Combined effects of extrinsic and intrinsic factors on otolith chemistry: implications for environmental reconstructions

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Running title: Extrinsic and intrinsic effects on otolith chemistry

Abstract

Otolith chemistry is widely used to understand patterns of fish movement and habitat use, with significant progress made in understanding the influence of environmental factors on otolith elemental uptake. However, few studies consider the interactive effect that environmental and genetic influences have on otolith chemistry. This study assessed the influence of salinity, temperature and genetics on the incorporation of three key elements (Sr, Ba and Mg) into the otoliths of two discrete stocks of *Argyrosomus japonicus* fingerlings reared in captivity. Elemental analysis via laser ablation ICPMS found that stock (genetics) had a significant interactive effect on otolith Sr:Ca (salinity × temperature × stock) and Ba:Ca (salinity × stock), but did not affect Mg:Ca incorporation. Mg:Ca showed a positive relationship with temperature for both stocks. The incorporation of some elements into the otoliths of fish is the result of complex interactions between extrinsic and intrinsic factors. These findings highlight the necessity to also consider stock along with environmental variables when using trace elemental signatures to reconstruct the environmental histories of fish.

Keywords: fish; salinity; temperature; genetics; stock; otolith; ICPMS; *Argyrosomus japonicus*; mulloway; kob; environmental; hypersalinity.

Introduction

Understanding patterns of movement and stock structure is vital for the development of spatially appropriate management and conservation action (Campana et al. 1999; Thorrold et al. 2001). It is well recognised that tag and recapture methods for assessing species movement and population structure only provide information on tagged fish, require large numbers of fish to be tagged to get meaningful returns and can be expensive. Otolith chemistry provides an alternate approach since all fish contain a natural tag. Furthermore, unlike conventional tag and recapture technologies, otolith chemistry is applicable to all life history stages (Gillanders 2005).

Otoliths are calcium carbonate structures that act as chronometers of environmental change by incorporating information from the surrounding environment into their matrix (Elsdon et al. 2008). This incorporation is permanent (Campana 1999), and has been widely used to reconstruct the environmental histories of fish and to delineate discrete stocks of fish (Campana et al. 2000; Morris et al. 2003; Ferguson et al. 2011). However, a growing body of literature indicates that otolith chemistry is influenced by a range of intrinsic (i.e. physiological and genetic) (e.g. Clarke et al. 2011) and extrinsic (i.e. environmental: salinity and temperature) (e.g. Elsdon and Gillanders 2002) factors. These factors have been shown to interact (Elsdon and Gillanders 2002) or act independently (Martin et al. 2004) on various elements that are incorporated into otoliths in a species-specific manner (Diouf et al. 2006; Martin and Wuenschel 2006; Dorval et al. 2007). Species-specific responses make generalised predictions of environmental effects on otolith chemistry difficult, potentially impeding the ability to reconstruct the habitat use of fish. A greater understanding of how both intrinsic and extrinsic factors affect otolith trace element incorporation is needed.

Inter and intraspecific genetic differences may affect otolith chemistry but have not been extensively tested (Thresher 1999). Inter or species-specific differences in otolith chemistry has been demonstrated through tank rearing experiments (Elsdon and Gillanders 2003) and similarly in the

54 wild (Gillanders and Kingsford 2003; Hamer and Jenkins 2007). However, it is possible that within
55 species (intra) genetic differences (stock or population differences) may also cause differences in
56 otolith chemistry. One previous study has reported intraspecific effects (genetics) on otolith
57 chemistry of a teleost (*Menidia menidia*) (Clarke et al. 2011). Similarly extrinsic factors also require
58 further investigation into their influence on otolith chemistry since variation among species is
59 reported (Elsdon and Gillanders 2003).
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61 This study sought to determine if otolith chemistry varied between two genetic stocks of mulloway
62 (kob) *Argyrosomus japonicus*, a commercially important species, and whether any variation was
63 influenced by extrinsic factors, namely temperature and salinity. Specifically, we aimed to determine
64 the relative and interactive effects of stock (genetic component), salinity and temperature
65 (environmental component) on otolith elemental chemistry in a controlled laboratory experiment.
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Materials and Methods

