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Batch marking of otoliths and fin spines to assess the stock enhancement of *Argyrosomus japonicus*

M. D. Taylor*†, D. S. Fielder‡ and I. M. Suthers*

**School of Biological, Earth and Environmental Science, University of New South Wales, 2052, Australia and ‡Port Stephens Fisheries Centre, New South Wales Fisheries, Taylors Beach, NSW, 2316, Australia*

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Juvenile mulloway *Argyrosomus japonicus* (54·6 ± 4·6 mm total length, mean ± s.e.) were immersed in a range of oxytetracycline (OTC) solutions ranging between 0–600 mg l^{-1} in salinities of 5 (diluted sea water) and 35 (undiluted sea water), and alizarin complexone (ALC) solutions ranging between 0–60 mg l^{-1} in undiluted sea water, for 6, 12 and 24 h. Optimal marking conditions were 600 mg l^{-1} OTC for 24 h in a salinity of 5, and 30 mg l^{-1} ALC for 12 h respectively. Mark quality (MQ) was assessed using a score of 0–3 in both otoliths and anal fin spines, with a score >2 found to be acceptable for adequate mark identification. Acceptable marks were not produced using OTC in undiluted sea water. Immersion in OTC or ALC, or reduced salinity had no effect on survival relative to controls. Transverse sections of vertebrae from the ALC and OTC treatments with the highest otolith mark quality showed no discrete marks. Optimal marking techniques were used to produce double marks with a 3 day interval between marking, and marking techniques were applied to 130 000 juvenile mulloway in batch mode with minimal mortality. A numerical model of the chemical behaviour of OTC in sea water describes the decline of available OTC in increasing salinity, so that a species' salinity tolerance and successful marking can be optimized. © 2005 The Fisheries Society of the British Isles

Key words: batch marking; *Argyrosomus japonicus*; oxytetracycline; salinity.

INTRODUCTION

Mulloway or jewfish *Argyrosomus japonicus* (Temminck & Schlegel) is a highly prized sportfish and important commercial species in south-eastern Australia. The commercial catch of mulloway in Australian waters has been declining since the mid 1970s (Kailola *et al*., 1992). Recently, commercial catches in New South Wales have fallen from 120 t in 1983–1984 to 40 t in 1999–2000 in ocean waters and from *c*. 47 to 29 t over the same time interval in estuarine waters (D. Makin, pers. comm.). The decrease in catches have been attributed to habitat degradation, overfishing and bycatch of juvenile mulloway in school prawn *Metapenaeus macleayi* trawls (Broadhurst & Kennelly, 1994). The recreational catch of mulloway in Australia far exceeds commercial catches, with recreational fishers taking >975 t in 2000 (Henry & Lyle, 2003). Due to the

†Author to whom correspondance should be addressed. Tel.: +612 9385 2118; fax: +612 9385 1558; email: mattytaylor@unsw.edu.au

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high recreational demand for this species, and recent declines in commercial catch, the potential for stock enhancement of mulloway is under investigation. Extensive rearing techniques for this species exist, such that juveniles can now be cultured in large numbers for release to enhance fisheries (Fielder & Bardsley, 1999).

A responsible approach to marine stock enhancement (Blankenship & Leber, 1995) recommends that effective marking techniques are applied to stocked fishes. This allows estimation of survival and growth rates, and the contribution of stocked fishes to the fishery. Several methods are available for marking populations of juvenile hatchery-reared fishes, however, few encompass both low cost and ease of application to large numbers of individuals. External tags are bulky and cumbersome for fingerlings, and generally have only been used in stock enhancement studies on fishes >50 g (Svåsand *et al.*, 1990). Micro wire tags are regularly used to identify stocked fishes (Buckley & Blankenship, 1990), but they can involve high initial set up costs and tag application can be slow. Genetic markers are also used, however these can also be expensive to isolate, and may result in a decrease in the effective population size of the hatchery population (Utter, 1998).

Marking of fishes by immersion involves a compromise between cost, concentration, immersion time, salinity and mortality to produce the best mark. Batch marking by immersion in chemicals that fluoresce under UV light and bind to the growing edge of bone allows the marking of large numbers of juveniles with relatively low effort, and generally gives good mark retention over time and minimal treatment associated mortality (Brooks *et al.*, 1994; Beckman & Schulz, 1996; Frenkel *et al.*, 2002). Otolith marks have been produced in juvenile fishes using chemicals such as oxytetracycline hydrochloride (OTC) (McFarlane & Beamish, 1987; Paragamian *et al.*, 1992; Isermann *et al.*, 1999; Hining *et al.*, 2000), alizarin complexone (ALC) (Reichert *et al.*, 2000; Rogers *et al.*, 2001; Sanchez-Lamadrid, 2001), alizarin red (Blom *et al.*, 1994; Beckman & Schulz, 1996) and calcein blue (Brooks *et al.*, 1994; Frenkel *et al.*, 2002). Whilst there is a danger of fish mortality when using any chemical for immersion marking, OTC and ALC have been shown to produce high-quality marks with low or no mortalities. Alizarin red and calcein blue have both resulted in mortalities of larvae or juveniles being stained (Blom *et al.*, 1994; Brooks *et al.*, 1994), and calcein blue has also been shown to have inferior mark quality (Tsukamoto, 1988; Brooks *et al.*, 1994). Oxytetracyline hydrochloride and ALC can also be used to create double marks (Tsukamoto, 1988; Tsukamoto *et al.*, 1989; Iglesias & Rodriguez-Ojea, 1997), which allow the differentiation of fishes from different cohorts or treatments such as release site or size at release.

Oxytetracycline molecules form complexes with magnesium and calcium ions in sea water, which decreases the lipid solubility of OTC, inhibiting absorption across the gut wall of the fish (Lunestad & Goksøyr, 1990). Also, chelation of OTC with magnesium and calcium can prevent OTC from binding to calcium in bone forming tissue (Hettler, 1984). Magnesium and calcium ions are present in sea water at concentrations up to 53·6 and 10·2 mM respectively (Potts & Parry, 1964), and can account for a reduction in OTC available for uptake by as much as 95% (Lunestad & Goksøyr, 1990). Although cheaper, OTC is not as

commonly used as ALC for marking marine finfishes. Quantification of the relationship between the decay of OTC with increasing salinity may allow OTC to be used for marking marine and estuarine species where ALC is too expensive. Mulloway are an ideal species for testing the effect of different salinities on OTC immersion, since brackish water and riverine habitats are typical for juvenile mulloway (Gray & McDonall, 1993).

