<tr>
<td>Vic</td>
<td>Fisheries Victoria (Snobs Creek)</td>
<td>Neil Hyatt</td>
<td>923Z06</td>
</tr>
<tr>
<td>NSW</td>
<td>I&amp;I NSW (Grafton)</td>
<td>Gavin Butler</td>
<td>923Z08</td>
</tr>
<tr>
<td>NSW</td>
<td>I&amp;I NSW (Narrandera)</td>
<td>Stephen Thurstan</td>
<td>923Z03</td>
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<tr>
<td>QLD</td>
<td>DEEDI (Cairns)</td>
<td>Malcolm Pearce</td>
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</tr>
<tr>
<td>QLD</td>
<td>DEEDI (Bribie Island/Deception Bay)</td>
<td>Steven Brooks</td>
<td>923Z01</td>
</tr>
<tr>
<td>SA</td>
<td>SARDI Aquatic Sciences</td>
<td>Brenton Zampatti</td>
<td>923Z04</td>
</tr>
<tr>
<td>ACT</td>
<td>ACT Parks Conservation and Lands</td>
<td>Matthew Beitzel</td>
<td>923Z05</td>
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Table 7.3: Uptake of calcein marking following this study.

<table>
<thead>
<tr>
<th>Agency</th>
<th>Species</th>
<th>Numbers</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>I&amp;I NSW</td>
<td>Golden perch</td>
<td>210,000</td>
<td>Large scale hatchery marking for stocking</td>
</tr>
<tr>
<td>I&amp;I NSW</td>
<td>Murray cod</td>
<td>144,000</td>
<td>Large scale hatchery marking for stocking</td>
</tr>
<tr>
<td>I&amp;I NSW</td>
<td>Trout cod</td>
<td>83,000</td>
<td>Large scale hatchery marking for stocking</td>
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<tr>
<td>I&amp;I NSW</td>
<td>Australian bass</td>
<td>1,500</td>
<td>Hatchery trials</td>
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<tr>
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<tr>
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<td>Rainbow trout</td>
<td>230</td>
<td>Hatchery trials</td>
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<td>Vic DPI</td>
<td>Macquarie perch</td>
<td>50</td>
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<td>Qld DEEDI</td>
<td>Murray cod</td>
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Figure 7.2: Calcein field detection kit: includes a field fluorometer for quantitative measurement of calcein fluorescence and a specialised torch and glasses set for visual identification of marked fish.
8 Conclusions and recommendations

The research described in this report has provided a series of practical techniques for discriminating between hatchery and wild-bred fish. Regulatory issues relating to the use of these methods have been addressed in detail, and the methods have been tested at commercial hatchery scales to ensure that implementation is feasible. A field study that utilised some of the chemical marking methods was conducted to determine the contribution of stocked fish to Golden perch populations. This study provided the first detailed information on the outcomes of native fish stocking in rivers of the MDB, and showed that stocked Golden perch comprised a variable, but at times dominant, proportion of the Golden perch populations.

Given such findings, it reasonable to conclude that stocking has the potential to affect multi-species fish assemblages in rivers via trophic interactions, particularly as the stocked species are typically large-bodied, high order predators (e.g. Golden perch and Murray cod). This phenomenon is well known in modified environments such as impoundments, where top-down control of the trophic status of these environments has been demonstrated experimentally and has generated a wealth of literature on, and practical examples of, biomanipulation as a management tool. In riverine environments in the MDB, where native fish stocking is commonly used for enhancement of recreational fishing opportunities, alterations to trophic interactions that might result from stocking are not well understood.

Whilst the project has made available practical methods for marking hatchery fish and has endeavoured to facilitate uptake of the methods, it should be noted that the project's scope was confined to addressing the technical and regulatory aspects of chemical marking of hatchery fish and assessing the outcomes of native fish stocking in rivers. Ultimately, it is the responsibility of State and Territory agencies to consider these methods along with relevant financial, social and political issues relating to the use of chemicals to mark fish, and to determine whether and/or how such methods will be used in the future.