Experimental design

A controlled laboratory experiment was conducted to test the effects of salinity and temperature on elemental chemistry of otoliths of *Argyrosomus japonicus* from two different genetic stocks of hatchery fish. The salinity and temperature ranges were chosen to represent natural conditions experienced by the species (e.g. brackish to hypersaline). Limitations with obtaining juvenile fish at the same time meant that experimental rearing of each genetic stock of *A. japonicus* was conducted separately. The first experiment focused on a New South Wales (NSW) stock and used seven nominal salinity levels (10, 20, 30, 35, 40, 45 and 50 ‰) at a single temperature (20 °C). A further experiment used four of these salinities (10, 30, 40 and 50 ‰), each replicated at three different temperatures (16, 20 and 24 °C) to investigate the interactive effect of temperature and salinity. The Western Australian (WA) stock was exposed to four salinity (10, 30, 40 and 50 ‰) and two temperature (20 and 24 °C) treatments; treatment levels were reduced due to decreased numbers of fish being available.

Fish rearing

Both stocks of *A. japonicus* were sourced from hatcheries. The NSW stock came from the New South Wales Department of Primary Industries (NSW DPI) hatchery at Port Stephens and fish were ~0.8 g at the start of the experiment. The WA stock was sourced from Challenger TAFE in Perth, Western Australia as larvae and reared at the South Australian Research and Development Institute (SARDI) Aquatic Sciences hatchery at West Beach until the fish attained an approximate weight of 0.8 g.

Fish were initially housed in 250 L polypropylene tanks and held for at least one week to acclimate or in the case of larvae to metamorphose and grow to the desired size. During acclimation, temperature was maintained at a nominal 21.5 °C to encourage growth. Fresh UV filtered seawater was sourced from SARDI Aquatic Sciences (40 ‰) and supplied to the holding tanks in half volume

104 water changes twice weekly. Fish were fed daily on commercially available pellets (Grobest Pty Ltd;
105 barramundi feed - floating, 0.75 mm and 2mm diameter), except during the larval development
106 phase where the diet initially comprised rotifers and *Artemia* spp. until fish were pellet weaned
107 (consistent with standard hatchery practice) (e.g. Battaglene and Talbot 1994).

108

109 On completion of the acclimation or development period, all fish were fasted for 24-h and then
110 immersed in an alizarin complexone ($C_{19}H_{15}NO_8$) bath at a concentration of 35 $mg \cdot L^{-1}$ for 24-h to
111 mark the otoliths (de Vries et al. 2005). The alizarin mark distinguished the experimental growth
112 from the hatchery growth.

113

114 Fish were randomly assigned to experimental tanks at a nominal density of either ten fish per tank
115 (NSW) or seven fish (WA) and reared under experimental conditions for one month. The differences
116 in density were a result of less fish being available from the WA stock. There were two experimental
117 tanks per treatment for both stocks. The experimental tanks were 40 L in volume and manufactured
118 from high density polypropylene. Experimental tanks were covered with clear plexiglass lids to allow
119 light penetration, stop jumping mortalities and minimize evaporation thereby keeping experimental
120 salinities constant. Light was supplied as a timer controlled 12-h photoperiod by metal halide grow
121 lights. Water aeration was provided constantly by filtered compressed air. Water quality was
122 maintained during the course of the experiment by regular 50 % water changes.

123

124 Fish were gradually acclimatised to experimental salinities, which were raised or lowered at a rate of
125 5 ‰ every 24-h. For the NSW stock, hypersaline solution (~ 75 ‰) was sourced from Adelaide pilot
126 desalination plant (Adelaide Aqua). The brine was mixed with sea water (40 ‰) at appropriate
127 concentrations to produce the two hypersaline treatments (45 and 50 ‰). For the WA stock, Red
128 Sea Salt © was added to seawater (as desalination brine was unavailable) and allowed to stabilize
129 for 24-h with aeration before use to produce a single hypersaline solution (50 ‰). All experiments

130 used straight sea water for the ambient seawater (40 ‰) treatments. Treatments with salinities
131 below 40 ‰ were achieved by diluting seawater with bore water (1 – 2 ‰) sourced from SARDI
132 Aquatic Sciences.