Although marks are generally detected in the otoliths of fishes treated by immersion in OTC or ALC, the mark can be missed if the core of the otolith is not precisely sectioned, which can become increasingly difficult in larger fishes. In an effort to overcome this problem, the possibility of using immersion to obtain a mark in *A. japonicus* vertebrae was explored. Also, if fluorescent marks can be detected in fin spines or scales of the fish, it may allow non-lethal detection of marks.

This study aimed to determine the optimal immersion duration, concentration and salinity for marking juvenile *A. japonicus* otoliths with two fluorescent chemicals, OTC and ALC. Once determined, these conditions were used to evaluate the success of double marking, batch marking and the occurrence of fluorescent marks in the fin spines and vertebrae. Finally, by using the equilibrium properties of OTC-cation complexes described by Lunestad & Goksøyr (1990), the theoretical decline of available OTC in sea water was evaluated for a range of seawater and OTC concentrations.

METHODS

MARKING WITH OTC AND ALC

Juvenile mulloway were grown to $54{\cdot}6 \pm 4{\cdot}6$ mm total length (L_T, mean ± S.E.) at a commercial hatchery using extensive rearing techniques as described by Fielder & Bardsley (1999). A total of 1200 juvenile mulloway were transported from the hatchery to aquaria at the Port Stephens Fisheries Centre (PSFC) on the 28 January 2003, or the Cronulla Fisheries Centre (CFC) on the 10 February 2003. Transport was in 120 l tanks filled with sea water (salinity of 32, pH *c.* 7·5), supplied with pure oxygen (1–2 l min^{-1}). Mulloway were acclimatized to sea water in the aquaria (salinity of 35, pH *c.* 8) by exchanging water in the transport tank over 30 min. Mulloway were undisturbed for 2 days to adjust to the new tank environment. Fish were fed to satiation with 1–2 mm fishmeal-based pellets (Skretting, Cambridge, Australia) twice a day before and after treatments, and starved for 24 h prior to treatment.

Juvenile mulloway were immersed in a range of OTC and ALC concentrations for 6, 12 and 24 h (Table I). The OTC immersion was carried out in both undiluted sea water (salinity of 35) and sea water diluted to a salinity of 5 with fresh water (diluted sea water). Sea water was diluted by adding purified town water to both the experiment tanks and holding tanks containing fish for the diluted seawater treatments over 1·5 h until salinity was reduced to 5. The OTC was dissolved in the static undiluted and diluted seawater experiment tanks, and buffered to between pH 7 and pH 8 using sodium carbonate. The ALC was dissolved in 0·1 M KOH and added to the experiment tanks to attain the target concentration (Table I). It was not necessary to buffer the pH, which remained constant at pH 8 during the experiment. Twenty fish were then removed from each respective holding tank and added to each 60 l experiment tank ($n = 3$). At each immersion time point, five fish were removed from each experiment tank by dip net, and placed into individual 60 l tanks filled with the same salinity sea water as the experiment tank. The salinity of tanks with diluted sea water was increased from 5 to 35 over 1·5 h by adding fresh sea water. All fish were fed to satiation twice daily for 7 days.

TABLE I. Treatment conditions for chemical marking of juvenile *Aryrosomus japonicus*

Single marking					
Chemical	Concentration ($mg\,l^{-1}$)	Salinity	Immersion duration (h)	Aquarium	Number of fish per replicate
OTC	0, 25, 50, 100 and 200	35	6, 12 and 24	Recirculating (PSFC)	5
OTC	0, 200 and 600	5*	6, 12 and 24	Flow through (CFC)	5
ALC	0, 10, 30 and 60	35	6, 12 and 24	Flow through (CFC)	5

*Diluted sea water was used for OTC treatments after quality marks were not produced in OTC treatments using full strength sea water.

ALC, alizarin complexone; OTC, oxytetracycline hydrochloride; CFC, Cronulla Fisheries Centre; PSFC, Port Stephens Fisheries Centre.

Fifty fish of 61·3 ± 3·3 mm L_T (mean ± S.E.) were exposed to a double marking regime to test the feasibility of multiple marking of juvenile mulloway. Each immersion in the double marking experiment followed the protocol described for OTC and ALC marking, using optimal conditions (Table II). All double marking experiments were completed in a flow through aquarium system at CFC.

GROWTH AND SURVIVAL

During each treatment, water quality was checked every 6 h and any dead fish removed. Fish were grown in aquaria for 7 days after final immersion to monitor growth and survival, and to allow circuli deposition over the otolith mark to reduce the interference from otolith autofluorescence. Seven days after the final immersion treatment fish were euthanazed by immersion in ice slurry. Specific growth rates (G,%) were calculated using the formula (Lagardère *et al.*, 2000): $G = 100\ (\ln L_{Tf}\ \ln L_{Ti})\ (t_f - t_i)^{-1}$, where L_{Tf} and L_{Ti} are the final and initial mean L_T of each treatment, and $t_f - t_i$ is the duration (days) over which growth took place. Specific growth rates were converted to mm day^{-1} by multiplying the initial mean L_T for the experiment. Initial mean L_T were calculated from a randomly selected subsample of 50 fish. Differences in survival between chemical concentrations were tested for each single marking treatment using a single-factor ANOVA.

TABLE II. Double marking treatment conditions and interval between immersions for juvenile *Aryrosomus japonicus*

Double Marking			
Immersion A	Immersion B	Interval (days)	Number of fish
60 $mg\,l^{-1}$ ALC for 24 h	60 $mg\,l^{-1}$ ALC for 24 h	3	10
60 $mg\,l^{-1}$ ALC for 24 h	60 $mg\,l^{-1}$ ALC for 24 h	6	10
600 $mg\,l^{-1}$ OTC in diluted sea water for 24 h	60 $mg\,l^{-1}$ ALC for 24 h	86	15
600 $mg\,l^{-1}$ OTC in diluted sea water for 24 h	600 $mg\,l^{-1}$ OTC in diluted sea water for 24 h	86	15

ALC, alizarin complexone; OTC, oxytetracycline hydrochloride.

