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Detecting and tracing farmed salmon with natural geo-element otolith 'fingerprint' tags: **developing and validating tag delivery techniques**

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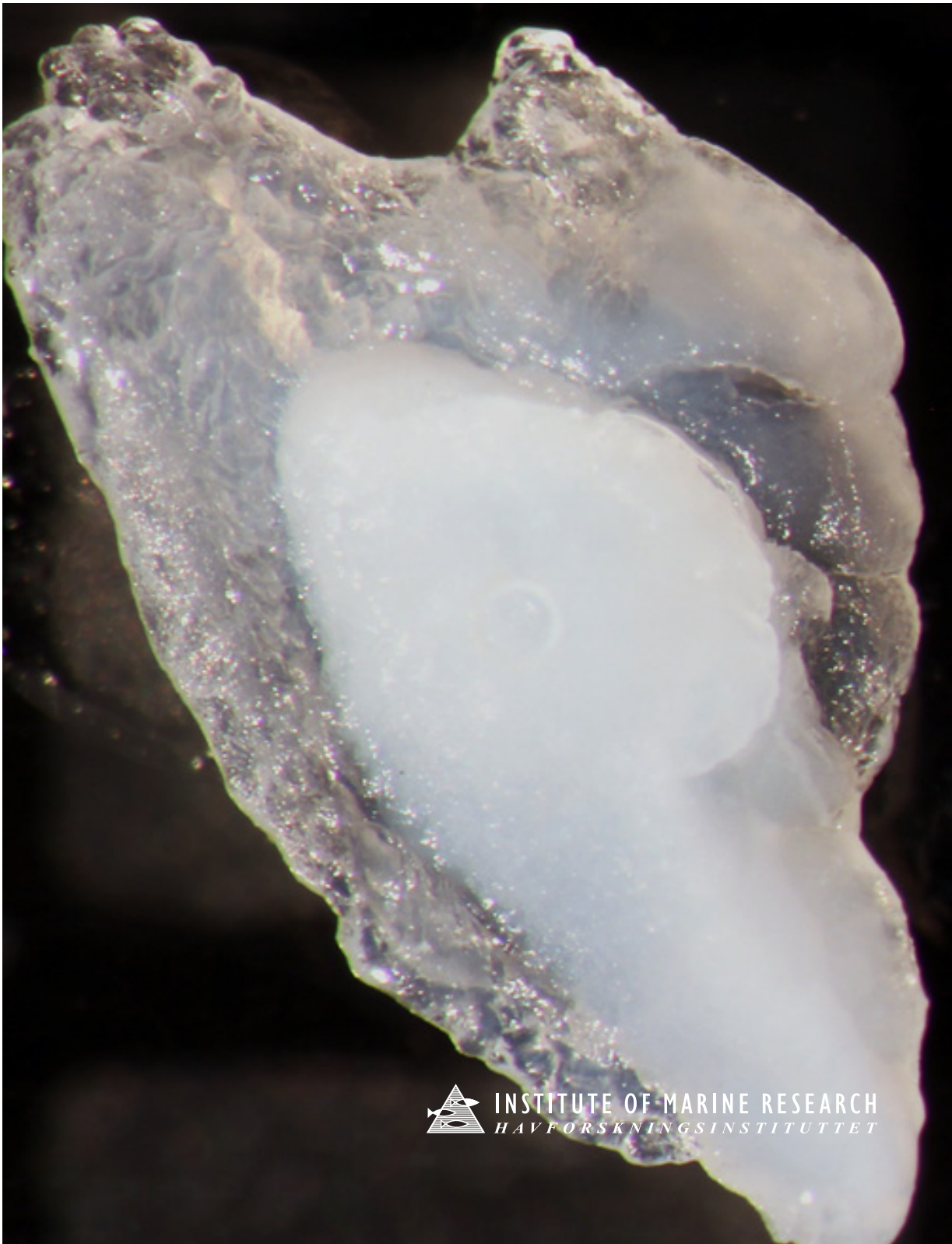

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FINAL REPORT

Detecting and tracing farmed salmon with natural geo-element otolith 'fingerprint' tags: developing and validating tag delivery techniques

Project # 900710

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1.1 Executive summary

Farmed fish escape and enter the environment with possible effects on wild populations. Attempts to reduce the incidence of escape could be assisted if individuals can be traced back to the point of escape, so that escape causes can be identified and technical standards improved.

We tested if permanent marks could be created on the otoliths of salmon that could be detected throughout the lives of the fish. Marks were created by altering the natural ratios of the natural elements barium (Ba) and strontium (Sr) in the otolith. By combining several natural geo-elements, unique fingerprints can be created, which can act as codes that enable tracing of fish back to the point of escape. The marks are detected by laser ablation inductively-coupled plasma mass spectrometry (LA-ICP-MS).

We delivered marks to salmon in 4 different ways: 1) injecting female broodstock with geo-element markers prior to spawning so that the marks can be passed from the mother to all eggs in a clutch, with all larvae receiving a mark in the core of their otolith; 2) incorporating small amounts of geo-element markers into the vaccination serum for co-delivery of the marks into the fish along with the vaccine; 3) bathing eggs of salmon in geo-element solution immediately after fertilisation; and 4) bathing late stage yolk-sac larvae in an geo-element solution.

All methods were successful in creating unique, permanent marks on the otoliths of salmon that could be detected throughout the lifetime of the fish. For all four delivery techniques, marking was 100% successful with Ba at concentrations as low as 0.001 µg. g^{-1} fish and for Sr at 1 µg. g^{-1} fish. Detection limits with the laser were set at 99.94%, so the techniques delivered a very high degree of accuracy. In all methods, we developed procedures that minimized marking and analysis costs, so that methods could be implemented at full-scale commercial application.

After marking, fish were ongrown under standard commercial conditions to harvest size (approx. 4 kg). Marking methods had no effect on all standard production parameters, such as survival, growth, and the incidence of larval deformities compared to unmarked control fish. Further, the amounts of natural

strontium and barium used to create the marks are so small that they pose no problem for food safety, as they represent less than 0.1–1% of the amounts that naturally occur in salmon tissues.

Our results indicate single marking with one of the techniques can create up to 63 unique fingerprint marks at low cost using Ba (0.0002–0.02 $US per mark) and Sr (0.46–0.82 $US per mark). Double marking in different parts of the otolith using two of the different code delivery methods was also successful (e.g. maternal transfer and vaccination combined). This means 63 x 15 unique codes, or 1023 codes are possible, which is sufficient to provide a unique code for every site in the sea in Norway.

We conclude that fingerprinting with natural geo-elements is feasible for commercial application. 94% of the salmon in Norway could be marked for as little as 0.02 $US per fish with a single marking method with traceability back to the top 63 producing companies. Double marking would enable more detailed tracing of every fish back to its site in the sea.

1.2 Norsk sammendrag

Oppdrettslaks rømmer og kan påvirke de ville populasjonene. Antallet rømminger kunne vært redusert hvis den rømte fisken kunne vært sporet tilbake til rømningsstedet slik at årsaken til rømning kunne blitt identifisert.

Vi testet om det er mulig å lage kjemiske merker på laksens ørestein som kan identifiseres gjennom hele livssyklusen. Disse merkene ble laget ved å endre forholdet mellom naturlige geoelementer (stabile isotoper av barium (Ba) og strontium (Sr)) i øresteinen. Ved å kombinere flere av disse naturlige geoelementene kan en lage unike 'fingeravtrykk/strekkoder' som kan brukes for å spore en fisk tilbake til rømningsstedet. Merkene leses ved en teknikk hvor en brenner hull i øresteinen med laser og måler sammensetningen ved hjelp av massespektrometri.

Merkene i øresteinen ble etablert ved hjelp av fire metoder: 1) ved å injisere morfisken med geoelementer i forkant av gytingen slik at disse kan overføres til alle eggene og at merket blir avsatt i øresteinens kjerne; 2) ved å tilsette små mengder av geoelementene i fiskens vaksine slik at fisken merkes ved vaksineringen; 3) ved å tilsette geoelementer i svellevannet som tilsettes rett etter befruktningen av eggene; 4) ved å bade plommesekkyngelen i en løsning med geoelementer.

Ved alle metodene klarte vi å lage unike, permanente merker i øresteinen som kunne finnes og kjennes igjen gjennom hele fiskens livssyklus. Alle metodene ga 100 % sikker merking med Ba-konsentrasjoner så lave som 0,001 µg. g^{-1} fisk og for Sr-konsentrasjoner ved 1 µg. g^{-1} fisk. Laserens deteksjonsgrense ble satt til 99,94 %, så metodene er meget nøyaktige og følsomme. Ved alle metodene ble det etablert prosedyrer som minimerer merke- og analysekostnadene slik at metodene kan overføres til fullskala oppdrett.

Etter merking ble fisken oppdrettet under standard oppdrettsbetingelser til slaktestørrelse (ca. 4 kg). Merkemetodene påvirket ingen produksjonsparametre som overlevelse, vekst eller innslag av deformiteter. Mengden naturlig strontium og barium som ble brukt er mindre enn 1 % av den mengden som finnes naturlig i laks og påvirker ikke matvaresikkerheten.

Våre resultater viser at en ved enkel merking med en av teknikkene kan lage opptil 63 unike 'fingeravtrykk' til en lav kostnad ved bruk av Ba (0,0002–0,02 $US per merke) og Sr (0,46–0,82 $US per

merke). Dobbelmerking; dvs. å merke i ulike deler av øresteinen ved hjelp av to av metodene, var også mulig (for eksempel injeksjon av morfisk og vaksinering på parrstadiet). Dette betyr at en kan lage 63 x 15 = 1023 unike koder, nok til å gi hver lokalitet i Norge en unik kode.

Vi konkluderer med at merking med naturlige geoelementer er en mulig løsning for å merke oppdrettsfisk. 94 % av laksen i Norge kan merkes med en merkemetode for 0,02 $US per fisk og med mulighet til å spore den tilbake til de 63 største produsentene. Dobbelmerking gir mulighet for sporing tilbake til lokalitet.

2 Background

The application of 'natural tags' or 'direct markers' to salmon to identify whether they are farmed or wild with certainty upon recapture months or years after an escape is complicated. All existing natural tag or direct marking techniques fail one or both of FHF's criteria of 100% accurate differentiation of farmed and wild salmon and traceability back to the owner/location. Many techniques have side effects which makes them impractical for mass marking production as growth and welfare outcomes are paramount (Table 1).

Table 1. Summary of existing marking/tracing techniques to differentiate farmed and wild salmonids, their ability to separate farmed from wild fish and their ability to enable traceability of fish back to the owner/ location

Marking/ tracing method	Marking or detectability issues	Farmed-wild separation	Traceability to owner/ location	Source
T-bar tags	Tag loss; may affect growth, health and survival; risk of infections	100% separation for tagged individuals, but no separation if tag loss	possible	Roberts *et al.* 1973 a,b,c, Serafy *et al.* 1995
Coded-wire tags	10% tag loss in fish 5 mo to 3 yr after release; detectability issues	100% separation for tagged individuals, but no separation if tag loss	possible	Munro *et al.* (2003)
Adipose fin removal	Welfare issues; reduced swimming performance of salmonids in turbulent flow	possible	impossible	Reimchen & Temple (2004), Buckland-Nicks *et al.* (2011)
Fluorescent markers	Handling stress; poor mark retention; uncertainty in mark detectability	possible, but not 100% accurate	impossible	Thorrold *et al.* (2006), Munro *et al.* (2009), Kuroki *et al.* (2010)
Genetic techniques	Expense; genetic variability in wild salmon populations	accurate, but not 100% in all cases	possible, but not in all cases	Glover *et al.* (2010)

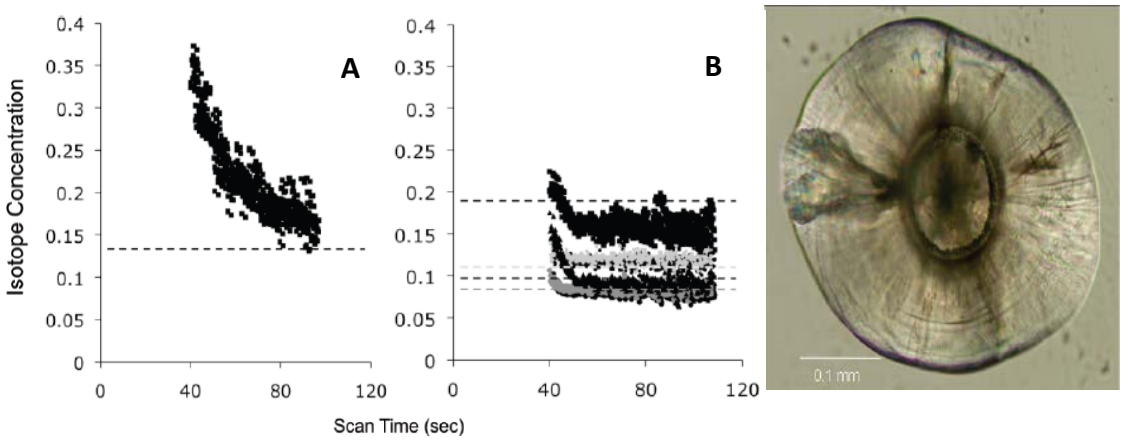
Indirect natural markers, such as the trace elements incorporated into otoliths, provide some capacity to separate farmed and wild salmon. However, natural variability in these elements in wild populations means this technique cannot offer 100% certainty in discriminating between wild and farmed salmon. Similarly, direct chemical marking by osmotic induction, injection or immersion of fish eggs, embryos, juveniles and adults in fluorescent compounds (Calcein, Alizarin Red S and Oxytetracycline), and in enriched strontium and barium geo-elements, has been used to successfully mass mark calcified structures of marine and freshwater organisms (Tsukamoto, 1988; Jones et al., 1999, 2005; Almany et al., 2007; Buckley et al., 2007; Munro et al., 2009; Crook et al., 2009., Smith & Swearer 2011). Applying these techniques to marking large numbers of fish remains difficult, due to cost, stress due to physical handling, mark retention and autofluorescence (Thorrold et al., 2006; Munro et al., 2009; Kuroki et al., 2010).

Trans-generational marking is a new method for mass marking larvae *in situ* (Thorrold et al. 2006). It involves the injection of an enriched, natural geo-element solution into gravid females, to induce unique marks by maternal transfer of the elements into the otoliths of the developing offspring. The trans-generational process can mark thousands of eggs or larvae with a single injection into the maternal parent, offering a quicker and more efficient alternative to manual marking or fluorescent chemical marking methods. While not yet tested for salmonids, trans-generational marking has been tested and validated by injecting barium and/or strontiumgeo-elements into both freshwater and marine fish species (Thorrold et al., 2006; Almany et al. 2007; Buckley et al., 2007; Munro et al., 2009; Williamson et al., 2009a; Kuroki et al., 2010; Smith & Swearer 2011 – see Fig. 1).

A technique that can be efficiently and economically administered to the hundreds of millions of salmon produced each year is essential for discrimination of wild and farmed fish when they mix in rivers. Incorporating natural geo-elements into the otoliths of fish represents a technique that can provide a permanent mark in all production fish which can be detected with 100% accuracy upon recapture. At present, all other techniques cannot guarantee 100% of fish are marked and/or 100% of fish are detected at whatever life history stage or size they are recaptured (Table 1). By combining several enriched natural geo-elements, unique fingerprints can be created (see Fig. 1b), which may enable tracing of fish back to the owner.

Natural geo-elements of barium and strontium occur naturally in aquatic ecosystems and are detectable in the otoliths of wild fish. Bariumgeo-elements 130, 132, 134, 135, 136, and 137 are rare (0.1–11 % prevalence) compared to barium 138, which is the dominantgeo-element at 72% prevalence. Similarly, strontiumgeo-elements 84, 86 and 87 are rare (0.6–10 % prevalence), while strontium 88 (83% prevalence) is common. By introducing small levels of these raregeo-elements into the fish, they are transferred to the otolith, where they are laid down as a permanent marker. This method can create unique elemental codes or fingerprints which can be detected in the otolith no matter what size or life history stage the fish is recaptured. With the use of barium and strontiumgeo-elements alone, the potential exists to generate several hundred unique otolith fingerprints. Natural geo-elements are known to be a safe marking technique; research has shown that they can be used at low dosages to mark commercially important marine fishes without adverse effects on the health of the fishes or on humans who may consume them (Williamson et al. 2009b).

Fig. 1 Natural geo-element 'fingerprint' markers visible in the otolith core of mosquito fish larvae. The core of the otolith analysed by laser ablation inductively coupled plasma mass spectrometry is visible as a dark circle in the otolith (far right). A: a single mark of ^{137}Ba. B: a fingerprint with 4 natural geo-elements: ^{137}Ba, ^{135}Ba, ^{86}Sr and ^{87}Sr. The left hand side of each profile corresponds to the edge of the otolith analysed (far right). The dashed lines are 3 standard deviations above the meangeo-element ratio recorded in controls (untagged fish). This represents a detection accuracy of 99.73%. By



increasing this to 3.3 standard deviations above the mean, a likelihood of accurate detection of 99.9% can be achieved.

While this method shows promise for application to the accurate discrimination of farmed and wild fish, development, testing and validation of techniques is required to effectively deliver the natural geo-element marker to all salmonids produced in industrial hatcheries.

3 Project objectives

1. Develop natural geo-element otolith 'fingerprint' tags to enable 100% accurate differentiation of farmed salmon and tracing back to the owner.
2. Develop, test and validate inter-generational delivery of natural geo-element tags for farmed salmon.
3. Develop, test and validate vaccine-based delivery of natural geo-element tags for farmed salmon.
4. Develop, test and validate egg immersion for delivery of natural geo-element tags for farmed salmon.
5. Develop, test and validate larval immersion for delivery of natural geo-element tags for farmed salmon.

4 Project delivery

We developed, tested and validated four separate, novel methods to deliver stable otolith markers to hatchery salmon. These novel methods include: 1) injecting broodstock with natural geo-element markers prior to spawning so that the markers can be passed from the mother to all eggs in a clutch, with all larvae receiving a natural geo-element tag in the core of their otolith; 2) incorporating small amounts of natural geo-element markers into the vaccination serum for co-delivery of the tag into the fish along with the vaccine; 3) bathing eggs of salmon in angeo-element solution immediately after fertilisation; and 4) bathing eggs in angeo-element solution during the larval stage. In all methods, we will seek to develop procedures that minimize marking and analysis costs.

Our goal was to deliver the natural geo-element fingerprint tag with no extra handling during the production process. In all methods, we tested dosage rates to ensure 100% of tagged fish were marked. Further, marked fish were tested throughout the production phase to ensure that tags can be detected at all production stages and fish sizes. The fish were monitored for commonly used production markers such as growth performance, mortality, maturity level and occurrence of skeletal deformities throughout the production cycle.

The experiments were carried out at IMR Matre, with all analyses of otoliths performed with laser ablation inductively-coupled plasma mass spectrometry (LA-ICP-MS) to detect the elemental fingerprints at the University of Melbourne.

5 Project results

WP 1 Mass marking farmed Atlantic salmon with transgenerational geo-elemental fingerprints

ABSTRACT: Farmed fish sometimes escape and enter natural environments, where they mix with wild fish populations and can have negative effects. Marking farmed fish is a prerequisite for the identification of the cause of escape, in order to improve farming practices. Here, we test transgenerational marking with enriched natural geo-elements to assess its effectiveness as an accurate, feasible, and cost effective marking method for Atlantic salmon grown in sea-cage aquaculture. We injected a combination of seven natural geo-elements, ^{134}Ba, ^{135}Ba, ^{136}Ba, ^{137}Ba, ^{86}Sr, ^{87}Sr and ^{26}Mg over a range of concentrations (2, 0.2, 0.02, and 0.002 µg. g^{-1} broodfish) into the abdominal cavity of mature female Atlantic salmon broodstock. Mark success was assessed in the otoliths of the resulting yolk sac larvae using Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS). Marking was 100% successful with Bageo-elements at concentrations as low as 0.002 µg and for Srgeo-elements at 2 µg, when there was at least 3 weeks between the day of injection and spawning. Our results demonstrate that 63 unique fingerprint marks can be made at low cost using enrichedgeo-elements of Ba ($0.0002–0.002 USD per mark) and Sr ($0.05–0.13 USD per mark). Compared to other mass marking techniques, transgenerational marking of farmed Atlantic salmon is an economically feasible method for tracing escapees with similarly low costs to delivery by egg bathing or vaccines, and an order of magnitude or more lower than other conventional marking methods.

INTRODUCTION

The rise of modern industrial aquaculture has introduced millions of selectively bred fish into environments where they are co-located with wild conspecifics. When they escape from aquaculture facilities, farmed fish can cause damaging ecological impacts when mixing with wild fish (Fleming et al. 2000, McGinnity et al. 2003, Hindar et al. 2006, Hutchings & Fraser 2008, Tuledo-Guedes et al. 2011, Glover et al. 2013b). Efforts to reduce escape events first requires detection of where the escape event occurred, so that subsequent engineering investigations can determine the cause of the escape event and make recommendations to improve the technical standards of containment systems (Jensen et al. 2010).

Atlantic salmon (*Salmo salar*) escape from sea-cage aquaculture farms in every country in which they are produced. Detecting escaped farmed salmon once they become mixed within wild populations and tracing escapees back to their farm of origin remains problematic. Although the point of escape is often possible to be determine through DNA-based methods (Glover et al. 2010) a fail-safe identification technique is still lacking. As an alternative to DNA-based approaches, a permanent tag or coded mark applied to all farmed fish would enable effective tracing. However, current mass marking methods, for example, fluorescent markers (Mohler 2003, Taylor et al. 2005), fin clipping and physical tags (Vander Haegen et al. 2005) or visible implant tags (FitzGerald et al. 2004), are unsuccessful with one or more aspects related to the ability to deliver 100% traceability to point of origin, fish welfare considerations or cost-effectiveness at industry scale.

Recently, new methods have been developed that enable 100% traceability of farmed salmon, are cost effective, and have no impact on fish welfare right throughout the production cycle. These methods involve the use of natural geo-elements to code the otoliths of fish with uniquegeo-element fingerprint marks during the hatchery stages of production (e.g. de-Braux et al. 2014, Warren-Myers et al. 2014, 2015a,b). To date, otolith marking with enriched natural geo-elements of Ba and Sr has been highly successful in many species and marks have been created using a range of delivery techniques, for example, via injection (Thorrold et al. 2006, Williamson et al. 2009b, Warren-Myers et al. 2014, 2015a), immersion (Munro et al. 2008, Woodcock et al. 2011b; de Braux et al. 2014; Warren-Myers et al. 2015b) or food supplementation (Woodcock et al. 2013).

The potential for identifying the origin of escaped farmed salmon with natural geo-element marking is clear; between 7 and 63 mark combinations were created when Atlantic salmon parr were successfully marked with a combination of 6 geo-elements mixed with a vaccine and delivered via injection (Warren-Myers et al. 2015a), and salmon embryos were marked with a combination of 3geo-elements during their egg swelling phase immediately after fertilisation (Warren-Myers et al. 2015b). Another method with the potential to create additional multiple mark combinations with enriched natural geo-elements in farmed salmon is transgenerational marking (Thorrold et al. 2006, Almany et al. 2007). This technique which can successfully mark both freshwater (Munro et al. 2009, Starrs et al. 2014b) and marine fish species (Thorrold et al. 2006, Williamson et al. 2009b) requires an injection of enriched natural geo-element into the abdominal cavity of mature females prior to spawning, which is then passed on *in situ* to the offspring. Marks are detectable in the core of otoliths of the resulting larvae (Thorrold et al. 2006). Many studies claim transgenerational marking to be a successful technique for field applications to assess population connectivity (Thorrold et al. 2006, Williams et al. 2009b, Huelga-Suarez et al. 2012), yet only one study (Almany et al. 2007) has demonstrated that transgenerational making is feasible for mass marking 10s to 100s of females. In fish farming, transgenerational marking would allow all eggs of a single broodfish to be marked with a single injection several weeks prior to stripping and fertilization. For hatcheries, this means that no extra labour or protocol steps would be required to mark fish from the day of stripping onwards. Marking prior to stripping may also be an advantage over marking during the egg swelling (Warren-Myers et al. 2015b), larval (de Braux et al. 2014), or parr stage (Warren-Myers et al. 2015a) as it would ensure all fish are marked prior to any movement of eggs or fish within or between hatcheries.

Past studies on transgenerational marking have shown that timing between spawning and injection, and the concentration required for 100% mark success varies greatly among species. For example, concentrations of 0.5 to 23 µg. g^{-1} female have been successful in saltwater species (Thorrold et al. 2006, Williamson et al. 2009b), and 0.3 to 40 µg. g^{-1} female in freshwater species (Munro et al. 2009, Huelga-Suarez et al. 2013) with spawning occurring anywhere between 1 and 170 days post injection in freshwater species (Munro et al. 2009, Starrs et al. 2014b) and 2 to 108 days in saltwater species (Cuif et al. 2014). Hence, time between spawning and injection and the concentration required to achieve 100% mark success in farmed salmon requires optimising, to assess whether the technique will be suitable for large scale application in aquaculture.

Here, we investigate whether transgenerational marking with enriched natural geo-elements is a viable option for mass marking farmed Atlantic salmon by testing transgenerational marking on Atlantic salmon broodstock females using seven enriched natural geo-elements ^{134}Ba, ^{135}Ba, ^{136}Ba,^{137}Ba, ^{86}Sr, ^{87}Sr and ^{26}Mg at four concentrations (2, 0.2, 0.02, 0.002 µg. g brood $fish^{-1}$). We assess mark success, mark strength and mark intensity in the otoliths of the resulting offspring. In addition, growth and mortality of offspring were monitored from hatching though to harvest size to check for any potential long term

effects of transgenerational marking with enriched natural geo-elements. Finally, we make cost estimates for the amount of geo-element required to produce all successful fingerprint combinations.

MATERIALS AND METHODS

Experimental design

The experiment was conducted at the Institute of Marine Research field station, at Matre, in Masfjorden, western Norway (60°N) using Atlantic salmon broodfish (AquaGen strain) that had been transferred from sea-cages to onshore freshwater tanks buffered with saltwater to a salinity of 0.7 g $NaCl.L^{-1}$ two months prior to the experiment. We tested transgenerational making by injecting mature Atlantic salmon females (Mass: 9.15 ± 0.26 kg [mean ± SE]) in the intraperitoneal cavity using a hypodermic syringe with a standard volume of 60 ml prior to spawning. Each injection contained a combination of the enriched natural geo-elements, $^{134}BaCl$, $^{135}BaCl$, $^{136}BaCl$, $^{137}BaCl$, $^{86}SrCl$, $^{87}SrCl$ and $^{26}MgCl$ (Oak Ridge National Laboratory; www.ornl.gov) at either one of 4 different enriched geo-element concentrations or a 5% NaCl (control) solution (Table 1). Females were checked once a week post injection for ripeness and any females ready to spawn had their eggs stripped and a subsample of eggs fertilised with 2 ml of sperm from 2 males (1 ml each).

Fertilised egg batches were kept at a constant temperature of 6 °C throughout the egg incubation period (81 days) and yolk sac larval stage (52 days). Immediately prior to first feeding (Day 133), a subsample of 10 yolk sac larvae from each female's egg batch was collected and euthanized by anaesthetic overdose for otolith analysis. Sagittal otoliths from the subsampled larvae were dissected and removed, cleaned of any adhering tissue, air dried, and stored individually in plastic tubes for otolith analysis. All remaining larvae from each egg batch were transferred to separate first feeding tanks, with a subsample of 50 fish from each batch randomly selected at the pre-smolt stage to be grown on to 4 kg harvest size.

Otolith preparation

Sagittal otoliths were cleaned as per Warren-Myers et al. (2014). Briefly, any remaining organic tissue was removed by immersing otoliths in a solution of ultrapure 15% H_2O_2 buffered with 0.1 M NaOH. Following immersion, otoliths were ultra-sonicated (Sonic Clean 250HT) for 5 minutes and then left for 6 hours in the cleaning solution. The cleaning solution was then aspirated off and the otoliths were transferred through three Milli-Q water rinses, each of which consisted of 5 minutes of ultra-sonification and 30 minutes resting time. Otoliths were then air dried in a laminar flow bench for at least 24 hours. Once dry, one otolith per fish was fixed onto gridded microscope slides using quick dry cyanoacrylate glue.

Otolith analysis

Natural geo-element analyses were done on a Varian 7700x Inductively Coupled Plasma Mass Spectrometer (ICP-MS) fitted with a HelEx (Laurin Technic and the Australian National University) laser ablation (LA) system constructed around a Compex 110 (Lambda Physik) excimer laser operating at 193 nm. 612 and 610 NIST (National Institute of Standards and Technology) glass standards doped with trace elements at known concentrations were used to calibrate the system. Otoliths were run in blocks of 16 samples selected randomly from all treatments and bracketed by analyses of the standards. Samples and standards were analysed in time-resolved mode, using a spot size of 157 µm, a laser energy setting of ~ 60 mJ and a laser repetition rate of 10 Hz. Spot ablation was performed under pure He (200 ml/min) to minimise re-deposition of ablated material and the sample was then entrained into the Ar (0.95 ml/min) carrier gas flow to the ICP-MS. Using this method, we were able to quantify the geo-element

ratios for ^{134}Ba:^{138}Ba, ^{135}Ba:^{138}Ba, ^{136}Ba:^{138}Ba, ^{137}Ba:^{138}Ba, ^{86}Sr:^{88}Sr, ^{87}Sr:^{88}Sr, ^{24}Mg:^{26}Mg and ^{55}Mn:^{43}Ca, from the edge to the core of salmon yolk sac larval otoliths (^{55}Mn:^{43}Ca was used to identify when the laser had hit the core; Barbee & Swearer 2007). Data were processed off-line using a specialised MS Excel template which involved a low pass filter to remove any spikes (a single acquisition value >2x the median of the adjacent acquisitions), smoothing (a running average of 3 acquisitions) and blank subtracting functions. A correction factor ($K = R_{true}/R_{obs}$, where R_{true} is the naturally occurring geo-element ratio and R_{obs} is the average geo-element ratio measured in the NIST 612 or 610 standard run before and after each set of 16 samples) was applied to all sample acquisitions to correct for mass bias.

Statistical Analysis

Mark success for each treatment was evaluated using a mark detection limit (Warren-Myers et al. 2014). Briefly, detection limits for the geo-element ratios ^{134}Ba:^{138}Ba, ^{135}Ba:^{138}Ba, ^{136}Ba:^{138}Ba, ^{137}Ba:^{138}Ba, ^{86}Sr:^{88}Sr, ^{87}Sr:^{88}Sr and ^{26}Mg:^{24}Mg were calculated from the average geo-element ratios of all control treatment fish (i.e. 0 µg. L^{-1} treatment). To ensure a correct classification probability of 99.94%, mark detection limits were set at 3.3 standard deviations (SDs) above the mean observed ratio in control fish for each enriched geo-element used. Because of the inherent instability in geo-elemental ratios measured on single-detector, ICP-based mass spectrometers, we conservatively set the criteria for detecting a successful mark in the otolith as at least 3 consecutive acquisitions with ratios above the detection limit.

Mark strength and mark intensity for each enriched geo-element used was analysed using 2 factor ANOVAs with geo-element concentration and number of weeks between injection and spawning treated as fixed factors. An interaction term was not included as two combinations of concentration by weeks post injection (Week 1, 0.002 µg and Week 2, 2 µg) had no females spawn and hence no data. The response variables used was the mean maximum geo-element ratio value (mark strength) and the mean proportion of acquisitions between the otolith edge and otolith core with ratio values above the detection limit (mark intensity) measured from the otoliths of the 10 subsampled fish for each egg batch. The effect of treatment on total hatchery mortality per egg batch and the number of larval deformities observed at first feeding per egg batch were analysed with one-way ANOVAs. The effect of treatment on length, weight, Fulton's condition factor (*k*) (Ricker, 1975), and survival of harvest size fish was analysed with one-way ANOVAs.

RESULTS

Mark success

Natural geo-element enrichment concentration and the number of weeks between injection and spawning influenced the degree of mark success (Table 2). The highest concentration (2 µg .g^{-1} fish) achieved 100% mark success in the shortest time period for the Ba (1 week: ^{134}Ba, ^{135}Ba ^{136}Ba, ^{137}Ba) and Sr geo-elements (3 weeks ^{86}Sr, ^{87}Sr), but only 30% for ^{26}Mg by week 3. When the concentration was reduced (0.2 and 0.02 µg .g^{-1} fish), 100% mark success for the Ba geo-elements was achieved when spawning occurred at least 2 weeks post injection for ^{135}Ba and ^{137}Ba, or at least 3 weeks post injection for ^{134}Ba and ^{136}Ba. Mark success was poor for ^{86}Sr, ^{87}Sr and ^{26}Mg (0 to 10%) at a concentration of 0.2 µg .g^{-1} fish or less, regardless of the number of weeks between injection and spawning. 75% and 80% mark success was achieved for ^{135}Ba and ^{137}Ba (respectively) when spawning occurred 3 weeks post injection at the lowest concentration (0.002 µg .g^{-1} fish).

Mark strength – maximum acquisition ratios

Mark strength, assessed using the maximum acquisition ratios, showed that the 2 μg $.g^{-1}$ fish concentration produced the highest maximum ratios (Fig. 1, Table 3) and that maximum geo-element ratios increased as the period between injection date and spawning date lengthened (Fig. 2).

Mark strength for ^{134}Ba, ^{135}Ba, ^{136}Ba, and ^{137}Ba (Fig. 1) showed the average maximum ratios were higher in the 2 and 0.2 μg treatments ($F_{4,26}$ = 83, 88, 92, 29, respectively, P < 0.001 for all; Pairwise Comparisons: 2 μg > 0.2 μg > 0.02 μg = 0.002 μg = 0 μg, P < 0.05 for all). Ratios for the Ba geo-elements ranged between 6 and 21 times greater than the threshold limit in the 2 μg treatment and between 2 and 10 times greater than the threshold limit in the 0.2 μg treatment (Table 3).

For ^{135}Ba and ^{137}Ba, the third week had the highest average max ratios, but this was only different to week 2, not week 1 (Fig. 2; $F_{2, 26}$ = 7.2, 6.4, respectively, P < 0.01 for both; Pairwise comparisons: WK3 $\underline{>}$ WK1 = WK2, P < 0.05). For ^{134}Ba and ^{136}Ba, the third week had higher average max ratios compared to week 2 and week 1 (Fig. 2; $F_{2, 26}$ = 7.8, 7.9 respectively, P < 0.01 for both; Pairwise Comparisons: WK3 >WK2 = WK1, P < 0.05).

Average max ratios for mark strength for ^{86}Sr and ^{87}Sr were higher in the 2 μg treatment (Fig. 1; $F_{4, 26}$ = 29, 24 respectively, P < 0.001 for both; Pairwise Comparisons: 2 μg > 0.2 μg = 0.02 μg = 0.002 μg > 0 μg, P < 0.05 for both) and max ratios were 1.1 times greater than the threshold limit (Table 3). The third week had higher average max ratios compared to week 2 and week 1 (Fig. 2; $F_{2, 26}$ = 9.0 and 6.4, P = 0.003 and 0.01, respectively; Pairwise Comparisons: WK3 >WK2 = WK1, P < 0.05 for both).

Mark strength for ^{26}Mg showed no effect of concentration or week (Fig. 1 & Fig. 2; $F_{4, 26}$ = 1.8, P = 0.2 and $F_{2, 26}$ = 0.6, P = 0.6, respectively).

Mark intensity – % of acquisition counts above detection limit

Mark intensity, assessed by the proportion of an otolith marked with acquisition counts above the detection limit, showed that the higher concentrations marked a greater proportion of the otolith (Fig. 3, Table 3). In addition, the proportion of otolith marked increased as the period between injection and spawning lengthened (Fig. 4).

Acquisition counts for the Ba geo-elements indicated that the 2 μg, 0.2 μg and 0.02 μg treatments had a greater proportion of otolith marked with enriched Ba compared to the 0.002 μg treatment (Fig. 3; $F_{3, 21}$ = 21, 35, 35, 177 for ^{134}Ba, ^{135}Ba, ^{136}Ba, ^{137}Ba respectively, P < 0.001 for all; for pairwise comparisons see Fig. 3). Otoliths from offspring spawned 3 weeks post injection had a greater proportion of otolith marked compared to weeks 1 and 2 (Fig. 4; $F_{2, 21}$ = 12, 10, 18, 57 for ^{134}Ba, ^{135}Ba, ^{136}Ba, ^{137}Ba, respectively, P < 0.01 for all; Pairwise Comparisons: WK3 >WK2 = WK1, P < 0.05 for all).

For ^{86}Sr and ^{87}Sr the 2 μg treatment produced a greater proportion of otolith marked compared to all lower concentrations (Fig. 3; $F_{3, 21}$ = 88 & 134, respectively, P < 0.001 for both; for pairwise comparisons see Fig. 3). Week 3 had a greater proportion of the otolith marked compared to weeks 1 and 2 (Fig. 4; $F_{2, 21}$ = 50 & 34 respectively, P < 0.001 for both; Pairwise Comparisons: WK3 >WK2 = WK1, P < 0.05 for both).

The number of count ratios above the detection limit for ^{26}Mg was insufficient to justify conducting mark intensity analysis on the proportion of otoliths marked.