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134 Fish were gradually acclimatised to experimental temperatures at a rate of 2 °C over 48-h, using
135 aquarium heaters in tanks to increase temperatures or flow through chillers to decrease
136 temperatures. All tanks were immersed in water baths to maintain constant temperatures. The 16 °C
137 treatments were maintained by external chillers (Carrier ©), split system air-conditioning and back
138 up portable chillers (necessary during the hot South Australian summer). The 20 °C and 24 °C
139 treatments were maintained for both experiments with water baths chilled to 18 °C and individual
140 tanks raised by aquarium heaters to the appropriate levels.

141

142 Temperature and salinity were periodically measured in each tank using an electronic water quality
143 unit (YSI Sonde - 556 MPS). The meter was calibrated once a week using a solution of known salinity
144 and temperature chosen to represent the mid range of experimental salinities and temperatures.

145

146 Chemical analysis of water samples
147 Water samples were collected for elemental analysis at the beginning, midway and at the
148 completion of the rearing period. Water samples were collected using a 25 ml syringe, filtered (40
149 µm) and nitric acid preserved (pH < 2) before refrigeration. Experimental water samples were
150 analysed for ambient elemental concentrations, which were determined using dual-view inductively
151 coupled plasma–atomic emission spectrometer (DV ICP-AES; Perkin-Elmer 3000) for the analysis of
152 calcium (Ca) and magnesium (Mg) and a dynamic reaction cell inductively coupled plasma–mass
153 spectrometer (DRC ICP-MS; Perkin-Elmer 6000) for the analysis of strontium (Sr) and barium (Ba).
154 Water concentration data were converted to molar concentrations and ratioed to calcium.

155

156 Otolith preparation and analysis

157 At the end of the experiments (28 days), all fish were euthanized in an ice and seawater slurry, and
158 then stored frozen until the otoliths were extracted. For each fish, standard lengths were recorded
159 before dissection. The sagittal otoliths were extracted, washed in ultrapure water and allowed to dry
160 before being stored in microcentrifuge tubes. Otoliths were embedded in two part epoxy 'Epofix'
161 (Struers) spiked with 40 ppm indium and sectioned transversely through the nucleus to 0.35 ± 0.05
162 mm using an Isomet low speed diamond saw (Buehler Ltd). Otolith sections were polished using
163 lapping film lubricated with ultra pure water to produce a surface finish appropriate for chemical
164 analysis (~0 3 µm). Otolith sections from different treatments were mounted randomly on
165 microscope slides using 40 ppm indium spiked thermoplastic cement (Crystalbond 509). Slides were
166 then cleaned by sonication in ultrapure water for five minutes and allowed to dry in a laminar flow
167 hood. Otolith sections were examined under a fluorescent microscope with transmitted light (Leica
168 model - DMLB), which highlighted the alizarin mark thereby indicating experimental growth. Rough
169 otolith drawings indicating the alizarin mark were made to facilitate the targeting of experimental
170 growth during elemental analysis.

171

172 The concentrations of Sr, Ba, Mg and Ca (the trace elements that record the environmental
173 conditions) in the otolith samples were determined using an Agilent 7500cs Inductively Coupled
174 Plasma-Mass Spectrometer (ICP-MS) coupled to a Merchantek UP213 (New Wave Research) Nd: YAG
175 deep UV laser microprobe, with a pulse rate of 5.00 Hz. The outer otolith edge of all experimental
176 fish was analysed using a 30 µm diameter laser beam. The beam was centred approximately 20 µm
177 from the outer edge of the otolith but within the experimental growth region. Sample gases were
178 extracted from the chamber through a smoothing manifold facilitated by a helium and argon stream.
179 Analysis involved a 30-s background count to determine detection limits followed by a 100-s ablation
180 of the experimental otolith growth.

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To correct for machine drift a glass reference sample (National Institute of Standards and Technology, NIST 612) was analysed at the beginning and end of the sampling sessions, and after every 10 to 12 samples. All elements in otoliths were at least one order of magnitude greater than the background. The element (Sr, Ba, Mg and Ca) mass count data were converted to concentrations (ppm) using Glitter software (Macquarie University – www.accessmq.com.au). Concentrations (ppm) were then converted to molar concentrations and standardised to calcium for statistical analysis (as per the water samples). Elements were standardised to calcium as these elements substitute for Ca in the $CaCO_3$ matrix of the otoliths. The average analytical accuracy of elements ranged from 99% (Mg) to 100% (Ba). The precision was < 4.3 % for all elements.