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BATCH MARKING AND MARK RETENTION

In April 2003, >100 000 juvenile mulloway (mean ± S.E. 79·8 ± 0·9 mm L_T) were marked using 600 mg l^{-1} OTC dissolved in diluted sea water at PSFC. Two 10 000 l tanks on a flow-through system were set up, one containing fish in undiluted sea water and one containing the 600 mg l^{-1} OTC solution in diluted sea water buffered to pH 8. Fish were starved for 24 h before marking. The tank containing fish was diluted with fresh water over 3 h until salinity was 5–8, drained to *c.* 1000 l, and then refilled with the buffered OTC solution. The tank was covered to reduce degradation of the stain in UV light, and to maintain fish in darkness to reduce stress. The stain was left for 24 h, and provided with diffused pure oxygen at 7–8·5 mg l^{-1}. Water quality was monitored hourly and buffer applied as required to maintain pH 8. After 24 h fresh sea water was run through the tank to discard OTC and salinity increased to 35 over 3 h.

In March 2004, *c.* 6000 juvenile mulloway (mean ± S.E. 77·2 ± 0·8 mm L_T) were marked in a target concentration of 30 mg l^{-1} ALC at PSFC. Three 10 000 l tanks on a flow-through system were set up, two containing fish to be marked and one containing 2000 l of a 45 mg l^{-1} ALC seawater solution, pH 7.5 (buffering was not necessary). Fish were starved for 24 h before marking. The tanks containing fish were drained to *c.* 500 l and 1000 l of the ALC solution immediately pumped into these tanks to achieve the target concentration of 30 mg l^{-1}. The treatment tanks were covered and dissolved oxygen maintained at 7 mg l^{-1} by bubbling pure oxygen at 1–2 l min^{-1}. Water quality was monitored hourly and buffer applied as required. After 24 h the treatment tanks were drained to 500 l, and filled to 10 000 l with sea water using a flow through system. Waste treatment water was held for 7 days in settlement ponds, allowing chemical breakdown in sunlight.

A number of batch marked fish were released into Georges River (33°58′ S; 151°00′ E) and Botany Bay (34°01′ S; 151°07′ E) over 2003 and 2004 (Table III), as part of a stocking trial with *A. japonicus*. Fish were held in the flow through system at PSFC (salinity of 35, pH *c.* 8) at a density of 15 kg m^{-3} in 10 000 l tanks until transport to the release site. Fish were removed from the tanks with buckets and placed at a density of 20–30 kg m^{-3}into a 6000 l transporter (for the 2003 cohort) or two 600 l tranporters (for the 2004 cohort) supplied with pure oxygen at 1–2 l min^{-1}. Water quality conditions were checked for changes every 30 min whilst in transit from Port Stephens to the Georges River and Botany Bay. Fish were acclimatized to conditions at the release site (salinity of 25, pH 7·5), by exchanging water in the transport tank over 60 min. Fish were then removed from the transporters using buckets and released into the estuary. As part of an ongoing monitoring programme, released mulloway were opportunistically sampled to evaluate mark retention after the experiment. These fish were recaptured at the release site in the Georges River using an otter trawl (6 m mouth, 6 mm cod-end mesh-size) towed at *c.* 1 m s^{-1}, up to 120 days (ALC marked fish) or 425 days post staining (OTC marked fish).

MARK DETECTION

Sagittal otoliths and anal fin spines were extracted from all fish from each single and double marking experiment (Tables I and II). Vertebrae were also extracted from all fish stained using the highest concentration of each chemical, and all control fish (Table I).

TABLE III. Releases of batch marked *Argyrosomus japonicus* into Botany Bay and the Georges River

Cohort	Mark	Number Released	Release Site
April 2003	OTC	54000	Botany Bay and Georges River
March 2004	ALC	5200	Georges River

ALC, alizarin complexone; OTC, oxytetracycline hydrochloride.

Otoliths, fin spines and vertebrae were embedded in epoxy resin (Streurs Epofix, Copenhagen, Denmark), and sectioned using a diamond edge wafering saw. Sectioned otoliths, fin spines and vertebrae were mounted on glass slides and polished with 3 μm grade paper. Sections were examined using an Olympus BH2 microscope with RFC fluorescence attachment using excitation and emission wavelengths for OTC and ALC (Table IV). Mark quality was assessed using a scale of 0–3 (0, no mark present; 1, a faint mark; 2, a medium mark brightness; 3, a clear, bright mark). Photographs were taken using an Olympus SC-35 Type 12, 35 mm film camera mounted on an Olympus MOM-L camera adapter.

MODELLING THE DECLINE OF UNBOUND OTC IN SEA WATER

Equilibrium constants for the formation of calcium- (K_{Ca}) and magnesium (K_{Mg})-OTC complexes were determined spectrophotometrically by Lunestad & Goksøyr (1990) using the equations: $K_{Ca} = C_{Ca\ OTC}(C_{Ca}\,C_{OTC})^{-1}$ and $K_{Mg} = C_{Mg\ OTC}(C_{Mg}\,C_{OTC})^{-1}$, where K_{Ca} and K_{Mg} were the complex constants for the calcium- and magnesium-OTC complexes respectively, $C_{Ca\text{-}OTC}$ and $C_{Mg\text{-}OTC}$ were the concentrations of calcium- and magnesium-OTC complexes in solution, C_{Ca} and C_{Mg} were the concentrations of free Ca^{2+} and Mg^{2+} ions in solution and C_{OTC} was the concentration of unbound OTC in solution. The initial concentrations of OTC (I_{OTC}), Ca^{2+} (I_{Ca}) and Mg^{2+} (I_{Mg}) ions in solution were calculated from: $I_{OTC} = C_{Mg\,OTC} + C_{Ca\,OTC} + C_{OTC}$, $I_{Ca} = C_{Ca\,OTC} + C_{Ca}$ and $I_{Mg} = C_{Mg\,OTC} + C_{Mg}$. The equations were solved simultaneously for $C_{Mg\text{-}OTC}$ in Maple (Maplesoft, Ontario, Canada). $C_{Mg\text{-}OTC}$ was calculated using $K_{Ca} = 190\ M^{-1}$ and $K_{Mg} = 290\ M^{-1}$ (Lunestad & Goksøyr, 1990) for a matrix of I_{OTC}, I_{Ca} and I_{Mg} values using MatLab (The Mathworks Inc., MA, U.S.A.). $C_{Ca\text{-}OTC}$ was calculated for each $C_{Mg\text{-}OTC}$ using the equation: $C_{Ca\,OTC} = I_{OTC} - C_{Mg\,OTC} - C_{Mg\,OTC}\,[K_{Mg}\,(I_{Mg} - C_{Mg\,OTC}]^{-1}$ and the subsequent amount of unbound OTC in solution was calculated for each $C_{Mg\text{-}OTC}$ and $C_{Ca\text{-}OTC}$ using the equation: $C_{OTC} = I_{OTC} - C_{Mg\ OTC} - C_{Ca\,OTC}$.