Brood stock health, hatchery mortality, larval deformities, and condition at harvest

Of the 30 females injected, 3 fish were unsuccessfully spawned. These consisted of one fish that died 10 days after injection for unknown reasons, a second having overripe eggs due to being stripped too late, and the third not reaching spawning ripeness in the time frame of egg collection (within 6 weeks post injection). All other females in the experiment produced viable eggs, although there was some variation in the degree of egg ripeness when spawned. Offspring mortality between egg fertilisation and first feeding stage (mean ± SE) averaged 15.7 ± 3 % per egg batch and there was no treatment effect of geo-element enrichment ($F_{4, 26}$ = 1.2, P = 0.4). Yolk sac larval deformities observed between hatching and first feeding averaged 0.25 ± 0.07% per egg batch, with no treatment effect of geo-element enrichment ($F_{4, 26}$ = 0.5, P = 0.7). Fish harvested at 2 1/4 years post hatch (weight 3.79 ± 0.02 kg, fork-length 62.9 ± 2.5 cm, condition factor (k) 1.39 ± 0.06) showed no difference in length, weight or condition among treatments (weight: $F_{4, 27}$ = 0.88, p = 0.5; fork length: $F_{4, 26}$ = 0.81, p = 0.5; Fulton's condition factor (k): $F_{4, 26}$ = 1.59, p = 0.2). Mortality per treatment during the sea cage stage averaged 8 ± 0.5%, with no difference among treatments ($F_{4, 26}$ = 1.79, p = 0.9).

DISCUSSION

We have demonstrated that producing unique geo-elemental fingerprint marks in the otoliths of Atlantic salmon larvae via transgenerational marking is highly successful with Ba and Sr enriched natural geo-elements. This means it is possible to mass mark farmed Atlantic salmon at the earliest possible point in the life cycle, prior to spawning. Ensuring 100% mark success is dependent on the concentration of enriched geo-element used and the length of time between injection date and spawning date.

Mark success

A six marker fingerprint with 100% mark success was achieved using a combination of four Ba and two Sr enriched natural geo-elements in the 2 µg g^{-1} broodfish treatment when injection date and spawning date were at least 3 weeks apart. This is the first reported successful six mark geo-element combination using the transgenerational marking technique. Only one other study has successfully marked fish with a six geo-element combination (Warren-Myers et al. 2015a), but marks were delivered by an injection of natural geo-elements directly into salmon parr, not via broodstock. 100% mark success for concentrations lower than 2 µg g^{-1} female were successful using the four Ba geo-elements, but not the two Sr geo-elements, with all Ba geo-elements achieving 100% mark success at 0.02 µg g^{-1} female when injection date and spawning date were at least 3 weeks apart. Ba concentrations as low as 0.5 µg. g^{-1} female have been successful in saltwater species (Thorrold et al. 2006, Williamson et al. 2009b) and 0.3 µg. g^{-1} female in freshwater species (Huelga-Suarez et al. 2013), yet these are 15 to 25 times higher than we used in this study to achieve 100% mark success. However, compared to our study, the minimum time between injection and spawning was generally shorter in saltwater species (3 days: Thorrold et al. 2006; 13 days: Williamson et al. 2009b) and longer in freshwater species (1 to 2 months, Huelga-Suarez et al. 2013).

Mark strength and intensity

^{135}Ba produced the strongest (maximum ratio) and most intense (proportion of otolith marked) tags (Table 3). On average, 80% of acquisitions in the otolith were marked with ^{135}Ba in the lowest successful concentration (0.02 µg. g^{-1} female) with a maximum value 4.6 times higher than the threshold limit. ^{137}Ba produced marks of similar strength an intensity with 71% of acquisitions marked and an average maximum value 2.8 times above the threshold limit. ^{137}Ba is the most commonly used Ba geo-element for marking fish otoliths (Thorrold et al. 2006, Munro et al. 2009, Cuif et al. 2014) and ^{135}Ba less so

(Williamson et al. 2009b, Almany et al. 2007), yet our results suggest ^{135}Ba has the potential to produce slightly stronger marks than ^{137}Ba, potentially due to differences in purity of the two enriched geo-elements used (^{137}Ba 81.7% vs. ^{135}Ba 93.4%, Oak Ridge National Laboratory; www.ornl.gov).

^{134}Ba and ^{136}Ba mark strength and intensity were ~50% lower compared to ^{135}Ba and ^{137}Ba in the 0.02 µg. g^{-1} female concentration (Table 3), likely due to the higher detection limits for these geo-elements resulting from isobaric interference from Xe in the carrier gases. On average, 34 and 35% of acquisitions in the otoliths were marked with ^{134}Ba and ^{136}Ba with maximum values 2 and 2.3 times higher than the threshold limits. Although strength and intensity were ~50% lower, marks created with ^{134}Ba and ^{136}Ba were clearly definable at a concentration of 0.02 µg. g^{-1} female when the timing between injection and spawning surpassed 3 weeks and therefore should be highly useful for creating fingerprint combinations using 1, 2, 3 or 4 Ba geo-elements. Prior to this study, neither of these geo-elements have been tested or demonstrated to be 100% successful in marking otoliths using transgenerational marking. However, Warren-Myers et al. (2015a) successfully used ^{136}Ba and ^{134}Ba mixed with a vaccine and delivered via injection in Atlantic salmon parr and produced slightly higher mark strength values (3.6 times the relative threshold limit for both). In addition, Woodcock et al. (2011a) achieved 93% mark success with ^{136}Ba in Golden perch (*Macquaria ambigua*) using a larval immersion technique, but reported neither mark strength nor intensity.

^{86}Sr and ^{87}Sr produced well defined marks in the otoliths of offspring that came from broodstock females injected with a concentration of 2 µg. g^{-1} female that spawned 3 weeks post injection. Mark strength maximum values were 1.1 times higher than the threshold limit for both ^{86}Sr and ^{87}Sr and 37 to 43% of acquisitions in the otoliths were marked with ^{86}Sr and ^{87}Sr, respectively. Mark success with ^{86}Sr and ^{87}Sr at a concentration of 2 µg. g^{-1} female has not been demonstrated prior to this study using LA-ICP-MS detection methods. However, 100% success has been achieved with ^{87}Sr at a concentration of 20 µg. g^{-1} female (Starrs et al. 2014b). Relative to the concentration of 0.02 µg g^{-1} female of all four Ba geo-elements required to inject 10 kg Atlantic salmon broodstock to ensure successful marking of offspring, 2 µg. g^{-1} female for Sr geo-elements is high. Sr geo-elements are therefore less financially feasible for mass marking programs. Sr geo-elements may be more suitable if applied to smaller sized species (e.g. *Melanotaenia splendid*; Starrs et al. 2014b), or in alternate geo-element mark delivery methods, such as immersion (Munro et al. 2008, Smith & Whitledge 2011, de-Braux et al. 2014).

Brood stock health, hatchery mortality, larval deformities, and condition at harvest

Injecting brood stock with enriched natural geo-elements had no effect on spawning success or brood stock survival till spawning. However, when eggs were stripped, some internal bleeding in the abdominal cavity had occurred around the injection site in some females. Smaller injection volumes may help prevent this from occurring, and should be tested in the future, particularly as the process of injection has been reported to kill broodfish in other species (e.g. Starrs et al. 2014b). Offspring of all successfully spawned females (27 from 30) showed there was no effect of geo-element marking on egg survival or larval deformity rates, which is consistent with other studies that have marked with natural geo-elements at concentrations equivalent to 2 µg. g^{-1} female or less (Thorrold et al. 2006, Cuif et al. 2014, Warren-Myers et al. 2015b). No effect of marking on harvest size fish was found which is consistent with results observed in fish that have been vaccinated with natural geo-elements and grown to 5 kg (Warren-Myers et al. 2015a). Based on our results and previous research, transgenerational marking with natural geo-elements of Ba and Sr is a safe, effective method for mass marking farmed fish.

Transgenerational marking as a mass marking tool

Mass marking millions of fish can be an expensive exercise, hence quick, accurate and cost effective techniques that instantly batch mark numerous fish are preferred. Here, we have shown transgenerational marking with enriched natural geo-elements is another useful tool for mass marking salmon offspring prior to spawning in commercial hatcheries with 63 unique codes possible (Table 4) Marks using Ba geo-elements are cheaper to apply ($0.0002 to 0.002 USD per fish) compared to Sr geo-elements ($0.05 to 0.13 USD per fish), but Sr geo-elements may still be useful if used on small numbers of brood fish.

To date, transgenerational marking with enriched natural geo-elements has been validated in 13 species (Table 5), including freshwater, diadromous, and marine fish. Both Sr and Ba enriched geo-elements work well for freshwater species, yet Sr is not as successful as Ba for marine species. This may be because the natural abundance of Sr increases with salinity (Walther and Limburg, 2012). Hence, the higher abundance of Sr in marine waters may be reflected in the maternal Sr levels in marine fish, or fish with a marine growth phase, which mask any effects of the enriched Sr geo-element introduced. Broodstock in this study were transferred from seawater cages two months prior to spawning and held in freshwater tanks buffered with 0.7% NaCl thereafter, which may have reduced the seawater Sr signal. However, determining whether this occurred would require daily or weekly measurements of total Sr levels in broodfish for several months prior to spawning.

Analysis of all transgenerational marking studies with enriched natural geo-elements conducted to date reveals that Ba geo-elements have been the most successful across all fish species tested (Table 5). For Atlantic salmon, this is also the case for different delivery methods that have tested geo-element marking across a range of life history stages, for example; bathing of freshly fertilised eggs (Warren-Myers et al. (2015b), immersion of yolk-sac larvae (de-Braux et al. 2014), or injection of parr (Warren-Myers et al. 2015a). In Atlantic salmon, geo-elements of Ba produce strong, easily identifiable marks at concentrations 100 times lower than Sr geo-elements and therefore are the most suitable and cost effective geo-elements to use for mass marking farmed fish. Transgenerational marking with Ba geo-elements is another successful method to effectively mass mark fish that pinpoints the pre-spawning stage in the production lifecycle.

LITERATURE CITED

Almany GR, Berumen ML, Thorrold SR, Planes S, Jones GP (2007) Local replenishment of coral reef fish populations in a marine reserve. Science 316:742-744

Barbee N, Swearer SE (2007) Characterizing natal source population signatures in the diadromous fish *Galaxias maculatus*, using embryonic otolith chemistry. Mar Ecol Prog Ser 343:273-282

Cuif M, Keller F, Chateau O, Kaplan DM, Labonne M, Lett C, Vigliola L (2014) Evaluation of transgenerational isotope labelling of embryonic otoliths in a coral reef damselfish with single and repeated injections of enriched ^{137}Barium. J Exp Mar Biol Ecol 459:151-159

de Braux E, Warren-Myers F, Dempster T, Fjelldal PG, Hansen T, Swearer SE (2014) Osmotic induction improves batch marking of larval fish otoliths with enriched stable isotopes. ICES J Mar Sci 71:2530-2538

FitzGerald JL, Sheehan TF, Kocik JF (2004) Visibility of visual implant elastomer tags in Atlantic salmon reared for two years in marine net-pens. N Am J Fish Manag 24:222-227

Fleming IA, Hindar K, Mjolnerod IB, Jonsson B, Balstad T, Lamberg A (2000) Lifetime success and interactions of farm salmon invading a native population. Proc R Biol Sci 267:1517–1523

Glover KA (2010) Forensic identification of fish farm escapees: the Norwegian experience. Aquacult Environ Interact 1:1-10

Glover KA, Sørvik AGE, Karlsbakk E, Zhang Z, Skaala Ø (2013b) Molecular genetic analysis of stomach contents reveals wild Atlantic cod feeding on Piscine Reovirus (PRV) infected Atlantic salmon originating from a commercial fish farm. PloS One 8(4):e60924

Hindar K, Fleming IA, McGinnity P, Diserud O (2006) Genetic and ecological effects of salmon farming on wild salmon: modelling from experimental results. ICES J Mar Sci 63:1234-1247

Huelga-Suarez G, Moldovan M, Garcia-Valiente A, Garcia-Vazquez E, Garcia Alonso JI (2012) Individual-specific transgenerational marking of fish populations based on a barium dual-isotope procedure. Anal Chem 84:127-133

Huelga-Suarez G, Fernandez B, Moldovan M, Garcia Alonso JI (2013) Detection of transgenerational barium dual-isotope marks in salmon otoliths by means of LA-ICP-MS. Anal Bio Chem 405:2901-2909

Hutchings JA, Fraser DJ (2008) The nature of fisheries- and farming- induced evolution. Mol Ecol 17:294-313

Jensen Ø, Dempster T, Thorstad EB, Uglem I, Fredheim A (2010) Escapes of fishes from Norwegian sea-cage aquaculture: causes, consequences and prevention. Aquacult Environ Interact 1:71-83

McGinnity P, Prodohl P, Ferguson A, Hynes R, Maoileidigh N, Baker N, Cotter D, O'Hea B, Cooke D, Rogan G, Taggart J, Cross T (2003) Fitness reduction and potential extinction of wild populations of Atlantic salmon, *Salmo salar*, as a result of interactions with escaped farm salmon. Proc R Soc Lond B Biol Sci 270:2443-2450

Mohler JW (2003) Producing fluorescent marks on Atlantic salmon fin rays and scales with calcein via osmotic induction. N Am J Fish Manag 23:1108-1113

Munro AR, Gillanders BM, Elsdon TS, Crook DA, Sanger AC (2008) Enriched stable isotope marking of juvenile golden perch (*Macquaria ambigua*) otoliths. Can J Fish Aquat Sci 65:276-285

Munro AR, Gillanders BM, Thurstant S, Crook DA, Sanger AC (2009) Transgenerational marking of freshwater fishes with enriched stable isotopes: a tool for fisheries management and research. Fish Biol 75:668-684

Ricker WE (1975) Computation and interpretation of biological statistics of fish populations. Bull Fish Res Board Can 191:1-382

Roy A-S, Frisch AJ, Syms C, Thorrold SR, Jones GP (2013) Retention of a transgenerational marker (^{137}Barium) in tissues of adult female anemonefish and assessment of physiological stress. Environ Biol Fish 96:459-466

Smith KT, Whitledge GW (2011) Evaluation of a stable-isotope labelling technique for mass marking fin rays of age-0 lake sturgeon. Fish Manag Ecol 18:168-175

Starrs D, Davis JT, Schlaefer J, Ebner BC, Eggins SM, Fulton CJ (2014a) Maternally transmitted isotopes and their effects on larval fish: a validation of dual isotopic marks within a meta-analysis context. Can J Fish Aquat Sci 71:387-397

Starrs D, Ebner BC, Eggins S, Fulton CJ (2014b) Longevity in maternal transmission of isotopic marks in a tropical freshwater rainbowfish and the implications for offspring morphology. Mar Freshw Res. 65:400-408

Taylor MD, Fielder DS, Suthers IM (2005) Batch marking of otoliths and fin spines to assess the stock enhancement of *Argyrosomus japonicus*. J Fish Biol 66:1149-1162

Thorrold S, Planes S, Hare J (2006) Transgenerational marking of embryonic otoliths in marine fishes using barium stable isotopes. Can J Fish Aquat Sci. 63:1193-1197

Toledo-Guedes K, Scanchez-Jerez P, Mora-Vidal J, Girard D, Brito A (2011) Escaped introduced sea bass (*Dicentrarchus labrax*) infected by *Sphaerospora testicularis* (Myxozoa) reach maturity in coastal habitats off Canary Islands. Mar Ecol 31:26-31

Vander Haegen GE, Blankenship HL, Hoffmann A, Thompson DA (2005) The effects of adipose fin clipping and coded wire tagging on the survival and growth of spring chinook salmon. N Am J Fish Manag 25:1161-1170

Walther BD, Limburg KE (2012) The use of otolith chemistry to characterize diadromous migrations. J Fish Biol 81:796–825

Warren-Myers F, Dempster T, Jensen A, Fjelldal PG, Hansen T, Swearer SE (2014) Stable isotope marking of otoliths during vaccination: A novel method for mass marking fish. Aquacult Environ Interact 5:143-154

Warren-Myers F, Dempster T, Fjelldal PG, Hansen T, Swearer SE (2015a) An industry-scale mass marking technique for tracing farmed fish escapees. PLoS ONE 10(3): e0118594

Warren-Myers F, Dempster T, Fjelldal PG, Hansen T, Swearer SE (2015b) Rapid uptake of stable isotope markers in salmonids during egg swelling. Can J Fish Aquat Sci 72:1-6

Williamson DH, Jones GP, Thorrold SR (2009b) An experimental evaluation of transgenerational isotope labelling in a coral reef grouper. Mar Biol 156:2517-2525

Williamson DH, Jones GP, Thorrold SR Frisch AJ (2009a) Transgenerational marking of marine fish larvae: stable-isotope retention, physiological effects and health issues Journal of Fish Biology 74: 891–905

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

Woodcock SH, Gillanders BM, Munro AR, Crook DA, Munro AR (2011b) Determining mark success of 15 combinations of enriched stable isotopes for the batch marking of larval otoliths. N Am J Fish Manag 31:843-851

Woodcock SH, Grieshaber CA, Walther BD (2013) Dietary transfer of enriched stable isotopes to mark otoliths, fin rays and scales. Can J Fish Aquat Sci 70:1-4

Zitek A, Irrgeher J, Kletzl M, Weismann T, Prohaska T (2013) Transgenerational marking of brown trout *Salmo trutta* f.f., using an ^{84}Sr spike. Fish Manag Ecol 20:354-361

Zitek A, Irrgeher J, Cervicek M, Horsky M, Kletzl M, Weismann T, Prohaska T (2014) Individual-specific transgenerational marking of common carp *Cyprinus carpio*, L., using $^{86}Sr/^{84}Sr$ double spikes. Mar Freshw Res 65: 978-986

Figure 1. Effect of concentration on the strength of enriched geo-element marks. Maximum observed geo-element ratios in the otoliths of Atlantic salmon yolk sac larvae marked via transgenerational marking. Concentrations were 2, 0.2, 0.02, 0.002 and 0 (control) µg of each geo-element per g brood fish weight. Error bars represent ± 1 Standard Error.

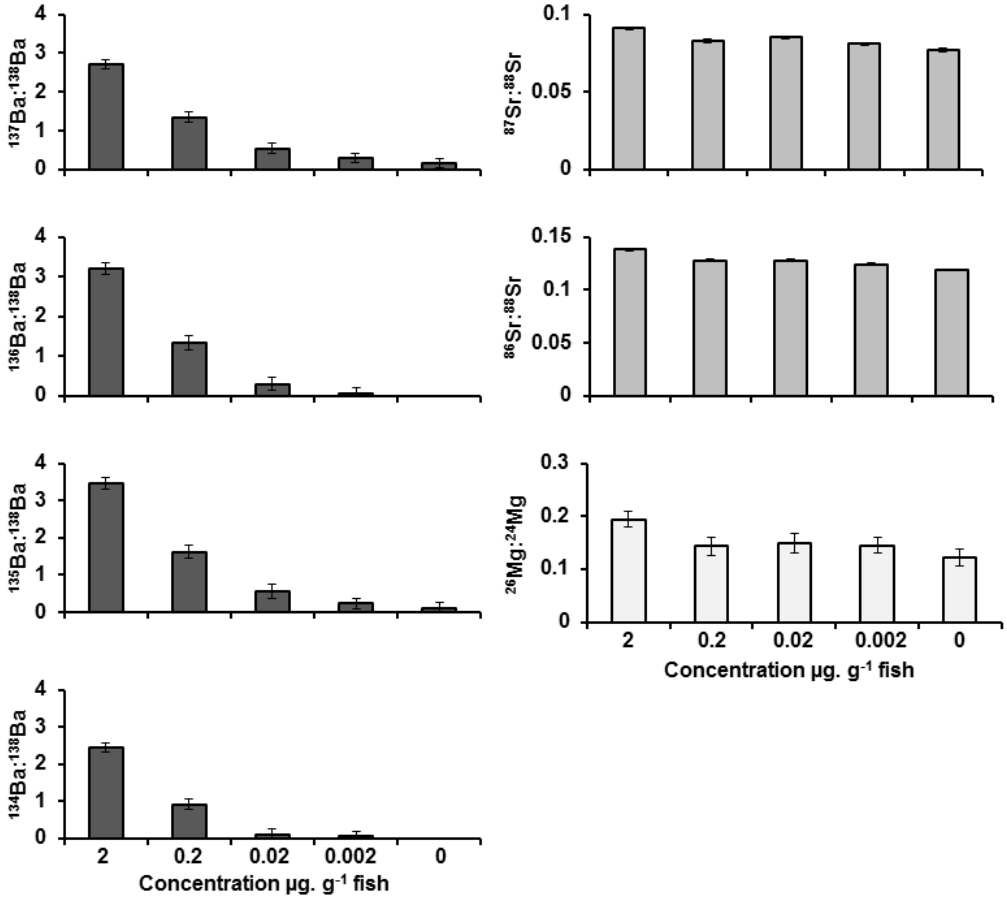


Figure 2. Effect of time interval between injection and spawning on the strength of enriched geo-element marks. Maximum observed geo-element ratios in the otoliths of Atlantic salmon yolk sac larvae marked via transgenerational marking categorized by number of weeks between injection date and spawning date. Error bars represe nt ± 1 Standard Error.

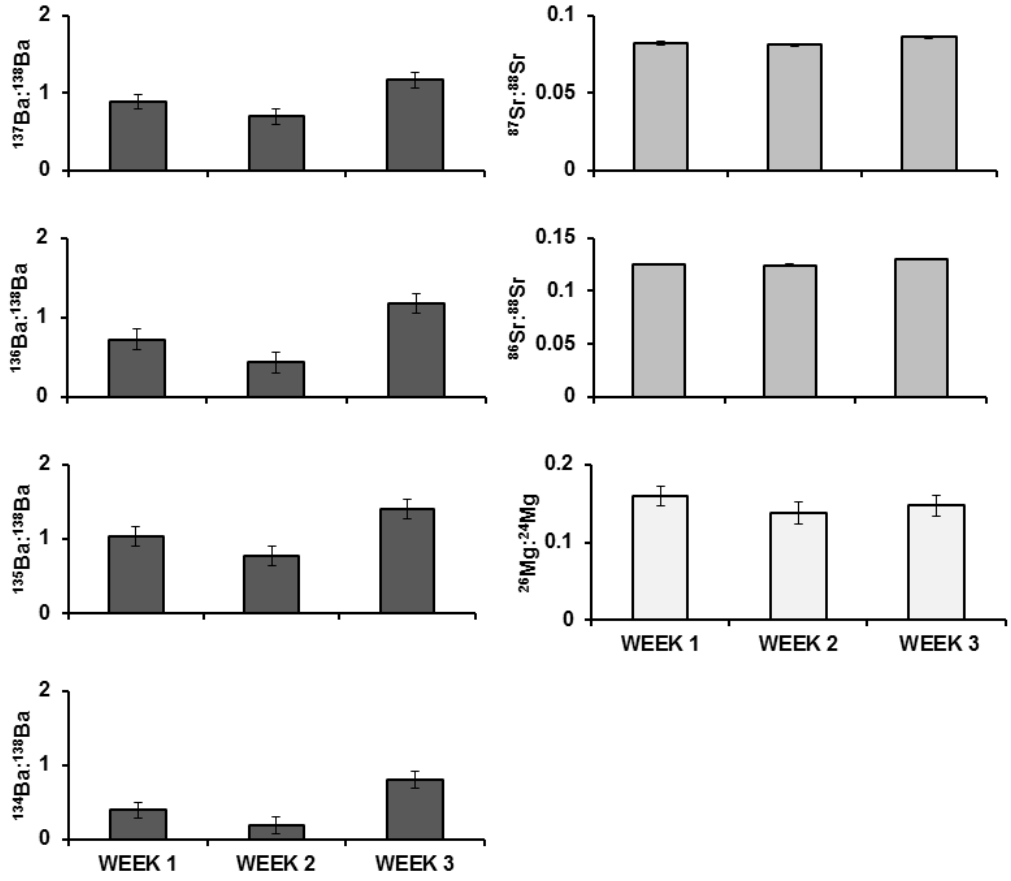


Figure 3. Effect of concentration on the intensity of enriched geo-element marks. Percentage of otolith marked with enriched barium geo-elements between the edge and the core of Atlantic salmon yolk sac larval otoliths marked via transgenerational marking. Concentrations were 2, 0.2, 0.02, 0.002 µg of each geo-element per g brood fish weight. Error bars represent ± 1 Standard Error. Different letters above bars indicate difference among concentrations using Pairwise Comparisons, $P < 0.05$.

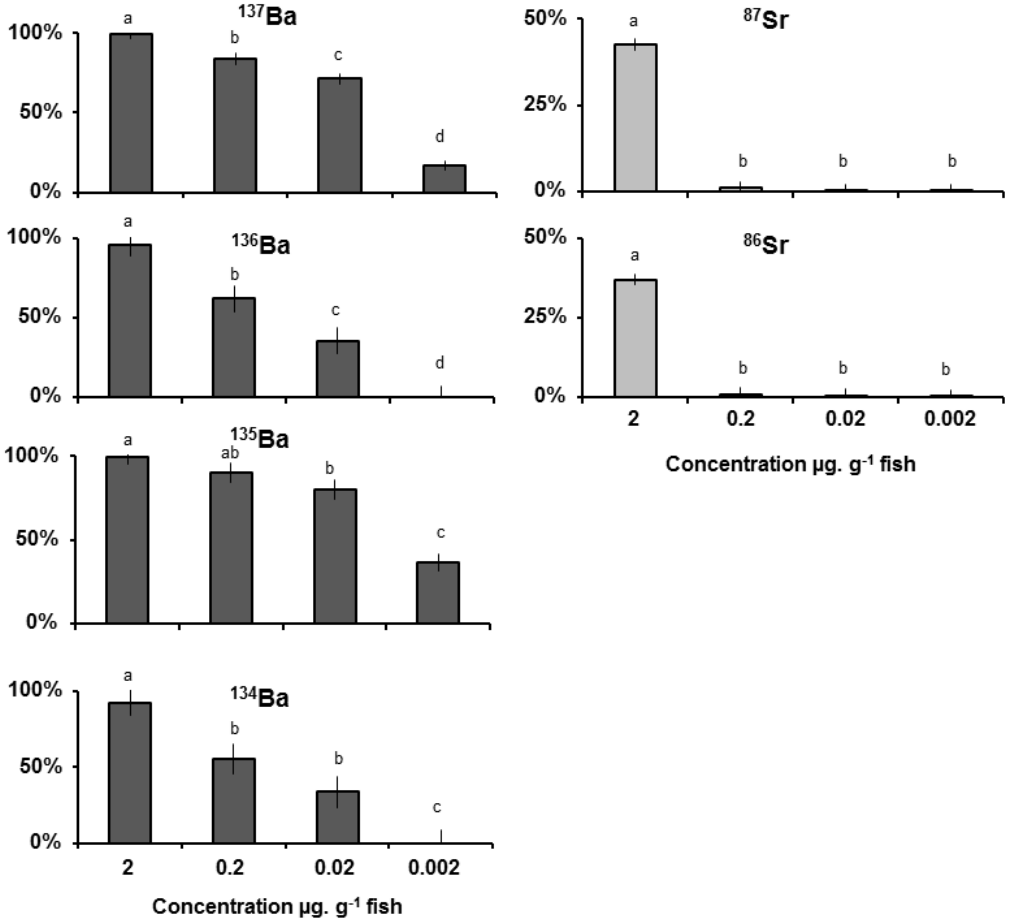


Figure 4. Effect of time interval between injection and spawning on the intensity of enriched geo-element marks. Percentage of otolith marked with enriched barium geo-elements between the edge and the core of Atlantic salmon yolk sac larval otoliths marked via transgenerational marking categorized by number of weeks between injection date and spawning date. Error bars represent ± 1 Standard Error.

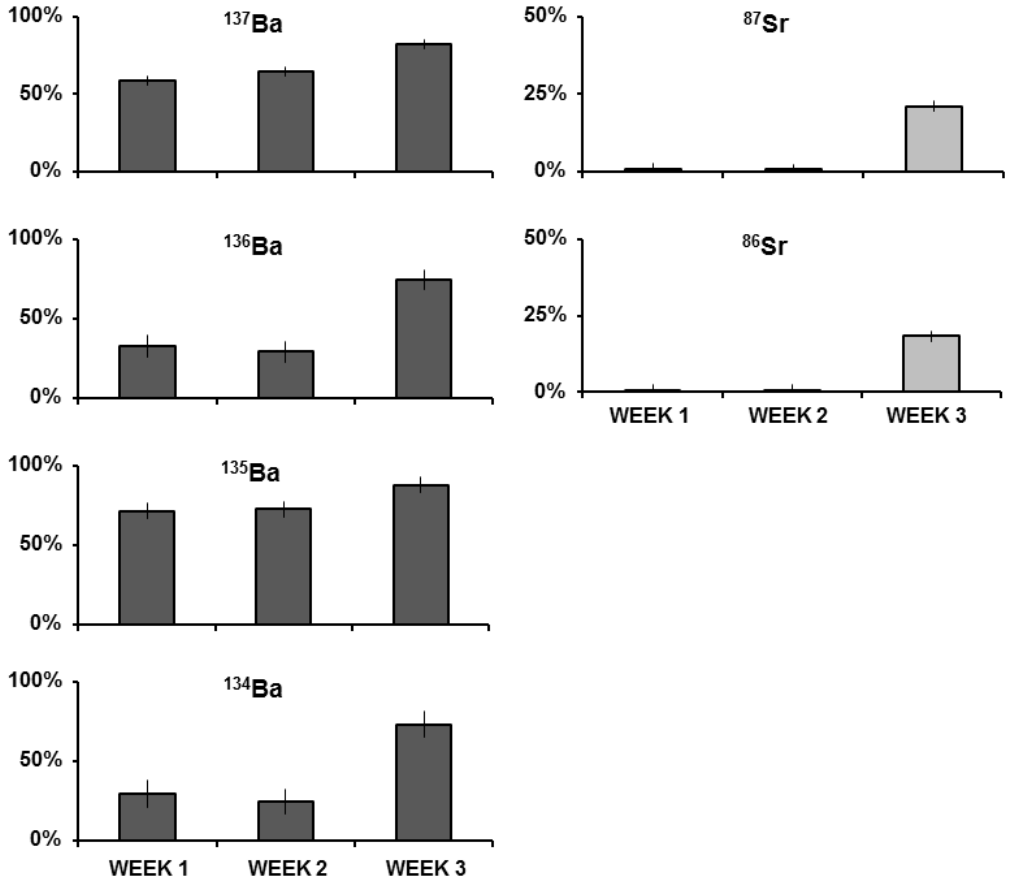


Table 1. Natural geo-element enrichment concentrations used for transgenerational marking.

Enriched geo-element concentration per treatment	**Treatment concentration**	**Brood fish per treatment**
^{134}Ba, ^{135}Ba, ^{136}Ba, ^{137}Ba, ^{26}Mg, ^{86}Sr, ^{87}Sr	Total amount of geo-element	Replicates
(µg. g^{-1} broodfish)	(µg. g^{-1} broodfish)	(N)
2	14	6
0.2	1.4	6
0.02	0.14	6
0.002	0.014	6
0	0	6

Table 2. Mark success. Percentage of Atlantic salmon yolk sac larval otoliths marked using a combination of seven enriched natural geo-elements delivered via transgenerational marking.

Spawning Date	**# Females Spawned**	**Concentration µg. g^{-1}**	**Mark success**						
			^{137}Ba	**^{136}Ba**	**^{135}Ba**	**^{134}Ba**	**^{87}Sr**	**^{86}Sr**	^{26}Mg
Week 1	4		**100%**	**100%**	**100%**	**100%**	15%	3%	10%
Week 2	0	2							
Week 3	2		**100%**	**100%**	**100%**	**100%**	**100%**	**100%**	30%
Week 1	1		95%	10%	**100%**	5%	0%	0%	0%
Week 2	4	0.2	**100%**	98%	**100%**	90%	5%	5%	8%
Week 3	1		**100%**	**100%**	**100%**	**100%**	10%	0%	0%
Week 1	2		95%	0%	**100%**	0%	0%	0%	0%
Week 2	1	0.02	**100%**	10%	**100%**	10%	0%	0%	10%
Week 3	1		**100%**	**100%**	**100%**	**100%**	0%	0%	0%
Week 1	0		0%	0%	0%	0%	0%	0%	0%
Week 2	4	0.002	30%	0%	65%	0%	0%	0%	8%
Week 3	2		75%	0%	80%	0%	0%	0%	0%

Table 3. **Comparison of geo-element mark strength and intensity**. Strength is the number of times the maximum geo-element ratio measured in a marked otolith is greater than the threshold limit. Intensity is the percentage of otolith marked with a geo-element ratio greater than the threshold limit. Colours indicate the minimum amount of weeks required between injection date and spawning date to reach 100% mark success for each geo-element. Cells with no colour indicate 100% mark success was not obtained.

	Concentration							
	2 $\mu g.g^{-1}$		0.2 $\mu g.g^{-1}$		0.02 $\mu g.g^{-1}$		0.002 $\mu g.g^{-1}$	
Geo-element	Strength	Intensity	Strength	Intensity	Strength	Intensity	Strength	Intensity
^{137}Ba	11.0	99.5%	5.5	83.7%	2.8	71.2%	1.2	17.1%
^{136}Ba	7.6	95.5%	3.2	61.7%	2.3	35.3%	0	0%
^{135}Ba	21.5	99.8%	10.0	89.9%	4.6	79.8%	1.5	36.2%
^{134}Ba	6.2	92.3%	2.3	55.4%	2.0	33.6%	0	0%
^{87}Sr	1.1	42.6%	0	0%	0	0%	0	0%
^{86}Sr	1.1	36.9%	1.0	0.8%	0	0%	0	0%

100% marked by week 1 | 100% marked by week 2 | 100% marked by week 3

Table 4. **Cost per code estimates for transgenerational marking of Atlantic salmon**. 63 natural geo-element marker codes and the estimated cost per code to mark using the transgenerational marking method. Code cost are calculated, based on a 10 kg brood fish producing at least 5000 viable offspring, injected with the minimum required amount of enriched geo-element to achieve 100% mark success, Geo-element pricing is based on cost at the time geo-elements where purchase from Oak Ridge National Laboratory; www.ornl.gov (June, 2012).

< $US 0.002 per fish		< $US 0.05 per fish		< $US 0.09 per fish		< $US 0.13 per fish	
Code	**Cost**	**Code**	**Cost**	**Code**	**Cost**	**Code**	**Cost**
		87Sr	$0.0452	86Sr	$0.0824	86Sr+87Sr	$0.1276
137Ba	$0.0002	137Ba+87Sr	$0.0454	137Ba+86Sr	$0.0826	137Ba+86Sr+87Sr	$0.1278
136Ba	$0.0003	136Ba+87Sr	$0.0455	136Ba+86Sr	$0.0827	136Ba+86Sr+87Sr	$0.1279
137Ba+136Ba	$0.0004	137Ba+136Ba+87Sr	$0.0456	137Ba+136Ba+86Sr	$0.0828	137Ba+136Ba+86Sr+87Sr	$0.1280
135Ba	$0.0005	135Ba+87Sr	$0.0457	135Ba+86Sr	$0.0829	135Ba+86Sr+87Sr	$0.1281
137Ba+135Ba	$0.0007	137Ba+135Ba+87Sr	$0.0459	137Ba+135Ba+86Sr	$0.0831	137Ba+135Ba+86Sr+87Sr	$0.1283
136Ba+135Ba	$0.0008	136Ba+135Ba+87Sr	$0.0460	136Ba+135Ba+86Sr	$0.0832	136Ba+135Ba+86Sr+87Sr	$0.1284
134Ba	$0.0008	134Ba+87Sr	$0.0460	134Ba+86Sr	$0.0832	134Ba+86Sr+87Sr	$0.1284
137Ba+136Ba+135Ba	$0.0010	137Ba+136Ba+135Ba+87Sr	$0.0462	137Ba+136Ba+135Ba+86Sr	$0.0834	137Ba+136Ba+135Ba+86Sr+87Sr	$0.1286
137Ba+134Ba	$0.0010	137Ba+134Ba+87Sr	$0.0462	137Ba+134Ba+86Sr	$0.0834	137Ba+134Ba+86Sr+87Sr	$0.1286
136Ba+134Ba	$0.0011	136Ba+134Ba+87Sr	$0.0463	136Ba+134Ba+86Sr	$0.0835	136Ba+134Ba+86Sr+87Sr	$0.1287
137Ba+136Ba+134Ba	$0.0012	137Ba+136Ba+134Ba+87Sr	$0.0464	137Ba+136Ba+134Ba+86Sr	$0.0836	137Ba+136Ba+134Ba+86Sr+87Sr	$0.1288
135Ba+134Ba	$0.0013	135Ba+134Ba+87Sr	$0.0465	135Ba+134Ba+86Sr	$0.0837	135Ba+134Ba+86Sr+87Sr	$0.1289
137Ba+135Ba+134Ba	$0.0015	137Ba+135Ba+134Ba+87Sr	$0.0467	137Ba+135Ba+134Ba+86Sr	$0.0839	137Ba+135Ba+134Ba+86Sr+87Sr	$0.1291
136Ba+135Ba+134Ba	$0.0016	136Ba+135Ba+134Ba+87Sr	$0.0468	136Ba+135Ba+134Ba+86Sr	$0.0840	136Ba+135Ba+134Ba+Sr86+Sr87	$0.1292
137Ba+136Ba+135Ba+134Ba	$0.0018	137Ba+136Ba+135Ba+134Ba+Sr87	$0.0470	137Ba+136Ba+135Ba+134Ba+86Sr	$0.0842	137Ba+136Ba+135Ba+134Ba+86Sr+87Sr	$0.1294

Table 5. Number of species marked. Species validated to have been marked via the transgenerational marking method with enriched Ba and Sr natural geo-elements. Concentrations and spawning times reflect the required minimums to achieve 100% mark success.

WP 2.1 Mass marking farmed Atlantic salmon with transgenerational geo-elemental fingerprints during vaccination – pilot sudy

ABSTRACT: Tagging or marking of fishes enables the collection of population-based information for ecological research, yet few techniques enable 100% mark detection success. We tested a new mass-marking technique: otolith marking with enriched natural geo-elements delivered during vaccination. Atlantic salmon (*Salmo salar*) parr were injected in either the abdominal cavity or muscle with a combination of enriched ^{137}Ba, ^{86}Sr and ^{26}Mg, using 1 of 3 carrier solutions (water, vaccine, vaccine mimic). Laser ablation inductively coupled plasma mass spectrometry of the otoliths indicated that ^{137}Ba and ^{86}Sr geo-element enrichment treatments achieved 100% mark success, with 0 to 34% success for ^{26}Mg, compared to experimental controls. Mark strength was greater when enriched geo-elements were injected into the abdominal cavity compared to muscle. Geo-element markers did not affect fish condition or survival. Marks could be differentiated with 100% success from the background levels present in wild parr collected from 22 Norwegian rivers. Natural geo-element marking via vaccination with enriched natural geo-elements is a mass-marking technique that, once optimised, could allow for cost-effective differentiation of wild and escaped farmed fish for each independent farming area.