Based on the findings of the study, we make the following recommendations in relation to the marking of hatchery fish and assessment of the outcomes of native fish stocking in the MDB:

**Continued development of fish marking methodologies**

*Trial methodologies on other species*

The development and evaluation of the various marking methodologies examined during this study have used Golden perch, and to a lesser extent Murray cod, as the model species. There is a need to trial and evaluate the methods (including mark quality, effects on mortality and growth, residue testing) on a wider range of species. As mentioned in Chapter 7, much of this work is currently underway.

*Identify cheaper sources of calcein*

The costs of calcein marking are currently quite prohibitive (AUD$20 per 1000 fish), in part due to the fact that analytical grade calcein has only been available to date. This product is typically used in biochemical and medical research projects, rather than in manufacturing or industrial settings. There is a need for research into the availability of cheaper sources of lower grade industrial calcein to reduce the costs associated with marking fish.

*Improve and validate field detection of calcein marked fish*

To date, the longevity of the calcein marks has only been examined over a relatively short period (approximately 2 years). Further research is required to determine the length of time over which calcein marks can be detected, particularly in field situations where environmental factors (e.g. ambient light) and growth of the stocked fish may impact on mark longevity and detection.
Additionally, there is scope for research to optimize the sensitivity and accuracy of the equipment used to detect calcein marks.

**Develop field detection techniques for fish marked with alizarin red S**

The fluorometer and torch/glasses methods were successfully developed for non-lethal field detection of calcein marked fish. We were unable to develop methods for detecting fish marked with ARS. Further research is required to develop a system for the non-lethal field detection of ARS marked fish.

**Incorporate detection of marked fish into research and survey programs**

Measures of abundance and species compositions of native fish populations are often used as indicators in assessments of river health (e.g. Sustainable Rivers Audit). At present, stocking has the potential to confound the results of studies that use fish assemblage data for such purposes. It is recommended that options be investigated to incorporate calcein mark detection into the Sustainable Rivers Audit and other routine fish survey programs in the MDB so that marked fish can be accounted for in calculations of fish abundance, recruitment and other metrics.

**Combine markers to increase information available from recaptured fish**

Further testing of combinations of chemical markers (e.g. calcein and isotope marking) is an important future direction to maximise the information available from recaptured fish. For example, it may be possible to use the calcein mark to detect fish in the field, an isotope mark to identify the source of the fish (hatchery or batch), and otolith increment analysis to determine the year of release. Further research on combined chemical markers is recommended.

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**A coordinated approach to marking hatchery fish**

**Use existing forums to coordinate marking activities**

Ideally, all hatchery-produced fish that are released into the wild would be marked to allow identification of stocked fish in all geographic regions. However, there are currently a number of logistical and organisational constraints that limit the capacity of relevant authorities to implement an all encompassing marking program. While acknowledging that each State will face different pressures in developing and implementing a policy on adopting these marking methodologies, it would be very beneficial if a coordinated, cross-jurisdictional approach was taken, as the waterways of the MDB are spread across four States and the ACT. It is recommended that appropriate representatives from MDBA and the relevant fisheries agencies raise the issue of marking hatchery fish at existing forums (e.g. Australian Fisheries Management Forum, MDBA Native Fish Advisory Panel) in order to develop high level strategies for coordinating policy on chemical marking of fish in hatcheries across Australia.

**Create a register of marked fish**

Due to the wide range of potential uses and users of chemical marking methods, there is a risk that implementation of chemical marking protocols will be fragmented and piecemeal. The release of chemically marked fish without careful consideration of other releases has the potential to undermine the integrity of research/management activities using marked fish. Therefore, it is recommended that a multi-jurisdictional database of all chemically marked fish be established and maintained by the relevant fisheries management agencies.

**Create a price incentive for operators to mark fish**

Although the marking techniques developed in this project have been designed to minimise impacts on routine hatchery protocols, there is still a need for incentives to encourage uptake by privately operated hatcheries and angling organisations. This could include a "premium" price paid
Methods for discriminating hatchery fish and outcomes of stocking in the Murray-Darling Basin

to hatcheries for calcein marked fish within programs such as the New South Wales dollar-for-dollar scheme. Such a scheme might also include a compliance monitoring program, where hatchery marking procedures and batches of fish for release are audited to maximise compliance with agreed quality standards.