RESULTS

IMMERSION DURATION, CONCENTRATION AND SALINITY

Mark quality >2 was deemed an acceptable mark as it could be readily detected in the structure under fluorescent light. Single marking trials using OTC and undiluted seawater solution produced either faint marks or no marks for all concentrations and immersion periods [Fig. 1(a)]. When present, fluorescent marks were difficult to detect, and rarely stood out against the background

TABLE IV. Dichroic mirror and filter combinations wavelengths for visualizing OTC and ALC

Chemical	Wavelength (nm)		
	Dichroic mirror	Excitation filter	Barrier filter
OTC and ALC*	500	490	515
ALC	570	545	590

*ALC could be visualized under both mirror and filter combinations; however, mark clarity was greater at lower wavelengths.

ALC, alizarin complexone; OTC, oxytetracycline hydrochloride.

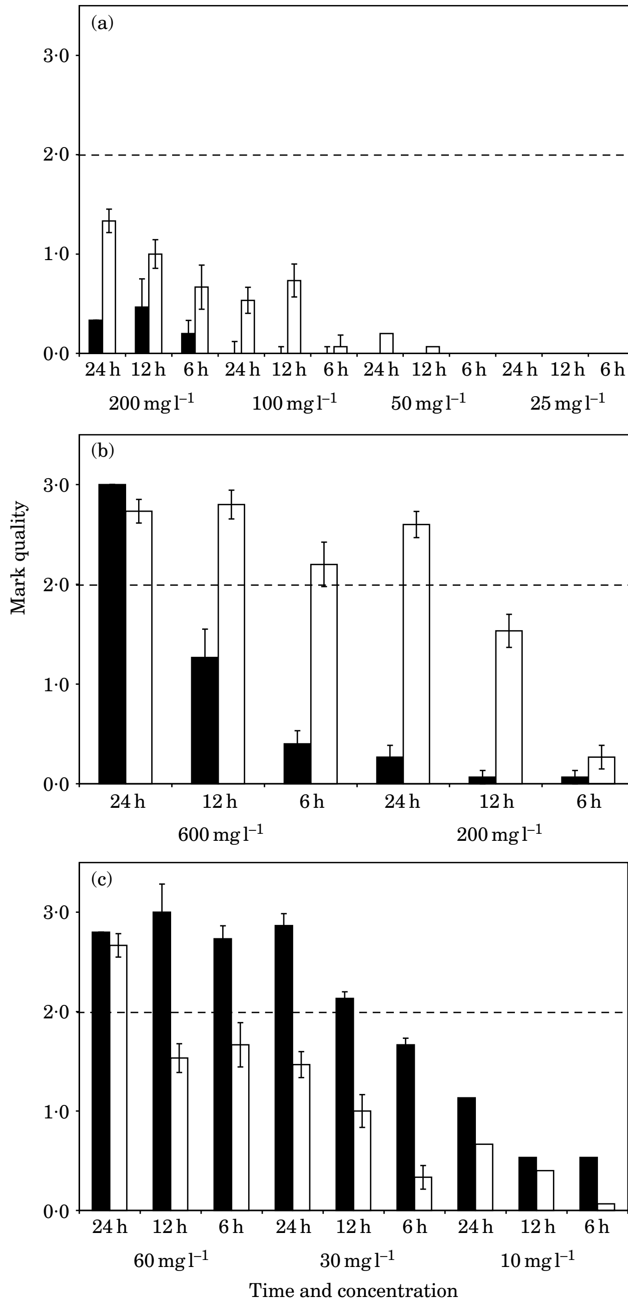

FIG. 1. Mark quality (mean ± S.E., $n = 3$) for immersion trials using oxytetracycline in (a) undiluted sea water of salinity 35, (b) oxytetracycline in diluted sea water of salinity 5 and (c) using alizarin complexone in sea water. Mark quality is shown for otoliths (■) and fin spines (□). (a) No marks were detected in otoliths for OTC in undiluted seawater solutions < 200 mg l^{-1}, and no marks were detected in fin spines for OTC concentrations < 50 mg l^{-1}. Marks were detected in otoliths and fin spines across all concentrations and immersion times for (b) OTC and diluted seawater solutions and (c) ALC solutions. Marks were produced using substantially lower ALC chemical concentrations compared to OTC treatments. - - -, the threshold for an acceptable mark.

fluorescence of the otolith or fin spine. Single marking trials using OTC in diluted seawater solution [Fig. 1(b)] and alizarin complexone [Fig. 1(c)] produced at least some marks in both fin spines and otoliths for all concentrations and immersion periods. When otoliths were exposed to the appropriate wavelengths of UV light (Table IV), OTC and alizarin marks appeared as a yellow and red fluorescent ring respectively.

Acceptable marks in otoliths were produced using ALC concentrations of $\geq$30 mg l^{-1} for $\geq$12 h, and 600 mg l^{-1} OTC in diluted sea water for 24 h. In some cases, acceptable OTC marks appeared in fin spines at lower chemical concentrations and immersion times than for otoliths [Fig. 1(a), (b)]. Marks in fin spines were more obvious at 490 nm [Fig. 2(a), (b)], however, detecting marks in the spines required the intensity of fluorescent light to be adjusted for each sample, due to the high level of autofluorescence of the tissue [Fig. 2(c), (d)]. Mark quality was compared for the 24 h and 200 mg l^{-1} OTC in diluted sea water and 24 h and 200 mg l^{-1} OTC in undiluted seawater treatments with a single-factor ANOVA, and the effect of diluting sea water to salinity of 5 produced significantly better marks in otoliths ($P << 0{\cdot}01$, d.f. = 1) and fin