Introduction

Fish identification markers, whether artificial or natural, are an essential tool for population-based ecological research, particularly for studies of population connectivity (Swearer et al. 1999, Thorrold et al. 2006, Almany et al. 2007), stock identification (Campana 2005, Barnett-Johnson et al. 2007), fish migratory patterns (Kalish 1990, Jones et al. 1999, Kennedy et al. 2002, Elsdon & Gillanders 2004, Walther & Limburg 2012) and stock discrimination (Adey et al. 2009, Glover 2010). However, the reliability of a mark or marker-based data can be uncertain depending on the type of identification used. For example, uncertainty may arise due to poor mark retention, mark misidentification, low recapture rates, or marker-related effects on growth and survival. As no single marking technique is suitable for all situations, it is important to choose a marker that minimises the uncertainty in fish identification for the particular research question and application.

Markers may be categorised into 2 general groups: natural or artificial. Natural markers include genetic sequences (Glover et al. 2008), elemental composition of otoliths (Kennedy et al. 2000, 2002, Gillanders 2005, Barbee & Swearer 2007) or scales (Adey et al. 2009), or differences in fish morphology. Natural markers are most suited for investigating population structure in fish species that have enough spatial, biological or environmental variability to effectively differentiate among groups of fish. Natural markers are effective in that they already exist within a fish population; however, identification and discrimination of groups of fish using natural markers is often limited by the requirement of a large and comprehensive baseline data library to accurately discriminate among groups (e.g. Glover et al. 2008).

Artificial markers, in contrast, require intervention to create the mark and are most suited for marking small numbers of fish (<1000 individuals). These include physical markers that are inserted into fish (e.g. anchor: Serafy et al. 1995; disk: Collins et al. 1994; and coded wire tags: Munro et al. 2003) or removal of some part of the fish that does not regrow, e.g. barbels (Collins et al. 1994) and adipose fins (Vander Haegen et al. 2005). These marking techniques, however, can cause physical stress, lesions and compromised swimming ability, with subsequent increases in mortality (Collins et al. 1994, Serafy et al. 1995, Buckland-Nicks et al. 2012). In addition, they are costly and labour-intensive to apply.

The alternative to marking fish individually is to mass-mark. Mass-marking is preferable when marking large numbers of fish (>1000 individuals) is a high priority, as it is less labour-intensive and reduces individual handling stress for fish. Mass-marking has been achieved through otolith thermal

marking (Volk et al. 1999); chemical marking by immersion in fluorescent dyes such as tetracycline (Jones et al. 1999), calcein and alizarin red S (Crook et al. 2009); and elemental marking (Farrell & Campana 1996, Bath et al. 2000). These marking techniques also have issues, such as poor longevity of some chemical dyes (Crook et al. 2009), and up to 40% inaccuracy in identification of multiple thermal marks (Volk et al. 1999).

Marking with enriched natural geo-elements (Thorrold et al. 2006) is a relatively new mass-marking method that can create unique single and multiple markers with 100% accuracy. Artificial natural geo-element 'fingerprint' marks can be created when enough enriched geo-element is introduced to significantly change the relative geo-elemental abundance in the otolith compared to the natural background geo-element ratio. Natural geo-elements have been used to successfully mark fish embryos (Thorrold et al. 2006, Williamson et al. 2009b), larvae (Woodcock et al. 2011a), and juveniles (Munro et al. 2008, Smith & Whitledge 2011) by changing the geo-elemental ratios of barium (Ba) and strontium (Sr) in their otoliths. Natural geo-elements of Ba and Sr occur naturally in aquatic ecosystems and are detectable in wild fish in ratios that are largely invariant (see reviews by Campana 2005, Gillanders 2005). The one exception is Sr, where low levels of variation in geo-element ratios within otoliths have been used to trace movement patterns of fish within freshwater catchments (Kalish 1990, Kennedy et al. 2000, 2002, Elsdon & Gillanders 2004, Barnett-Johnson et al. 2005). Natural geo-element fingerprint marking using Ba and/or Sr geo-elements can be applied to the individual, or groups of fish, and has been validated in both marine and freshwater fish species using a variety of delivery methods, e.g. via: (1) maternal transfer, where enriched natural geo-elements injected into brood stock is passed on to the offspring (Thorrold et al. 2006, Munro et al. 2009, Huelga-Suarez et al. 2012); (2) immersion of larvae or juveniles in an geo-element-enriched solution (Smith & Whitledge 2011, Woodcock et al. 2011a,b); or (3) delivery via geo-element-enriched feeds (Woodcock et al. 2013). Marking with enriched natural geo-elements of magnesium (Mg) has shown poor mark success via immersion and dietary uptake (Woodcock et al. 2011a, 2012). However, the incorporation of Mg-enriched natural geo-elements via direct injection has yet to be investigated and may provide a more successful marking application for Mg geo-elements.

To date, there are no studies on marking with natural geo-elements of individual fish during vaccination, or on how injection site or carrier solution affects marking success. Current geo-element marking techniques indicate that the delivery method, duration of exposure, and the amount of geo-element received influence the uptake of enriched geo-elements, and consequently, mark success (Munro et al. 2009, Williamson et al. 2009b, Woodcock et al. 2011a). In addition to validating a natural geo-element mark delivery method, knowledge of the natural variability in geo-elemental ratios for a given species and study system is required before a natural geo-element fingerprinting method can be considered to be an effective and accurate individual- or mass-marking tool.

Here, we tested a novel enriched natural geo-element marking technique by investigating whether natural geo-element otolith fingerprint markers can be combined with routine vaccination of Norwegian farmed Atlantic salmon *Salmo salar*. We explored this delivery method because escaped fish from aquaculture are a significant environmental problem (Jensen et al. 2010), and accurate methods to differentiate escaped farmed fish from wild fish and the farm of origin would enhance compliance measures. In addition, all ~300+ million farmed salmon grown in the sea in Norway each year (Jensen et al. 2010) are vaccinated in the abdominal cavity with an oil-adjuvant vaccine. Hence, during vaccination, geo-element markers may be administered in a controlled amount, to individual fish at a specific point in the life history stage, with no extra manual handling in the production process. Consequently, all marking issues, such as the period of geo-element enrichment, the amounts of geo-element received, and mark effectiveness, have the potential to be controlled and evaluated at a whole-of-industry scale.

First, we tested whether we could create unambiguous marks through introducing enriched natural geo-elements during routine vaccination via 2 injection sites, using a vaccine, a vaccine mimic, or

water as a carrier solution to determine whether mark success and strength varied with injection site and carrier solution. Second, we determined if otolith fingerprinting via injection had any adverse side effects by comparing condition and survival of injected fish 10 wk after marking. Finally, we generated a baseline database of variation in the geo-elemental ratios of Ba, Sr and Mg by sampling Atlantic salmon parr from 22 rivers across the latitudinal extent of Norway, which we could use to assess if the artificial otolith fingerprint marks we created could be unambiguously detected relative to wild fish.

Materials and Methods

Experimental location and fish

The experiment was conducted at the Institute of Marine Research field station, at Matre, in Masfjorden, western Norway (60° N). Atlantic salmon (Aqua-Gen strain) parr (standard length: 16.2 ± 0.02 cm [mean ± SE]; mass: 57.1 ± 0.07 g) were used in the experiment. All fish were passive integrated transponder (pit) tagged with 11 mm Trovan ID 101 tags (BTS Scandinavia AB) 2 mo prior to the experiment and reared in standard commercial hatchery conditions. Fish in all treatments were in similar condition (Fulton's condition factor K; $F_{11,71}$ = 0.9, p = 0.5) at Day 1 of the experiment. All work was conducted in accordance with the laws and regulations of the Norwegian Regulation on Animal Experimentation 1996.

Experimental design

We tested if the level of natural geo-element enrichment, carrier solution and injection location affected otolith mark success and detectability of Ba, Sr and Mg geo-element fingerprints (Table 1). Atlantic salmon parr were injected with either no geo-element, or a combination of 3 enriched natural geo-elements, ^{137}Ba, ^{86}Sr and ^{26}Mg (Oak Ridge National Laboratory; www.ornl.gov), each at a concentration of 2 µg of geo-element per gram of parr average mass. One of 3 carrier solutions was used: (1) water-based (W) carrier solution, which consisted of 100% Milli-Q water; (2) oil-based vaccine (V) carrier solution, which consisted of 3.5% Milli-Q water and 96.5% multi vaccine MINOVA 6 (NORVAX® MINOVA 6, Global Aquatic Animal Health, Thormøhlensgate 55, 5008 Bergen, Norway); and (3) oil emulsion-based vaccine mimic (VM) carrier solution, which consisted of 50% Milli-Q water and 50% paraffin oil. Natural geo-elements in powder chloride form used for the geo-element enrichment treatments were first dissolved in water and then mixed into the final carrier solutions. The VM final carrier solution required the addition of soy lecithin (130 mg ml^{-1} VM solution) and vortexing for 1 min at 13000 rpm (Ultra-Turrax T25, IKA©-Labortechnik) to obtain a stable emulsion. Injections were given into the abdominal cavity (AC), approximately 20 mm behind the pectoral fin on the ventral side of parr, or into the musculature (M), approximately 10 mm below the dorsal fin on the lefthand side of each fish. Parr were injected with a hypodermic syringe using a 5 mm, 27 gauge vaccination needle with a standard vaccination volume of 0.1 ml.

Fish were anaesthetised with Benzoak VET (dose 0.2 ml l^{-1} of clean hatchery water), identified by their PIT tag number, then weighed, measured (fork length) and injected. After injection, fish were placed into one of three 1000 l tanks with equal interspersion of individuals among treatments within each tank (i.e. 4 fish from each treatment per tank). The fish were reared under a 12 h light:12 h dark photoperiod for the first 2 wk post-injection before being switched to 24 h continuous light for the next 8 wk to induce smoltification. Two weeks after injection, a randomly selected sub-sample of 6 parr per treatment were anaesthetised and identified by their PIT tag number before being weighed, measured (fork length) and then euthanised by anaesthetic overdose. Sagittal otoliths from each fish were

dissected and removed, mechanically cleaned of any adhering tissue, air-dried, and stored individually in plastic tubes. The remaining fish (n = 6 per treatment) were grown for a further 8 wk before they were anaesthetised, weighed, and measured (fork length) to test for differences in growth and condition among treatments. Remaining fish were euthanised by anaesthetic overdose at the final endpoint of the experiment (10 wk post-injection date).

Table 1. Design of the experiment to test mark success and strength through introducing enriched natural geo-elements during routine vaccination of *Salmo salar* via 2 injection sites (muscle [M] or abdominal cavity [AC]), using a vaccine (V), a vaccine mimic (VM), or water (W) as a carrier solution. Enrichment: yes (Y) or no (N)

Factors			Sample sizes (n)		
Injection location	Carrier solution	Geo-element enrichment	Fish per treatment	Growth analysis	Otolith analysis
M	W	Y	12	6	6
M	W	N	12	6	6
M	V	Y	12	6	6
M	V	N	12	6	5
M	VM	Y	12	6	6
M	VM	N	12	6	5
AC	W	Y	12	6	6
AC	W	N	12	6	6
AC	V	Y	12	6	6
AC	V	N	12	6	6
AC	VM	Y	12	6	6
AC	VM	N	12	6	6

Baseline geo-element ratios for Atlantic salmon in Norwegian rivers

Samples of Atlantic salmon parr from 22 rivers spanning the latitudinal extent of Norway were used to determine natural baseline variation in the ratios of ^{134}Ba:^{138}Ba, 135Ba:138Ba, ^{136}Ba:^{138}Ba, ^{137}Ba:^{138}Ba, ^{86}Sr:^{88}Sr, ^{87}Sr:^{88}Sr and ^{26}Mg:^{24}Mg. These samples had been collected by the Norwegian Institute for Nature Research between 1986 and 2010 and preserved in ethanol. In addition to determining spatial variability, temporal variability was assessed between 1990 and 2010 using samples from 6 randomly selected years from each of the Saltdalselva and Strynselva rivers. Sagittal otoliths from 3 parr per location or year were used for the assessment of baseline ratios.

Otolith preparation

Sagittal otoliths were cleaned of any remaining organic tissue by immersing in a solution of ultrapure 15% H_2O_2 buffered with 0.1 M NaOH. Following immersion, otoliths were ultra-sonicated (Sonic Clean 250HT) for 5 min and then left for 6 h in the cleaning solution. The cleaning solution was then aspirated off and the otoliths were transferred through 3 Milli-Q water rinses, each of which consisted of 5 min of ultra-sonification and 30 min resting time. Otoliths were then air-dried in a laminar

flow bench for at least 24 h. Once dry, 1 otolith per fish was fixed, sulcus side down, onto gridded microscope slides using quick-dry cyanoacrylate glue.

Otolith analysis

Natural geo-element analyses were done on a Varian 7700x inductively coupled plasma mass spectrometer (ICP-MS) fitted with a HelEx (Laurin Technic and the Australian National University) laser ablation (LA) system constructed around a Compex 110 (Lambda Physik) excimer laser operating at 193 nm. National Institute of Standards and Technology (NIST) 612 and 610 glass standards doped with trace elements at known concentrations were used to calibrate the system. External precision estimates (%RSD, Relative Standard Deviation) based on 20 analyses of a MACS3 microanalytical carbonate standard were as follows: ^{134}Ba:^{138}Ba = 7.37; ^{135}Ba:^{138}Ba = 0.81, ^{136}Ba:^{138}Ba = 4.51, ^{137}Ba:^{138}Ba = 0.72, ^{86}Sr:^{88}Sr = 0.94, ^{87}Sr:^{88}Sr = 1.16 and ^{26}Mg:^{24}Mg = 0.60. Otoliths were run in blocks of 16 samples selected randomly from all treatments and bracketed by analyses of the standard. Samples and standard were analysed in time resolved mode, using a spot size of 157 µm, a laser energy setting of ~60 mJ and a laser repetition rate of 5 Hz. Spot ablation was performed under pure helium (He) (200 ml min−1) to minimise re-deposition of ablated material, and the sample was then entrained into the argon (Ar) (0.95 ml min−1) carrier gas flow to the ICP-MS. Using this method, we were able to quantify the concentrations of ^{134}Ba, ^{135}Ba, ^{136}Ba, ^{137}Ba, ^{138}Ba, ^{86}Sr, ^{87}Sr, ^{88}Sr, ^{24}Mg, ^{26}Mg and ^{43}Ca in the outer region of salmon parr otoliths. Data were processed off-line using a specialised MS Excel template which involved a low-pass filter to remove any spikes (a single scan value >2× the median of the adjacent scans), smoothing (a running average of 3 scans) and blank subtracting functions. A correction factor ($C = R_{true}/R_{obs}$, where R_{true} is the naturally occurring geo-element ratio and R_{obs} is the average geo-element ratio measured in either the NIST 612 or NIST 610 standard run before and after each set of 16 samples) was applied to all sample scans to correct for mass bias. NIST 612 was used for ^{137}Ba, ^{135}Ba, ^{87}Sr, ^{86}Sr and ^{26}Mg, and NIST 610 for ^{134}Ba and ^{136}Ba. Geo-element ratios are expressed as the enriched geo-element divided by the most commonly abundant geo-element for each element used, so that the measure of enrichment is always expressed as an increase in the enriched geo-element relative to the most common geo-element.

Statistical analysis

Mark detection limits for the geo-element ratios ^{137}Ba:^{138}Ba, ^{86}Sr:^{88}Sr and ^{26}Mg:^{24}Mg were calculated from the average geo-element ratios of fish across all control treatments (i.e. non-enrichment treatments). To ensure a correct classification probability of 99.94%, mark detection limits were set at 3.3 standard deviations (SDs) above the mean observed ratio in all control fish for each enriched geo-element used. Because of the inherent instability in geo-elemental ratios measured on single-detector, ICP-based mass spectrometers, we conservatively set the criteria for detecting a successful mark in the otolith as at least 3 consecutive scans with ratios above the detection limit.

The effects of geo-element enrichment (0 and 2 µg geo-element g^{-1} fish), carrier solution (W, V and VM) and injection location (AC and M) on the ratios ^{137}Ba:^{138}Ba, ^{86}Sr:^{88}Sr and ^{26}Mg:^{24}Mg were analysed using 3-factor ANOVAs with data standardised for initial fish weight. The response variable used was the maximum geo-element ratio observed in each fish otolith.

Strength of ^{137}Ba:^{138}Ba, ^{86}Sr:^{88}Sr and ^{26}Mg:^{24}Mg mark success for only the geo-element enrichment treatments (2 µg geo-element g^{-1} fish) was assessed by testing the effects of carrier solution (W, V and VM) and injection site (AC and M) with 2-factor ANOVAs using data standardised for initial fish weight. The response variables used for each fish were the total number of scans with ratios above the

detection limit and the average geo-element ratio of all scans above the detection limit. A scan is defined as a single laser ablation data point.

The effect of treatment on change in fish condition over the experimental period was analysed with a factorial ANOVA. Carrier solutions (W, V and VM), injection location (AC and M) and natural geo-element enrichment (0 and 2 µg geo-element g^{-1} fish) were treated as fixed factors. The response variable used was change in fish condition and was estimated using Fulton's condition factor *K*. Statistical significance was determined at $\alpha = 0.05$ for all ANOVAs.

The baseline ratios ^{134}Ba:^{138}Ba, ^{135}Ba:^{138}Ba, ^{136}Ba:^{138}Ba, ^{137}Ba:^{138}Ba, ^{86}Sr:^{88}Sr, ^{87}Sr:^{88}Sr and ^{26}Mg:^{24}Mg for each of the 22 rivers in Norway were expressed as the geo-element ratio value (mean ± SE) analysed from 3 fish from each river and each year.

Results

Mark success

A mark success of 100% was achieved with the natural geo-elements ^{137}Ba and ^{86}Sr across all enriched geo-element treatments, irrespective of injection location, or carrier solution (Figs. 1 & 2). Mark success for ^{26}Mg in the enriched treatments was poor by comparison. ^{26}Mg mark success ranged from 0 to 34% and varied among injection location and carrier solutions (Fig. 3). No aberrant ^{137}Ba, ^{86}Sr or ^{26}Mg geo-element marks were observed above the threshold limit in the non-enriched (control) treatments.

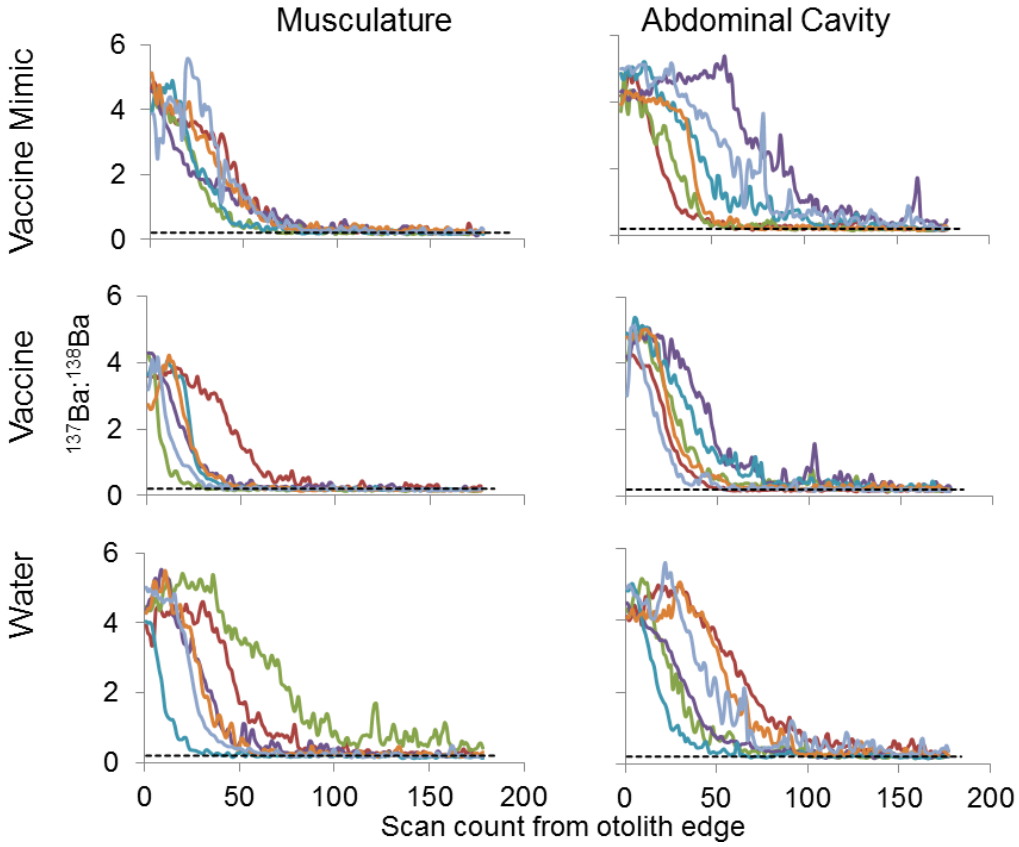


Fig. 1. *Salmo salar.* Mark success with ^{137}Ba:^{138}Ba for the 6 enrichment treatments. Treatments differed in the geo-element carrier solution used (water, vaccine, vaccine mimic) and the injection location (musculature or abdominal cavity). Coloured lines represent individual Atlantic salmon. Black dotted line represents average control ratio + 3.3 SDs. A scan is defined as a single laser ablation data point

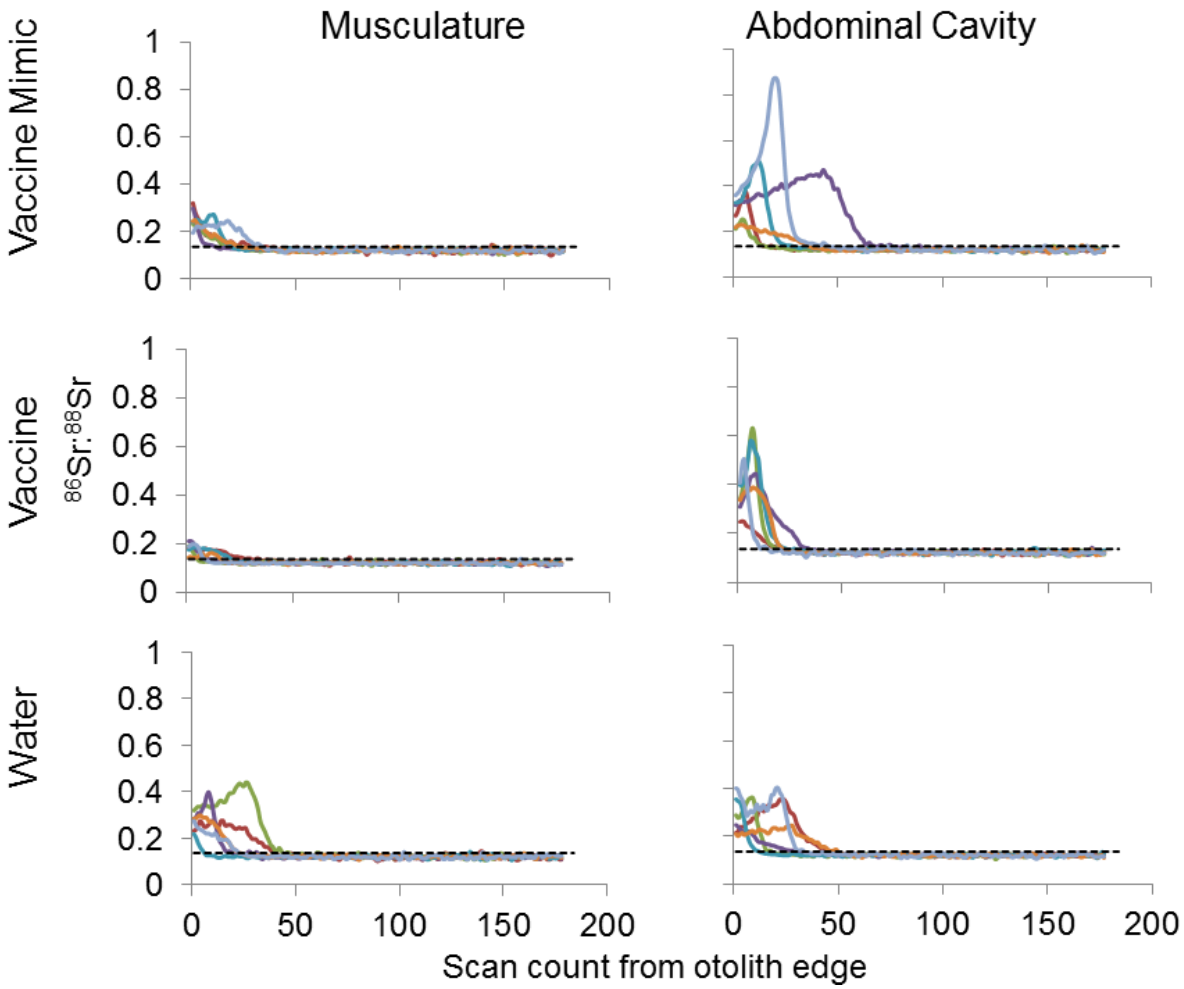


Fig. 2. *Salmo salar.* Mark success with ^{86}Sr:^{88}Sr for the 6 enrichment treatments. Treatments differed in the geo-element carrier solution used (water, vaccine, vaccine mimic) and the injection location (musculature or abdominal cavity). Coloured lines represent individual Atlantic salmon. Black dotted line represents average control ratio + 3.3 SDs. A scan is defined as a single laser ablation data point

Effect of treatment on geo-element ratios

Maximum recorded geo-element ratios were 22 times higher for ^{137}Ba:^{138}Ba and 2.4 times higher for ^{86}Sr:^{88}Sr in the enriched treatments compared to the non-enriched treatments (^{137}Ba enrichment ratio: 4.84 ± 0.05 [mean ± SE], non-enrichment ratio: 0.22 ± 0.05, $F_{1,69}$ = 4164, p < 0.001; ^{86}Sr enrichment ratio: 0.33 ± 0.02, non-enrichment ratio: 0.14 ± 0.02, $F_{1,69}$ = 80, p < 0.001). Maximum ratios did not differ between enriched and non-enriched treatments for ^{26}Mg: ^{24}Mg (^{26}Mg enrichment ratio: 0.19 ± 0.02, non-enrichment ratio: 0.16 ± 0.02, $F_{1,69}$ = 1.2, p = 0.3).

An effect of carrier solution was found for ^{137}Ba:^{138}Ba ($F_{2,69}$ = 3.5, p = 0.04), with maximum ratio values higher for VM compared to V (VM: 2.75 ± 0.06; V: 2.53 ± 0.06; W: 2.60 ± 0.06; post hoc Tukey HSD: VM >

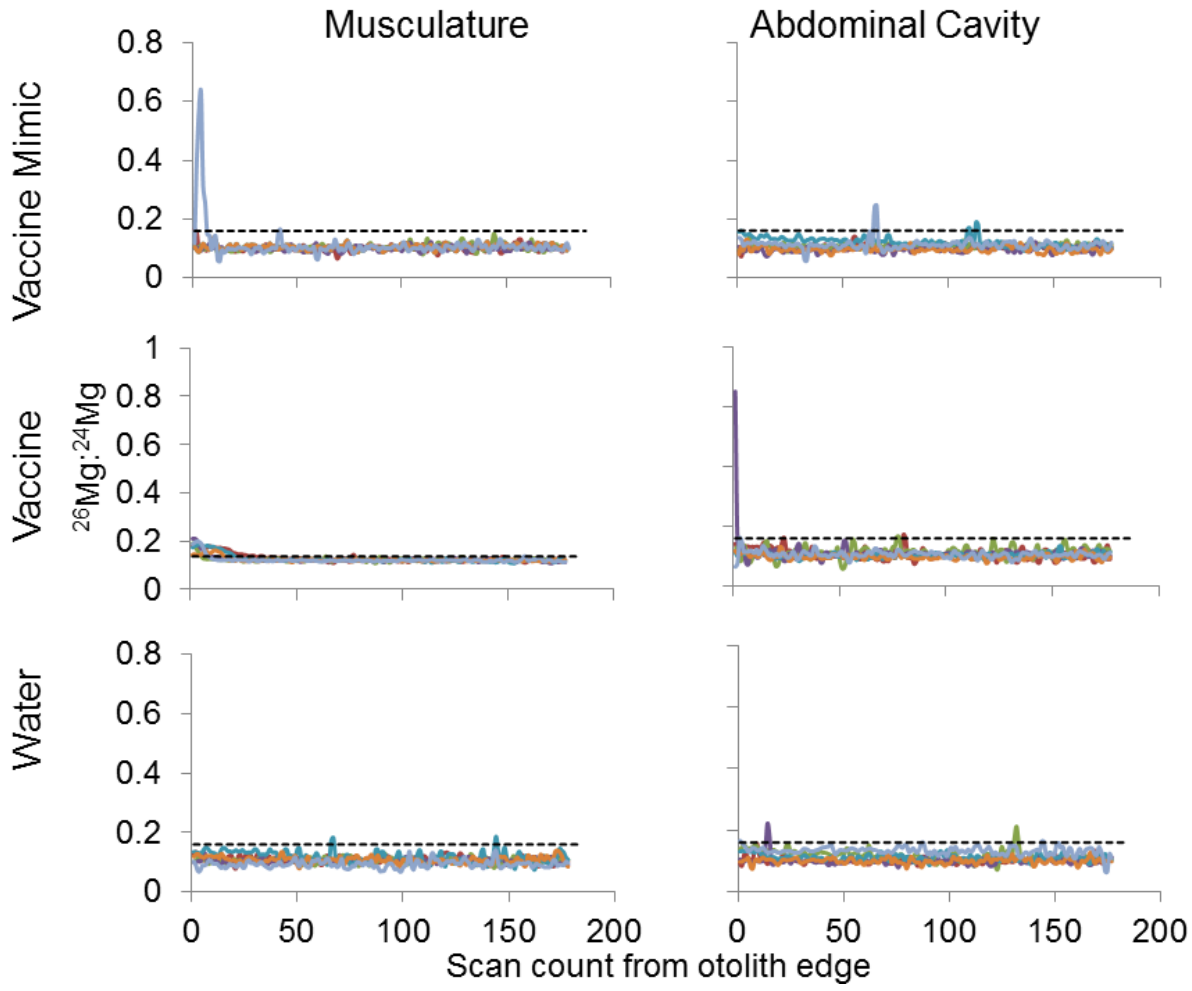


Fig. 3. *Salmo salar.* Mark success with ^{26}Mg:^{24}Mg for the 6 enrichment treatments. Treatments differed in the geo-element carrier solution used (water, vaccine, vaccine mimic) and the injection location (musculature or abdominal cavity). Coloured lines represent individual Atlantic salmon. Black dotted line represents average control ratio + 3.3 SDs. A scan is defined as a single laser ablation data point

V; p = 0.04). Conversely, there was no effect of carrier solution on ^{86}Sr:^{88}Sr ($F_{2,69}$ = 0.34, p = 0.7) or ^{26}Mg:^{24}Mg ratios ($F_{2,69}$ = 0.6, p = 0.6).

Injection location influenced the maximum geo-element ratios values for ^{86}Sr:^{88}Sr and ^{137}Ba:^{138}Ba ($F_{1,69}$ = 12, p = 0.001; and $F_{1,69}$ = 5.3, p = 0.03; respectively). For Sr, the maximum ratio was 1.4 times higher in otoliths from AC- than M-injected fish (AC: 0.27 ± 0.02; M: 0.20 ± 0.02). For Ba, the maximum ratio was 1.07 times higher for AC compared to M-injected fish (AC: 2.61 ± 0.05; M: 2.44 ± 0.05). There was no difference in maximum ratios between injection locations for ^{26}Mg:^{24}Mg ($F_{1,69}$ = 0.03, p = 0.9).

Several 2-way interactions occurred between factors for the Sr and Ba maximum ratios. An Enrichment × Injection location interaction occurred for both ^{86}Sr:^{88}Sr ($F_{1,69}$ = 12, p = 0.001) and ^{137}Ba:^{138}Ba ($F_{1,69}$ = 5.9, p = 0.02), with AC-injected enrichment treatments returning higher ratios compared to M-injected enrichment treatments (Figs. 4 & 5). In addition, there was an Enrichment × Carrier solution interaction for ^{137}Ba:^{138}Ba ($F_{2,69}$ = 4.1, p = 0.02), with higher maximum ratios occurring in enrichment treatments for carrier solutions VM and W compared to V.

Strength of geo-element enrichment

Analysis of the number of scans and average ratio of scans above the detection limit were only analysed for ^{137}Ba:^{138}Ba and ^{86}Sr:^{88}Sr, as the ^{26}Mg:^{24}Mg enrichment did not produce enough scans with ratios above the detection limit to warrant further analyses. For the number of scans above the detection limit, carrier solution and injection location affected the strength of the ^{137}Ba:^{138}Ba geo-element enrichment (carrier solution: $F_{2,35}$ = 6.4, p = 0.005; injection location: $F_{1,35}$ = 5.6, p = 0.03; Fig. 4). AC returned a greater number of scans above the detection limit than M injection, and the VM and W carrier solutions returned a greater number of scans above the detection limit compared to V (post hoc Tukey HSD: VM > V, p = 0.02; W > V, p = 0.006). No difference was found for the number of scans between enrichment treatments for carrier solution or injection location for 86Sr:88Sr (carrier solution: $F_{2,35}$ = 0.1, p = 0.09; injection location: $F_{1,35}$ = 3.1, p = 0.09; Fig. 5).

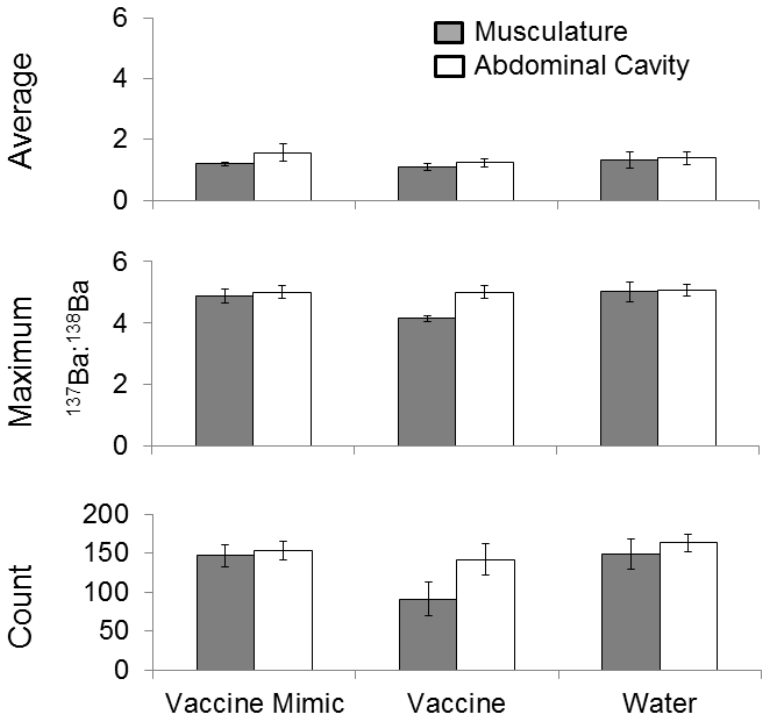


Fig. 4. *Salmo salar*. Strength of mark uptake with ^{137}Ba enrichment. Bar graphs show: (a) average ratio, (b) maximum ratio for scans above detection limit, and (c) number of scans above detection limit. Error bars represent ± 1 SE

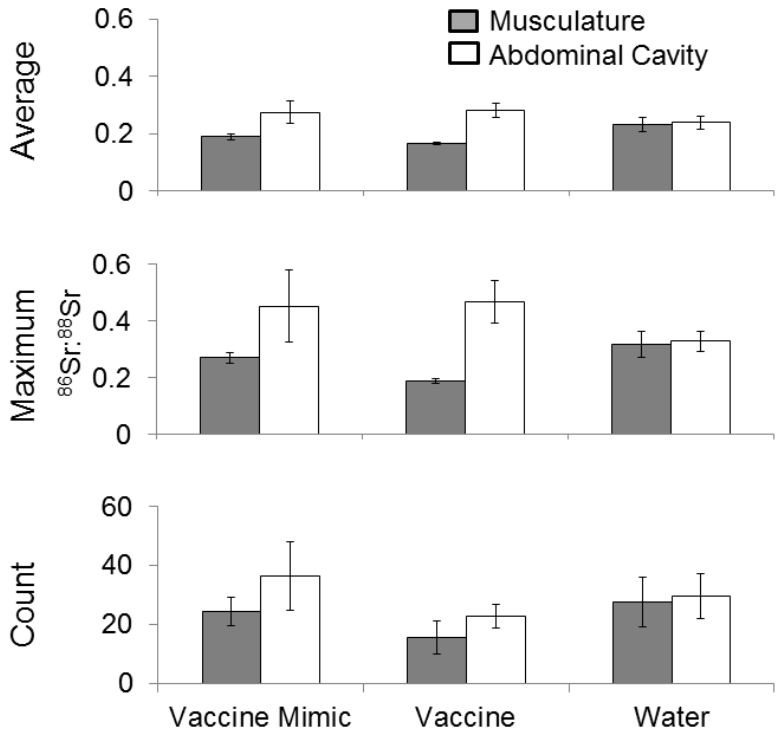


Fig. 5. *Salmo salar*. Strength of mark uptake with ^{86}Sr enrichment. Bar graphs show: (a) average ratio, (b) maximum ratio for scans above detection limit, and (c) number of scans above detection limit. Error bars represent ± 1 SE

Average ratios for scans above the detection limit highlighted the importance of injection location when using Sr geo-element enrichment; AC produced a higher mean ratio than M injection for ^{86}Sr:^{88}Sr (AC: 0.26 ± 0.11 [mean ± SE]; M: 0.19 ± 0.11; $F_{1,35}$ = 18, p < 0.001). In addition, an interaction between carrier solution and injection location for ^{86}Sr:^{88}Sr ($F_{2,35}$ = 3.7, p = 0.04) showed there was higher average geo-element uptake for VM and V compared to W with AC compared to M injection (Fig. 5). No differences in the average ratio for scans above the detection limit were found between carrier solutions or injection locations for ^{137}Ba:^{138}Ba (carrier solution: $F_{2,35}$ = 1.3, p = 0.3; injection location: $F_{1,35}$ = 3.2, p = 0.09) (Fig. 4).