Statistical analysis

Univariate, permutational analysis of variance (ANOVA) was used to test whether significant differences occurred in the concentration of trace elements Sr:Ca, Ba:Ca, Mg:Ca (the response variables) between stocks and among temperatures and salinities. Four separate multifactorial ANOVAs were used to test (i) the influence of salinity (seven levels at one temperature) on the NSW stock, the combined effects of salinity and temperature for the (ii) NSW stock (four salinity levels at each of three temperatures) and the (iii) WA stock (four salinity levels at each of two temperatures) and (iv) the influence of stock for the common temperature and salinity levels (four salinities and two temperatures) across both stocks. Four separate designs were used because the same treatments of temperature and salinity were not possible for both stocks. The same response variables (Sr:Ca, Ba:Ca, Mg:Ca) were tested in each design. Salinity, temperature and stock were treated as fixed factors and tank as a random factor nested in either: salinity, temperature and stock (comparison among stocks experiment (Test 1)), salinity (NSW salinity experiment (Test 2)) or salinity and temperature (other experiments (Tests 3a and 3b)). If significant differences were detected ($P < 0.05$), a posteriori pair-wise tests were used to determine which treatments (e.g. temperature, salinity, stock or their interactions) differed.

For the stock comparisons between NSW and WA fish our analyses were restricted to the two temperatures (20 and 24 °C) and four salinities (10, 30, 40, 50 ‰), which overlapped between stocks. The results report outcomes of the full analyses, but restrict post-hoc pairwise comparisons to between stock comparisons since this was the main factor of interest for this analysis. In addition, the salinity and temperature results for each stock were similar to the findings of the designs discussed below (i.e. where there was overlap in statistical tests).

Results

Rearing environment and fish growth

Experimental salinities and temperatures generally conformed to the treatment conditions, with only minor variation between treatment tanks (Table S1[1]). Each treatment was significantly different for both temperature and salinity. Salinities were sometimes slightly elevated from the nominal level, which was likely due to concentration from evaporation (Table S1[1]). Trace elemental concentrations of rearing waters were not manipulated and were generally consistent between tanks (Table S1[1]). However, there was some minor fluctuation in Ba:Ca concentration in the most saline treatments (50 ‰) compared to the other salinity treatments. Standard lengths of experimental *A. japonicus* revealed slight (non-significant) variation in fish size at the time of sacrifice (Table S1[1]).

Comparison of NSW and WA stocks at different temperatures and salinities (Test 1)

A significant interactive effect of salinity × temperature × stock was found for Sr:Ca concentrations in *A. japonicus* otoliths (Figure 1a, Table 1). For Ba:Ca in otoliths, a significant salinity × stock, plus salinity × temperature interaction was identified (Figure 1b, Table 1). Mg:Ca in otoliths only showed a significant effect of temperature, where Mg concentration increased with an increase in temperature (Figure 1c, 3b, Table 1). For the three way interaction (salinity × temperature × stock) found for otolith Sr:Ca, there were three significant differences between stocks. At 24 °C, the WA stock had significantly higher Sr:Ca concentrations for 30 and 40 ‰, but not for 10 and 50 ‰ (Figure 1a), than the NSW stock. At 20°C, there were only significant differences between stocks at 50 ‰ with the WA stock again incorporating more Sr:Ca (Figure 1a). The otolith Ba:Ca interaction between salinity and stock was due to the two stocks differing at two salinities (30 and 50 ‰), but not at the