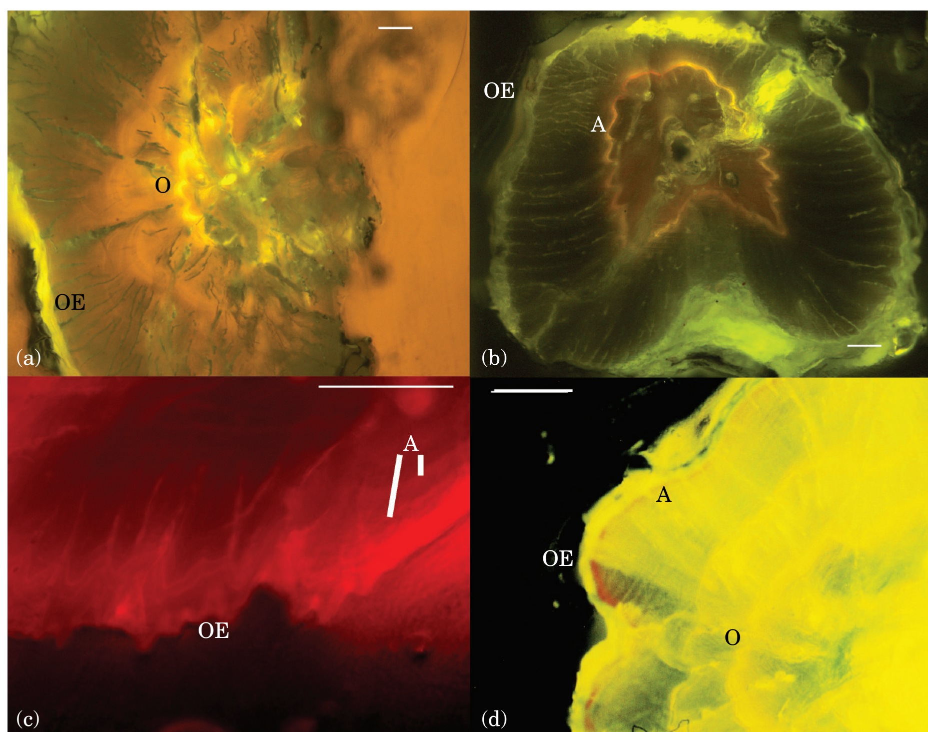

FIG. 2. Transverse sections of anal fin spine (OE, outer edge of otolith; O, OTC mark; A, ALC mark), showing (a) an OTC mark 320 days after marking, (b) an ALC mark 120 days after marking, (c) two ALC marks with a 3 day-interval and (d) one OTC and one ALC mark with an 86 day interval. All marks are shown using a 490 nm excitation filter except (c) (545 nm excitation filter). Scale bar: 100 µm.

spines ($P << 0{\cdot}01$, d.f. = 1). No marks were detected in vertebrae for any treatments examined.

Double marking was successful for all treatments. The minimum period between marking events of 3 days produced two rings that were clearly separate around the whole otolith [Fig. 3(a)]. Similar results were achieved with 6 days between marking events [Fig. 3(b)], but with approximately twice the distance between marks. Double OTC marking produced two distinct yellow marks in the otolith [Fig. 3(c)], and double marking with OTC then ALC produced a yellow and red band in the otolith that could be visualized simultaneously at 490 nm excitation wavelength [Fig. 3(d)]. Double marks were also detected in the fin spine for all treatments, however, the marks were harder to distinguish with a 3 [Fig. 2(c)] or 6 day interval. Double marking with OTC and ALC also produced a red and yellow mark in the fin spine when viewed at 490 nm [Fig. 2(d)].

GROWTH AND MORTALITY

There were no significant differences in mortality between controls and treatments for OTC in undiluted sea water, OTC in diluted sea water or ALC. A maximum mortality of 10% occurred in a control for the OTC in undiluted seawater experiment, however, there was no significant difference in

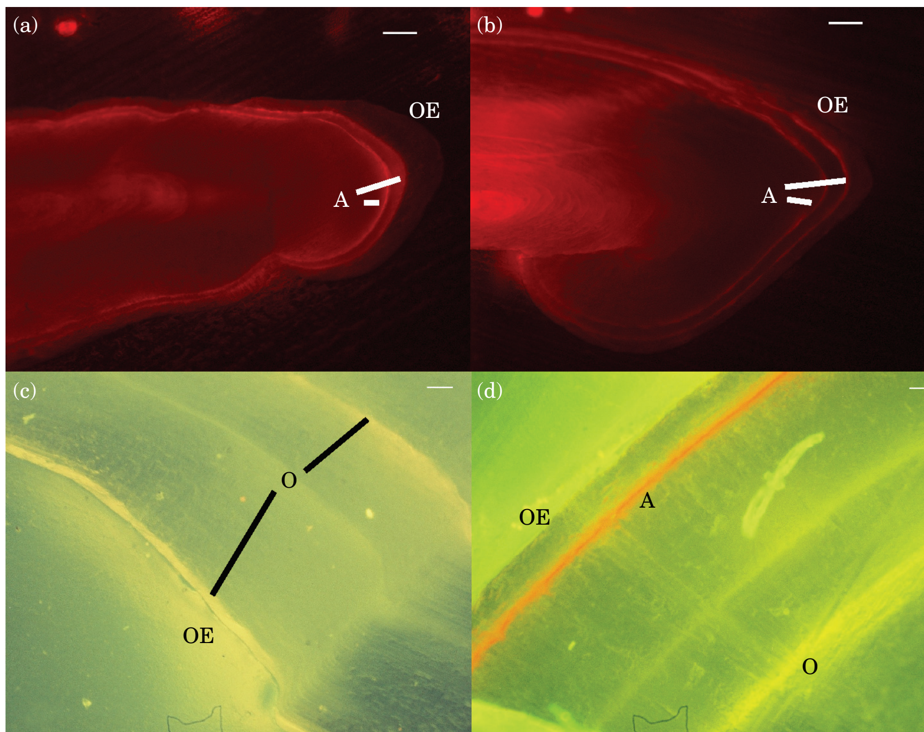

FIG. 3. Sections of double marked otoliths (OE, outer edge of otolith; O, OTC mark; A, ALC mark), showing (a) two ALC marks with a 3 day interval, (b) two ALC marks with a 6 day interval, (c) two OTC marks with an 86 day interval (the growth check under the 'O' is not an OTC mark) and (d) one OTC and one ALC mark with an 86 day interval. Scale bar: 100 μm.

mortality between treatments and controls for this experiment ($P = 0{\cdot}30$, d.f. $= 4$). No mortality occurred in controls or treatments for OTC in diluted sea water, ALC or the double marking experiments. Fish grew at an average daily growth rate of $1{\cdot}6 \pm 0{\cdot}1$ mm day^{-1} (mean $\pm$ S.E.) for 7 days after treatment.