Formal guidelines for standardised chemical marking

This project has developed and evaluated a suite of protocols for chemically marking hatchery fish. However, further refinement of protocols will undoubtedly occur as uptake increases and as methods are trialed on new species. To ensure high levels of quality are maintained, it is recommended that the responsible agencies produce formal guidelines for standardised chemical marking of each species. These guidelines should be distributed to all hatcheries undertaking chemical marking and should be updated regularly as further research and trials are conducted.

Communication

Develop strategies for ongoing communication

Adoption of the techniques developed as part of this study is reliant upon effective and sustained communication between researchers, fisheries managers, hatchery managers and recreational fishers. The Chemical Marking Workshop and presentations at the Native Fish Strategy Forum proved to be excellent formats for achieving this type of interaction. Whilst a second full scale workshop is not considered necessary in the immediate future, regular demonstrations of chemical marking techniques would provide an ongoing means of ensuring that interested stakeholders are provided with opportunities to learn about chemical marking techniques and interact with scientists and hatchery staff. Thus, it is recommended that strategies for undertaking and supporting regular demonstrations of chemical marking techniques be developed.

Develop targeted communication tools

Although direct engagement with stakeholders at workshops and other forums is a very effective means of communication, it is not possible to reach all stakeholders via this form of communication. Similarly, whilst the CD produced for the workshop includes a large amount of information in the form of text and still images, it may be more effective to use a more visual medium to demonstrate the chemical marking techniques.

To more effectively communicate the study's outputs, it is recommended that more sophisticated and targeted communication tools be developed. These might include:

- Production of a DVD containing video footage and detailed narratives of chemical marking techniques and mark detection protocols. The DVD could also include detailed supplementary information similar to that provided in the workshop CD. The DVD could be made widely available and videos posted on MDBA and agency websites.

- Development of web pages containing summary information, frequently asked questions (FAQs) and links to project outputs on MDBA and agency websites. For a good example of this type of communication tool, see the "Riparian Restoration Experiment" web pages at: http://www.mdba.gov.au/riparian-restoration-experiment/
Addressing knowledge gaps on outcomes of fish stocking

There has been a long standing need for research on the outcomes of fish stocking. The marking methodologies developed as part of this project make such research feasible. Priority areas for research on the outcomes of native fish stocking in the MDB include:

Determine the environmental and biological factors that affect survival of stocked fish

The experimental stocking study conducted as part of this project demonstrated that stocking can have a significant impact upon fish density and population structure in rivers of the MDB. However, the impacts of stocking were highly variable among rivers and between years. This suggests that environmental and biological factors influence survival of stocked fish. Stocking frequency is also likely to affect the survival of cohorts of stocked fish due to density dependant factors such as food and habitat availability. Developing an understanding of the factors that influence stocking outcomes in different waterways and for different species is critical to optimising stocking programs and minimising the risks of adverse outcomes.

Examine impacts of stocking on food web dynamics and resident biota

The introduction of large numbers of top order predators, such as Murray cod and Golden perch, has the potential to alter food web dynamics, predator-prey relationships, and inter- and intra-specific competitive interactions. There is a need for quantitative information on the impacts of stocking on riverine food webs and any flow-on effects upon riverine biota.

Undertake research on genetic impacts of fish stocking in rivers

It is clear that stocking has the potential to drastically increase the abundance of the stocked species, and potentially, to fundamentally alter fish assemblage composition. However, very little is known of the impacts of stocking on genetic integrity of resident populations, particularly at fine scales (i.e. local variation in allelic frequencies). Further research is required to understand the genetic impacts of stocking and to determine the ecological importance of any impacts. As any impacts will occur over generations, specialised genetic analyses (mitochondrial DNA, microsatellite DNA) will be required to address this knowledge gap.

Conduct studies on other species and locations

Conclusions regarding the findings of our experimental stocking study are limited to the outcomes of Golden perch stocking in rivers of the southern MDB. There is a need for further research on the outcomes of stocking for other species and in other geographic localities.
References


Methods for discriminating hatchery fish and outcomes of stocking in the Murray-Darling Basin