[1] Supplementary information: Table S1

239 other two salinities (Figure 1b, 3a). At 30 ‰, otolith Ba:Ca was significantly greater in the NSW stock,
240 whereas at 50 ‰ it was significantly greater in the WA stock (Figure 1b, 3a).
241
242 NSW stock: salinity effects (Test 2)
243 A significant effect of salinity was detected on the concentration of NSW *A. japonicus* otoliths for
244 Sr:Ca, but not Ba:Ca or Mg:Ca (Figure 2a, Table 2). Otolith Sr:Ca for the 50 ‰ salinity treatment
245 differed from all salinities less than 35 ‰. An increase in otolith Sr:Ca concentration was observed
246 with increasing salinity (Figure 1a). Significant tank effects were detected in otoliths of NSW fish for
247 all three elemental ratios (Table 2). Pairwise tests showed that one to two treatments had significant
248 tank effects (Figure 2).
249
250 NSW Stock: Salinity and temperature effects (Test 3a)
251 A significant interactive effect of salinity and temperature was detected for Sr:Ca and Ba:Ca
252 concentrations in *A. japonicus* otoliths, but not for Mg:Ca (Figure 1, Table 3). At all three
253 temperatures, Sr:Ca concentrations differed among salinities, but the same salinities did not
254 necessarily differ for each temperature. At 16 °C the difference in Sr:Ca otolith concentration was
255 due to the low salinity treatment (10 ‰), which was significantly lower than all other treatments
256 (Figure 1a). At 20 °C, Sr:Ca showed an increase with increasing salinity, but only differed among the
257 lowest salinity treatments (10 and 30 ‰) and the highest salinity treatment (50 ‰) (Figure 1a). At 24
258 °C the hypersaline treatment (50 ‰) was significantly greater than all other salinity treatments
259 (Figure 1a). For Ba:Ca, no differences were found between salinities at low temperature, but there
260 was some variation at higher temperatures (Figure. 1b). At 20 and 24 °C the difference in Ba:Ca
261 otolith concentration was due to the 40 ‰ treatment, which was significantly less than all other
262 treatments, with the exception of the hypersaline (50 ‰) treatment at 20 °C (Figure 1b).
263

264 Temperature differences in otolith Sr:Ca incorporation were only found at the low salinity (10 ‰)
265 (Figure 1a). At low salinity, the general trend was for an increase in otolith Sr:Ca with increasing
266 temperature, but only the highest and lowest temperatures differed from each other (Figure 1a). For
267 Ba:Ca in otoliths, differences among temperatures were only found at the 10 to 40 ‰ salinities and
268 also showed a positive increasing trend (Figure 1b). For Mg:Ca in otoliths all temperature treatments
269 were significantly different; concentration increased with increasing temperature (Figure 1c). Salinity
270 did not influence Mg:Ca incorporation into otoliths (Figure 1c, Table 3).
271
272 NSW *A. japonicus* otoliths showed a significant tank effect for two of the three elements analysed,
273 Ba:Ca and Mg:Ca (Table 3). For Ba:Ca, pairwise tests showed that the tank effect was due to a single
274 treatment (Figure 1b). For Mg:Ca, significant tank effects were found for four treatments (Figure 1c).
275
276 WA stock: Salinity and temperature effects (Test 3b)
277 A significant interaction between salinity and temperature was found for Sr:Ca in otoliths, whereas
278 Ba:Ca and Mg:Ca were only significantly affected by salinity and temperature, respectively (Figure 1,
279 Table 4). For otolith Sr:Ca at 20 °C, the hypersaline (50 ‰) treatment was significantly greater than
280 all other salinity treatments (Figure 1a). The two lowest salinities were similar (10 and 30 ‰), as
281 were the two mid level salinities (30 and 40 ‰). This contrasted with the 24 °C treatment, where the
282 lowest salinity (10 ‰) was significantly lower than all other salinities, but the 30 to 50 ‰ salinities
283 had similar otolith Sr:Ca concentrations. For otolith Ba:Ca, the hypersaline treatment was
284 significantly higher than all other salinities (Figure 1b). For otolith Mg:Ca, temperature treatments
285 were significantly different where higher concentrations were found at higher temperatures, which
286 was similar to the NSW experiment (Figure 1c).
287

288 A significant tank effect was also found for the elemental composition of *A. japonicus* otoliths from
289 the WA stock for two of the elements analysed, Ba:Ca and Mg:Ca (Table 4). Significant tank effects
290 were due to differences among duplicate tanks for one Ba:Ca treatment and two Mg:Ca treatments.
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Discussion

Our findings suggest that Sr:Ca and Ba:Ca in otoliths record not only environmental conditions, but may also be influenced by intrinsic factors such as genetics. Environmental reconstructions for *A. Japonicus* are more complex than anticipated, but stock differences may enhance the method as a stock identification tool. For example, differences in otolith chemistry among stocks of wild *A. japonicus* have been attributed to different environmental conditions (e.g. salinity and temperature) and thus been used to delineate stock structuring (Ferguson et al. 2011). Our findings indicate that differences in the otolith chemistry of *A. japonicus* may reflect both environmental and genetic differences among populations. Hence, the differences in otolith chemistry detected among populations of *A. japonicus* by Ferguson (2011) may indicate highly distinct groupings. Mitochondrial DNA support the existence of genetically discrete stocks of *A. japonicus* (Farmer 2008). As such, it may be possible that subtle genetic differences may interact with environmental variables to affect otolith trace elemental signatures, which could enhance differences among stocks.