BATCH MARKING AND MARK RETENTION

Batch marking in OTC was carried out in three consecutive batches, at a maximum density of *c.* 35 000 fish per 10 000 l tank (20 kg m^{-3}). The pH decreased gradually over the period of staining, however, this was buffered with Na_2CO_3. No evidence of stress was observed in fish and mortality was negligible (<1%), and mainly due to cannibalism.

Batch marking in alizarin complexone was carried out simultaneously in two batches of 3000 fish. In both batches water quality remained constant over the period of staining, and there was 5% mortality in one batch and <1% mortality in the other. The high mortality in one of the marked batches of fish was attributed to stress and scale loss associated with an extra handling of these fish during harvest and transport from the commercial hatchery. Bright, high quality marks were detected in all otoliths that were sub-sampled from each batch marking method ($n = 80$).

Recaptured fish from releases in 2003 and 2004 (Table III) showed marks were still present in otoliths and fin spines 425 days after marking for OTC [Fig. 2(a); mark after 320 days], and 120 days after marking for ALC [Fig. 2(b)]. There was no reduction in mark quality for either stain observed over this time period, with all marks retaining a mark quality rating of 3.

MODELLING THE DECLINE OF UNBOUND OTC IN SEA WATER

The modelled relationship between the OTC available for uptake into the fish declines with the salinity of sea water [Fig. (4)]. This relationship supports the

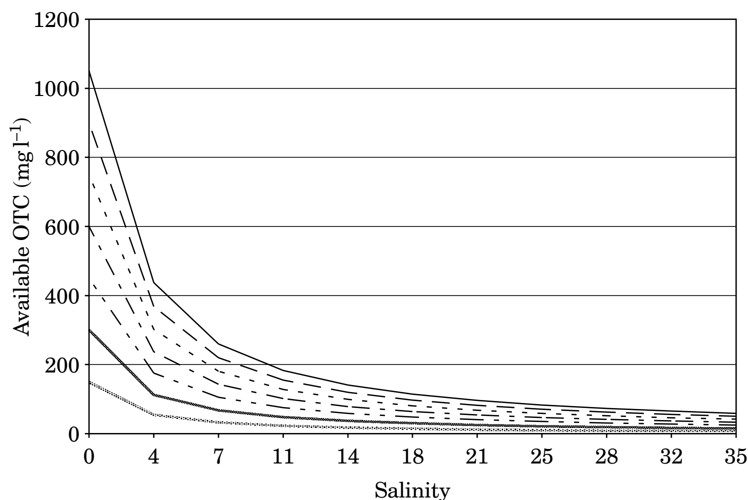

FIG. 4. Decay of available OTC in solution with increasing salinity. Initial concentrations of OTC are 1050 (——), 900 (— —), 750 (- - -), 600 (— - —), 450 (- - — - -), 300 (—) and 150 (---) mg l^{-1}.

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experimental results observed in this study. Concentrations of available OTC in 35 salinity sea water are negligible regardless of how much OTC is added. For $200\,mg\,l^{-1}$ OTC treatments, in a salinity of 5 and 35, *c*. $113\,mg\,l^{-1}$ and *c*. $16\,mg\,l^{-1}$ OTC was available for uptake respectively.

DISCUSSION

Stocked mulloway marked with OTC and ALC were successfully re-identified after nearly 18 months at liberty in an estuary. Successful marking of juvenile fishes in $<100\,mg\,l^{-1}$ ALC has been achieved for several marine or anadromous species, including red sea bream *Pagrus major* Temminck & Schlegel (Tsukamoto *et al*., 1989), Atlantic cod *Gadus morhua* L. (Blom *et al*., 1994), Japanese flounder *Paralichthys olivaceus* Temminck & Schlegel (Yamashita *et al*., 1994), ayu *Plecoglossus altivelis* Temminck & Schlegel (Tsukamoto, 1988), turbot *Psetta maxima* (L.) (Iglesias & Rodriguez-Ojea, 1997), gilthead sea bream *Sparus aurata* L. (Sanchez-Lamadrid, 2001) and rainbow trout *Onchorynchus mykiss* (Walbaum) (van der Walt & Faragher, 2003). There are few cases, however, where marking of marine fishes using OTC in undiluted sea water has been successful. Although, Cermeno *et al*. (2003) produced multiple detectable marks in *Engraulis encrasicolus* (L.) using multiple immersions in $400\,mg\,l^{-1}$ OTC for 24 h, recent studies show that marking is more successful in salinities <15 and OTC concentrations $>500\,mg\,l^{-1}$ (Jenkins *et al*., 2002; Butcher *et al*., 2003). Alternatively, fishes can be marked by direct injection with OTC (Babaluk & Craig, 1990; Bumguardner, 1991; Murphy & Taylor, 1991; Ross *et al*., 1995), although this method is impractical for marking large numbers of fishes. The present results demonstrate that a combination of diluted sea water and appropriate concentration of OTC can produce high quality marks in *A. japonicus*. Many species of marine fishes, however, do not have a broad salinity tolerance, or can only handle gradual changes in salinity, which may make the marking exercise too time consuming. Immersion marking of marine species with OTC generally requires concentrations of chemical $\geq\times10$ that of ALC (Tsukamoto, 1985; Butcher *et al*., 2003), however, OTC is 10 to 40 times cheaper than alizarin complexone. Otolith marking using OTC incorporated into feed has been shown to be unsuccessful (Pedersen & Carlsen, 1991).

Multiple ALC and OTC marks can be clearly resolved with a 3 day interval between marks [Figs 2(c) and 3(a), (b)]. Van der Walt & Faragher (2003) argue that a longer period between markings would be required to distinguish multiple marks given the intensity of the ALC mark, however, the present data show that marks could be clearly distinguished with only 3 days between immersions. This interval could be reduced to even 1 or 2 days depending on growth rate, although this may result in loss of clarity. Multiple OTC and ALC induced otolith marks have been detected up to 3 years after immersion (Tsukamoto *et al*., 1989; Reinert *et al*., 1998; Jenkins *et al*., 2002).