Effect of treatment on mortality and condition

No signs of morbidity or mortalities were recorded during the experiment and there were no detectable changes in fish condition due to geo-element enrichment, injection location, or carrier solution (Fulton's condition factor *K*: enrichment: $F_{1,71}$ = 0.4, p = 0.5; injection location: $F_{1,71}$ = 1.2, p = 0.3; carrier solution: $F_{2,71}$ = 0.9, p = 0.4).

Baseline geo-element ratios for Atlantic salmon in Norwegian rivers

Baseline ratios for ^{137}Ba:^{138}Ba, ^{86}Sr:^{88}Sr and ^{26}Mg:^{24}Mg varied little across the 22 rivers surveyed (Fig. 6; Table A1 in the Appendix). Among-river ratios ranged from 0.156 to 0.159 for ^{137}Ba:^{138}Ba, 0.108 to 0.121 for ^{86}Sr:^{88}Sr, and 0.086 to 0.136 for ^{26}Mg:^{24}Mg. In addition, baseline ratios varied little among the 6 random years between 1990 and 2010 analysed from each of the Saltdalselva and Strynselva rivers (Table A2 in the Appendix). Among-year ratios ranged from 0.157 to 0.158 for ^{137}Ba:^{138}Ba, 0.109 to 0.121 for ^{86}Sr:^{88}Sr, and 0.094 to 0.131 for ^{26}Mg:^{24}Mg. The range of baseline ratios, among rivers and years, were all within the mean ± 2.5 SD of control ratios observed in the vaccination trial (vaccine control ratios: ^{137}Ba:^{138}Ba: 0.158 ± 0.037; ^{86}Sr:^{88}Sr: 0.121 ± 0.012; ^{26}Mg:^{24}Mg: 0.103 ± 0.035). This suggests that unmarked farmed Atlantic salmon parr have similar geo-elemental ratios to that of wild Atlantic salmon in the rivers of Norway. Therefore, all wild salmon had otolith geo-element ratios that would be scored as unmarked using our method.

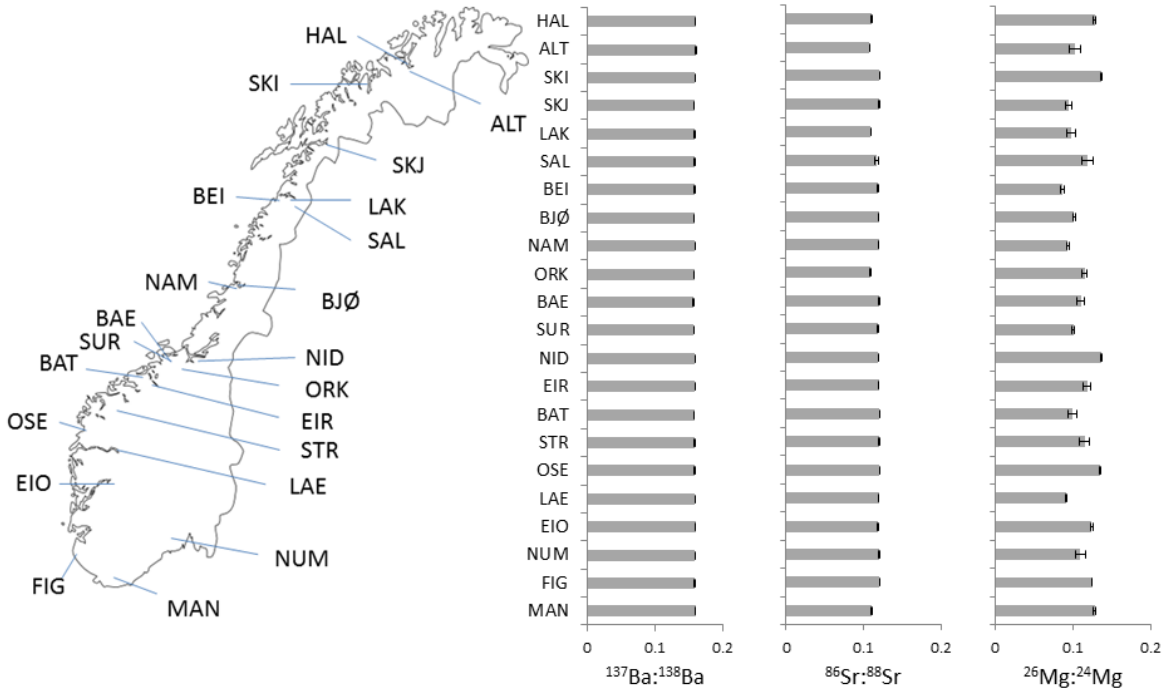


Fig. 6. Baseline geo-element ratios of Norwegian *Salmo salar* parr surveyed from 22 rivers across Norway. Bars represent the average ratios of 3 fish from each river sampled, except for Saltdalselva (SAL) and Strynselva (STR), where bars represent the average ratios of 18 fish sampled between 1990 and 2010. Error bars represent ± 1 SE. See Table A1 in the Appendix for abbreviations

Discussion

Injecting with enriched ^{137}Ba and ^{86}Sr was 100% effective in significantly changing the ^{137}Ba:^{138}Ba and ^{86}Sr:^{88}Sr ratios in the otoliths of farmed Atlantic salmon parr for both injection locations (AC and M)

and with all 3 carrier solutions (W, V and VM). In addition, natural geo-element enrichment appears to have no short-term effects on fish condition or survival rate. Furthermore, the Ba and Sr geo-element ratios created in marked experimental farmed fish in this study were uniquely different from observed natural ratios of Ba and Sr in wild salmon parr from the 22 rivers across Norway. These findings indicate that mass-marking with Ba and Sr natural geo-elements via vaccination injection has the potential to be a 100% effective fish-identification technique. Furthermore, this technique could be developed to produce multi-elemental fingerprint codes in otoliths. If adopted at a whole-of-industry scale, this technique could be used to differentiate farmed and wild fish and to identify the source farm of escaped Atlantic salmon.

Mark success

An unambiguous mark is critical for accurate fish identification, particularly when low numbers of tagged fish are caught during mark-recapture surveys. Here, 100% mark success was achieved using enriched ^{137}Ba and ^{86}Sr at a concentration of 2 µg of geo-element per gram of average fish weight. Other research has shown 100% mark success can be achieved using lower concentrations of natural geo-element when using a transgenerational geo-element marking technique, such as 0.5 µg of ^{137}Ba per gram brood fish in brown-marbled groper *Epinephelus fuscoguttatus* (Williamson et al. 2009b) and 0.3 µg of ^{137}Ba per gram brood fish in brown trout *Salmo trutta* (Huelga-Suarez et al. 2012). Hence, it may be possible to use ^{137}Ba and ^{86}Sr geo-elements at 10 to 100 times lower concentrations and still achieve 100% mark success via vaccination, which would greatly reduce the amount of geo-element, and thus cost, required for marking.

Mark success for ^{26}Mg was poor and varied greatly across treatments (0 to 34%), indicating that ^{26}Mg enrichment is not suitable for marking parr. The mark success rate for Mg observed in this experiment is lower compared to that of Woodcock et al. (2011a), who tagged golden perch *Macquaria ambigua* with ^{26}Mg and achieved approximately 60% mark success using a larval immersion technique. Mg appears to be self-regulated in salmonids and may be sourced from either food or water (Shearer & Åsgård 1992). In addition, Mg has a slow exchange rate in body tissue compared to calcium, and only 1 to 2% of Mg ions are transported into the endolymph fluid (Maguire & Cowan 2002) in which otoliths are encapsulated. A combination of these factors and the likelihood that farmed fed salmon parr used in our experiment were not deficient in total Mg suggests that either a concentration of 2 µg g^{-1} fish mass of ^{26}Mg was insufficient for achieving 100% mark success, or the time between injection and sampling of the otoliths may have been too short (14 d) for sufficient uptake of ^{26}Mg to occur.

Mark strength

We quantified the strength of the geo-element markers by comparing the average ratio and total number of scans above the threshold ratio in each enrichment treatment. Overall, injection into the abdominal cavity returned stronger and more consistent marks for ^{137}Ba and ^{86}Sr compared to injecting into the musculature. This may simply be due to better retention of the carrier solutions in the abdominal cavity compared to the musculature. Leakage of the solution from the musculature injection site was observed post-injection, whereas no visible leakage occurred for the abdominal cavity injection site (F. Warren-Myers & T. Dempster pers. obs.). An alternate possibility is that the biological pathways for Ba and Sr ion transport from the abdominal cavity to the endolymph fluid surrounding the otolith may be more efficient or direct compared to ion transport from musculature tissue.

The strength of mark uptake for ^{137}Ba and ^{86}Sr enrichment was influenced by carrier solution in addition to injection location by the number of scans with ratios above detection, but not the average

ratio of scans above detection. The number of scans with ratios above the detection limit suggests carrier solutions that contained 50% (VM) and 100% (W) water produced a stronger mark than V (3.5% water) for ^{137}Ba:^{138}Ba, which may imply that water is a more efficient medium for delivering barium geo-elements via injection. However, the opposite effect was found for ^{86}Sr:^{88}Sr, with carrier solutions showing no difference in number of scans with ratios above the detection limit, but average ratios indicating carrier solutions with lower water content (VM: 50%, and V: 3.5%) may enhance strontium geo-element enrichment.

Fish condition and survival

A mass-marking method that does not compromise fish health or growth rate is an ideal prerequisite for a marking program. Parameters monitored in this trial to assess fish health (Fulton's condition factor *K*, survival rate) indicated there were no negative health effects of enriched natural geo-element marking on Atlantic salmon parr 70 d post-injection. Previous experiments that have used natural geo-elements to mark fish by other delivery methods, such as transgenerational and immersion, have similarly detected no negative short-term effects of natural geo-element treatments on survival and growth (Munro et al. 2009, Williamson et al. 2009a, Woodcock et al. 2011a,b), although possible effects may occur for different fish species (Starrs et al. 2014). While we have no *a priori* reason to expect that natural geo-element marking via vaccination injection should have any detrimental long-term effects on fish growth and condition in salmon, longer-term, larger-scale trials are required before the technique can be adopted as a mass-marking method for use on millions of fish.

Baseline ratio comparisons

Geo-element ratios of ^{137}Ba:^{138}Ba, ^{86}Sr:^{88}Sr and ^{26}Mg:^{24}Mg are typically highly conserved in natural waters. This was reflected in the ratios observed in wild salmon parr collected from Norwegian rivers in both space (22 rivers) and time (1990 to 2010; Saltdalselva and Strynselva). Ratios varied by less than 2% for ^{137}Ba:^{138}Ba and less than 10% for ^{86}Sr:^{88}Sr. As natural variation of less than 3% in the geo-element ratios of ^{86}Sr:^{87}Sr has been used to separate natal habitats in some fish species with up to 80% correct assignment (e.g. Kennedy et al. 2000, 2002, Veinott & Porter 2005), our results suggest that Sr geo-element ratios could be a useful tool for investigating migratory behaviour and the degree of philopatry in wild Atlantic salmon populations in Norway. Importantly, all natural background ratios were within 2.5 SDs of control fish analysed in the vaccination trial, indicating that no wild salmon would have been falsely assessed as being a marked farm-reared escapee. Conservatively, to ensure an artificial geo-elemental mark is not mistaken for a natural geo-elemental signature, the ratios in marked fish otoliths should be well above that of natural background variation to guarantee correct fish identification.

Optimisation of enriched natural geo-element otolith fingerprinting during vaccination

In the present study, the enriched geo-element treatments shifted the geo-elemental ratios of ^{137}Ba:^{138}Ba and ^{86}Sr:^{88}Sr by 2 to 3 orders of magnitude compared to the experimental controls and the natural baseline ratios. This is well above the conservative threshold of 3.3 SDs which we set as the level to determine mark success with 100% accuracy, which suggests the amount of geo-element used for enrichment could be reduced. Optimisation of the minimum required concentration of geo-elements needed to create a marker is required to confirm if this method is cost-effective for mass-marking, while still ensuring marks are uniquely different from wild salmon. Further investigation using the commercial vaccine MINOVA 6 with other geo-elements, e.g. ^{134}Ba, ^{135}Ba, ^{136}Ba and ^{87}Sr, would determine the feasibility of creating multiple combinations of natural geo-element markers (e.g. Munro et al. 2008, Woodcock et al. 2011a,b) using the vaccination-based delivery method.

Application of enriched natural geo-element otolith fingerprinting during vaccination

Farmed fish, including salmon, escape from aquaculture facilities and enter the wild (Ø. Jensen et al. 2010, Jackson et al. 2012, A. J. Jensen et al. 2013), with subsequent ecological and/or evolutionary effects on wild fish populations (Fleming et al. 2000, McGinnity et al. 2003). A marking technique that enabled tracing of escapees back to the farm of origin would provide greater insight into the causes of escape events (Jensen et al. 2010), better capacity for regulatory bodies to determine the level of under-reporting, and improvement of enforcement of compliance measures (Fiske et al. 2006). An ideal marking technique should meet the following criteria: (1) sufficient unique marks to be useful at a whole-of-industry scale; (2) 100% correct mark detection; (3) an efficient and cost-effective method of application; and (4) no negative side effects on production parameters or fish health. The natural geo-element marking via vaccination technique trialed in this study has the potential to meet these criteria. If all fish in the salmon farming industry were vaccinated, geo-element markers could be added during the vaccine production phase prior to being delivered to commercial farms, thus ensuring no extra manual labour costs to fish farmers for the purpose of marking and monitoring all farmed Atlantic salmon.

References

Adey EA, Black KD, Sawyer T, Shimmield TM, Trueman CN (2009) Scale microchemistry as a tool to investigate the origin of wild and farmed Salmo salar. Mar Ecol Prog Ser 390:225–235 doi:10.3354/meps08161

Almany GR, Berumen ML, Thorrold SR, Planes S, Jones GP (2007) Local replenishment of coral reef fish populations in a marine reserve. Science 316:742–744 PubMed doi:10.1126/science.1140597

Barbee NC, Swearer SE (2007) Characterizing natal source population signatures in the diadromous fish Galaxias maculatus, using embryonic otolith chemistry. Mar Ecol Prog Ser 343:273–282 doi:10.3354/meps06886

Barnett-Johnson R, Ramos FC, Grimes CB, MacFarlane RB (2005) Validation of Sr isotopes in otoliths by laser ablation multicollector inductively coupled plasma mass spectrometry (LA-MC-ICPMS): opening avenues in fisheries science applications. Can J Fish Aquat Sci 62:2425–2430 doi:10.1139/f05-194

Barnett-Johnson R, Grimes CB, Royer CF, Donohoe CJ (2007) Identifying the contribution of wild and hatchery Chinook salmon (Oncorhynchus tshawytscha) to the ocean fishery using otolith microstructure as natural tags. Can J Fish Aquat Sci 64:1683–1692 doi:10.1139/f07-129

Bath GE, Thorrold SR, Jones CM, Campana SE, McLaren JW, Lam JWH (2000) Strontium and barium uptake in aragonitic otoliths of marine fish. Geochim Cosmochim Acta 64:1705–1714 doi:10.1016/S0016-7037(99)00419-6

Buckland-Nicks JA, Gillis M, Reimchen TE (2012) Neural network detected in a presumed vestigial trait: ultrastructure of the salmonid adipose fin. Proc R Soc Lond B Biol Sci 279:553–563 PubMed doi:10.1098/rspb.2011.1009

Campana SE (2005) Otolith elemental composition as a natural marker for fish stocks. In: Cadrin SX, Frieland KD, Waldman JR (eds) Stock identification methods: applications in fishery science. Elsevier Academic Press, Burlington, MA, p 227–245

Collins MR, Smith TIJ, Heyward LD (1994) Effectiveness of six methods for marking juvenile shortnose sturgeons. Prog Fish-Cult 56:250–254 doi:10.1577/1548-8640(1994)056<0250:EOSMFM>2.3.CO;2

Crook DA, O'Mahony DJ, Sanger AC, Munro AR, Gillanders BM, Thurstan S (2009) Development and evaluation of methods for osmotic induction marking of golden perch Macquaria ambigua with calcein and alizarin red S. N Am J Fish Manage 29:279–287 doi:10.1577/M07-224.1

Elsdon TS, Gillanders BM (2004) Fish otolith chemistry influenced by exposure to multiple environmental variables. J Exp Mar Biol Ecol 313:269–284 doi:10.1016/j.jembe.2004.08.010

Farrell J, Campana SE (1996) Regulation of calcium and strontium deposition on the otoliths of juvenile tilapia, Oreochromis niloticus. Comp Biochem Physiol A 115:103–109 doi:10.1016/0300-9629(96)00015-1

Fiske P, Lund RA, Hansen LP (2006) Relationships between the frequency of farmed Atlantic salmon, Salmo salar L., in wild salmon populations and fish farming activity in Norway, 1989-2004. ICES J Mar Sci 63:1182–1189 doi:10.1016/j.icesjms.2006.04.006

Fleming IA, Hindar K, Mjølnerød IB, Jonsson B, Balstad T, Lamberg A (2000) Lifetime success and interactions of farm salmon invading a native population. Proc R Soc Lond B Biol Sci 267:1517–1523 PubMed doi:10.1098/rspb.2000.1173

Gillanders BM (2005) Using elemental chemistry of fish otoliths to determine connectivity between estuarine and coastal habitats. Estuar Coast Shelf Sci 64:47–57 doi:10.1016/j.ecss.2005.02.005

Glover KA (2010) Forensic identification of fish farm escapees: the Norwegian experience. Aquacult Environ Interact 1:1–10 doi:10.3354/aei00002

Glover KA, Skilbrei OT, Skaala Ø (2008) Genetic assignment identifies farm of origin for Atlantic salmon Salmo salar escapees in a Norwegian fjord. ICES J Mar Sci 65:912–920 doi:10.1093/icesjms/fsn056

Huelga-Suarez G, Moldovan M, Garcia-Valiente A, Garcia-Vazquez E, Garcia Alonso JI (2012) Individual-specific transgenerational marking of fish populations based on a barium dual-isotope procedure. Anal Chem 84:127–133 PubMed doi:10.1021/ac201946k

Jackson D, Drumm A, McEvoy S, Jensen Ø and others (2012) Chapter 2. A pan-European evaluation of the extent, causes and cost of escape events from sea-cage fish farming. In: PREVENT ESCAPE Project Compendium. 7th Research Framework Program, Commission of the European Communities, Brussels. www.preventescape.eu

Jensen Ø, Dempster T, Thorstad EB, Uglem I, Fredheim A (2010) Escapes of fishes from Norwegian sea-cage aquaculture: causes, consequences and prevention. Aquacult Environ Interact 1:71–83 doi:10.3354/aei00008

Jensen AJ, Karlsson S, Fiske P, Hansen LP, Hindar K, Østborg GM (2013) Escaped farmed Atlantic salmon grow, migrate and disperse throughout the Arctic Ocean like wild salmon. Aquacult Environ Interact 3:223–229 doi:10.3354/aei00064

Jones GP, Milicich MJ, Emsile MJ, Lunow C (1999) Self-recruitment in a coral reef fish population. Nature 402:802–804 doi:10.1038/45538

Kalish JM (1990) Use of otolith microchemistry to distinguish the progeny of sympatric anadromous and non-anadromous salmonids. Fish Bull 88:657–666

Kennedy BP, Blum JD, Folt CL, Nislow KH (2000) Using natural strontium isotopic signatures as fish markers: methodology and application. Can J Fish Aquat Sci 57:2280–2292 doi:10.1139/f00-206

Kennedy BP, Klaue A, Blum JD, Folt CL, Nislow KH (2002) Reconstructing the lives of fish using Sr isotopes in otoliths. Can J Fish Aquat Sci 59:925–929 doi:10.1139/f02-070

Maguire ME, Cowan JA (2002) Magnesium chemistry and biochemistry. Biometals 15:203–210 PubMed doi:10.1023/A:1016058229972

McGinnity P, Prodohl P, Ferguson A, Hynes R and others (2003) Fitness reduction and potential extinction of wild populations of Atlantic salmon, Salmo salar, as a result of interactions with escaped farm salmon. Proc R Soc Lond B Biol Sci 270:2443–2450 PubMed doi:10.1098/rspb.2003.2520

Munro AR, McMahon TE, Leathe SA, Liknes G (2003) Evaluation of batch marking small rainbow trout with coded wire tags. N Am J Fish Manag 23:600–604 doi:10.1577/1548-8675(2003)023<0600:EOBMSR>2.0.CO;2

Munro AR, Gillanders BM, Elsdon TS, Crook DA, Sanger AC (2008) Enriched stable isotope marking of juvenile golden perch (Macquaria ambigua) otoliths. Can J Fish Aquat Sci 65:276–285 doi:10.1139/f08-010

Munro AR, Gillanders BM, Thurstant S, Crook DA, Sanger AC (2009) Transgenerational marking of freshwater fishes with enriched stable isotopes: a tool for fisheries management and research. J Fish Biol 75:668–684 PubMed doi:10.1111/j.1095-8649.2009.02352.x

Serafy JE, Lutz SJ, Capo TR, Ortner PB, Lutz PL (1995) Anchor tags affect swimming performance and growth of juvenile red drum (Sciaenops ocellatus). Mar Freshw Behav Physiol 27:29–35 doi:10.1080/10236249509378951

Shearer KD, Åsgård T (1992) The effect of water-borne magnesium on the dietary magnesium requirement of the rainbow trout (Oncorhynchus mykiss). Fish Physiol Biochem 9:387–392 PubMed doi:10.1007/BF02274219

Smith KT, Whitledge GW (2011) Evaluation of a stable-isotope labelling technique for mass marking fin rays of age-0 lake sturgeon. Fish Manag Ecol 18:168–175 doi:10.1111/j.1365-2400.2010.00771.x

Starrs D, Davis JT, Schlaefer J, Ebner BC, Eggins SM, Fulton CJ (2014) Maternally transmitted isotopes and their effects on larval fish: a validation of dual isotopic marks within a meta-analysis context. Can J Fish Aquat Sci 71:387–397 doi:10.1139/cjfas-2013-0416

Swearer SE, Caselle JE, Lea DW, Warner RR (1999) Larval retention and recruitment in an island population of a coral reef fish. Nature 402:799–802 doi:10.1038/45533

Thorrold SR, Jones GP, Planes S, Hare JA (2006) Transgenerational marking of embryonic otoliths in marine fishes using barium stable isotopes. Can J Fish Aquat Sci 63:1193–1197 doi:10.1139/f06-048

Vander Haegen GE, Blankenship HL, Hoffmann A, Thompson DA (2005) The effects of adipose fin clipping and coded wire tagging on the survival and growth of spring chinook salmon. N Am J Fish Manag 25:1161–1170 doi:10.1577/M04-011.1

Veinott G, Porter R (2005) Using otolith microchemistry to distinguish Atlantic salmon (Salmo salar) parr from different natal streams. Fish Res 71:349–355 doi:10.1016/j.fishres.2004.09.004

Volk EC, Schroder SL, Grimm JJ (1999) Otolith thermal marking. Fish Res 43:205–209 doi:10.1016/S0165-7836(99)00073-9

Walther BD, Limburg KE (2012) The use of otolith chemistry to characterize diadromous migrations. J Fish Biol 81:796–825 PubMed doi:10.1111/j.1095-8649.2012.03371.x

Williamson DH, Jones GP, Thorrold SR, Frisch AJ (2009a) Transgenerational marking of marine fish larvae: stable isotope retention, physiological effects and health issues. J Fish Biol 74:891–905 PubMed doi:10.1111/j.1095-8649.2008.02176.x

Williamson DH, Jones GP, Thorrold SR (2009b) An experimental evaluation of transgenerational isotope labelling in a coral reef grouper. Mar Biol 156:2517–2525 doi:10.1007/s00227-009-1276-0

Woodcock SH, Gillanders BM, Munro AR, McGovern F, Crook DA, Sanger AC (2011a) Using enriched stable isotopes of barium and magnesium to batch mark otoliths of larval golden perch (Macquaria ambigua, Richardson). Ecol Freshw Fish 20:157–165 doi:10.1111/j.1600-0633.2010.00475.x

Woodcock SH, Gillanders BM, Munro AR, Crook DA, Sanger AC (2011b) Determining mark success of 15 combinations of enriched stable isotopes for the batch marking of larval otoliths. N Am J Fish Manag 31:843–851 doi:10.1080/02755947.2011.623760

Woodcock SH, Munro AR, Crook DA, Gillanders BM (2012) Incorporation of magnesium into fish otoliths: determining contribution from water and diet. Geochim Cosmochim Acta 94:12–21 doi:10.1016/j.gca.2012.07.003

Woodcock SH, Grieshaber CA, Walther BD (2013) Dietary transfer of enriched stable isotopes to mark otoliths, fin rays and scales. Can J Fish Aquat Sci 70:1–4 doi:10.1139/cjfas-2012-0389

WP 2.1 Mass marking farmed Atlantic salmon with transgenerational geo-elemental fingerprints during vaccination – An industry-scale mass marking technique for tracing farmed fish escapees

ABSTRACT: Farmed fish escape and enter the environment with subsequent effects on wild populations. Reducing escapes requires the ability to trace individuals back to the point of escape, so that escape causes can be identified and technical standards improved. Here, we tested if natural geo-element otolith fingerprint marks delivered during routine vaccination could be an accurate, feasible and cost effective marking method, suitable for industrial-scale application. We tested seven natural geo-elements, ^{134}Ba, ^{135}Ba, ^{136}Ba, ^{137}Ba, ^{86}Sr, ^{87}Sr and ^{26}Mg, on farmed Atlantic salmon reared in freshwater, in experimental conditions designed to reflect commercial practice. Marking was 100% successful with individual Ba geo-elements at concentrations as low as 0.001 µg. g^{-1} fish and for Sr geo-elements at 1 µg. g^{-1} fish. Our results suggest that 63 unique fingerprint marks can be made at low cost using Ba (0.0002 – 0.02 $US per mark) and Sr (0.46 – 0.82 $US per mark) geo-elements. Natural geo-element fingerprinting during vaccination is feasible for commercial application if applied at a company level within the world's largest salmon producing nations. Introducing a mass marking scheme would enable tracing of escapees back to point of origin, which could drive greater compliance, better farm design and improved management practices to reduce escapes.

Introduction

Farmed fish escapees from sea-cage aquaculture are perceived as a serious threat to wild fish populations as they can cause damaging ecological impacts. These include transfer of diseases to wild fish (Glover et al. 2013b), introduction and establishment of escapees as exotic species (Toledo-Guedes et al. 2001), competition between escapees and wild stocks (Fleming et al. 2000, McGinnity et al. 2003), and outbreeding depression through genetic mixing of wild and farmed populations from hybrid crosses (Hindar et al. 2006, Hutchings & Fraser 2008).

Atlantic salmon (*Salmo salar*) is the most commonly occurring farmed fish escapee from sea-cage aquaculture (Jensen et al. 2010). For instance, 4.6 million salmon were reported to have escaped from Norwegian fish farms from 2001-2012 (http://www.fiskeridir.no/) and escapes occur in all salmon farming countries. Although most farmed escaped salmon disappear, never to be observed again (Hansen 2006, Skilbrei 2010a,b), some survive and migrate into rivers and onto the spawning grounds of native populations (Fiske et al. 2006). As a result of farmed salmon successfully spawning with wild salmon, genetic changes have been observed in native salmon populations in Ireland (Crozier 2000) and Norway (Glover et al. 2012), with introgression of farmed salmon estimated at 0-47% for 20 native populations spanning the entire Norwegian coastline (Glover et al. 2013a). Introgression of farmed salmon in native populations is of significant concern because their offspring display reduced survival in the wild compared to wild salmon (McGinnity et al. 1997, Fleming et al. 2000, Skaala et al. 2012), and may also disrupt local adaptations (Garcia de Leaniz et al. 2007).

Although fish farmers in many jurisdictions are obliged to report escapes, in some cases, escapes of farmed fish are not reported to the authorities. Under reporting is problematic, as without an understanding of why fish escape through technical investigations of escape causes, improvements to technical standards cannot be made rapidly (Jensen et al. 2010). Detecting escapees and determining the farm they originated from is possible through DNA-based methods (Glover 2010) or fatty acid profiling (Martinez et al. 2009), although these methods do not identify the farm in all cases. As an alternative, a permanent coded mark or tag for all farm fish applied at an industry scale would allow for a fail-safe method to trace escapees to their point of origin. Numerous methods currently exist to mark fish (e.g. adipose fin clipping and physical tags (Vander Haegen et al. 2005); otolith thermal marking (Volk et al. 1999); fluorescent markers (Crook et al. 2009)), but all fail in one or more aspects related to the ability to deliver 100% traceability to point of origin, fish welfare considerations or cost-effectiveness at industry scale.

Here, we advance a recently developed marking technique for identifying and tracing farmed Atlantic salmon escapees using natural geo-element otolith fingerprint markers, delivered during vaccination (Warren-Myers et al. 2014), by testing multiple combinations of seven enriched geo-elements over a concentration gradient, to determine if the technique can be feasibly applied at full industrial scale where up to 500 million fish require marking each year. Marking during routine vaccination could effectively and accurately mark all farmed fish in commercial facilities with no additional manual handling or labour costs. Typically, Atlantic salmon are routinely vaccinated during the parr stage with multi-vaccines against a range of pathogens (Bjørn et al. 2013). Otolith fingerprinting during vaccination is 100% successful using enriched natural geo-elements ^{137}Ba and ^{86}Sr at high concentrations, and marginally successful (0 to 35%) with enriched ^{26}Mg (Warren-Myers et al. 2014). Otolith fingerprinting via larval immersion on other species suggests that the use of additional natural geo-elements of Ba, Sr and Mg could produce over 100 possible otolith fingerprint combinations via vaccination (Woodcock et al. 2011a,b). Whether these combinations produce viable marks and what minimum dosages are possible for cost-effectiveness for marking during vaccination must be determined to make this marking technique financially feasible for industry-scale application.

Here, we tested seven enriched natural geo-elements (^{134}Ba, ^{135}Ba, ^{136}Ba, ^{137}Ba, ^{86}Sr, ^{87}Sr, and ^{26}Mg) at 4 concentration levels (1, 0.1, 0.01 and 0.001 µg. g^{-1} fish) in fingerprint combinations of 1, 4 or 7, which could provide 127 unique marks. To make the experiment industry-relevant, we followed standard commercial farming practices for salmon. Moreover, we monitored the health and welfare of all marked fish until they grew to harvest size (5 kg) to determine if marked fish had similar condition and welfare to unmarked control fish and that the concentrations of natural geo-elements of Ba, Sr and Mg used in this study are harmless for farmed salmon.

Methods

Ethics statement

This study was conducted in accordance with the laws and regulations of the Norwegian Regulation on Animal Experimentation 1996. The protocol was approved by the Norwegian Animal Research Authority (Ethics permit number: 6176).

Experimental location and fish

The experiment was conducted at the Institute of Marine Research, Matre Research Station, in Masfjorden, western Norway (60°N). A total of 650 Atlantic salmon (AquaGen strain) parr (mean ± SE: fork length = 19.8 ± 0.04 cm; weight = 103 ± 0.6 g) were used in the experiment. All fish were pit tagged with 11 mm Trovan ID 101 tags (BTS Scandinavia AB, Sweden) four months prior to the experiment, and reared in freshwater tanks buffered with saltwater to a salinity of 0.7 g NaCl.L^{-1} in standard commercial hatchery conditions. Fish in all treatments were of similar length and weight at day 1 of the experiment (one-way ANOVA; length; $F_{12, 649} = 1.32$, $p = 0.2$, weight; $F_{12, 649} = 0.87$, $p = 0.6$).

Experimental design

We tested three combinations of the enriched natural geo-elements, ^{134}Ba, ^{135}Ba, ^{136}Ba, ^{137}Ba, ^{86}Sr, ^{87}Sr and ^{26}Mg (Oak Ridge National Laboratory; www.ornl.gov) at four concentrations (1, 0.1, 0.01, or 0.001 µg of each geo-element per g of parr average weight) to determine the minimum geo-element concentrations required to ensure 100% mark success of geo-element fingerprint tags delivered during vaccination (Warren-Myers et al. 2014). Atlantic salmon parr (50 per treatment) were injected with the multi vaccine MINOVA 6 (NORVAX MINOVA 6, Global Aquatic Animal Health, Bergen, Norway) that contained either: 1) no geo-element enrichment; 2) enriched ^{137}Ba; 3) a combination of enriched ^{135}Ba, ^{136}Ba, ^{137}Ba and ^{86}Sr; or 4) a combination of enriched ^{134}Ba, ^{135}Ba, ^{136}Ba, ^{137}Ba, ^{86}Sr, ^{87}Sr and ^{26}Mg (Table 1).

Enriched natural geo-elements in powder chloride form ($BaCl_2$, $SrCl_2$ & $MgCl_2$) used for the geo-element enrichment treatments were first dissolved in Milli-Q water to make standard stock solutions of each geo-element combination (i.e., 1, 4, or 7 geo-element markers). The required geo-element combination-by-concentration solutions were then mixed firstly by pipetting the appropriate amounts from the standard stock solutions into a 1 ml eppendorf tube and then mixing this solution with the MINOVA 6 vaccine on the day of vaccination. Final solutions were agitated for 30 seconds using a Virvel Mixer (Heidolph Instruments Gmbh & Co.KG) to ensure a stable solution. Injections (0.1 ml per fish) were given into the abdominal cavity, approximately 20 mm behind the pectoral fin on the ventral side of parr using a standard commercial vaccination gun (Socorex Swiss-167; www.socorex.com) fitted with a 5 mm, 27 gauge vaccination needle.

On the day of vaccination, fish were anaesthetised with Benzoak VET (dose 0.2 ml L^{-1} of clean hatchery water), identified by their PIT tag number, then weighed, measured (fork length) and injected. After injection, fish were placed into one of five 1000 litre tanks with equal interspersion of individuals among treatments within each tank (i.e. 130 fish per tank, 10 from each treatment). The fish were reared under a 12 h light : 12 h dark photoperiod for the first six weeks post injection before being switched to 24 hours continuous light to induce smoltification. To monitor differences in growth and condition among treatments, 90 days post-injection, all fish (n = 50 per treatment) were anaesthetised, weighed, and measured (fork length). At this time, a randomly selected sub-sample of 10 fish per treatment, were euthanized by anaesthetic overdose and their otoliths were removed for geo-element analysis. Sagittal

otoliths (mean ± SE: maximum diameter = 3.3 ± 0.1 mm) were cleaned of any adhering tissue, air dried, and stored individually in plastic tubes. Remaining fish were transferred to a sea cage farm and grown to commercial harvest size (~ 5 kg, 570 days post-injection), then humanely culled with a quick blow to the head, measured (fork length) and weighed, to assess condition at harvest.

Table 1. Experimental design

Factors		**Injection**	**Sample sizes**		
Geo-element fingerprint mark	Concentration (µg. g^{-1} fish)	Total geo-element used (µg) in 0.1 ml injection per 40 g fish	Total treatment (N)	Growth analysis (N)	Otolith analysis (N)
No fingerprint (control)	0	0	50	50	10
^{137}Ba	1	40	50	50	10
^{137}Ba	0.1	4	50	50	10
^{137}Ba	0.01	0.4	50	49	10
^{137}Ba	0.001	0.04	50	50	10
^{137}Ba, ^{136}Ba, ^{135}Ba, ^{86}Sr	1	160	50	50	10
^{137}Ba, ^{136}Ba, ^{135}Ba, ^{86}Sr	0.1	16	50	50	9
^{137}Ba, ^{136}Ba, ^{135}Ba, ^{86}Sr	0.01	1.6	50	50	10
^{137}Ba, ^{136}Ba, ^{135}Ba, ^{86}Sr	0.001	0.16	50	50	10
^{137}Ba, ^{136}Ba, ^{135}Ba, ^{134}Ba, ^{87}Sr, ^{86}Sr, ^{26}Mg	1	280	50	50	10
^{137}Ba, ^{136}Ba, ^{135}Ba, ^{134}Ba, ^{87}Sr, ^{86}Sr, ^{26}Mg	0.1	28	50	49	9
^{137}Ba, ^{136}Ba, ^{135}Ba, ^{134}Ba, ^{87}Sr, ^{86}Sr, ^{26}Mg	0.01	2.8	50	50	10
^{137}Ba, ^{136}Ba, ^{135}Ba, ^{134}Ba, ^{87}Sr, ^{86}Sr, ^{26}Mg	0.001	0.28	50	49	10

Design of the experiment to test mark success and strength through introducing geo-element fingerprint combinations of one, four or seven enriched natural geo-elements at four concentrations during routine vaccination. Sample sizes of fish per treatment and those used for growth analyses and otoliths analyses are shown.

Otolith preparation

Otoliths were prepared as per Warren-Myers et al. (2014). Sagittal otoliths were cleaned of any remaining organic tissue by immersing in a solution of ultrapure 15% H_2O_2 buffered with 0.1 M NaOH.