The appearance of OTC and ALC marks in fin spines after shorter immersion times than otoliths [Fig. 1(a), (b), (c)] makes them an ideal structure to target when marking high densities of fishes, since water quality problems are more likely to arise during extended immersions. Fin spines retain the same form throughout life which make them appropriate for long-term assessments (Brown

et al., 2002), although the high autofluorescence of tissue in fin spines may complicate detection. The positive mark retention results observed in this experiment [Fig. 2(a), (b)], mean that fin spines can be used as a non-lethal method of mark detection for ongoing monitoring of mulloway. Vertebrae have rarely produced fluorescent marks using immersion protocols (Rojas-Beltran *et al.*, 1995). The OTC and ALC may have been incorporated into the vertebrae in this study, but the high level of natural fluorescence of the vertebrae sections prevented their detection. Oxytetracycline marks have been detected in the vertebrae of marine fishes, but only when the chemical was administered through feed (Pedersen & Carlsen, 1991; Nørdeide *et al.*, 1992).

The relationship between available OTC and salinity (Fig. 4) will allow future investigators to trade-off the concentration of OTC used for marking against the minimum salinity the target species can tolerate. In general, the variability of research conclusions when using OTC to mark marine fishes is maybe explained by the fact that little of the OTC added to solution is available to be incorporated into otolith or fin spine of the fish (Fig. 4). Further work using OTC to mark otoliths should address the threshold concentration of available OTC necessary to produce a mark, however, this may differ between species. Using the present model to calculate available OTC, Jenkins *et al.* (2002) was estimated to have produced OTC marks in red drum *Sciaenops occelatus* (L.) with only *c.* 170 mg l^{-1} available OTC for 4 h. In addition, Butcher *et al.* (2003) used a concentration of *c.* 850 mg l^{-1} available OTC for 12 h to produce a mark in dusky flathead *Platycephalus fuscus* Cuvier, while detectable OTC marks in the present study were produced using *c.* 500 mg l^{-1} available OTC for 24 h. Hettler (1984) used a 1% NaCl solution in place of sea water with 500 mg l^{-1} OTC for 3 h in spot croaker *Leiostomus xanthurus* Lacepède and pinfish *Lagodon rhomboides* (L.), however, this resulted in 6·5% mortality. Although the potential use of OTC may be limited for completely marine fish species, for those species that use estuarine habitats and have broader salinity tolerances it is a cheaper alternative to ALC to produce a comparable mark.

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References

Babaluk, J. A. & Craig, J. F. (1990). Tetracycline marking studies with pike, *Esox lucius* L. *Aquaculture and Fisheries Management* **21,** 307–315.

Beckman, D. W. & Schulz, R. G. (1996). A simple method for marking fish otoliths with alizarin compounds. *Transactions of the American Fisheries Society* **125,** 146–149.

Blankenship, H. L. & Leber, K. M. (1995). A responsible approach to marine stock enhancement. *American Fisheries Society Symposium* **15,** 167–175.

Blom, G., Nørdeide, J. T., Svåsand, T. & Borge, A. (1994). Application of two fluorescent chemicals, alizarin complexone and alizarin red S, to mark otoliths of Atlantic cod, *Gadus morhua* L. *Aquaculture and Fisheries Management* **25,** 229–243.

Broadhurst, M. K. & Kennelly, S. J. (1994). Reducing the by-catch of juvenile fish (mulloway *Argyrosomus hololepidotus*) using square-mesh panels in codends in the Hawkesbury River prawn-trawl fishery, Australia. *Fisheries Research* **19,** 321–331.

Brooks, R. C., Heidinger, R. C. & Kohler, C. C. (1994). Mass-marking otoliths of larval and juvenile walleyes by immersion in oxytetracycline, calcein, or calcein blue. *North American Journal of Fisheries Management* **14,** 143–150.

Brown, M. L., Powell, J. L. & Lucchesi, D. O. (2002). In-transit oxytetracycline marking, nonlethal mark detection, and tissue residue depletion in yellow perch. *North American Journal of Fisheries Management* **22,** 236–242.

Buckley, R. M. & Blankenship, H. L. (1990). Internal extrinsic identification systems: Overview of implanted wire tags, otolith marks and parasites. *American Fisheries Society Symposium* **7,** 183–202.

Bumguardner, B. W. (1991). Marking subadult red drums with oxytetracycline. *Transactions of the American Fisheries Society* **120,** 537–540.

Butcher, A., Mayer, D., Willet, D., Johnston, M. & Smallwood, D. (2003). Scale pattern analysis is preferable to OTC marking of otoliths for differentiating between stocked and wild dusky flathead, *Platycephalus fuscus*, and sand whiting, *Sillago ciliata*. *Fisheries Management and Ecology* **10,** 163–172.

Cermeno, P., Uriarte, A., de, M. A. M. & Morales-Nin, B. (2003). Validation of daily increment formation in otoliths of juvenile and adult European anchovy. *Journal of Fish Biology* **62,** 679–691. doi: 10.1046/j. 0022-1112.2003.00056.x

Fielder, D. S. & Bardsley, W. J. (1999). A preliminary study on the effects of salinity on growth and survival of mulloway *Argyrosomus japonicus* larvae and juveniles. *Journal of the World Aquaculture Society* **30,** 380–387.

Frenkel, V., Kindschi, G. & Zohar, Y. (2002). Noninvasive, mass marking of fish by immersion in calcein: evaluation of fish size and ultrasound exposure on mark endurance. *Aquaculture* **214,** 169–183.

Gray, C. A. & McDonall, V. C. (1993). Distribution and growth of juvenile mulloway, *Argyrosomus hololepidotus* (Pisces, Sciaenidae), in the Hawkesbury River, South-Eastern Australia. *Australian Journal of Marine and Freshwater Research* **44,** 401–409.

Henry, G. W. & Lyle, J. M. (2003). *The National Recreational and Indigenous Fishing Survey*. Canberra: Australian Government Department of Agriculture, Fisheries and Forestry.

Hettler, W. F. (1984). Marking otoliths by immersion of marine fish in tetracycline. *Transactions of the American Fisheries Society* **113,** 370–373.