Following immersion, otoliths were ultra-sonicated (Sonic Clean 250HT) for 5 minutes and then left for 6 hours in the cleaning solution. The cleaning solution was then aspirated off and the otoliths were transferred through three Milli-Q water rinses, each of which consisted of 5 minutes of ultra-sonification and 30 minutes resting time. Otoliths were then air dried in a laminar flow bench for at least 24 hours. Once dry, one otolith per fish was fixed, sulcus side down, onto gridded microscope slides using quick dry cyanoacrylate glue.

Otolith analysis

Natural geo-element analyses were done on a Varian 7700x Inductively Coupled Plasma Mass Spectrometer (ICP-MS) fitted with a HelEx (Laurin Technic and the Australian National University) laser ablation (LA) system constructed around a Compex 110 (Lambda Physik) excimer laser operating at 193 nm. 612 and 610 NIST (National Institute of Standards and Technology) glass standards doped with trace elements at known concentrations was used to calibrate the system. External precision estimates (%RSD) based on 20 analyses of a MACS3 microanalytical carbonate standard were as follows: ^{134}Ba:^{138}Ba = 7.37; ^{135}Ba:^{138}Ba = 0.81, ^{136}Ba:^{138}Ba = 4.51, ^{137}Ba:^{138}Ba = 0.72, ^{86}Sr:^{88}Sr = 0.94, ^{87}Sr:^{88}Sr = 1.16 and ^{26}Mg:^{24}Mg = 0.60. Otoliths were run in blocks of 16 samples selected randomly from all treatments and bracketed by analyses of the standards. Samples and standards were analysed by vertically profiling in time-resolved mode, using a stationary laser with a spot size of 157 µm, an energy setting of ~ 60 mJ and a repetition rate of 10 Hz. Ablation was performed under pure He (200 ml/min) to minimise re-deposition of ablated material and the sample was then entrained into the Ar (0.95 ml/min) carrier gas flow to the ICP-MS. Using this method, we were able to quantify the concentrations of ^{134}Ba, ^{135}Ba, ^{136}Ba, ^{137}Ba, ^{138}Ba, ^{86}Sr, ^{87}Sr, ^{88}Sr, ^{24}Mg, ^{26}Mg and ^{43}Ca in the outer region of salmon pre-smolt otoliths. Data were processed off-line using a specialised MS Excel template which involved a low pass filter to remove any spikes (a single acquisition value >2x the median of the adjacent acquisitions), smoothing (a running average of 3 acquisitions) and blank subtracting functions (an acquisition = a single measure of an geo-element ratio while vertically profiling in time-resolved acquisition mode). A correction factor ($K = R_{true}/R_{obs}$, where R_{true} is the naturally occurring geo-element ratio and R_{obs} is the average geo-element ratio measured in the NIST 612 and 610 standards run before and after each set of 16 samples) was applied to all sample acquisitions to correct for mass bias. The NIST 612 was used for ^{137}Ba, ^{135}Ba, ^{87}Sr, ^{86}Sr and ^{26}Mg and NIST 610 for ^{134}Ba and ^{136}Ba. Geo-element ratios are expressed as the enriched geo-element divided by the most commonly abundant geo-element for each element used so that the measure of enrichment is always expressed as an increase in the enriched geo-element relative to the most common geo-element. Statistical analyses were conducted on the final post-processed acquisition data values.

Statistical analysis

Mark success for each treatment was evaluated using a mark detection threshold set by Warren-Myers et al. (2014). Briefly, the mark detection threshold for the geo-element ratios ^{134}Ba:^{138}Ba, ^{135}Ba:^{138}Ba, ^{136}Ba:^{138}Ba, ^{137}Ba:^{138}Ba, ^{86}Sr:^{88}Sr, ^{87}Sr:^{88}Sr and ^{26}Mg:^{24}Mg were calculated from the average geo-element ratios of fish across the control treatment (i.e. non-enrichment treatment) (S1 dataset). To ensure a correct classification probability of 99.94%, mark detection thresholds were set at 3.3 standard deviations above the mean observed ratio in control fish for each enriched geo-element used. Because of the inherent instability in geo-elemental ratios measured on single-detector, ICP-based mass spectrometers, we conservatively set the criteria for detecting a successful mark in the otolith as at least three consecutive acquisitions with ratios above the detection threshold.

Mark strength of ^{134}Ba:^{138}Ba, ^{135}Ba:^{138}Ba, ^{136}Ba:^{138}Ba, ^{137}Ba:^{138}Ba, ^{86}Sr:^{88}Sr, ^{87}Sr:^{88}Sr and ^{26}Mg:^{24}Mg for each geo-element enrichment concentration used (1, 0.1, 0.01, & 0.001 µg geo-element g^{-1} fish) was analysed using a series of ANOVAs with geo-element concentration and combination treated as fixed factors. The response variables used were the maximum geo-element ratio observed (intensity) and the numbers of acquisitions above detection (spatial extent), in each fish otolith. The number of acquisitions above detection were ln (count + 1) transformed to improve ANOVA assumptions of equal variances.

The effects of treatment on fish length (fork length), weight and condition over the experimental period were analysed with one-way ANOVAs. The response variables used were change in fish length, weight and condition over the time frame of the experiment (sampling at 90 days and harvest 570 days). Fish condition was estimated using Fulton's condition factor (*K*) calculated with the formula K = ((W/L3)×100), where W is the live body weight (g), and L is the fork length (cm) (Ricker 1975).

Results

Mark success

A six marker fingerprint combination using the enriched natural geo-elements ^{137}Ba, ^{136}Ba, ^{135}Ba, ^{134}Ba, ^{86}Sr and ^{87}Sr was successfully created by marking during vaccination (Fig. 1). However, mark success was dependent on enrichment concentration and geo-element combination (Table 2). ^{137}Ba achieved 100% mark success with a minimum concentration of 0.001 µg. g^{-1} fish when used as a single geo-element marker and 0.01 µg. g^{-1} fish when used in a combination of 4 or 7 geo-elements. Marking with ^{135}Ba and ^{136}Ba was 100% successful with a minimum concentration of 0.01 µg. g^{-1} fish when used in combinations of 4 or 7 geo-elements. Marking with ^{134}Ba was 100% successful with a minimum concentration of 0.01 µg. g^{-1} fish when used in a 7 geo-element combination. Sr geo-elements were only successful at a concentration of 1 µg. g^{-1} fish. Mark success using ^{86}Sr was 100% successful in combinations of 4 or 7 geo-elements, and ^{87}Sr was 100% successful in the 7 geo-element combination. ^{26}Mg used in a combination of 7 geo-elements produced no successful marks at any concentration level.

Intensity of geo-element enrichment

^{137}Ba, ^{136}Ba, ^{135}Ba and ^{134}Ba max geo-element ratios

For ^{137}Ba, an interaction between marker combination and the concentration of geo-element used, showed that as concentration decreased and marker combination increased, mark strength decreased (interaction term: concentration x combination, $F_{6, 127} = 3.01$, $p = 0.009$). Post hoc test for the interaction term highlighted that ^{137}Ba used as a singular marker produced higher maximum ratios than combinations of 4 or 7 geo-elements, depending on the enrichment concentration used (Fig. 2A, Tukey HSD: 1 µg, 1 marker > 4 markers = 7 markers; 0.1 µg, 1 marker > 7 markers; $p < 0.05$).

There was no interaction between combination and concentration for ^{136}Ba or ^{135}Ba ($F_{3, 87} = 0.2$ & 1.2 respectively, $p > 0.3$ for both). Mark strength for ^{136}Ba and ^{135}Ba used in combinations of 4 or 7 geo-elements decreased as concentration decreased (Fig. 2B, $F_{3, 87} = 341$, $p < 0.001$ and Fig. 2C, $F_{3, 87} = 337$, $p < 0.001$ respectively; Tukey HSD: 1 µg > 0.1 µg > 0.01 µg > 0.001 µg = 0 µg, $p < 0.05$ for both). However, there was no difference in mark strength for either geo-element when they were used in combinations of 4 or 7 (^{136}Ba, $F_{1, 87} = 2.11$, $p = 0.15$; ^{135}Ba, $F_{1, 87} = 2.24$, $p = 0.14$). ^{134}Ba produced a similar pattern as observed with the other Ba geo-elements of decreased mark strength as concentration decreased when used in a 7 marker combination (Fig. 2D, $F_{4, 48} = 178$, $p < 0.001$; Tukey HSD: 1 µg > 0.1 µg > 0.01 µg > 0.001 µg = 0 µg, $p < 0.05$).

^{86}Sr and ^{87}Sr max geo-element ratios

There was no interaction between combination and concentration for ^{86}Sr ($F_{3, 87} = 0.2$, $p = 0.8$). Mark strength for ^{86}Sr was 1.6 times stronger in the highest concentration (1 µg. g^{-1} fish) compared to the 3 lower concentrations and the control (Fig. 2E, $F_{3, 87} = 229$, $p < 0.001$; Tukey HSD: 1 µg > 0.1 µg = 0.01 µg = 0.001 µg = 0 µg, $p < 0.05$) and there was no difference in mark strength between the 4 or 7 geo-element combinations ($F_{1, 87} = 0.098$, $p = 0.76$). Similarly, ^{87}Sr used in a 7 marker combination produced 1.9 times stronger marks in the highest concentration (1 µg. g^{-1}fish weight) compared to the 3 lower concentrations and the control (Fig. 2F, $F_{4, 48} = 74$, $p < 0.001$; Tukey HSD: 1 µg > 0.1 µg = 0.01 µg = 0.001 µg = 0 µg, $p < 0.05$).

^{26}Mg max geo-element ratios

No difference in mark strength across concentrations was observed for ^{26}Mg when used in the 7 marker combination enrichment (Fig. 2G, $F_{4, 48} = 0.17$, $p = 0.96$).

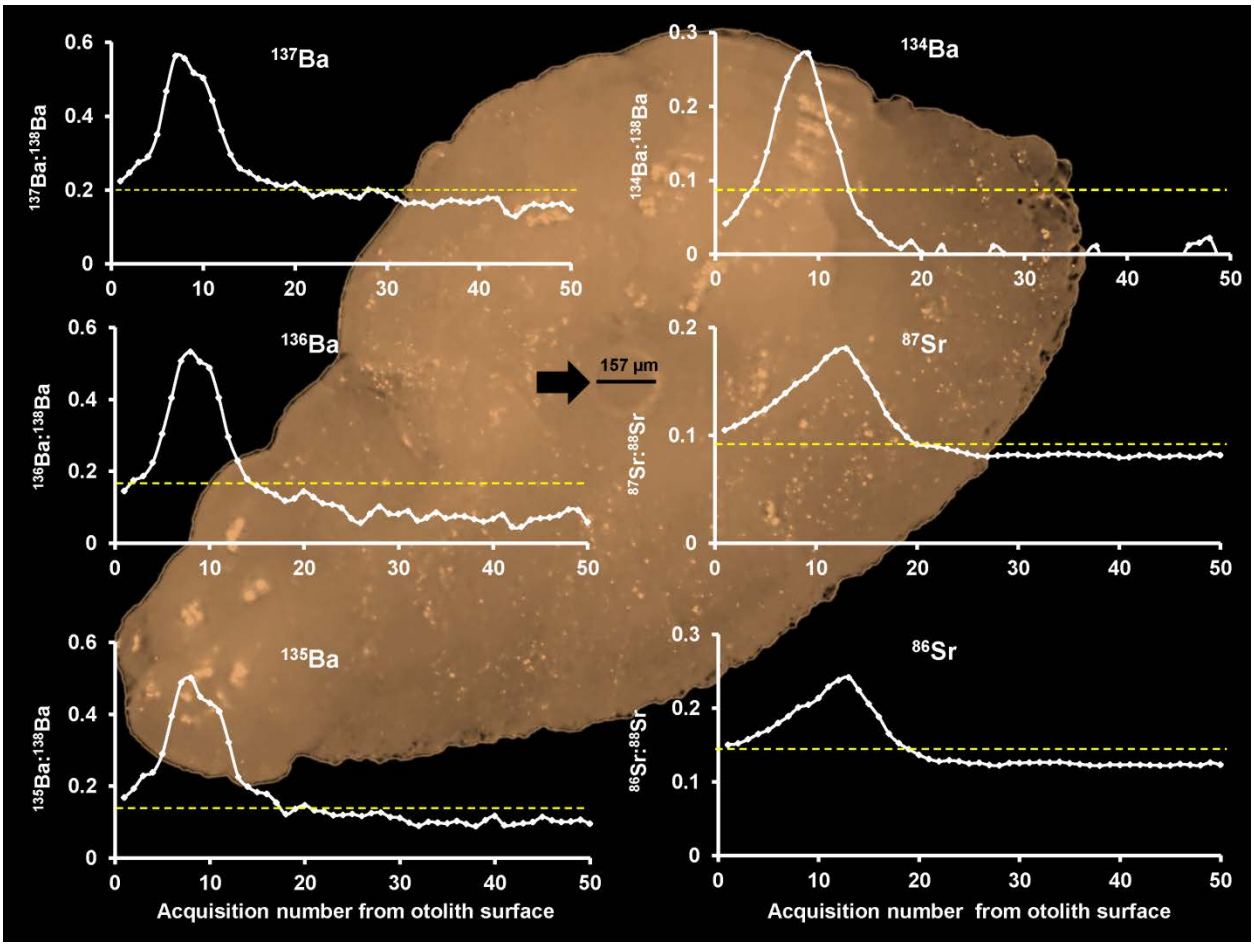


Fig. 1. Six mark enriched natural geo-element fingerprint. Scans of six enriched geo-element markers, ^{137}Ba, ^{136}Ba, ^{135}Ba, ^{134}Ba, ^{86}Sr and ^{87}Sr in the otolith of Atlantic salmon parr successfully delivered during vaccination at a concentration of 1 µg. g^{-1} fish for Sr geo-elements and 0.01 µg. g^{-1} fish for Ba geo-elements. White lines represent the first 50 acquisition values recorded for each geo-element analysed

using LA-ICP spot ablation with a spot size diameter of 157 µm (depicted by black arrow). Ablation began from the surface of the otolith and ablated towards to core. Yellow dotted lines show the 99.94% mark detection threshold for determining a unique mark for each geo-element used.

Table 2. Mark success during vaccination using multiple combinations of geo-element markers

	Conc.	Geo-element mark success						
Combination	**(µg. g^{-1} fish)**	**^{26}Mg**	**^{86}Sr**	**^{87}Sr**	**^{134}Ba**	**^{135}Ba**	**^{136}Ba**	**^{137}Ba**
7	1	0%	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**
	0.1	0%	22%	56%	**100%**	**100%**	**100%**	**100%**
	0.01	0%	0%	0%	**100%**	**100%**	**100%**	**100%**
	0.001	0%	0%	0%	0%	70%	20%	70%
4	1	-	**100%**	-	-	**100%**	**100%**	**100%**
	0.1	-	30%	-	-	**100%**	**100%**	**100%**
	0.01	-	0%	-	-	**100%**	**100%**	**100%**
	0.001	-	0%	-	-	80%	20%	80%
1	1	-	-	-	-	-	-	**100%**
	0.1	-	-	-	-	-	-	**100%**
	0.01	-	-	-	-	-	-	**100%**
	0.001	-	-	-	-	-	-	**100%**

Mark success during vaccination using combinations of 1, 4 or 7 geo-elements at one of four concentrations (1, 0.1, 0.01 & 0.001 µg per g fish weight). Mark success was classed as three consecutive geo-element ratios 3.3 standard deviations above control ratios for each geo-element used.

Spatial extent of geo-element enrichment

Analysis of the total number of acquisitions observed above the detection threshold were only analysed for the Ba and Sr enrichment treatments. Mg enrichment did not produce enough acquisitions above the detection threshold to warrant further analyses. No control fish had 3 consecutive acquisitions above the detection threshold for any Ba or Sr geo-element, so the analysis was restricted to the geo-element enriched vaccine treatments.

^{137}Ba, ^{136}Ba, ^{135}Ba and ^{134}Ba acquisitions above detection

There were no interactions between concentration and combination for ^{137}Ba ($F_{6, 117} = 0.85$, $p = 0.5$), ^{136}Ba or ^{135}Ba ($F_{3, 87} = 0.04$ & 0.29 respectively, $p > 0.5$ for both). Separately, geo-element concentration and combination did influenced the total number of acquisitions above the detection threshold for ^{137}Ba ($F_{3, 117} = 77.9$, $p < 0.001$ and $F_{2, 117} = 7.56$, $p = 0.001$, respectively). Count ratios were highest for ^{137}Ba when used as a single marker at the highest concentration (1 µg. g^{-1} fish), but decreased

as enrichment concentration decreased (Fig. 3A; Tukey HSD: 1 µg > 0.1 µg > 0.01 µg > 0.001 µg, $p < 0.05$) and as marker combination increased from 1 to 4 or 7 geo-elements (Tukey HSD: 1 marker > 4 markers = 7 markers, $p < 0.05$). Total number of acquisitions for ^{136}Ba, ^{135}Ba and ^{134}Ba used in geo-element combinations of 4 and 7, or only 7, were affected by concentration ($F_{3,77} = 95.9$, $F_{3,77} = 51.1$, $F_{3,38} = 38.1$, respectively; $p < 0.001$ for all). As concentration decreased, total count ratios decreased accordingly (Figs. 3B-3D; Tukey HSD: 1 µg > 0.1 µg > 0.01 µg > 0.001 µg, $p < 0.05$).

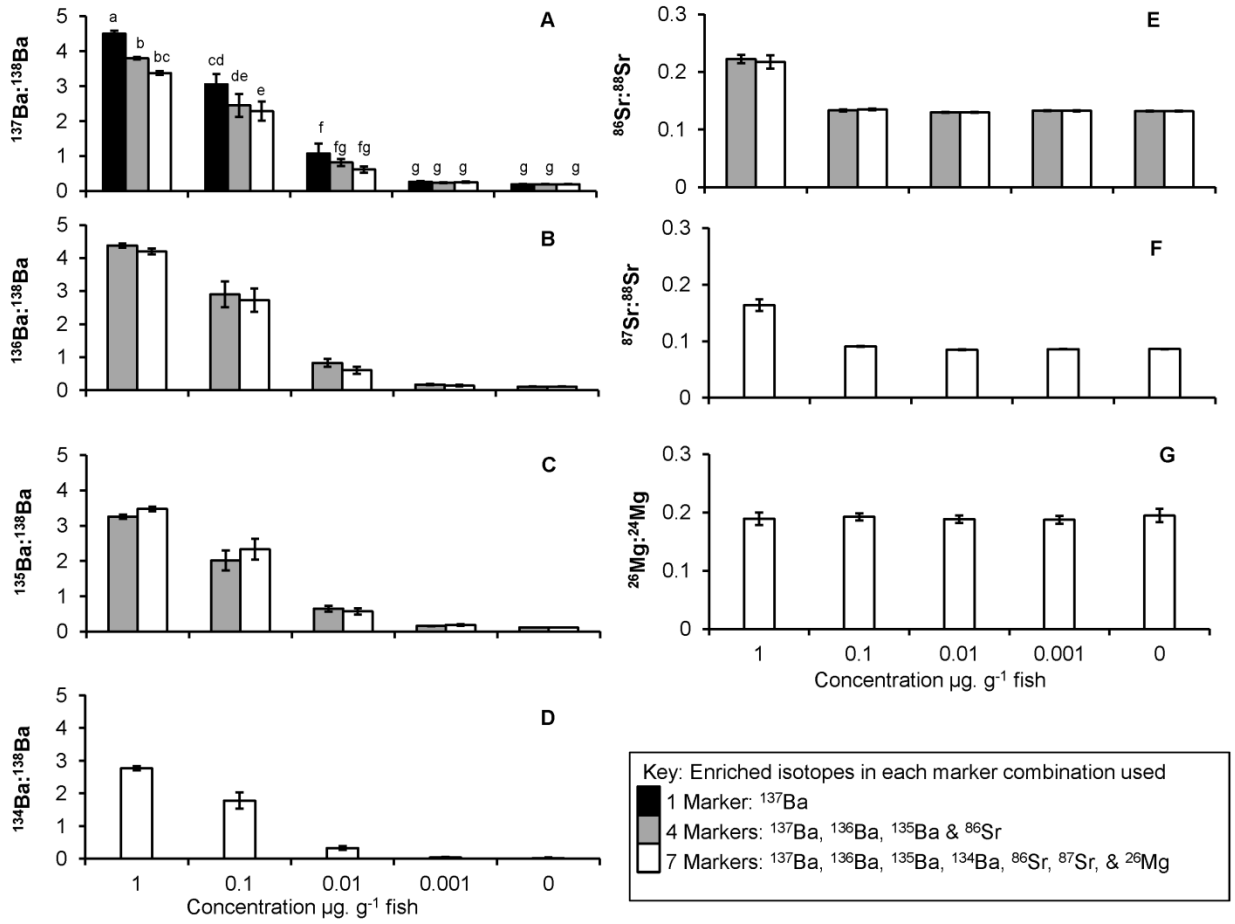


Fig. 2. Intensity of mark using multiple combinations of geo-elements. Maximum geo-element ratios for ^{137}Ba (A), ^{136}Ba (B), ^{135}Ba (C), ^{134}Ba (D), ^{86}Sr (E), ^{87}Sr (F) and ^{26}Mg (G) when used either singularly or in combination with 4 or 7 geo-element markers. Bars represent mean maximum ratio for each concentration by geo-element combination treatment. Error bars represent ± 1 SE. Concentrations were 1, 0.1, 0.01, 0.001 or 0 (control) µg geo-element g^{-1} fish for each geo-element used in a treatment. Letters above bars for ^{137}Ba (A) show the Post Hoc Tukey HSD for the interaction term (Concentration*Combination, $p < 0.05$), different letters mean bars are significantly different.

^{86}Sr and ^{87}Sr acquisitions above detection

There was no interaction between combination and concentration for ^{86}Sr ($F_{3,\ 77} = 0.2$, $p = 0.8$).The number of acquisitions above detection for ^{86}Sr were 10 times higher in the high concentration (1µg. g^{-1} fish) compared to the 3 lower concentrations (Fig. 3E, $F_{3,\ 77} = 86.6$, $p < 0.001$; Tukey HSD: 1 µg >

0.1 µg = 0.01 µg = 0.001 µg, $p < 0.05$). No difference was detected in the number of acquisitions between the 4 or 7 geo-element combinations ($F_{1, 77} = 1.09$, $p = 0.3$). Similarly, ^{87}Sr used in a 7 marker combination produced 10 times more acquisitions in the 1µg. g^{-1}fish concentration and 2.5 times higher in the 0.1µg. g^{-1}fish concentration compared to the two lowest concentrations (0.01 and 0.001 µg) (Fig. 3F, $F_{3, 38} = 56.8$, $p < 0.001$; Tukey HSD: 1 µg > 0.1 µg > 0.01 µg = 0.001 µg, $p < 0.05$).

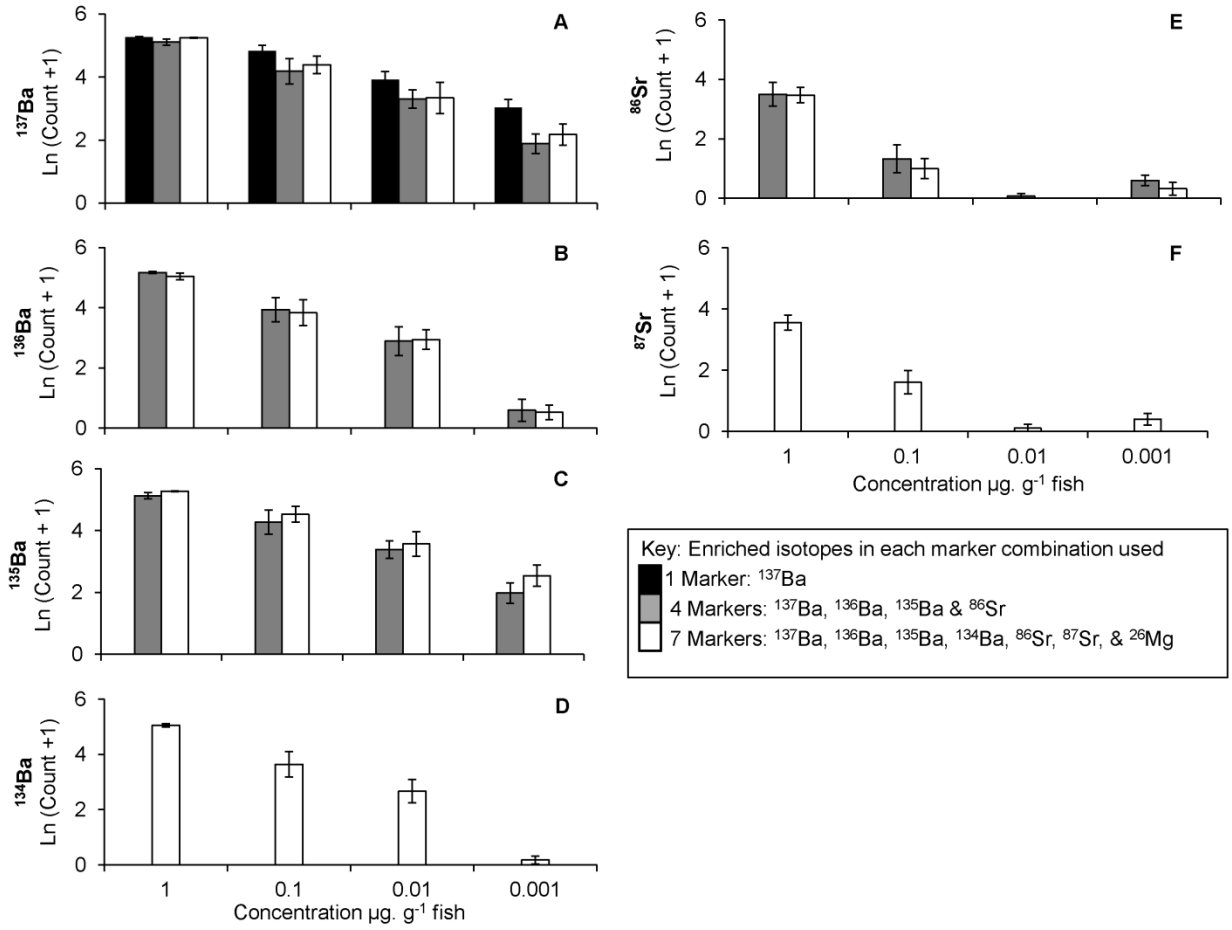


Fig. 3. Spatial extent of mark using multiple combinations of geo-elements. Number of acquisitions above detection for ^{137}Ba (A), ^{136}Ba (B), ^{135}Ba (C), ^{134}Ba (D), ^{87}Sr (E) and ^{86}Sr (F) when used singularly, or in a combination with 4 or 7 geo-element markers. Bars represent mean Ln (count +1) values for each concentration by geo-element combination treatment. Error bars represent ±1 SE. Note: there is no 0 concentration treatment as no control fish had 3 consecutive acquisitions above the detection threshold.

Mortality and growth

There was no effect of treatment on mortality, with only 3 mortalities out of a total of 650 injected fish during the first 90 days before sea-transfer. Prior to sea transfer, overall, experimental fish increased in weight by 53.9 ± 0.6 g (mean ± SE) and fork-length by 3.9 ± 0.02 cm. No differences were detected among treatments for fish growth (weight: $F_{12, 646} = 1.18$, $p = 0.3$; fork length: $F_{12, 646} = 1.27$, $p = 0.2$) or fish condition (Fulton's condition factor (k): $F_{12, 646} = 1.02$, $p = 0.4$). However, average condition of

fish across all treatments was approximately 10% lower at 90 days post injection compared to day 1 (Fulton's condition factor (*k*): Day 1 = 1.30 ± 0.002; Day 90 = 1.16 ± 0.003). Fish harvested at 570 days post injection (5.21 ± 0.06 kg, fork-length 72.2 ± 0.3 cm, condition factor (*k*) 1.36 ± 0.007) showed no difference in length, weight or condition among treatments (Fig. 4, weight: $F_{12, 408} = 1.11$, $p = 0.3$; fork length: $F_{12, 408} = 0.84$, $p = 0.6$; Fulton's condition factor (*k*): $F_{12, 408} = 0.82$, $p = 0.6$); $p = 0.4, 0.6, 0.6$ respectively). Mortality per treatment during the sea cage stage averaged 7.4 ± 1%, with no difference among treatments ($\chi^2_{12} = 7.2$, $p > 0.1$).

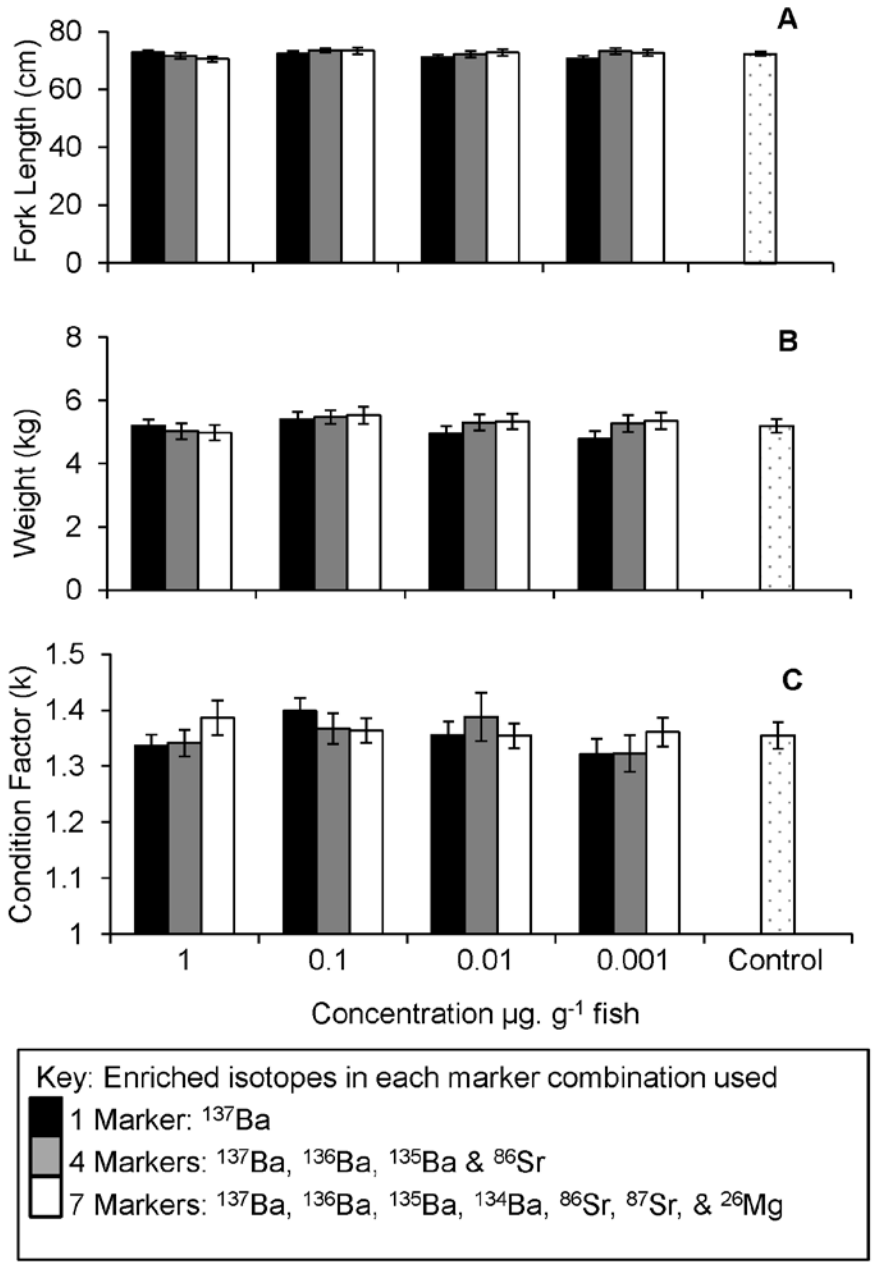


Fig. 4. Comparison of growth parameters at harvest. Graphs show average fork length (A), weight (B), and Fulton's condition factor K (C), for all fish at harvest in each of the treatments and the control. Error bars show ± 1 Standard Error.

Discussion

Mark success and strength

We have successfully produced a six marker natural geo-element fingerprint and effectively determined the minimum optimal concentrations required for the 63 possible combinations of four Ba geo-elements and two Sr geo-elements for fingerprint marking Atlantic salmon otoliths during vaccination (Fig 1.). Creating a single Ba geo-element fingerprint mark using ^{137}Ba with 100% mark success is achievable at a concentration of 0.001 µg. g^{-1} fish. However, as the number of geo-elements used in a fingerprint combination increases to four (^{137}Ba, ^{136}Ba, ^{135}Ba & ^{86}Sr) or seven (^{137}Ba, ^{136}Ba, ^{135}Ba, ^{134}Ba, ^{87}Sr, ^{86}Sr & ^{26}Mg) the required concentration of each Ba geo-element needed to ensure 100% mark success increases to 0.01 µg. g^{-1} fish. Sr geo-elements used in multiple fingerprint marks required higher concentrations in comparison to Ba geo-elements (1 µg vs 0.01 µg. g^{-1} fish, respectively) to guarantee 100% mark success, and for Sr there was no difference between fingerprint combinations of four or seven. These results demonstrate that the minimum concentration of geo-element required to mark an individual fish during vaccination is 2 (for Sr) to 2000 (for ^{137}Ba) times lower than the initial concentrations of 2 µg. g^{-1} fish trial by Warren-Myers et al. (2014), depending on the geo-element used and the geo-element combination. This suggests that natural geo-element marking during vaccination with Ba geo-elements, in particular, has the potential to be economically feasible at an industry scale where costs per fish must be as low as possible.

Mark strength, measured using maximum geo-element ratios (intensity) and number of acquisitions above detection (spatial extent) declined as geo-element concentration was reduced. However, ratios did not decrease by an order of magnitude as one would predict, and for the Ba geo-elements the intensity of marks was higher for single mark compared to 4 or 7 multiple marks at the highest concentration (1 µg. g^{-1} fish). This would suggest there is a possible facilitation, competition, or dilution effect influencing the degree of marker incorporation in multiple marker fingerprints above a threshold concentration. Facilitation of Ba uptake when Sr is present is known to occur in some fish species when the Sr:Ca ambient concentration in brackish or sea water is greater than 20 µmol. mol^{-1} (de Vries et al. 2005). However, if facilitation was occurring this should have increased the intensity of Ba marks when Sr was present in multiple mark fingerprints, not decreased mark intensity. Reduced mark intensity when using multi-geo-elements markers compared to a single marker due to competition has not been reported before, but maybe a plausible explanation if 1 µg. g^{-1} fish (highest concentration tested) is a threshold at which competition among geo-elements of the same element occurs. Dilution is most likely the cause for reduced mark intensity when multiple geo-elements from the one element (e.g. ^{137}Ba, ^{136}Ba, ^{135}Ba, ^{134}Ba) are used together. A dilution effect could result from the residual amount of ^{138}Ba impurities in the enrich geo-elements used (^{138}Ba impurities in: ^{137}Ba = 17.4%; ^{136}Ba = 2.4%; ^{135}Ba = 3.6%; ^{134}Ba = 5.3 %, Oak Ridge National Laboratory; www.ornl.gov). A dilution effect of added residual ^{138}Ba would also explain why mark success for ^{137}Ba, which was 100% when used as a single geo-element marker at the lowest concentration (0.001 µg. g^{-1} fish), dropped to 80% when used in combination with 2 other Ba geo-element markers (^{135}Ba, ^{136}Ba) and to 70% when used with 3 other Ba geo-element markers (^{135}Ba, ^{136}Ba, ^{134}Ba). Hence, an increase in marker concentration from 0.001 to 0.01 µg. g^{-1} fish for multiple Ba marks is required to ensure mark intensity and spatial extent is strong enough that 100% unique marks are created.

Mark success with ^{26}Mg was unsuccessful even at the highest concentration of 1 µg. g^{-1} fish. Poor mark success with Mg geo-elements has been reported for marking via vaccination (Warren-Myers et al. 2014), or larval immersion (Woodcock et al. 2011a) and may be due to self-regulation of Mg in fish (Shearer & Åsgård 1992) or a slow exchange rate of Mg ions into the endolymph fluid that surrounds the otolith (Maguire & Cowan 2002). Alternatively, natural levels of Mg in water and food may be too high for the introduction of an enriched Mg spike to significantly shift the natural Mg geo-element ratios at the concentrations Mg has been tested. Greater concentrations than used in this study, even if successful, would make Mg too costly and hence unsuitable for marking farmed salmon.

Fish condition and survival

Natural geo-element marking with Ba, Sr and Mg did not affect growth, condition or survival, among treatments over the 570 days between injection date and harvest date. This is consistent with other natural geo-element marking studies that used transgenerational and larval immersion techniques, and which similarly detected no negative effects on survival and growth due to marking (Munro et al. 2009, Williamson et al. 2009a, Woodcock et al. 2011a,b). Although average condition of all fish in the experiment dropped initially (~10%) over the first 90 days, the photoperiod regime used in the trial typically induces a decrease in condition factor similar to that normally seen during the parr–smolt transformation of Atlantic salmon and other salmonid species (McCormick et al. 1997, Bjørnsson et al. 2000). In addition, growth rates often reduce in the short-term in vaccinated Atlantic salmon (Aunsmo et al. 2008, Grini et al. 2011), which is associated with loss of appetite post vaccination (Sørum & Damsgård 2004). At harvest, fish condition was slightly higher compared to condition at injection date and no differences were found among treatments suggesting there are no long-term detrimental effects of natural geo-elements on Atlantic salmon when delivered during vaccination.

Application of otolith fingerprinting with enriched natural geo-elements during vaccination

We have demonstrated that geo-element marking delivered during vaccination can effectively mark farmed salmon and enable detection of the mark with 99.94% accuracy. Moreover, the concentrations required are sufficiently low that cost-effectiveness is high compared to all other common salmonoid mass marking techniques (Table 3). The amount of geo-element required to mark a fish delivered during vaccination is between 0.01 and 0.001 µg. g^{-1} fish for Ba geo-elements and 1 µg. g^{-1} fish weight for Sr geo-elements. Typically, Atlantic salmon parr average 40 g at vaccination time, meaning the total amount of enriched geo-element required for marking ranges between 0.04 and 1.6 µg of Ba geo-element per fish depending on the fingerprint combination used (Table 4) and 40 µg per fish for a single Sr geo-element. The optimal geo-element concentration delivered during vaccination used in the present study is lower for Ba, but higher for Sr when compared with a larval immersion study on Murray cod (*Maccullochella peelii*) (Woodcock et al. 2011b), which used the equivalent of 2 to 3 µg of Ba, and 5 to 8 µg of Sr per individual. Hence, for marking during vaccination at the concentrations we have demonstrated, geo-elements of Ba are the most suitable and cost effective given current prices (geo-element source = Oak Ridge National Laboratory; www.ornl.gov), which range from $US 0.0002 to $US 0.02 per fish depending on the combination of Ba geo-elements used. Concentrations at which Sr geo-elements are effective render them less economically viable for delivery during vaccination (from $US 0.48 to $US 1.72 per fish depending on the combination of Sr geo-element). Sr geo-elements may be more cost effective for marking using other techniques, such as immersion with osmotic induction (e.g. de Braux et al. 2014).

In addition to marking, there is an additional analysis cost of identifying marked fish. The cost per sample to analyse based on this study this was between $US 15 to 20 per fish. Hence, analytical

costs for monitoring for compliance of correct application of marks at an individual fish farm, or an assessment of a group of fish thought to have come from an escape event could be done for as little as $US 300 to 400 (N = 20 fish) due to the high accuracy (99.94%) of the enriched natural geo-element marking method.

Table 3. Marker costs for mass marking Atlantic salmon

Method	Marker cost ($US) per fish	Product information source
Coded wire tag	0.09	http://www.nmt.us
Elastomer tags	0.09	http://www.nmt.us
Pit tags	2.50	http://bts-id.com
Adipose fin clipping	0.05	http://wdfw.wa.gov
Ba geo-element marking during vaccination	0.0002 - 0.02	http://www.ornl.gov

Marker cost per fish refers to the material cost of the marker or tag, except in the case of adipose fin clipping were the cost relates to the cost of removing the adipose fin per fish.

Industry-scale marking with geo-elements of Ba

For a mass marking technique to work at an industry scale and to be successful in driving compliance, escaped marked fish need to be traceable to a point that assigns accountability for an escape event. Natural geo-element fingerprint marking could ensure accountability if each company within major producer nations was assigned its own unique marker combination. Mass marking would also allow for an accurate assessment of the level of integration between escapees and wild fish. For example, the Scottish salmon industry produces 180 000 tonnes of salmon a year from 13 main companies, hence, using the 13 cheapest of a possible 15 Ba marker combinations (Table 4) would enable each company in Scotland to have its own unique salmon identification mark at a median cost of $US 0.012 per fish. Canadian salmon farming produces 100 000 tonnes of salmon per year from 6 main companies, meaning only the 6 cheapest of a possible 15 Ba marker combinations are required to mark at the company level for the Canadian salmon industry at a median cost of $US 0.0008 per fish. The Faroe Islands produces 61 000 tonnes of salmon per year from just 3 companies, requiring just 3 unique Ba markers at a median cost of $US 0.0003 per fish.

Table 4. Ba geo-element otolith fingerprinting

		Required amount of geo-element (µg per 40g fish)				Total	Cost ($US)
Code #	Geo-element combination	^{137}Ba	^{136}Ba	^{135}Ba	^{134}Ba	per fish	per fish
1	^{137}Ba	0.04	-	-	-	0.04	0.0002
2	^{137}Ba, ^{136}Ba	0.4	0.4	-	-	0.8	0.0055

3	^{137}Ba, ^{135}Ba	0.4	-	0.4	-	0.8	0.0086
4	^{137}Ba, ^{134}Ba	0.4	-	-	0.4	0.8	0.0122
5	^{137}Ba, ^{136}Ba, ^{135}Ba	0.4	0.4	0.4	-	1.2	0.0119
6	^{137}Ba, ^{136}Ba, ^{134}Ba	0.4	0.4	-	0.4	1.2	0.0155
7	^{137}Ba, ^{135}Ba,^{134}Ba	0.4	-	0.4	0.4	1.2	0.0187
8	^{137}Ba,^{136}Ba,^{135}Ba,^{134}Ba	0.4	0.4	0.4	0.4	1.6	0.0219
9	^{136}Ba	-	0.04	-	-	0.04	0.0003
10	^{136}Ba,^{135}Ba	-	0.4	0.4	-	0.8	0.0098
11	^{136}Ba, ^{134}Ba	-	0.4	-	0.4	0.8	0.0133
12	^{136}Ba, ^{135}Ba,^{134}Ba	-	0.4	0.4	0.4	1.2	0.0198
13	^{135}Ba	-	-	0.04	-	0.04	0.0006
14	^{135}Ba,^{134}Ba	-	-	0.4	0.4	0.8	0.0165
15	^{134}Ba	-	-	-	0.04	0.04	0.0010

Minimum required amounts (µg) and estimated raw material cost per fish ($US, Oak Ridge National Laboratory; www.ornl.gov) for natural geo-element marking of 40 g Atlantic salmon parr during vaccination.

Country production and data source: Scotland: Annual production 180 000 tonnes, 13 companies, *Scottish Salmon Producers' Organisation Limited (SSPO)* Website; www.scottishsalmon.co.uk. **Canada:** Annual production 100000 tonnes, 6 companies, *Canadian Aquaculture Industry Alliance (CAIA)* Website; www.aquaculture.ca **Faroe Islands***:* Annual production 61000 tonnes, 3 companies*, Faroe Fish Farmers Association (FFFA)* Website; www.salmon.for **Norway:** Annual production 1.28 Million tonnes, 83 companies*, The Norwegian Ministry of Fisheries and Coastal Affairs* (*NMFCA*) Website; www.fisheries.

Currently the biggest producer of salmon worldwide is Norway, with an estimated annual production of 1.28 million tonnes per year from 83 companies. 15 Ba markers are insufficient to assign a unique mark at the company level. More markers would need to be tested, for example ^{132}Ba and ^{131}Ba to ensure enough unique combinations. However, recent legalisation in Norway now allows for greater industry amalgamation; individual stakeholders may now obtain up to a 40% share of Norway's total production (increased from 25%). Hence, if the total number of companies is reduced in the future to less than 60 through the amalgamation of smaller industry partners, marking with Ba and Sr natural geo-elements during vaccination would become viable for the Norwegian salmon industry. An alternate solution that could produce hundreds of marks using only the most cost-effective barium geo-elements would be to combine marking during vaccination at the parr stage (this study) with marking via immersion during the larval stage (de Braux et al. 2014) to produce multiple fingerprint marks in different parts of the otolith. Although confirmation that this approach doesn't cause cross-contamination of marks is required, it would allow for a possible 255 unique fingerprints.

We have shown that natural geo-element fingerprint marking during vaccination using geo-elements of Ba is an economically viable method for uniquely identifying fish to the company level for

the major salmon production regions worldwide. Importantly, the marks are permanent, unique, relatively easy to detect, and can be incorporated into standard salmon hatchery production with no additional production or welfare issues for fish grown to full commercial production size.

References

Glover KA, Sørvik AGE, Karlsbakk E, Zhang Z, Skaala Ø (2013b) Molecular genetic analysis of stomach contents reveals wild Atlantic cod feeding on Piscine Reovirus (PRV) infected Atlantic salmon originating from a commercial fish farm. Plos One 8(4): e60924

Toledo-Guedes K, Scanchez-Jerez P, Mora-Vidal J, Girard D, Brito A (2001) Escaped introduced sea bass (*Dicentrarchus labrax*) infected by *Sphaerospora testicularis* (Myxozoa) reach maturity in coastal habitats off Canary Islands. Mar Ecol 31:26-31

Fleming IA, Hindar K, Mjolnerod IB, Jonsson B, Balstad T, Lamberg A (2000) Lifetime success and interactions of farm salmon invading a native population. Proc R Biol Sci 267:1517–1523

McGinnity P, Prodohl P, Ferguson A, Hynes R, Maoileidigh N, Baker N, et al. (2003) Fitness reduction and potential extinction of wild populations of Atlantic salmon, *Salmo salar*, as a result of interactions with escaped farm salmon. Proc R Biol Sci 270:2443-2450

Hindar K, Fleming IA, McGinnity P, Diserud O (2006) Genetic and ecological effects of salmon farming on wild salmon: modelling from experimental results. ICES J Mar Sci 63:1234-1247

Hutchings JA, Fraser DJ (2008) The nature of fisheries- and farming- induced evolution. Mol Ecol 17:294-313

Jensen Ø, Dempster T, Thorstad EB, Uglem I, Fredheim A (2010) Escapes of fishes from Norwegian sea-cage aquaculture: causes, consequences and prevention. Aquacult Environ Interact 1:71-83

Hansen LP (2006) Migration and survival of farmed Atlantic salmon (*Salmo salar* L.) released from two Norwegian fish farms. ICES J Mar Sci 63:1211-1217

Skilbrei OT (2010a) Adult recaptures of farmed Atlantic salmon post-smolts allowed to escape during summer. Aquacult Environ Interact 1:147-153

Skilbrei OT (2010b) Reduced migratory performance of farmed Atlantic salmon post-smolts from a simulated escape during autumn. Aquacult Environ Interact 1:117-125

Fiske P, Lund RA, Hansen LP (2006) Relationships between the frequency of farmed Atlantic salmon, *Salmo salar* L., in wild salmon populations and fish farming activity in Norway, 1989-2004. ICES J Mar Sci 63:1182-1189

Crozier WW (2000) Escaped farmed salmon, *Salmo salar L*., in the Glenarm River, Northern Ireland: genetic status of the wild population 7 years on. Fish Manag Ecol 7:437-446

Glover KA, Quintela M, Wennevik V, Besnier F, Sørvik AGE, Skaala O (2012) Three decades of farmed escapees in the wild: A spatio-temporal analysis of population genetic structure throughout Norway. Plos One 7(8): e43129

Glover KA, Pertoldi C, Besnier F, Wennevik V, Kent M, Skaala Ø (2013a) Atlantic salmon populations invaded by farmed escapees: quantifying genetic introgression with a Bayesian approach and SNPs. BMC Genet 14:1-19

McGinnity P, Stone C, Taggart JB, Cooke D, Cotter D, Hynes R, et al. (1997) Genetic impact of escaped farmed Atlantic salmon (*Salmo salar* L.) on native populations: use of DNA profiling to assess freshwater performance of wild, farmed, and hybrid progeny in a natural river environment. ICES J Mar Sci 54:998-1008

Skaala Ø, Glover KA, Barlaup BT, Svåsand T, Besnier F, Hansen MM, et al. (2012) Performance of farmed, hybrid, and wild Atlantic salmon (*Salmo salar*) families in a natural river environment. Can J Fish Aquat Sci 69:1994-2006

Garcia de Leaniz C, Fleming IA, Einum S, Verspoor E, Jordan WC, Consuegra S, et al. (2007) A critical review of adaptive genetic variation in Atlantic salmon: implications for conservation. Biol Rev 82:173-211

Glover KA (2010) Forensic identification of fish farm escapees: the Norwegian experience. Aquacult Environ Interact 1:1-10

Martinez A, Standal IB, Axelson DE, Finstad B, Aursand M (2009) Identification of the farm of origin of salmon by fatty acid and HR ^{13}C NMR profiling. Food Chem 116:766-773

Vander Haegen GE, Blankenship HL, Hoffmann A, Thompson DA (2005) The effects of adipose fin clipping and coded wire tagging on the survival and growth of spring chinook salmon. N Am J Fish Manag 25:1161-1170

Volk EC, Schroder SL, Grimm JJ (1999) Otolith thermal marking. Fish Res 43:205-219

Crook DA, O'Mahony DJ, Sanger AC, Munro AR, Gillanders BM, Thurstan S (2009) Development and evaluation of methods for osmotic induction marking of golden perch *Macquaria ambigua* with calcein and alizarin red S. N Am J Fish Manag 29:279-287

Warren-Myers F, Dempster T, Jensen A, Fjelldal PG, Hansen T, Swearer SE (2014) Stable isotope marking of otoliths during vaccination: A novel method for mass marking fish. Aquacult Environ Interact 5:143-154

Bjørn EB, Rune W, Børge N, Fredriksen KL, Knut-Egil L, Marianne B, et al. (2013) Status and future perspectives of vaccines for industrialised fin-fish farming. Fish Shellfish Immunol 35:1759-1768

Woodcock SH, Gillanders BM, Munro AR, Crook DA, Sanger AC (2011b) Determining mark success of 15 combinations of enriched stable isotopes for the batch marking of larval otoliths. N Am J Fish Manage 31:843-851

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

Ricker WE (1975) Computation and interpretation of biological statistics of fish populations. Bull Fish Res Board Can 191:1-382

de Vries MC, Gillanders BM, Elsdon TS (2005) Facilitation of barium uptake into fish otoliths: influence of strontium concentration and salinity. Geochim Cosmochim Acta **69**:4061-4072

Shearer KD, Åsgård T (1992) The effect of water-borne magnesium on the dietary magnesium requirement of the rainbow trout (*Oncorhynchus mykiss*). Fish Physiol Biochem 9:387-392

Maguire ME, Cowan JA (2002) Magnesium chemistry and biochemistry. BioMetals 15:203-210

Munro AR, Gillanders BM, Thurstan S, Crook DA, Sanger AC (2009) Transgenerational marking of freshwater fishes with enriched stable isotopes: a tool for fisheries management and research. J Fish Biol 75:668-684

Williamson DH, Jones GP, Thorrold SR, Frisch AJ (2009a) Transgenerational marking of marine fish larvae: stable isotope retention, physiological effects and health issues. J Fish Biol 74:891-905

Bjørnsson BTh, Hemre G-I, Bjørnevik M, Hansen T (2000) Photoperiod regulation of plasma growth hormone levels during induced smoltification of underyearling Atlantic salmon. Gen Comp Endocrin 119:17-25

McCormick SD, Saunders RL, Henderson EB, Harmon PR (1987) Photoperiod control of parr–smolt transformation in Atlantic salmon (*Salmo salar*): Changes in salinity tolerance, gill Na1, K1-ATPase activity, and plasma thyroid hormones. Can J Fish Aquat Sci 44:1462–1468

Aunsmo A, Guttvik A, Midtlyng PJ, Larssen RB, Evensen O, Skjerve E (2008) Association of spinal deformity and vaccine-induced abdominal lesions in harvest-sized Atlantic salmon, *Salmo salar.* L. J Fish Dis 31:515-524

Grini A, Hansen T, Berg A, Wargelius A, Fjelldal PG (2011) The effect of water temperature on vertebral deformities and vaccine-induced abdominal lesions in Atlantic salmon, *Salmo salar* L. J Fish Dis 34:531-546

Sørum U, Damsgård B (2004) Effects of anaesthetisation and vaccination on feed intake and growth in Atlantic salmon (*Salmo salar* L.). Aquaculture 232:333-341

de Braux E, Warren-Myers F, Dempster T, Fjelldal PG, Hansen T, Swearer SE (2014) Osmotic induction improves batch marking of larval fish otoliths with enriched stable isotopes. ICES J Mar Sci 71:2530-2538

WP 3 Egg immersion for delivery of natural geo-element tags for farmed salmon

ABSTRACT: Determining the value of re-stocking wild fisheries with hatchery reared fish requires the ability to identify and quantify the survival of hatchery fish after release. However, to obtain accurate estimates of survival rates, multiple fish identification techniques are often used, making the monitoring of re-stocking inefficient and costly. Here we test a new immersion marking method to determine its efficiency and cost effectiveness for marking millions of hatchery reared Atlantic salmon. Salmon eggs were marked during the egg swelling stage by immersing eggs in a solution containing seven enriched natural geo-elements (^{134}Ba, ^{135}Ba, ^{136}Ba, ^{137}Ba, ^{86}Sr, ^{87}Sr & ^{26}Mg) for two hours immediately after fertilisation. 100% successful marks were detected in the otoliths of resulting larvae at a concentration of 1000 µg. L^{-1} for ^{136}Ba and 100 µg. L^{-1} for ^{135}Ba and ^{137}Ba with no detrimental effects on survival or health of egg and yolk sac larvae. We estimate that 7 unique mark combinations can be made at a cost of $0.0001 to $0.0017 (US) per egg and conclude that marking via egg immersion is suitable for low cost, accurate marking of hatchery reared salmonids destined for restocking purposes.

Introduction

Salmonids are the most produced anadromous fish species in the world, through hatchery production for fish farming and re-stocking purposes. For example, hatchery facilities release approximately $5x10^9$ juvenile salmon (pink, chum, Coho, Chinook, sockeye, steelhead, and masu) annually into the North Pacific Ocean for the purpose of restocking salmon fisheries (http://www.npafc.org), yet current estimates suggest survival of hatchery bred salmon in the wild is low and may vary between 1 and 15% (Beamish et al. 2012; Morre et al. 2012). Assessing what happens to the few survivors and what aspects of hatchery production and release could be changed to improve survival rates, requires the ability to accurately identify and trace large numbers of fish from different cohorts released from hatcheries. To achieve this, hatcheries have resorted to combining multiple marking methods, such as adipose fin clipping, DNA analysis, coded wire tagging, and otolith marking (e.g. Beamish et al. 2012; Daly et al. 2012). Although having a variety of marking techniques may improve mark detection rates, marking fish with multiple marks is a costly exercise, with the added financial and handling costs of having to apply multiple marks combined with the requirement for maintaining multiple databases and identification techniques. If a single, standardised, accurate marking method was possible for all hatchery released salmon, this would: (1) simplify the mark-identification process, (2) allow for better estimates of hatchery fish survival within and among years, and (3) reduce cost.

Of the current mass marking techniques available, batch marking of eggs, larvae, or juveniles is the most feasible way to mark large numbers of fish, because individual handling of fish is not necessary. Batch marking techniques typically mark the otoliths, scales, or fin rays of fish. Examples of batch marking methods are: otolith thermal marking, where hatchery water temperature is manipulated to create recognisable dark and light bands in the otoliths; fluorescent markers, where fish are immersed in oxytetracycline, alizarin, or calcein, creating a visible band of colour in the otolith, scales, or fin rays; and natural geo-element marking, where larvae or juveniles are immersed in a solution containing barium or strontium geo-elements to change the geo-elemental ratios in the otolith. Otolith thermal marking (Volk et al. 1994, 1999) is permanent, has no reported side effects on fish and is inexpensive. However, it can take several weeks to apply marks and correct mark detection is not guaranteed, usually due to mistaking unmarked for marked fish (Hagen et al. 1995; Volk et al. 1999). Fluorescent markers are effective for batch marking large numbers of fish with a single identification mark. However,

oxytetracycline mark success is not always 100%, can result in bone deformities (Toften & Jobling 1996), and mark retention decreases with age (Reinert et al. 1998). Alizarin and calcein can be used at high concentrations to ensure 100% mark success, but this can result in high mortality in some species (Brooks et al. 1994, Bumguardner & King 1996; Crook et al. 2009). Natural geo-element marking of hatchery fish, first suggested by Trefethen & Novotny (1963), has none of the above mentioned drawbacks of thermal or fluorescent otolith marking methods, and is capable of creating multiple unique combinations of marks that are permanent and harmless to fish (Thorrold et al. 2006). However, the practical and economic feasibility of using natural geo-elements for marking millions of hatchery reared salmon produced for restocking purposes has not been evaluated.

Here we investigate whether natural geo-element marking is a viable option for marking millions of hatchery reared salmon. Presently, natural geo-element marking is possible using barium (Ba) and strontium (Sr) geo-elements administered via maternal transfer (Thorrold et al. 2006; Almany et al. 2007; Munro et al. 2009; Williamson et al. 2009; Kuroki et al. 2010), through the immersion of larvae or juvenile fish in geo-element enriched solutions (Munro et al. 2008; Smith & Whitledge 2011;Woodcock et al. 2011a,b; de Braux et al. 2014), through dietary transmission (Woodcock et al. 2013), or via direct injection with enriched geo-elements during vaccination (Warren-Myers et al. 2014a). Concentrations of enriched geo-elements required to achieve 100% mark success varies greatly among delivery techniques. In the case of immersion, the time required for fish to be immersed in an geo-element solution varies greatly as well (e.g. 1 hour (de Braux et al. 2014) to 70 days (Walther & Thorrold 2006)). If marking requires high concentrations and/or long immersion times, this can result in unacceptable financial costs or mortality rates (e.g. Walther & Thorrold 2006), respectively. Hence, if natural geo-element marking is to be feasible, the time spent marking, the associated marking costs, and fish welfare issues need to be minimized.

This study tests the feasibility of natural geo-element otolith marking via egg immersion in hatchery-reared Atlantic salmon (*Salmo salar*). A hatchery marking method should complement hatchery production procedures; hence, we evaluated the fish husbandry procedures for Atlantic salmon (*Salmo salar*) in a hatchery facility to identify when marking via immersion could be undertaken without interrupting the fish rearing process. We determined that the egg swelling stage (Eddy & Talbot 1983), that is the period immediately following fertilisation where eggs are left to swell in water for several hours before being transferred to hatchery rearing tanks, was an optimal point for natural geo-element marking via immersion. We immersed batches of freshly fertilised eggs in a solution containing 7 enriched natural geo-elements (^{134}Ba, ^{135}Ba, ^{136}Ba,^{137}Ba, ^{86}Sr, ^{87}Sr, and ^{26}Mg) at five different concentrations (Ba: 1000, 100, 10, 1, and 0 µg. L^{-1}; Mg and Sr: 2500, 250, 25, 2.5 , and 0 µg. L^{-1} each). With this experiment, we aimed to determine the minimum concentrations required to achieve 100% mark success, and assess the strength of marks and any effects of marking on egg and larval survival. We show that batch marking via egg immersion can be an effective, low cost, hatchery applicable mass marking method for fish, such as salmonids, that have an egg swelling stage.

Methods

Experimental design

The experiment was conducted at the Institute of Marine Research field station, at Matre, in Masfjorden, western Norway (60°N) using eggs and sperm from Atlantic salmon broodfish (AquaGen strain) held in freshwater tanks buffered with saltwater to a salinity of 0.7 g NaCl.L^{-1} for 2 months prior to spawning. We tested natural geo-element otolith marking via egg immersion during the egg swelling stage by immersing batches of freshly fertilised eggs in Milli-Q water that contained a combination of

the enriched natural geo-elements, ^{134}Ba, ^{135}Ba, ^{136}Ba, ^{137}Ba, ^{86}Sr, ^{87}Sr and ^{26}Mg (Oak Ridge National Laboratory; www.ornl.gov) at five different concentrations (Table 1) . Replicate batches of eggs (N = 3) for each treatment consisted of 150 ml of eggs selected from three females (~50 ml each) fertilised with 2 ml of sperm from two males (1 ml each).

Table 1. Natural geo-element enrichment concentrations used for marking via egg immersion.

Enriched geo-element concentration per treatment		**Egg batches**
^{134}Ba, ^{135}Ba, ^{136}Ba, ^{137}Ba	^{26}Mg, ^{86}Sr, ^{87}Sr	Replicates
(µg. L^{-1})	(µg. L^{-1})	(N)
1000	2500	3
100	250	3
10	25	3
1	2.5	3
0	0	3

Egg swelling

The majority of swelling in salmonid eggs occurs within the first hour of immersion in water (Eddy & Talbot 1983), and initial hardening begins 1-2 h after fertilisation (Zotin 1958). Hence, a minimum of 2 h immersion time was chosen for this experiment to ensure fluid uptake had occurred. Egg batches were kept at 4 °C during the egg swelling phase by placing egg containers on ice. Egg batches were fertilised and after 2 min the ovarian fluid was gently drained off. After draining, each batch was weighed, then 300 ml of immersion solution was gently added, before eggs were left to swell. After 2 - 2.5 h, the excess immersion solution was drained off and each batch weighed again to obtain an estimate of the amount of solution absorbed. Batches of eggs were then placed in individual hatchery rearing trays in a common hatchery rearing tank. Eggs were kept at a constant temperature of 6 °C throughout the egg incubation period (81 days) and yolk sac larval stage (52 days). Immediately prior to first feeding (Day 133), a subsample of 10 yolk sac larvae from each replicate egg batch was collected and euthanized by anaesthetic overdose for otolith analysis. Sagittal otoliths from the subsampled larvae were dissected and removed, cleaned of any adhering tissue, air dried, and stored individually in plastic tubes for otolith analysis.

Otolith preparation

Sagittal otoliths were cleaned as per Warren-Myers et al. (2014). Briefly, any remaining organic tissue was removed by immersing otoliths in a solution of ultrapure 15% H_2O_2 buffered with 0.1 M NaOH. Following immersion, otoliths were ultra-sonicated (Sonic Clean 250HT) for 5 minutes and then left for 6 hours in the cleaning solution. The cleaning solution was then aspirated off and the otoliths were transferred through three Milli-Q water rinses, each of which consisted of 5 minutes of ultra-sonification and 30 minutes resting time. Otoliths were then air dried in a laminar flow bench for at least 24 hours. Once dry, one otolith per fish was fixed, sulcus side down, onto gridded microscope slides using quick dry cyanoacrylate glue.

Otolith analysis

Natural geo-element analyses were done on a Varian 7700x Inductively Coupled Plasma Mass Spectrometer (ICP-MS) fitted with a HelEx (Laurin Technic and the Australian National University) laser ablation (LA) system constructed around a Compex 110 (Lambda Physik) excimer laser operating at 193 nm. To correct for mass bias, 612 and 610 NIST (National Institute of Standards and Technology) glass standards doped with trace elements at known concentrations were used. Otoliths (~300 µm in diameter along the transverse axis) were run in blocks of 16 samples selected randomly from all treatments and bracketed by analyses of the standards. Samples and standards were analysed in time-resolved mode, using a spot size of 157 µm, a laser energy setting of ~ 60 mJ and a laser repetition rate of 10 Hz. Spot ablation was performed under pure He (200 ml/min) to minimise re-deposition of ablated material and the sample was then entrained into the Ar (0.95 ml/min) carrier gas flow to the ICP-MS. Using this method, we were able to quantify the following geo-element ratios: ^{134}Ba:^{138}Ba, ^{135}Ba:^{138}Ba, ^{136}Ba:^{138}Ba, ^{137}Ba:^{138}Ba, ^{86}Sr:^{88}Sr, ^{87}Sr:^{88}Sr, ^{26}Mg:^{24}Mg, ^{55}Mn:^{43}Ca from the edge to the core of salmon yolk sac larval otoliths (^{55}Mn:^{43}Ca was used to identify when the laser had hit the core; Barbee & Swearer 2007). Data were processed off-line using a specialised MS Excel template which involved a low pass filter to remove any spikes (a single scan value >2x the median of the adjacent scans), smoothing (a running average of three scans) and blank subtracting functions. A correction factor ($K = R_{true}/R_{obs}$, where R_{true} is the naturally occurring geo-element ratio and R_{obs} is the average geo-element ratio measured in the NIST 612 or 610 standard run before and after each set of 16 samples) was applied to all sample scans to correct for mass bias.

Statistical Analysis

Mark success for each geo-element used in the enrichment treatments was evaluated using a mark detection limit calculated from the geo-element ratios in the otoliths of control fish (Warren-Myers et al. 2014). Firstly, the mean geo-element ratios for ^{134}Ba:^{138}Ba, ^{135}Ba:^{138}Ba, ^{136}Ba:^{138}Ba, ^{137}Ba:^{138}Ba, ^{86}Sr:^{88}Sr, ^{87}Sr:^{88}Sr and ^{26}Mg:^{24}Mg were calculated using the observed geo-element ratios measured in the otoliths of all fish in the control treatment (i.e. 0 µg. L^{-1} treatment). Following this, to ensure a correct classification probability of 99.94%, the mark detection limits were then set at 3.3 standard deviations above the mean observed ratios in control fish for each enriched geo-element used. Because of the inherent instability in geo-element ratios measured on single-detector, ICP-based mass spectrometers, we conservatively set a further criteria by requiring at least three consecutive acquisitions with ratios above the detection limit for a fish to be classified as being marked.

Mark strength (maximum geo-element ratio, and proportion of otolith marked) for each enriched geo-element used was analysed using a series of one-way ANOVAs with geo-element immersion solution concentration (Table 1) treated as a fixed factor. The response variables used were the average maximum geo-element ratio value for each egg batch and the average proportion of acquisitions with ratio values above the detection limit for each egg batch. Egg batch averages were calculated from the otoliths of the 10 subsampled fish from each egg batch. The effect of treatment on total mortality and number of larval deformities were analysed with one-way ANOVAs.

Results

Egg swelling

Egg swelling time was between 2 and 2.5 h after fertilisation for each egg batch. Mean increase in mass (eggs + absorbed immersion fluid) ranged from 8 % to 10.6 % across treatments (Fig. 1) with no difference in percentage increase in mass among treatments ($F_{4, 14} = 1.506$, $P = 0.273$).

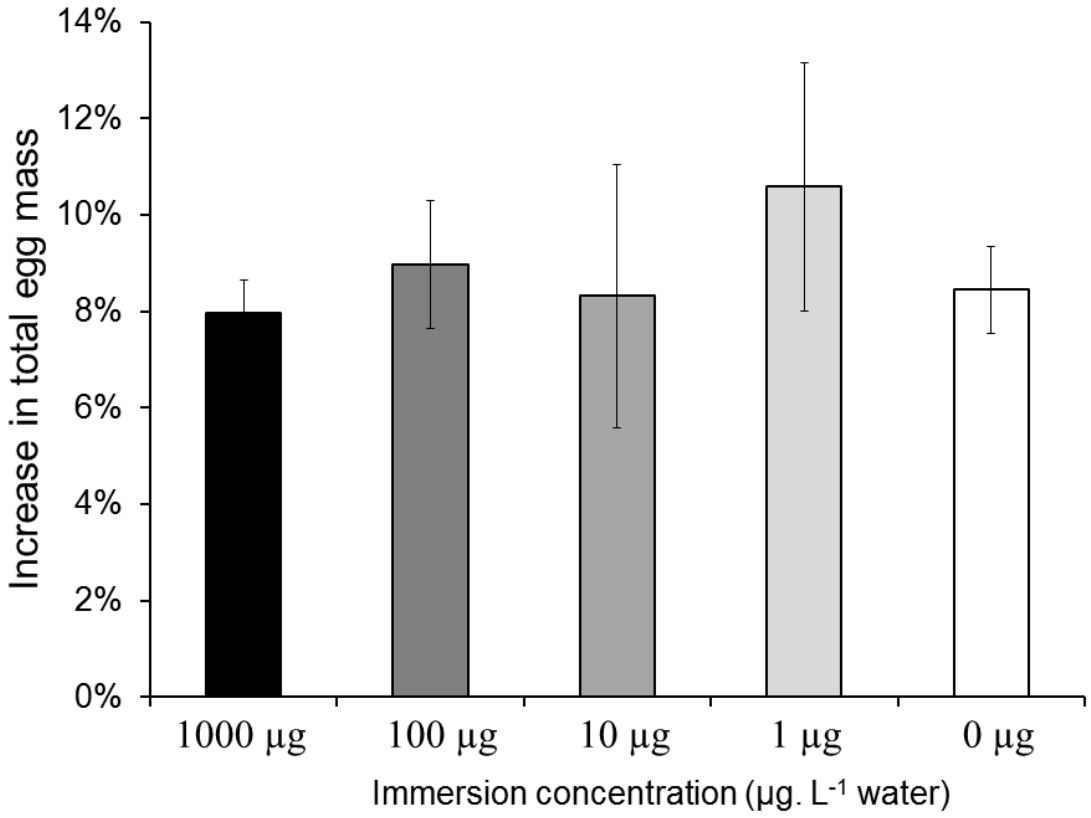


Figure 1. Increase in total weight during egg swelling. Increase in drained wet mass of eggs after immersion in natural geo-element immersion solutions for 2 hr 15 min ± 15 min. Each concentration contained three replicate egg batches with ~1200 eggs per batch. Error bars represent ± 1 SE.

Mark success

Mark success ranged from 0 to 100%, was dependent on concentration, and varied among the natural geo-elements used (Table 2). For the Ba geo-element enrichment, the highest concentration (1000 µg. L^{-1}) achieved 100% mark success with ^{135}Ba, ^{136}Ba and ^{137}Ba and 93% mark success with ^{134}Ba. One hundred percent mark success was also achieved for ^{135}Ba and ^{137}Ba at the next highest concentration (100 µg. L^{-1}). Mark success for ^{86}Sr, ^{87}Sr and ^{24}Mg was poor, ranging from 0 to 7% across all concentrations.

Table 2. Mark success in the otoliths of Atlantic salmon yolk sac larvae using a combination of seven enriched natural geo-elements delivered via egg immersion.

Marker Concentrations		**Mark success**						
^{134}Ba, ^{135}Ba, ^{136}Ba, ^{137}Ba	^{26}Mg, ^{86}Sr, ^{87}Sr	**^{137}Ba**	**^{136}Ba**	**^{135}Ba**	**^{134}Ba**	**^{87}Sr**	**^{86}Sr**	**^{26}Mg**
1000 µg. L^{-1}	2500 µg. L^{-1}	**100%**	**100%**	**100%**	93%	7%	0%	4%
100 µg. L^{-1}	250 µg. L^{-1}	**100%**	3%	**100%**	0%	0%	0%	0%

10 µg. L^{-1}	25 µg. L^{-1}	3%	0%	21%	0%	0%	0%	3%
1 µg. L^{-1}	2.5 µg. L^{-1}	0%	0%	0%	0%	0%	0%	0%

Mark strength – maximum ratios

Mark strength for ^{137}Ba (Fig. 2) showed the mean max ratios were 6 times greater at 1000 µg and 2 times greater at 100 µg compared with control max ratios ($F_{4,14}$ = 714, P < 0.001; Tukey honest significant difference (HSD): 1000 µg > 100 µg > 10 µg = 1 µg = 0 µg, P < 0.05). The ^{136}Ba mean max ratio was 16 times greater at 1000 µg compared with control max ratios ($F_{4,14}$ = 86, P < 0.001; Tukey HSD: 1000 µg > 100 µg = 10 µg = 1 µg = 0 µg, P < 0.05). The ^{135}Ba mean max ratio was 11 times greater at 1000 µg and three times greater at 100 µg compared with control max ratios ($F_{4,14}$ = 753, P < 0.001; Tukey HSD: 1000 µg > 100 µg > 10 µg = 1 µg = 0 µg, P < 0.05).The ^{134}Ba mean max ratio was 22 times greater at 1000 µg compared with control max ratios ($F_{4,14}$ = 71, P < 0.001; Tukey HSD: 1000 µg > 100 µg = 10 µg = 1 µg = 0 µg, P < 0.05). There was no difference in mean maximum ratio values among concentrations for ^{86}Sr, ^{87}Sr or ^{26}Mg ($F_{4,14}$ = 1.5, 3.1 and 0.7, P = 0.28, 0.06 and 0.61 respectively).

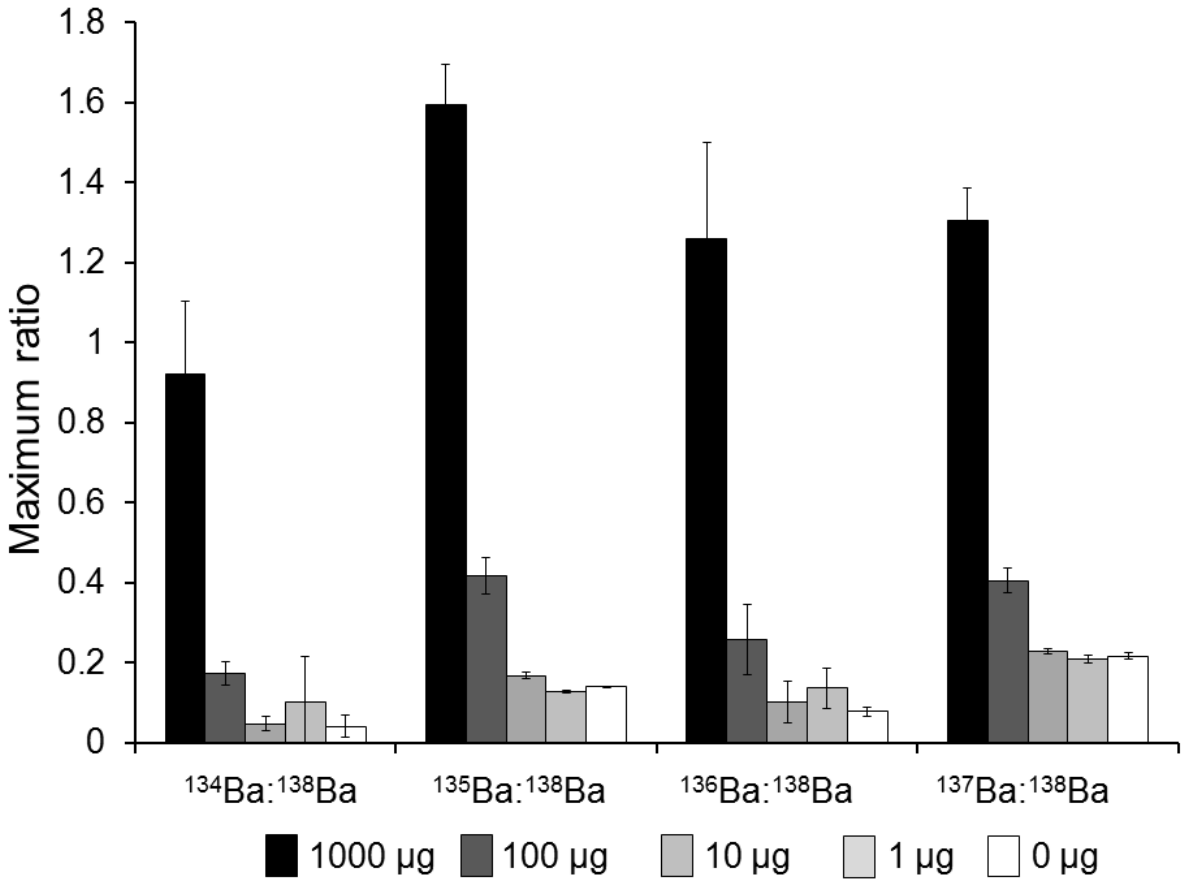


Figure 2. Mark strength for four Ba geo-elements. Maximum observed geo-element ratios ^{134}Ba:^{138}Ba, ^{135}Ba:^{138}Ba, ^{136}Ba:^{138}Ba and ^{137}Ba:^{138}Ba in the otoliths of Atlantic salmon yolk sac larvae marked via egg

immersion. Concentrations were 1000, 100, 10, 1 and 0 (control) µg of each geo-element per litre of water. Error bars represent ± 1 SE.