Hining, K. J., West, J. L., Kulp, M. A. & Neubauer, A. D. (2000). Validation of scales and otoliths for estimating age of rainbow trout from southern Appalachian streams. *North American Journal of Fisheries Management* **20,** 978–985.

Iglesias, J. & Rodriguez-Ojea, G. (1997). The use of alizarin complexone for immersion marking of the otoliths of embryos and larvae of the turbot, *Scophthalmus maximus* (L.): Dosage and treatment time. *Fisheries Management and Ecology* **4,** 405–417.

Isermann, D. A., Bettoli, P. W. & Sammons, S. M. (1999). Efficiency of identifying stocked crappies in a Tennessee reservoir through oxytetracycline marking. *North American Journal of Fisheries Management* **19,** 1122–1123.

Jenkins, W. E., Denson, M. R., Bridgham, C. B., Collins, M. R. & Smith, T. I. J. (2002). Retention of oxytetracycline-induced marks on sagittae of red drum. *North American Journal of Fisheries Management* **22,** 590–594.

Kailola, P. J., Williams, M. J., Stewart, P. C., Reichelt, R. E., McNee, A. & Grieve, C. (1992). *Australian Fisheries Resources*. Canberra: Fisheries Research and Development Corporation.

Lagardère, F. K., Thibaudeau, K. & Bégout Anras, M. L. (2000). Feasibility of otolith marking in large juvenile turbot, *Scophthalmus maximus*, using alizarin red S solutions. *ICES Journal of Marine Science* **57,** 1175–1181.

Lunestad, B. T. & Goksøyr, J. (1990). Reduction in the antibacterial effect of oxytetracycline in sea water by complex formation with magnesium and calcium. *Diseases of Aquatic Organisms* **9,** 67–72.

McFarlane, G. A. & Beamish, R. J. (1987). Selection of dosages of oxytetracycline for age validation studies. *Canadian Journal of Fisheries and Aquatic Sciences* **44,** 905–909.

Murphy, M. D. & Taylor, R. G. (1991). Direct validation of ages determined for adult red drums from otolith sections. *Transactions of the American Fisheries Society* **120,** 267–269.

Nørdeide, J. T., Holm, J. C., Otterå, H., Blom, G. & Borge, A. (1992). The use of oxytetracycline as a marker for juvenile cod (*Gadus morhua* L.). *Journal of Fish Biology* **41,** 21–30.

Paragamian, V. L., Bowles, E. C. & Hoelscher, B. (1992). Use of daily growth increments on otoliths to assess stockings of hatchery-reared kokanees. *Transactions of the American Fisheries Society* **121,** 785–791.

Pedersen, T. & Carlsen, B. (1991). Marking cod (*Gadus morhua* L.) juveniles with oxytetracycline incorporated into the feed. *Fisheries Research* **12,** 57–64.

Potts, W. T. W. & Parry, G. (1964). *Osmotic and Ionic Regulation in Animals*. Oxford: Pergamon Press.

Reichert, M. J. M., Dean, J. M., Feller, R. J. & Grego, J. M. (2000). Somatic growth and otolith growth in juveniles of a small subtropical flatfish, the fringed flounder, *Etropus crossotus*. *Journal of Experimental Marine Biology and Ecology* **254,** 169–188.

Reinert, T. R., Wallin, J., Griffin, M. C., Conroy, M. J. & Van, D. A. M. J. (1998). Long-term retention and detection of oxytetracycline marks applied to hatchery-reared larval striped bass, *Morone saxatilis*. *Canadian Journal of Fisheries and Aquatic Sciences* **55,** 539–543.

Rogers, J. S., Hare, J. A. & Lindquist, D. G. (2001). Otolith record of age, growth, and ontogeny in larval and pelagic juvenile *Stephanolepis hispidus* (Pisces: Monacanthidae). *Marine Biology (Berlin)* **138,** 945–953.

Rojas-Beltran, R., Gillet, C. & Champigneulle, A. (1995). Immersion mass-marking of otoliths and bone tissue of embryos, yolk-sac fry and fingerlings of Arctic charr *Salvelinus alpinus* (L). *Nordic Journal of Freshwater Research* **71,** 411–418.

Ross, J. L., Stevens, T. M. & Vaughan, D. S. (1995). Age, growth, mortality and reproductive biology of red drums in North Carolina Waters. *Transactions of the American Fisheries Society* **124,** 37–54.

Sanchez-Lamadrid, A. (2001). The use of alizarin complexone for immersion marking of otoliths of larvae of gilthead sea bream, *Sparus aurata* L. *Fisheries Management and Ecology* **8,** 279–281.

Svåsand, T., Jørstad, K. E. & Kristiansen, T. S. (1990). Enhancement studies of coastal cod in western Norway. Part I. Recruitment of wild and reared cod to a local spawing stock. *Journal du Conseil International Pour L'Exploration de la Mer* **47,** 5–12.

Tsukamoto, K. (1985). Mass-marking of ayu eggs and larvae by tetracycline-tagging of otoliths. *Nippon Suisan Gakkaishi* **51,** 903–911.

Tsukamoto, K. (1988). Otolith tagging of ayu embryo with fluorescent substances. *Nippon Suisan Gakkaishi* **54,** 1289–1295.

Tsukamoto, K., Kuwada, H., Hirokawa, J., Oya, M., Sekiya, S., Fujimoto, H. & Imaizumi, K. (1989). Size-dependant mortality of red sea bream, *Pagrus major*, juveniles released with fluorescent otolith-tags in News Bay, Japan. *Journal of Fish Biology* **35** (Suppl. A), 59–69.

Utter, F. (1998). Genetic problems of hatchery-reared progeny released into the wild, and how to deal with them. *Bulletin of Marine Science* **62,** 623–640.

van der Walt, B. & Faragher, R. A. (2003). Otolith marking of rainbow trout fry by immersion in low concentrations of alizarin complexone. *North American Journal of Fisheries Management* **23,** 141–148.

Yamashita, Y., Nagahora, S., Yamada, H. & Kitagawa, D. (1994). Effects of release size on survival and growth of Japanese flounder *Paralichthys olivaceus* in coastal waters off Iwate Prefecture, Northeastern Japan. *Marine Ecology Progress Series* **105,** 269–276.