Mark strength – proportion of otolith marked

The proportion of acquisitions between the edge and the core with ratios above the threshold limit for Ba geo-elements (Fig. 3) followed a similar pattern to max ratios. ^{137}Ba in the 1000 µg and 100 µg treatments marked a greater proportion of the yolk sac larval otolith (92% and 53%, respectively) compared with the 10 µg and 1 µg treatments (1% and 0.1%, respectively) ($F_{3,11}$ = 1266, p < 0.001; Tukey HSD:1000 µg > 100 µg > 10 µg = 1 µg, *P* < 0.05). ^{136}Ba marked 65% of the otolith in the 1000 µg treatment compared with < 1% in the 100, 10 and 1 µg treatments ($F_{3,11}$ = 75, p < 0.001: Tukey HSD: 1000 µg > 100 µg = 10 µg = 1 µg, *P* < 0.05). ^{135}Ba in the 1000 µg and 100 µg concentrations marked 95% and 72% (respectively) compared with 2.5% and 0% (respectively) in the 10 µg and 1 µg treatments ($F_{3,11}$ = 2011, p < 0.001; Tukey HSD:1000 µg > 100 µg = 10 µg =1 µg, *P* < 0.05) and ^{134}Ba marked 44% of the otolith in the 1000 µg treatment compared to < 1% in 100, 10 and 1 µg treatments ($F_{3,\ 11}$ = 12.4, p = 0.002; Tukey HSD: 1000 µg > 100 µg = 10 µg = 1 µg, *P* < 0.05). The number of acquisitions with ratios above the detection limits for ^{86}Sr, ^{87}Sr and ^{26}Mg was insufficient to justify conducting mark strength analysis on the average proportion of otolith marked.

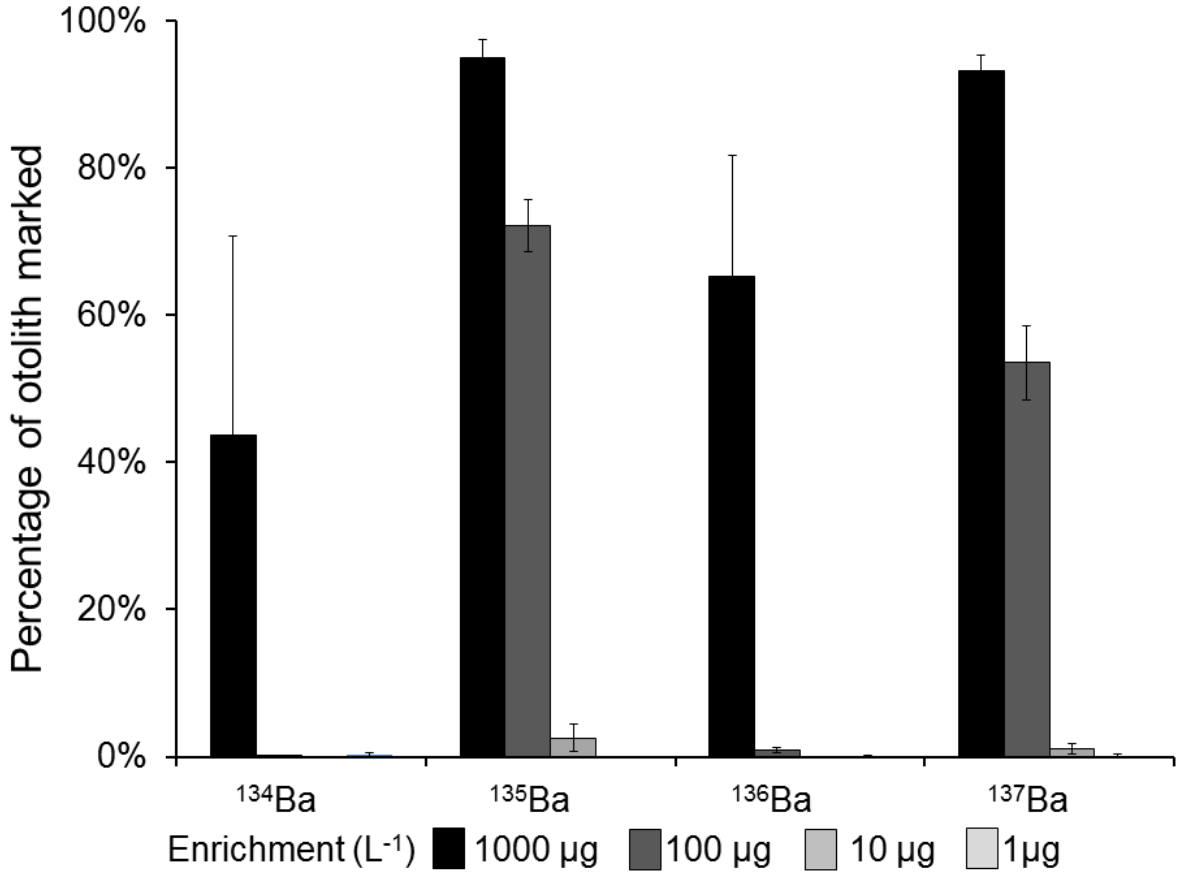


Figure 3. Mark strength for four Ba geo-elements. Proportion of otolith marked via egg immersion based on the number of acquisitions observed for ^{134}Ba:^{138}Ba, ^{135}Ba:^{138}Ba, ^{136}Ba:^{138}Ba and ^{137}Ba:^{138}Ba between the edge and core of Atlantic salmon yolk sac larval otoliths that were 3.3 SD above the overall

mean control ratio. Immersion solution concentrations were 1000, 100, 10, and 1 µg of each geo-element per litre of water. Error bars represent ± 1 SE.

Mortality and larval deformities

Total mortality over the time period between egg fertilisation and first feeding stage ranged from 11.4% to 13.6 % and there was no effect of geo-element enrichment ($F_{4,14}$ = 0.5, P = 0.7). Yolk sac larval deformities observed between hatching and first feeding ranged between 0.5% and 1.2% across treatments and there was no effect of geo-element enrichment. ($F_{4,14}$ = 2.3, P = 0.13).

Discussion

Batch marking fish may become a mandatory requirement for monitoring and biosecurity of hatchery reared fish that are used for stock enhancement programs or sea-cage aquaculture. Here, we have demonstrated a batch marking technique that is capable of effectively marking eggs using Ba geo-elements with a 99.94% guaranteed correct identification that can be implemented with no interruption to hatchery procedures. Marking via egg immersion can produce clear, identifiable marks in the otoliths of Atlantic salmon yolk sac larvae (Fig. 4) and was 100% successful for ^{135}Ba and ^{137}Ba geo-elements at a minimum concentration of 100 µg. L^{-1} and for ^{136}Ba at a minimum of 1000 µg. L^{-1}. These concentrations are similar to what has been used in other immersion methods using larvae (e.g. Woodcock et al. 2011a, b; de Braux et al. 2014). However, because of the small size of eggs, the number immersed per litre (~5000) and the point in the hatchery production cycle, egg immersion is potentially a more cost effective and efficient marking technique.

^{134}Ba, ^{86}Sr, ^{87}Sr and ^{26}Mg were not successful in achieving 100% mark success even at the highest concentrations tested (1000 & 2500 µg. L^{-1}). Although mark success may have been improved if concentrations were increased, this would make the cost of marking unviable for large scale hatchery marking of eggs. To date, only one study has demonstrated ^{134}Ba to be 100% successful, which was when ^{134}Ba was directly injected into Atlantic salmon parr during vaccination at a concentration of 0.01µg. g $^{-1}$ fish weight (Warren-Myers et al. 2015) which equates to approximately 0.3 to 0.5 µg per fish. The highest concentration for ^{134}Ba used in this experiment (1000 µg. L^{-1}), which achieved 93% mark success, is equivalent to approximately 0.4 µg per egg, suggesting the required concentration for achieving 100% mark success via egg immersion with ^{134}Ba is only marginally greater than 1000 µg. L^{-1}. One hundred percent mark success with enriched ^{86}Sr has been successful in other immersion studies (e.g. Munro et al. 2008; Smith & Whitledge 2011; de Braux et al. 2014), but all required longer immersion times and/or a higher concentration than what was used here. Although ^{26}Mg has been shown to have marginal success with marking Golden perch larvae (*Macquaria ambigua*) (Woodcock et al. 2011a), ^{87}Sr and ^{26}Mg are still yet to be proven 100% successful as geo-elemental markers using an immersion delivery method.

Maternally derived yolk sac Sr levels may explain why Sr geo-element enrichment was unsuccessful. Salmon eggs used in this study originated from broodfish that were reared in a sea cage farm and then transferred to onshore freshwater tanks (buffered with 0.7 g $NaCl.L^{-1}$), approximately 2 months prior to spawning. Hence, poor mark success observed with Sr geo-elements, but not Ba geo-elements, may be due to a higher amount of maternally derived Sr in the yolk sac compared with Ba. In support of this, a food-spiking validation experiment using enriched ^{138}Ba and ^{86}Sr (Woodcock & Walther, 2014) found that marking marine fish larvae in full strength seawater via spiked food to be successful for ^{138}Ba, but not ^{86}Sr. In addition, de Braux et al. (2014) marked yolk sac salmon larvae (reared in freshwater) 2 weeks prior to first feeding with ^{137}Ba and ^{86}Sr via immersion and found that a higher concentration of ^{86}Sr

relative to ^{137}Ba was required to achieve 100% successful marks in 1 h, although ^{86}Sr uptake was as successful as ^{137}Ba at longer immersion times (12 or 24 h). However, when marking salmon parr via vaccination (reared in freshwater) (Warren-Myers et al. 2015), no yolk sac maternal Sr should remain, yet 10 to 100 times more Sr than Ba was still necessary to achieve 100% successful marks. Marine maternal yolk sac Sr contribution may influence Sr mark success in larvae of some fish species. However, the reason why Sr was less successful than Ba in the present study is more likely due to either the rate of uptake or incorporation into the otolith being lower for Sr than Ba.

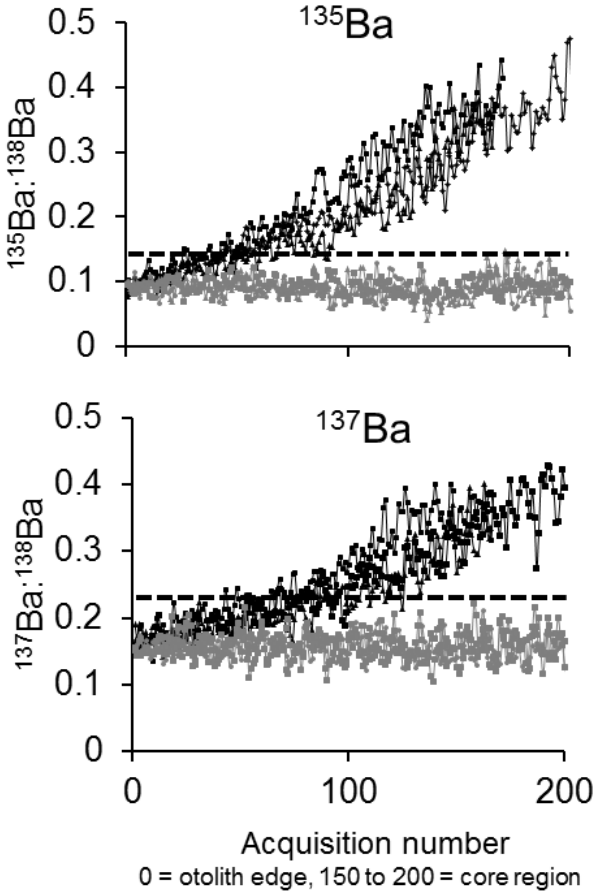


Figure 4. Signal intensity. Comparison of the change in ^{135}Ba:^{138}Ba and ^{137}Ba:^{138}Ba, between the otolith edge and otolith core in the otoliths of yolk sac larval Atlantic salmon. Black lines represent three successfully marked fish, grey lines represent three control fish, and the broke horizontal line represents the mark detection limit. Immersion concentrations used in marked fish were 100 µg. L^{-1} for both geo-elements.

During egg swelling, salmon eggs increased in mass by approximately 8 to 10% over a period of 2 to 2.5 h. meaning the time required for administering marks is greatly reduced compared with other immersion trials. Only one other study that used yolk sac Atlantic salmon larvae marked via immersion assisted with osmotic induction (de Braux et al. 2014) has managed to successfully mark otoliths via immersion in a shorter time period (1 h). All other trials using stable enriched geo-element markers have required a minimum immersion time of at least 24 h to ensure mark success (e.g. Woodcock et al. 2011a).

A full scale hatchery marking programme for the purpose of identifying the effectiveness of a restocking campaign would be feasible with Ba natural geo-element markers delivered during the egg swelling stage. We found no effects on egg or yolk sac larval survival, and the technique would be simple and easy to apply for both small and large hatchery facilities. In this study, we have validated seven geo-element combinations (Table 3). In the context of hatchery reared salmonids released into the North Pacific, this would mean each of the five major hatchery producing countries around the Pacific could have a unique geo-elemental signature produced in the core of the otolith of marked fish at a cost ranging from 0.0001–0.0017 $US per egg.

Table 3. Seven potential marker combinations validated using the egg immersion natural geo-element marking method. Costs are based on the immersion of 5000 eggs per litre. Raw material costs for geo-elements sourced from Oak Ridge National Laboratory; www.ornl.gov.

Geo-element combination	**Concentration (µg. L^{-1}) for 100% mark success**			**Cost ($US)**
	^{137}Ba	^{136}Ba	^{135}Ba	per egg
^{137}Ba	100	-	-	0.0001
^{135}Ba	-	-	100	0.0002
^{137}Ba + ^{135}Ba	100	-	100	0.0003
^{136}Ba	-	1000	-	0.0013
^{137}Ba + ^{136}Ba	100	1000	-	0.0014

A marking cost of 0.0001 to 0.0017 per egg is cheaper than all other available hatchery batch marking techniques, except otolith thermal marking at 0.001 $US per alevin (Hammer & Blankenship 2001). Cost for mark detection is also very similar for both techniques, with otolith thermal marking costing $10–$15 per otolith (Hammer & Blankenship 2001) and geo-element analysis (this study) costing $15 per otolith. However, detecting marks in the core of otoliths in older fish using laser ablation often requires sectioning and polishing, which would add to the cost of mark detection. To mitigate this potential cost, a large laser ablation spot size (157 µm) was used in the analyses to assess the feasibility of detecting geo-element marks in the core of adult salmonid otoliths without the need to section or polish. Correct mark identification rates for natural geo-element marking via egg immersion is 99.94% compared with otolith thermal marking rates that range from 65% to 95% (Hagen et al. 1995; Volk et al. 1999); less fish would be needed for sampling using the natural geo-element method to achieve the desired accuracy required for estimating the value of a hatchery re-stocking program.

References

Almany GR, Berumen ML, Thorrold SR, Planes S, Jones GP (2007) Local replenishment of coral reef fish populations in a marine reserve. Science 316:742-744

Barbee N, Swearer SE (2007) Characterizing natal source population signatures in the diadromous fish *Galaxias maculatus*, using embryonic otolith chemistry. Mar Ecol Prog Ser 343:273-282

Beamish RJ, Sweeting RM, Neville CM, Lange KL, Beacham TD, Preikshot D (2012) Wild chinook salmon survive better than hatchery salmon in a period of poor production. Environ Biol Fish 94:135-148

Brooks RC, Heidinger RC, Kohler CC (1994) Mass-marking otoliths of larval and juvenile walleyes by immersion in oxytetracycline, calcein, or calcein blue. N Am J Fish Manag 14:143-150

Bumguardner BW, King TL (1996) Toxicity of oxytetracycline and calcein to juvenile striped bass. Trans Am Fish Soc 125:143-145

Crook, D., O'Mahony, D., Sanger, A., Munro, A., Gillanders, B.M., and Thurstan, S. 2009. Development and evaluation of methods for osmotic induction marking of golden perch *Macquaria ambigua* with calcein and alizarin red S. N. Am. J. Fish. Manag. **29**: 279-287.

Daly, E.A., Brodeur, R.D., Fisher, J.P., Weitkamp, L.A., Teel, D.J., and Beckman, B.R. 2012. Spatial and trophic overlap of marked and unmarked Columbia River Basin spring Chinook salmon during early marine residence with implications for competition between hatchery and naturally produced fish. Environ. Biol. Fish. **94**: 117-134.

de Braux, E., Warren-Myers, F., Dempster, T., Fjelldal, P.G., Hansen, T., and Swearer, S.E. 2014. Osmotic induction improves batch marking of larval fish otoliths with enriched stable isotopes. ICES. J. Mar. Sci. 71(9): 2530-2538.

Eddy, F.B., and Talbot, C. 1983. Formation of the perivitelline fluid in Atlantic salmon eggs *(Salmo salar)* in fresh water and in solutions of metal ions. Comp. Biochem. Physiol. **75**: 1-4.

Hagen, P., Munk, K., Van Alen, B., and White, B. 1995. Thermal mark technology for inseason fisheries management: a case study. Alsk. Fish. Res. Bull. **2**: 143-155.

Hammer, S.A., and Blankenship, H.L. 2001. Cost comparisons of marks, tags, and mark with-tag combinations used in salmonid research. N. Am. J. Aquacult. **63**: 171-178.

Kuroki, M., Buckley, R., LeClair, L., and Hauser, L. 2010. Validation and efficacy of transgenerational mass marking of otoliths in viviparous fish larvae. J. Fish. Biol. **77**: 292-298.

Munro, A., Gillanders, B.M., Elsdon, T.S., Crook, D.A., and Sanger, A.C. 2008. Enriched stable isotope marking of juvenile golden perch (*Macquaria ambigua*) otoliths. Can. J. Fish. Aquat. Sci. **65**: 276-285.

Munro, A., Gillanders, B.M., Thurstant, S., Crook, D.A., and Sanger, A.C. 2009. Transgenerational marking of freshwater fishes with enriched stable isotopes: a tool for fisheries management and research. Fish. Biol. **75**: 668-684.

Moore M., Berejikian, B.A., and Tezak, E.P. 2012. Variation in the early marine survival and behavior of natural and hatchery-reared Hood Canal steelhead. PLoS One **7**(11): e49645.

Reinert, T.R., Wallin, J., Griffin, M.C., Conroy, M.J., and Van Den Avyle, M.J. 1998. Long-term retention and detection of oxytetracycline marks applied to hatchery-reared larval striped bass, *Morone saxatilis*. Can. J. Fish. Aquat. Sci. **55**: 539-543.

Smith, K.T., and Whitledge, G.W. 2011. Evaluation of a stable-isotope labelling technique for mass marking fin rays of age-0 lake sturgeon. Fish. Manag. Ecol. **18**: 168-175.

Thorrold, S., Planes, S., and Hare, J. 2006. Transgenerational marking of embryonic otoliths in marine fishes using barium stable isotopes. Can. J. Fish. Aquat. Sci. **63**: 1193-1197.

Toften, H., and Jobling, M. 1996. Development of spinal deformities in Atlantic salmon and Arctic charr fed diets supplemented with oxytetracycline. J. Fish. Biol. **49**: 668-677.

Trefethen, P.S., and Novotny, A.J. 1963. Marking fingerling salmon with trace elements and non-radioactive isotopes. North Atlantic fish marking symposium special publication No.4 **11**: 64-65.

Volk, E.C., Schroder, S.L., Grimm, J.J., and Ackley, H.S. 1994. Use of bar code symbology to produce multiple thermally induced marks. Trans. Am. Fish. Soc. **123**: 811-816.

Volk, E.C., Schroder, S.L., and Grimm, J.J. 1999. Otolith thermal marking. Fish. Res. **43**: 205-219.

Walther, B.D., and Thorrold, S.R. 2006. Water, not food, contributes the majority of strontium and barium deposited in the otoliths of a marine fish. Mar. Ecol. Prog. Ser. **311**: 125-130.

Warren-Myers, F., Dempster, T., Jensen, A., Fjelldal, P.G., Hansen, T., and Swearer, S.E. 2014. Stable isotope marking of otoliths during vaccination: A novel method for mass marking fish. Aquacult. Environ. Interact. **5**: 143-154.

Warren-Myers, F., Dempster, T., Fjelldal, P.G., Hansen, T., and Swearer, S.E. 2015. An industry-scale mass marking technique for tracing farmed fish escapees. PLoS One. 10(3): e0118594.

Williamson, D., Jones, G., and Thorrold, S. 2009. An experimental evaluation of transgenerational isotope labelling in a coral reef grouper. Mar. Biol. **156**: 2517-2525.

Woodcock, S.H., Gillanders, B.M., Munro, A.R., McGovern, F., Crook, D.A., and Sanger, A.C. 2011a. Using enriched stable isotopes of barium and magnesium to batch mark otoliths of larval golden perch (*Macquaria ambigua*, Richardson). Ecol. Freshw. Fish. **20**: 157-165.

Woodcock, S.H., Gillanders, B.M., Munro, A.R., Crook, D.A., and Sanger A.C. 2011b. Determining mark success of 15 combinations of enriched stable isotopes for the batch marking of larval otoliths. N. Am. J. Fish. Manag. **31**: 843-851.

Woodcock, S.H., Grieshaber, C.A., and Walther B.D 2013. Dietary transfer of enriched stable isotopes to mark otoliths, fin rays and scales. Can. J. Fish. Aquat. Sci. **70**: 1-4.

Woodcock, S.H., and Walther, B.D. 2014. Concentration-dependent mixing models predict values of diet-derived stable isotope ratios in fish otoliths. J. Exp. Mar. Biol. Ecol. **454**: 63-69.

Zotin, A.I. 1958. The mechanism of hardening of the salmonid egg membrane after fertilization or spontaneous activation. J. Embryol. Exp. Morph. **6**: 546-568.

WP 4 Larval immersion for delivery of natural geo-element tags for farmed salmon

ABSTRACT: Otolith marking with enriched natural geo-elements via immersion is a recent method of batch marking larval fish for a range of research and industrial applications. However, current immersion times and geo-element concentrations required to successfully mark an otolith limit the utility of this technique. Osmotic induction improves incorporation and reduces immersion time for some chemical markers, but its effects on geo-element incorporation into otoliths are unknown. Here, we tested the effects of osmotic induction over a range of different geo-element concentrations and immersion times on relative mark success and strength for ^{137}Ba:^{138}Ba, ^{86}Sr:^{88}Sr and ^{26}Mg:^{24}Mg on Atlantic salmon (*Salmo salar*) larvae. 100% and 71% mark success were achieved after 1 h of immersion for ^{137}Ba (30 µg L^{-1}) and ^{86}Sr (75 µg L^{-1}) geo-elements, respectively. Compared to conventional immersion, osmotic induction improved overall mark strength for ^{137}Ba and ^{86}Sr geo-elements by 26-116%, although this effect was only observed after 12 h of immersion and predominately for ^{86}Sr. The results demonstrate that osmotic induction reduces immersion times and the concentrations of geo-element required to achieve successful marks. Osmotically induced geo-element labels via larval immersion may prove a rapid and cost-effective way of batch marking fish larvae across a range of potential applications.

INTRODUCTION

Effective identification of fish through marking is essential for studies of movement and population connectivity (Swearer *et al*., 2002; Thorrold *et al*., 2002; Elsdon & Gillanders, 2003; Almany *et al*., 2007), demography (McFarlane *et al*., 1990; McCormick & Hoey, 2004), stock discrimination (Secor & Houde, 1995) and stock enhancement efforts (Hilborn *et al*., 1990; Reinert *et al*., 1998; Munro *et al.,* 2009). Batch marking approaches are often widely used as tagging fish individually is labor intensive, expensive, stressful to fish, and can be disruptive to hatchery operations (Nielson, 1992; Ennevor & Beames, 1993; Brown & Harris, 1995). Ideally, batch marking methods should: 1) easily, inexpensively and consistently tag fish *en masse* over most life history stages; 2) be applicable to multiple species; 3) minimise handling and stress; and 4) produce permanent marks. Currently available batch marking techniques, however, often fail to satisfy all criteria, particularly for juvenile fish. Physical tags have problems with poor tag retention (Kaill *et al*., 1990; Munro *et al*., 2003) or health and welfare issues (Serafy *et al*., 1995; Buckland-Nicks *et al*., 2011), fluorescent markers involve handling stress with risk of poor mark retention (Thomas *et al*., 1995; Reinert *et al*., 1998; van der Walt & Faragher, 2003; Thorrold *et al*., 2006); and thermal marking has a limited tagging window (eyed-egg to hatchling stage) and marks may be difficult to detect due to background variation (Volk *et al.,* 1999).

Otolith elemental marking using enriched natural geo-elements is a recently developed alternative to these existing batch marking methods. Otoliths are made of calcium carbonate within an organic matrix and their chemical composition reflects the physicochemical properties of the ambient water in which the fish lives (Edmonds *et al*., 1989; Campana 1999; Elsdon *et al*., 2008). Otolith marking using enriched natural geo-elements works by altering the relative abundance of certain natural geo-elements in the otolith, creating an artificial mark that is distinguishable from natural variations in geo-element ratios (Walther & Thorrold, 2006; Williamson *et al*., 2009a; Webb *et al*., 2012). In this way, unique geo-elemental “fingerprints” can be created which are detectable regardless of the size or life history stage of the recaptured fish. Several hundred unique otolith fingerprints can be potentially generated using different combinations of enriched barium and strontium geo-elements alone, allowing for marking in distinct batches using the same method. In addition, natural geo-elements can be used safely at low dosages to mark fish without adverse effects on their health or on humans who may consume them (Williamson *et al*., 2009a, b).

Although enriched natural geo-element marking has been validated in several fish species (Thorrold *et al*., 2006; Munro *et al*., 2009; Smith & Whitledge, 2011; Huelga-Suarez *et al*., 2012), mark strength often varies with species, the concentration and geo-element combination used, and at what stage in the life history the marker is delivered (Munro *et al*., 2008, 2009; Williamson *et al.,* 2009a; Woodcock *et al*., 2011a, b). Delivery methods for enriched natural geo-elements include immersion (Walther & Thorrold, 2006; Munro *et al.,* 2008; Woodcock *et al*., 2011a, b), maternal transfer (Thorrold *et al*., 2006; Almany *et al*., 2007; Kuroki *et al*., 2010), dietary transfer (Woodcock *et al*., 2013) and direct injection through vaccination (Warren-Myers *et al*., *in press*). Larval immersion has been successfully applied across several species with 100% mark success at relatively high concentrations of natural geo-element and long immersion times (Walther & Thorrold, 2006; Munro *et al.,* 2008; Woodcock *et al*., 2011a, b). As such, the technique remains expensive and time consuming, and may be impractical for many applications, unless innovations can be made to improve labor and financial costs.

Osmotic induction is a technique for accelerating uptake of fluorescent chemical marks via immersion (Alcobendas et al. 1991; Mohler 2003). The method is based on creating an osmotic potential between the target fish and the marking solution, thus assisting uptake of the marker. This involves momentarily immersing the fish in a hypersaline bath prior to immersion in a solution spiked with the desired chemical marker, in this case enriched natural geo-elements. Compared with conventional immersion techniques, this method promises to reduce marking time by significantly increasing marker uptake over a short period and has been successful with other chemical markers (Mohler, 2003; Negus & Tureson, 2004; Crook *et al*., 2007, 2009; Smith *et al*., 2010; Campanella *et al*., 2013).

Although osmotic induction has been used to accelerate the uptake of calcein and other fluorescent chemicals into calcified structures, whether the technique also reduces immersion times and improves uptake of natural geo-element tags into otoliths remains unknown. Here, we evaluate the interactive effects of osmotic induction and conventional larval immersion on mark success and mark strength and detectability over a range of different geo-element concentrations and immersion times, with the aim of improving the utility of enriched natural geo-element marking via larval immersion for scientific and commercial purposes.

MATERIALS & METHODS

Study species

Atlantic salmon (AquaGen strain) alevins from two mothers were sourced from the Institute of Marine Research (IMR) experimental farm located in Matre, Norway. All individuals were 33 days post hatch on the day of marking.

Experimental design

We tested the effects of osmotic induction (with or without), immersion time in geo-element solution (1 h, 12 h & 24 h) and geo-element concentration (low or high) on mark strength and detectability in larval Atlantic salmon (*Salmo salar*) otoliths. Thus, the fully crossed design had a total of 12 treatments (osmotic induction (2 levels) × geo-element concentration (2 levels) × immersion time (3 levels)). Additional fish were immersed for 24 h in unspiked water (control treatment) to determine the background geo-element ratios in unmarked fish.

The low and high concentrations of enriched natural geo-element were prepared using a triple geo-element combination of ^{137}Ba, ^{86}Sr, and ^{26}Mg (Oak Ridge National Laboratory, Oak Ridge, Tennessee, USA). The low concentration solution contained: ^{137}BaCl (30 µg L^{-1}), ^{86}SrCl (75 µg L^{-1}), and ^{26}MgCl (75 µg L^{-1}), while the high

concentration solution contained: ^{137}BaCl (100 µg L^{-1}), ^{86}SrCl (250 µg L^{-1}) and ^{26}MgCl (250 µg L^{-1}). Geo-element concentrations used were chosen based on previously used concentrations that achieved 100% mark success (Woodcock *et al*., 2011a, b; Warren-Myers *et al., in press*).

Prior to the experimental manipulations, all alevins were kept in a 10 L holding tank with a lid to reduce light stress. Each treatment consisted of 8 replicate fish. A replicate consisted of one randomly selected individual, which was transferred from the holding tank with a fine-mesh hand net into a 50 mL container. All 50 mL containers were partially submerged in water to regulate potential temperature fluctuations in the holding room (6°C) where they were kept for 24 hours. Fish were independent replicates as each treatment was separately applied to each fish.

For treatments with osmotic induction, a 5% NaCl solution was prepared from a mixture of pure water and non-iodized table salt. All treatment solutions were prepared the day before the experiment and kept at 6°C. Individuals undergoing osmotic induction were immersed in a saline bath for 3.5 minutes, rinsed briefly in pure water, and transferred to separate 50 mL clear plastic containers with geo-element solution. Individuals that did not undergo osmotic induction were immersed in a freshwater bath for 3.5 minutes and handled similarly to the osmotic induction treatment fish.

Immersion times in the geo-element solutions lasted for 1 h, 12 h or 24 h. Treatments running for less than 24 hours were removed from their treatment solution, rinsed, placed into another 50 mL container with pure water. After 24 hours, all individuals from each treatment were placed into a common hatchery tank, grouped by treatment, and grown for two weeks to ensure incorporation of the geo-element mark into the otolith.

Otolith preparation

Two weeks after geo-element marking, all individuals were collected and euthanized using tricane methanol sulfate (MS222) Finquel® before otolith extraction. Otoliths were prepared following the method of Warren-Myers *et al*. (*in press*). The left and right sagittal otoliths (~ 300 µm in diameter) were extracted from each larva and stored in clear eppendorf microtubes with the aid of a pair of fine-tipped tweezers and a dissecting microscope. Any remaining organic tissue was removed by immersing the otolith in a solution of ultrapure 15% H_2O_2 buffered with 0.1 M NaOH. Following immersion, otoliths were ultra-sonicated for 5 minutes and allowed to sit. After 6 hours, the cleaning solution was aspirated off and otoliths were transferred through 3 Milli-Q water rinses, each of which consisted of 5 minutes ultra-sonication and 30 minutes resting time. Otoliths were then allowed to air dry in a Class 100 laminar flow bench for at least 24 hours. Once dry, one randomly selected otolith from each alevin was fixed onto a gridded 8 x 8 microscope slide sulcus side down using cyanoacrylate glue.

Otolith analysis

Natural geo-element analyses were done on a Varian 7700x Inductively Coupled Plasma Mass Spectrometer (ICP-MS) fitted with a HelEx (Laurin Technic and the Australian National University) laser ablation (LA) system constructed around a Compex 110 (Lambda Physik) excimer laser operating at 193 nm. A 612 NIST (National Institute of Standards and Technology) glass standard doped with trace elements at known concentrations was used to calibrate the system. Otoliths were run in blocks of 16 samples selected randomly from all treatments and bracketed by analyses of the standard. Samples and the standard were analysed in time-resolved mode (where each 0.331 sec acquisition resulted in one mass scan of each geo-element), using a stationary laser with spot size of 157 µm, an energy setting of ~ 60 mJ and a repetition rate of 5 Hz. Ablation was performed under pure He (200 ml/min) to minimise re-deposition of ablated material and the sample was then entrained into the Ar (0.95 ml/min) carrier gas flow to the ICP-MS. Using this method, we were able to quantify the concentrations of ^{134}Ba, ^{135}Ba, ^{136}Ba, ^{137}Ba, ^{138}Ba, ^{86}Sr, ^{87}Sr, ^{88}Sr,

^{24}Mg, ^{26}Mg and ^{43}Ca in the outer layers above the core of salmon alevin otoliths. Data were processed off-line using a MS Excel template which involved a low pass filter to remove any spikes (a single scan value >2x the median of the adjacent scans), smoothing (a running average of 3 scans) and blank subtracting functions. A correction factor ($K = R_{true}/R_{obs}$, where R_{true} is the naturally occurring geo-element ratio and R_{obs} is the average geo-element ratio measured in the NIST 612 standard run before and after each set of 16 samples) was applied to all sample scans to correct for mass bias.

Statistical analyses

All tests were based on the ratios from the first 100 scans for each sample as this defined the limit of the region of the otolith that was potentially marked (Fig. 1). Beyond that, the laser was primarily sampling otolith material deposited prior to marking. Limits of detection (LODs) were calculated using the average geo-element ratios from all control fish. Detection limits were set at 3.3 standard deviations above the mean control observed ratio. Hence, fish with ^{137}Ba:^{138}Ba, ^{86}Sr:^{88}Sr and ^{26}Mg:^{24}Mg ratios that were greater than 3.3 standard deviations above the average ratio in control fish for at least three consecutive acquisitions were deemed successfully tagged. This criterion was chosen to ensure a mark success determination of > 99.94%.

We used a three-way Analysis of Variance (ANOVA) to determine if osmotic induction, geo-element concentration or immersion time affected the maximum geo-element ratio or the percentage of scans above the LOD. Post-hoc Tukey's tests were used for significant effects of immersion time and interactions. Log-transformations were applied where data did not meet assumptions of normality and homogeneity of variance.

RESULTS

Survival

Across all experimental treatments, 100% of the alevins survived the two-week period prior to sampling.

Mark success

Mark success varied with geo-element, concentration, immersion time and whether or not osmotic induction was used (Table 1). 100% mark success was achieved with ^{137}Ba across all treatments. For ^{86}Sr, 100% mark success was achieved in all 12 and 24 h treatments. However, in 1 h immersion treatments, mark success for ^{86}Sr depended on concentration and osmotic induction. The high ^{86}Sr concentration with osmotic induction produced 100% mark success, but only 88% mark success without osmotic induction. For the low ^{86}Sr concentration, mark success was 71% with and 38% without, osmotic induction. Mark success for ^{26}Mg ranged from 0 to 14% across treatments without any clear pattern between mark success and treatment.

Mark strength

Maximum geo-element ratio and number of scans above the limit of detection were only analysed for ^{137}Ba:^{138}Ba and ^{86}Sr:^{88}Sr. The ^{26}Mg:^{24}Mg enrichment did not produce enough scans with ratios above the detection limit to warrant further analysis.

Maximum geo-element ratio (MIR)

Across treatments, osmotic induction, higher geo-element concentrations, and increased immersion time (1h vs 12 and 24h) all increased, on average, the maximum ratio for ^{137}Ba observed within an otolith by 14%, 25% and 75%, respectively (Fig. 2, Table 2; $p < 0.001$ for all three factors). Maximum geo-element

ratios for ^{137}Ba showed no significant interactions occurring among osmotic induction, immersion time, and concentration (Table 2; $p > 0.1$ for all interaction terms).

Maximum geo-element ratios for ^{86}Sr varied depending on osmotic induction, immersion time, and concentration (Fig. 2, Table 2; $p = 0.008$ for 3-way interaction). The 3-way combination of osmotic induction, high geo-element concentration and the longer immersion times of 12 or 24 h produced significantly higher maximum ratios (255%, on average) compared to all other treatments (Table 2).

Scans above detection (SAD)

The percentage of scans above the limit of detection for ^{137}Ba showed an immersion time by concentration interaction (Table 3; $p = 0.03$), while osmotic induction had no effect ($p = 0.22$). All fish had 100% of scans above detection except for 5 individuals in the 1h, low concentration treatment.

The percentage of scans above detection for ^{86}Sr increased with both immersion time and concentration (Fig. 2, Table 3; $p < 0.001$ for both). The 12 h and 24 h immersion times improved mark strength by, on average, 219% (Table 3; $p < 0.05$, number of scans: 24 h: 87±4; 12 h: 78±4; 1 h: 26±4) and the high concentration improved mark strength by, on average, 54% (number of scans: high = 77±3; low = 50±3). No significant interactions occurred among osmotic induction, immersion time, and concentration (Table 3; $p > 0.1$ for all interaction terms).

DISCUSSION

We have demonstrated that osmotic induction, in conjunction with enriched geo-element immersion, can substantially improve geo-element marker uptake in otoliths. Furthermore, successful incorporation of ^{86}Sr and ^{137}Ba geo-element marks was achieved after an immersion time of only 1 h. The results support the viability of osmotically induced geo-element labels via larval immersion as a means of quickly and effectively marking larval fish *en masse* and paves the way for further investigations aimed at optimising natural geo-element larval immersion techniques.

No mortality occurred up to two weeks after marking. Although osmotic induction can cause buoyancy issues in larvae of other fish species (Crook *et al*., 2007, Campanella *et al*., 2013), we observed no such effect, nor any obvious differences in behaviour or condition among treatments (pers. obs). Although enriched geo-element marking of otoliths causes no long term negative effects on fish health in other species (Williamson *et al.,* 2009a; Roy *et al*., 2013), future work must assess major welfare and production parameters through the full life cycle to rule out any latent or long-term effects of this marking method.

Mark success

Osmotic induction, geo-element concentration and immersion time interacted to affect both the success rate and strength of mark delivered to the otoliths of Atlantic salmon alevins. 100% mark success was achieved for ^{137}Ba and 71% for ^{86}Sr geo-elements after 1 h of immersion using the low concentration geo-element mixture (30 and 75 µg L^{-1} of ^{137}Ba and ^{86}Sr, respectively) with osmotic induction. This is the shortest successful incorporation of a natural geo-element mark to date (Table 4). Only 25% of the otoliths that were unsuccessfully marked came from groups that underwent osmotic induction. This indicates the possibility that osmotic induction improves mark success for ^{86}Sr geo-elements, which were less readily incorporated into otoliths than ^{137}Ba geo-elements. Further investigation to quantify the effect of osmotic induction on mark success for both barium and strontium geo-elements is warranted; lower concentrations and/or shorter immersion times are required to detect differences in incorporation.

Mark strength

Osmotic induction

The use of osmotic induction contributed substantially to overall mark strength for both strontium and barium geo-elements compared to direct immersion, as has been demonstrated with other chemical markers (Mohler, 2003; Negus & Tureson, 2004; Crook *et al*., 2007, 2009; Smith *et al*., 2010; Campanella *et al*., 2013). The saline bath creates a hyperosmotic external environment, which results in water loss mainly across the gills and skin of the fish (Conte, 1969; Holliday, 1969). Subsequent immersion in the marker solution assists uptake due to the resulting osmotic difference between the fish and the solution during "rehydration" via osmosis (Mohler, 2003). The effect of osmotic induction could be enhanced by increasing the salt bath salinity (Negus & Tureson, 2004) or by lengthening the immersion time (Crook *et al*., 2009), as the larvae of many species can withstand relatively high salinity variations (Holliday, 1969). Optimal immersion time appears to lie between 1 and 12 h, but longer immersion times generally produced stronger marks.

Concentration of geo-element solution

Compared to low concentration geo-element solutions, high concentrations predictably enhanced mark strength for both strontium and barium geo-elements. Higher concentration marking solutions result in greater mark uptake for enriched geo-elements over longer periods (Munro *et al.,* 2008; Woodcock *et al*., 2011a, b). Depending on the marking application, the relative cost of the high concentration geo-element for Sr and Ba may diminish its benefits compared to osmotic induction and longer immersion times. Although geo-element concentrations directly affect marking costs and should be minimised, the practical limitations of certain marking efforts may allow for higher concentrations when time is a factor. The 100% marking success achieved for barium suggests that optimal marking concentrations are lower than the 30 µg L^{-1} used in this study. In addition, the potential exists to reuse the marker solution for multiple batches, although dilution rates after multiple immersions would need to be calculated.

Immersion times

As expected, longer immersion treatments substantially improved mark strength compared to 1 h immersion times for ^{86}Sr and ^{137}Ba across treatments. Although 1 h immersion times achieved < 90% mark success for strontium and barium, 12 h immersion time guaranteed 100% mark success and delivered greater mark strength. Among all other geo-element immersion studies, Woodcock *et al*. (2011a) achieved the fastest method that delivered 100% mark success for golden perch with 30 µg L^{-1} of ^{137}Ba after 1 day of immersion (Table 2). In this study, we cut the time to mark 100% of otoliths with 30 µg L^{-1} of ^{137}Ba to 1 hr. Moreover, we achieved 87.5% mark success for ^{86}Sr with 1 hr immersion times, indicating an immersion time between 1 and 12 h would reliably mark 100% of otoliths.

Combined effects

Uptake of ^{86}Sr and ^{137}Ba was positively influenced by the combined effects of osmotic induction, high concentration solutions and longer immersion times. Osmotic induction worked best with longer immersion times. However, there was no evidence to suggest that osmotic induction enhances mark strength for 1 h immersion times, even though the positive effects of this technique have been seen after less than 10 minutes of immersion with calcein and alizarin red S fluorescent markers (Mohler, 2003; Crook *et al.,* 2007, 2009; Campanella *et al*., 2013). This indicates that osmotic induction may be best employed in situations where immersion times exceed 12 h, unless the induction effect is somehow enhanced through optimisation. Longer immersion times with high concentration solutions combined made a significant difference to ^{86}Sr geo-element mark strength, a difference that was approximately 20-25% greater than osmotic induction combined with either longer immersion times or high concentration solutions. In addition, when compared individually for total individual contribution to ^{86}Sr and ^{137}Ba geo-element mark strength, longer immersion times accounted for 58% of the total contribution, while high concentration solutions and osmotic induction represented 25% and 17% of the total contribution, respectively. These findings suggest the following order for strength of influence on overall mark strength for ^{86}Sr and ^{137}Ba

geo-elements: Longer immersion times > High concentration solutions > Osmotic induction. The relative influence of each of these factors on mark strength suggests that optimisation may be achieved through osmotic induction with a low concentration solution and 12 h of immersion. These interacting effects should be further evaluated by modifying their relative strengths to attain 100% mark success without compromising mark strength and detectability. This could potentially be achieved by strengthening the osmotic induction effect, increasing the concentration of the geo-element solutions, or lengthening the immersion time.

Relative incorporation of different elements

Barium and strontium

Overall, barium incorporation was more successful than strontium, even given the lower concentrations in the spike solution, presumably due to greater uptake and incorporation into otoliths, with relatively little influence of the different treatments. The ^{86}Sr mark was often less diffuse and returned to background levels more quickly during depth profiling compared to ^{137}Ba, which was often a broad peak that was highly elevated and above background throughout the whole profile. Such differences could reflect different uptake and incorporation pathways, particularly as some studies have observed a lag in Sr incorporation in otoliths (e.g., Elsdon & Gillanders 2005). Alternatively, it could be a methodological artefact of a longer washout time for Ba given the higher enrichment observed. However, previous tests of the laser ablation system used in the present study have documented negligible cross-contamination while depth profiling through heterogeneous material (Woodhead *et al.*, 2004). High-resolution laser profiling along the growth axis of sectioned otoliths are necessary to fully resolve this question.

Magnesium

Treatments with ^{24}Mg geo-elements only marked 5% of otoliths, making magnesium unsuitable for enriched geo-element marking via immersion. Other geo-element immersion studies have encountered difficulties with ^{24}Mg incorporation (Munro *et al*., 2008; Woodcock *et al*., 2011a,b). As Mg is a physiologically essential element, it is tightly regulated by fish (Shearer & Asgard, 1992). It is also an abundant minor element in fish tissue, and only 1-2% of Mg ions are transported into the endolymph fluid due to a relatively slow exchange rate in body tissue compared to Ca (Maguire & Cowan, 2002). Consequently, changing the Mg geo-elemental composition of otoliths will likely require concentrations and immersion times that are not logistically feasible.

Potential applications

Osmotically induced geo-element labels via larval immersion is an efficient, reliable and practical way to batch mark fish, with the potential to create unique marks or “fingerprints” using different natural geo-element combinations. With only stable barium and strontium geo-elements, hundreds of unique combinations are possible, which makes the technique suitable for a wide range of applications, such as evaluating the success of restocking programs, the impacts of farmed fish escapees on wild stocks, and for mark-recapture studies investigating fish movement and mortality.

REFERENCES

Alcobendas, M., Lecomte, F., Castanet, J., Meunier, F. J., Maire, P., and Holt, E. M. 1991. Mass labeling of elvers with fast balneation in fluorochromes. Application to tetracycline labeling of 500kg of elvers. Bulletin Français de la Peche et de la Pisciculture 321:43–54.

Almany, G. R., Berumen, M. L., Thorrold, S. R., Planes, S., and Jones, G. P. 2007. Local replenishment of coral reef fish populations in a marine reserve. Science, 316: 742-744.

Brown, P., and Harris, J. H. 1995. Strontium batch-marking of golden perch (*Macquaria ambigua* Richardson) and trout cod (*Maccullochella macquariensis*) (Cuvier). *In* Recent developments in fish otolith research, pp. 693-701. Ed. by D. H. Secor, J. M. Dean, S. E. Campana. University of South Carolina Press, Columbia, S.C. 735 pp.

Buckland-Nicks, J. A., Gillis, M., and Reimchen, T. E. 2011. Neural network detected in a presumed vestigial trait: ultrastructure of the salmonid adipose fin. Proceedings of the Royal Society B – Biological Sciences, 279: 553-563.

Campanella, D., Garriz, A., Colautti, D. C., Somoza, G. M., and Miranda, L. A. 2013. Osmotic induction marking with Alizarin Red S on juveniles of pejerrey, *Odontesthes bonariensis* (Atherinopsidae). Neotropical Ichthyology, 11: 95-100.

Campana, S. E. 1999. Chemistry and composition of fish otoliths: pathways, mechanisms and applications. Marine Ecology Progress Series, 188: 263-297.

Conte, F. P. 1969. Salt secretion. *In* Fish physiology, volume 1, pp. 241-292. Ed. by W. S. Hoar and D. J. Randall. Academic Press, New York. 464 pp.

Crook, D. A., O'Mahony, D., Gillanders, B. M., Munro, A. R., and Sanger, A. C. 2007. Production of external fluorescent marks on Golden Perch fingerlings through osmotic induction marking with Alizarin Red S. North American Journal of Fisheries Management, 27: 670-675.

Crook, D. A., O'Mahony, D., Sanger, A. C., Munro, A. R., Gillanders, B. M., and Thurstan, S. 2009. Development and evaluation of methods for osmotic induction marking of golden perch *Macquaria ambigua* with Calcein and Alizarin Red S. North American Journal of Fisheries Management, 29: 279-287.

Edmonds, J. S., Moran, M. J., Caputi, N., and Morita, M. 1989. *Trace element analysis* of fish sagittae as an aid to stock identification: pink snapper (*Chrysophrys auratus*) in Western Australia waters. Canadian Journal of Fisheries and Aquatic Sciences, 46: 50-54.

Elsdon, T. S., and Gillanders, B. M. 2003. Relationship between water and otolith elemental concentrations in juvenile black bream *Acanthopagrus butcheri*. Marine Ecology Progress Series, 260: 263-272.

Elsdon, T. S., and Gillanders, B. M. 2005. Strontium incorporation into calcified structures: separating the effects of ambient water concentration and exposure time. Marine Ecology Progress Series, 285: 233-243.

Elsdon, T. S., Wells, B. K., Campana, S. E., Gillanders, B. M., Jones, C. M., Limburg, K. E., Secor, D. H., *et al*. 2008. Otolith chemistry to describe movements and life-history parameters of fishes: hypotheses, assumptions, limitations and inferences. Oceanography and Marine Biology: An Annual Review, 46: 297-330.

Ennevor, B. C., Beames, R. M. 1993. Use of lanthanide elements to mass mark juvenile salmonids. Canadian Journal of Fisheries and Aquatic Sciences, 50: 1039-1044.

Hilborn, R., Walters, C. J., and Jester, D. B. Jr. 1990. Value of fish marking in fisheries management. American Fisheries Society Symposium, 7: 5-7.

Holliday, F. G. T. 1969. The effects of salinity on the eggs and larvae of teleosts. *In* Fish physiology, volume 1, pp. 293.311. Ed. by W. S. Hoar and D. J. Randall. Academic Press, New York. 464 pp.

Huelga-Suarez, G., Moldovan, M., Garcia-Valiente, A., Garcia-Vazquez, E., and Garcia Alonso, J. I. G. 2012. Individual-specific transgenerational marking of fish populations based on a barium dual-isotope procedure. Analytical Chemistry, 84: 127-133.

Kaill, W. M., Rawson, K., and Joyce, T. 1990. Retention rates of half-length coded wire tags implanted in emergent pink salmon. *In* Fish-marking techniques, pp. 253-258. Ed. by N. C. Parker, A. E. Giorgi, R. C. Heidinger, D. B. Jr. Jester, E. D. Prince, and G. A. Winans. American Fisheries Society Symposium 7, Bethesda, Maryland. 879 pp.

Kuroki, M., Buckley, R. M., LeClair, L. L., and Hauser, L. 2010. Validation and efficacy of transgenerational mass marking of otoliths in viviparous fish larvae. Journal of Fish Biology, 77: 292-298.

Maguire, M. E., and Cowan, J. A. 2002. Magnesium chemistry and biochemistry. BioMetals, 15: 203-210.

McCormick, M. I., and Hoey, A. S. 2004. Larval growth history determines juvenile growth and survival in a tropical marine fish. Oikos, 106: 225-242.

McFarlane, G. A., Wydoski, R. S., and Prince, E. D. 1990. Historical review of the development of external tags and marks. American Fisheries Society Symposium, 7: 9-29.

Mohler, J. W. 2003. Producing fluorescent marks on Atlantic salmon fin rays and scales with calcein via osmotic induction. North American Journal of Fisheries Management, 23: 1108-1113.

Munro, A. R., McMahon, T. E., Leathe, S. A., and Liknes, G. 2003. Evaluation of batch marking small rainbow trout with coded wire tags. North American Journal of Fisheries Management, 23: 600-604.

Munro, A. R., Gillanders, B. M., Elsdon, T. S., Crook, D. A., and Sanger, A. C. 2008. Enriched stable isotope marking of juvenile golden perch (*Macquaria ambigua*) otoliths. Canadian Journal of Fisheries and Aquatic Sciences, 65: 276-285.

Munro, A. R., Gillanders, B. M., Thurstant, S., Crook, D. A., and Sanger, A. C. 2009. Transgenerational marking of freshwater fishes with enriched stable isotopes: a tool for fisheries management and research. Journal of Fish Biology, 75: 668-684.

Negus, M. T., and Tureson, F. T. 2004. Retention and nonlethal external detection of calcein marks in rainbow trout and Chinook salmon. North American Journal of Fisheries Management, 24: 741-747.

Reinert, T. R., Wallin, J., Griffin, M. C., Conroy, M. J., and Van den Avyle, 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.

Roy, A. S., Frisch, A. J., Syms, C., Thorrold, S. R., and Jones, G. P. 2013. Retention of a transgenerational marker (^{137}Barium) in tissues of adult female anemonefish and assessment of physiological stress. Environmental Biology of Fishes, 96: 459-466.

Secor, D. H., and Houde, E. D. 1995. Larval mark–release experiments: potential for research on dynamics and recruitment in fish stocks. *In* Recent developments in fish otolith research, pp. 423-444. Ed. by D. H. Secor, J. M. Dean, and S. E. Campana. University of South Carolina Press, Columbia, S.C. 735pp.

Serafy, J. E., Lutz, S. J., Capo, T. R., Ortner, P. B., and Lutz, P. L. 1995. Anchor tags affect swimming performance and growth of juvenile red drum (*Sciaenops ocellatus*). Marine and Freshwater Behaviour and Physiology, 27: 29-35.

Shearer, K. D., and Asgard, T. 1992. The effect of water-borne magnesium on the dietary magnesium requirement of the rainbow-trout (*Oncorhynchus mykiss*). Fish Physiology and Biochemistry, 9: 387-392.

Smith, J. E., Macreadie, P. I., and Swearer, S. E. 2010. An osmotic induction method for externally marking saltwater fishes, *Stigmatopora argus* and *Stigmatopora nigra,* with calcein. Journal of Fish Biology, 76: 1055-1060.

Smith, K. T., and Whitledge, G. W. 2011. Evaluation of a stable-isotope labelling technique for mass marking fin rays of age-0 lake sturgeon. Fisheries Management and Ecology, 18: 168-175.

Swearer, S. E., Shima, J. S., Hellberg, M. E., Thorrold, S. R., Jones, G. P., Robertson, D. R., Morgan, S. G., *et al*. 2002. Evidence of self-recruitment in demersal marine populations. Bulletin of Marine Science, 70S: 251-271.

Thomas, L. M., Holt, S. A., and Arnold, C. R. 1995. Chemical marking techniques for larval and juvenile red drum (*Sciaenops ocellatus*) otoliths using different fluorescent markers. *In* Recent developments in fish otolith research, pp. 703-717. Ed. By D. H. Secor, J. M. Dean, and S. E. Campana. University of South Carolina Press, Columbia, S.C. 735 pp.

Thorrold, S. R., Jones, G. P., Hellberg, M. E., Burton, R. S., Swearer, S. E., Neigel, J. E., Morgan SG, *et al.* 2002. Quantifying larval retention and connectivity in marine populations with artificial and natural markers. Bulletin of Marine Science, 70S: 291-308.

Thorrold, S. R., Jones, G. P., Planes, S., and Hare, J. A. 2006. Transgenerational marking of embryonic otoliths in marine fishes using barium stable isotopes. Canadian Journal of Fisheries and Aquatic Sciences, 63: 1193-1197.

van der Walt, B., and 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.

Volk, E. C., Schroder, S. L., and Grimm, J. J. 1999. Otolith thermal marking. Fisheries Research, 43: 205-219.

Walther, B. D., and Thorrold, S. R. 2006. Water, not food, contributes the majority of strontium and barium deposited in the otoliths of a marine fish. Marine Ecology Progress Series, 311: 125-130.

Warren-Myers, F., Dempster, T., Jensen, A., Fjelldal, P. G., Hansen, T., and Swearer, S. *in press.* Stable isotope marking of otoliths during vaccination: A novel method for mass marking fish. Aquaculture Environment Interactions.

Webb, S. D., Woodcock, S. H., and Gillanders, B. M. 2012. Sources of otolith barium and strontium in estuarine fish and the influence of salinity and temperature. Marine Ecology Progress Series, 453: 189-199.

Williamson, D. H., Jones, G. P., and Thorrold, S. R. 2009a. An experimental evaluation of transgenerational isotope labelling in a coral reef grouper. Marine Biology, 156: 2517-2525.

Williamson, D. H., Jones, G. P., Thorrold, S. R., and Frisch, A. J. 2009b. Transgenerational marking of marine fish larvae: stable isotope retention, physiological effects and health issues. Journal of Fish Biology, 74: 891-905.

Woodcock, S. H., Gillanders, B. M., Munro, A. R., McGovern, F., Crook, D. A., and Sanger, A. C. 2011a. Using enriched stable isotopes of barium and magnesium to batch mark otoliths of larval golden perch (*Macquaria ambigua* Richardson). Ecology of Freshwater Fish, 20: 157-165.

Woodcock, S. H., Gillanders, B. M., Munro, A. R., Crook, D. A., and Sanger, A. C. 2011b. Determining mark success of 15 combinations of enriched stable isotopes for the batch marking of larval otoliths. North American Journal of Fisheries Management, 31: 843-851.

Woodcock, S. H., Grieshaber, C. A., and Walther, B. D. 2013. Dietary transfer of enriched stable isotopes to mark otoliths, fin rays, and scales. Canadian Journal of Fisheries and Aquatic Sciences, 70: 1-4.

Woodhead, J., Hergt, J., Shelley, M., Eggins, S., and Kemp, R. 2004. Zircon Hf-isotope analysis with an excimer laser, depth profiling, ablation of complex geometries, and concomitant age estimation. Chemical Geology, 209: 121-135.

Figure 1. Example geo-element ratio profiles of the first 100 scans from a marked otolith (black line) and an unmarked otolith (grey line) for ^{137}Ba:^{138}Ba, ^{86}Sr:^{88}Sr and ^{26}Mg:^{26}Mg**.** One acquisition results in a scan of each geo-element. The otolith edge is located at the start of each analysis. Dashed black line represents the limit of detection for each geo-element ratio.

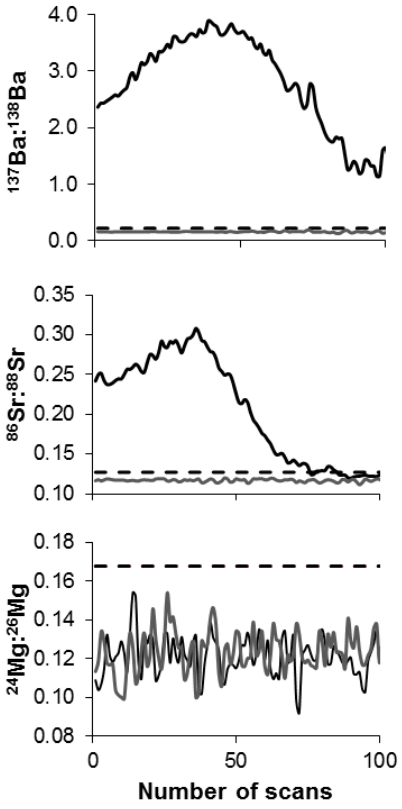


Figure 2. Relative mark strength among treatments for maximum geo-element ratio (MIR) and number of scans above the limit of detection (SAD). Each bar (grey- without osmotic induction; black- with osmotic induction) represents the mean of eight otoliths, except where asterisks indicate the mean of seven otoliths. Error bars are standard error. Note: statistical tests for MIR were performed on log-transformed data.

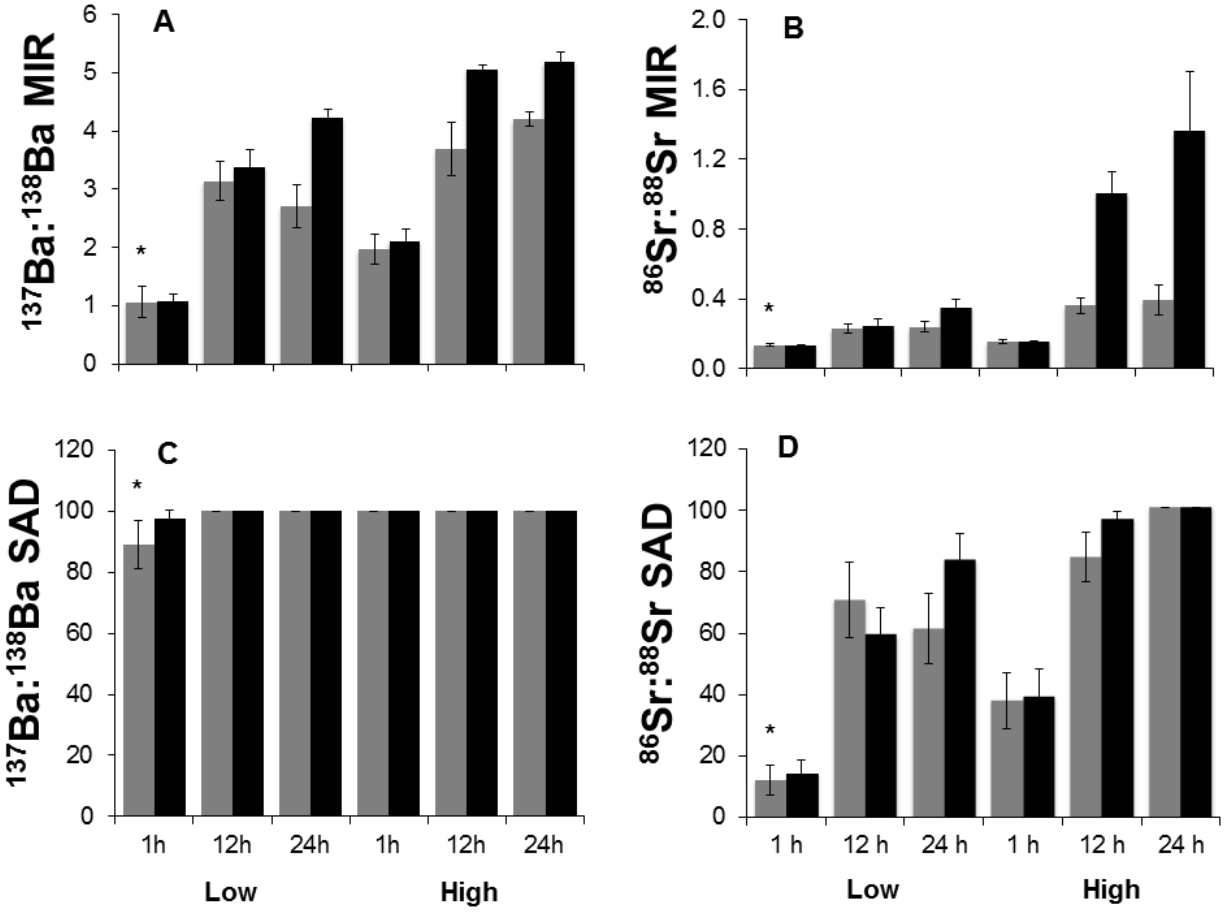


Table 1. Percentage of fish marked with ^{137}Ba, ^{86}Sr and ^{26}Mg per treatment. Treatments consisted of osmotic induction or no osmotic induction, low or high geo-element enrichment concentration, and 1, 12 or 24 hour immersion time. Successful marks were classified as 3 consecutive geo-elemental ratios in a treatment fish that were 3.3 times above the standard deviation of the average geo-elemental ratios in control fish. Low concentrations: ^{137}Ba 30 µg L^{-1}, ^{86}Sr and ^{26}Mg 75 µg L^{-1}. High concentrations: ^{137}Ba 100 µg L^{-1}, ^{86}Sr and ^{26}Mg 250 µg L^{-1}.

Treatments			**Isotope Mark Success**		
Osmotic Induction	Concentration	Immersion Time (h)	^{26}Mg	^{86}Sr	^{137}Ba
Yes	Low	1	14%	71%	**100%**
Yes	Low	12	0%	**100%**	**100%**
Yes	Low	24	0%	**100%**	**100%**
Yes	High	1	0%	**100%**	**100%**
Yes	High	12	13%	**100%**	**100%**
Yes	High	24	13%	**100%**	**100%**
No	Low	1	13%	38%	**100%**
No	Low	12	0%	**100%**	**100%**
No	Low	24	0%	**100%**	**100%**
No	High	1	13%	88%	**100%**
No	High	12	0%	**100%**	**100%**
No	High	24	0%	**100%**	**100%**

Table 2. Effects of osmotic induction, immersion time and geo-element concentration on maximum geo-element ratios for $^{137}Ba:^{138}Ba$ and $^{86}Sr:^{88}Sr$ ratios in the otoliths of *Salmo salar* alevins. Osm Ind = osmotic induction; Conc = concentration; Time = immersion time. For post-hoc Tukey HSD ($p < 0.05$): concentration = high or low; immersion time = 24h, 12h or 1h; osmotic induction = yes or no.

Geo-element	Source	df	MS	F	P	Tukey HSD
Log (Max)	Osm Ind	1	0.131	14.1	<**0.001**	
($^{137}Ba:^{138}Ba$)	Conc	1	0.382	41.1	<**0.001**	
	Osm Ind × Conc	1	0.000	0.03	0.87	
	Time	2	0.892	96.0	<**0.001**	24h = 12h > 1h
	Osm Ind × Time	2	0.019	2.02	0.14	
	Conc × Time	2	0.012	1.33	0.27	
	Osm Ind × Conc × Time	2	0.019	2.04	0.14	
	Error	81	0.009			
Log (Max)	Osm Ind	1	0.112	33.1	<**0.001**	
($^{86}Sr:^{88}Sr$)	Conc	1	0.180	53.2	<**0.001**	
	Osm Ind × Conc	1	0.071	21.1	<**0.001**	Yes-High > No-High = Yes-Low = No-Low
	Time	2	0.121	35.9	<**0.001**	24h = 12h > 1h
	Osm Ind × Time	2	0.031	9.22	**0.001**	Yes-24h = Yes-12h > No-12h = No-24h = Yes-1h = No-1h
	Conc × Time	2	0.038	11.3	<**0.001**	High-24h = High-12h > low-24h = Low-12h = High-1h = Low-1h
	Osm Ind × Conc × Time	2	0.018	5.19	**0.008**	Yes-High-24h = Yes-High-12h > No-High-12h = No-High-24h = Yes-Low-24h = No-Low-12h = Yes-Low-12h = No-Low-24h = Yes-High-1h = No-High-1h = No-Low-1h =Yes-Low-1h
	Error	81	0.003			

Table 3. Effects of osmotic induction, immersion time and geo-element concentration on the percentage of scans above the detection limit for ^{137}Ba:^{138}Ba and ^{86}Sr:^{88}Sr ratios in otoliths of *Salmo salar* alevins. Osm Ind = osmotic induction; Conc = concentration; Time = immersion time. For post-hoc Tukey HSD ($p < 0.05$): concentration = high or low; immersion time = 24h, 12h or 1h; osmotic induction = yes or no.

Geo-element	**Source**	**df**	**MS**	**F**	**P**	**Tukey HSD**
Log (Count +1)	Osm Ind	1	0.008	1.53	0.22	
^{137}Ba:^{138}Ba	Conc	1	0.019	3.50	0.06	
	Osm Ind × Conc	1	0.008	1.59	0.21	
	Time	2	0.019	3.53	**0.034**	24h = 12h > 1h
	Osm Ind × Time	2	0.008	1.48	0.23	
	Conc × Time	2	0.018	3.43	**0.037**	High-24h = High-12h = low-24h = Low-12h = High-1h > Low-1h
	Osm Ind × Conc × Time	2	0.008	1.54	0.22	
	Error	81	0.005			
Log (Count +1)	Osm Ind	1	0.351	0.72	0.39	
^{86}Sr:^{88}Sr	Conc	1	11.17	22.9	**<0.001**	
	Osm Ind × Conc	1	0.034	0.70	0.79	
	Time	2	24.50	50.2	**<0.001**	24h = 12h > 1h
	Osm Ind × Time	2	0.063	0.13	0.88	
	Conc × Time	2	1.423	2.91	0.06	
	Osm Ind × Conc × Time	2	0.226	0.46	0.63	
	Error	81	0.488			

Table 4. Study species, concentrations and immersion times used in natural geo-element immersion marking studies that achieved 100% mark success.

Source	**Species**	**Geo-element Concentration**	**Immersion Time**	**Osmotic induction**
Walther & Thorrold 2006	*Fundulus heteroclitus*	^{137}Ba (5 µg L^{-1}), ^{86}Sr (100 µg L^{-1})	70 d	No
Munro *et al*. 2008	*Macquaria ambigua*	^{137}Ba (15 µg L^{-1})	8 d	No
Woodcock *et al.* 2011a	*Macquaria ambigua*	^{137}Ba (30 µg L^{-1})	1 d	No
Woodcock *et al.* 2011b	*Maccullochella peelii*	^{137}Ba, ^{138}Ba, ^{88}Sr (all at 15 µg L^{-1})	6 d	No
Present study	*Salmo salar*	^{137}Ba (30 µg L^{-1}), ^{86}Sr (75 µg L^{-1})	1 hr, 12 hr	No
Present study	*Salmo salar*	^{137}Ba (30 µg L^{-1}), ^{86}Sr (250 µg L^{-1})	1 hr	Yes

6 Project deliverables

Published scientific articles:

de Braux E, Warren-Myers F, Dempster T, Fjelldal PG, Hansen T, Swearer SE (2014) Osmotic induction improves batch marking of larval fish otoliths with enriched stable isotopes. ICES. J. Mar. Sci. 71(9): 2530-2538.

Warren-Myers F, Dempster T, Jensen A, Fjelldal PG, Hansen T, Swearer SE (2014) Stable isotope marking of otoliths during vaccination: A novel method for mass marking fish. Aquacult. Environ. Interact. **5**: 143-154.

Warren-Myers F, Dempster T, Fjelldal PG, Hansen T, Swearer SE (2015a) An industry-scale mass marking technique for tracing farmed fish escapees. PLoS One. 10(3): e0118594.

Warren-Myers F, Dempster T, Fjelldal PG, Hansen T, Swearer SE (2015b) Rapid uptake of stable isotope markers in salmonids during egg swelling. Can J Fish Aquat Sci 72:1-6

Warren-Myers F, Dempster T, Fjelldal PG, Hansen T, Swearer SE (2015c) Mass marking farmed Atlantic salmon with transgenerational isotopic fingerprints to trace farm fish escapees. *Submitted*

Conference and workshop presentations:

Warren-Myers, F., T. Dempster, PG. Fjelldal, T. Hansen, and S. E. Swearer (2014) Stable isotope otolith fingerprint signatures: A mass marking technique for farmed Atlantic salmon *Salmo salar.* 5th International Otolith Symposium, Mallorca, Spain.

Warren-Myers, F., T. Dempster, PG. Fjelldal, T. Hansen, and S. E. Swearer (2014) Otolith fingerprint signatures: A mass marking technique for farmed Atlantic salmon *Salmo salar.* World Aquaculture Conference, Adelaide, Australia

7 References

Almany, G., and S. Thorrold (2007). Local replenishment of coral reef fish populations in a marine reserve. *Science* 316: 742-744.

Buckland-Nicks, J.A., Gillis M., and T.E. Reimchen (2011) Neural network detected in a presumed vestigial trait: ultrastructure of the salmonid adipose fin. *Proceedings of the Royal Society B – Biological Sciences*. (in press)

Buckley, R., LeClair, L., Volk, E., and S. Schroder (2007). Preliminary results of transgenerational marking of larval marine fish otoliths. *In Biology, Assessment and Management of Pacific Rockfishes* 3: 87-98.

Crook, D., O'Mahony, D., Sanger, A., Munro, A., Gillanders, B. and S. Thurstan (2009). Development and evaluation of methods for osmotic induction marking of golden perch *Macquaria ambigua* with calcein and alizarin red S. *North American Fisheries Management* 29: 279-287.

Fiske, P., Lund, R.A., and L.P. Hansen (2006) Relationships between the frequency of farmed Atlantic salmon, *Salmo salar* L., in wild salmon populations and fish farming activity in Norway, 1989-2004. *ICES Journal of Marine Science* 63: 1182-1189.

Glover, K.A. (2010) Forensic identification of fish farm escapees: the Norwegian experience. *Aquaculture Environment Interactions* 1: 1-10.

Jensen, Ø., Dempster, T., Thorstad, E.B., Uglem, I., and A. Fredheim (2010) Escapes of fishes from Norwegian sea-cage aquaculture: causes, consequences and prevention. *Aquaculture Environment Interactions* 1: 71-83.

Jones, G., Emsile, M., and C. Lunow (1999). Self-recruitment in a coral reef fish population. *Nature* 402: 802-804.

Jones, G., and S. Thorrold (2005). Coral reef fish larvae settle close to home. *Current Biology* 15: 1314-1318.

Kuroki., M., Buckley, R., LeClair, L., and L. Hauser (2010) Validation and efficacy of transgenerational mass marking of otoliths in viviparous fish larvae. *Journal of Fish Biology* 77: 292-298.

Munro, A.R., and T.E. McMahon (2003) Evaluation of batch marking small rainbow trout with coded wire tags. *North American Journal of Fisheries Management* 23: 600-604.

Munro, A., Gillanders, B., Thurstant, S., Crook, D., and A. Sanger (2009) Transgenerational marking of freshwater fishes with enriched stable isotopes: a tool for fisheries management and research. *Fish Biology* 75: 668-684.

Roberts R.J., McQueen A., Shearer W.M., and Young H. (1973a) The histopathology of salmon tagging. I. The tagging lesion in newly tagged parr. *Journal of Fish Biology* 5: 497-503.

Roberts R.J., McQueen A., Shearer W.M., and Young H. (1973b) The histopathology of salmon tagging. II. The chronic tagging lesion in returning adult fish. *Journal of Fish Biology* 5: 615-619.

Roberts R.J., McQueen A., Shearer W.M., and Young H. (1973c) The histopathology of salmon tagging. III. Secondary infections associated with tagging. *Journal of Fish Biology* 5: 621-623.

Reimchen, T., and N.F. Temple (2004) Hydrodynamic and phylogenetic aspects of the adipose fin in fishes. *Canadian Journal of* Zoology 82: 910-916.

Serafy J.E., Lutz S.J., Capo T.R., Ortner P.B., and Lutz P.L. (1995) Anchor tags affect swimming performance and growth of juvenile red drum (*Sciaenops ocellatus*). *Marine and Freshwater Behaviour and Physiology* 27: 29-35.

Smith, J., and S. Swearer (2011) Transgenerational marking of embryonic mosquitofish larvae via maternal transmission of enriched stable isotopes. Masters Thesis, University of Melbourne, Australia.

Swearer S., Caselle, J.E., Lea, D.W., and R.R. Warner (1999) Larval retention and recruitment in an island population of a coral-reef fish. *Nature* 402: 799-802.

Swearer, S.E., and J.S. Shima (2010) Regional variation in larval retention and dispersal drives recruitment patterns in a temperate reef fish. *Marine Ecology Progress Series* 417: 229-236.

Thorrold, S., Planes, S., and J. Hare (2006) Transgenerational marking of embryonic otoliths in marine fishes using barium stable isotopes. *Canadian Journal of Fisheries and Aquatic Sciences* 63: 1193-1197.

Torrissen, O.J. (2007) Status report for Norwegian aquaculture 2007. *Kyst og Havbruk* 2007: 11-12 (in Norwegian)

Tsukamoto, K. (1988). Mass-marking of ayu eggs and larvae by tetracycline-tagging of otoliths. *Fisheries Oceanography* 51: 903-911.

Williamson, D., Jones, G., and S. Thorrold (2009a) An experimental evaluation of transgenerational isotope labelling in a coral reef grouper. *Marine Biology* 156: 2517-2525.

Williamson, D., Jones, G., Thorrold S., and Frisch, A.J. (2009b) Transgenerational marking of marine fish larvae: stable-isotope retention, physiological effects and health issues. *Journal of Fish Biology* 74: 891-905.