The temperature and salinities used in our study are within those in which *A. japonicus* naturally occur. They are a euryhaline species found in sub-tropical to temperate near shore, surf zone and estuarine areas of the southern hemisphere. The species commonly encounter a wide range of salinities (NSW - 0 to 35 ‰ (Gray and McDonall 1993; Taylor et al. 2007) and WA - 2 to ~40 ‰ (Loneragan et al. 1987)). Estuaries in WA often have higher salinities than NSW mainly due to the affects of a lower annual rainfall (Loneragan et al. 1987; Taylor et al. 2007), exposing *A. japonicus* in WA to higher salinities. *A. japonicus* have been recorded in hypersaline conditions (~50 to 60 ‰) in the Coorong Estuary, South Australia during prolonged drought conditions (Ye, Q, 2007, SARDI Aquatic Sciences pers. comm.). Their natural reported temperature ranges in NSW are 14 to 26 °C (Taylor et al. 2007) and 13 to 25 °C in WA (Loneragan et al. 1987) depending on the season. Increased somatic growth in juvenile *A. japonicus* occurs where temperatures are above 20 °C (Bernatzeder and Britz 2007). No clear optimum growth pattern with salinity was found (Bernatzeder

et al. 2010; Fielder and Bardsley 1999), but juveniles are commonly found in low salinity environments (Ferguson et al. 2008).

The significant effect of population genetics (albeit influenced by environmental factors) suggests that environmental reconstructions using otolith chemistry are complicated, particularly for euryhaline fish. We found that stock (genetics) had a significant effect on the incorporation of strontium (Sr) and barium (Ba), but not magnesium (Mg) in the otoliths of *A. japonicus*, but this was often influenced by salinity or temperature. For Sr, stock showed a significant interaction with salinity and temperature, while Ba showed a significant stock and salinity interaction. These results suggest that the effects of salinity and temperature on Sr and Ba are at least modified by intrinsic factors such as intraspecific genetic differences. We are only aware of one previous study that investigated genetic effects on otolith chemistry. Similar to our results, Clarke et al. (2011) found a significant effect of genetic stock on Ba:Ca. However, they also found an effect on Mg:Ca, but not Sr:Ca. The findings show that studies that directly test the effect of population genetics on otolith chemistry suggest that traditional environmental influences (salinity and temperature) are modified slightly (but significantly) by intraspecific genetic differences. The same general of trends of elemental response to extrinsic factors are evident between stocks i.e. Ba:Ca concentration in otoliths increased as the salinity treatments departed from the ambient marine (40 ppk) to brackish or hypersaline. However the magnitude of incorporation differed i.e. the NSW stock incorporated more Ba at the brackish treatments and the WA stock incorporated more at the hypersaline treatment.

Mg may be an important temperature recorder for the study species since no interaction of stock or salinity was found. Increasing temperature caused an increase in Mg:Ca concentration in *A. japonicus* otoliths. Past studies have found mixed responses of Mg:Ca to temperature, with no effect of temperature (Hoff and Fuiman 1995; Elsdon and Gillanders 2002; Martin and Thorrold 2005) and

a negative effect on one species (Fowler et al. 1995a; Fowler et al. 1995b) reported. These include experiments on Sciaenids, suggesting that consistent temperature effects on otolith Mg:Ca concentrations do not occur at the family level at least over the temperatures tested. Mg thermometry is well established in calcitic skeletons of formanifera (which also display a positive relationship), but in teleosts Mg is likely under biological control (Martin and Thorrold 2005). Magnesium is in greater concentration in the blood of fish compared to the endolymphatic fluid that bathes the otoliths and therefore controls their composition (Melancon et al. 2009). As such it is likely that physiological fractionation of Mg occurs between the blood and endolymph (Woodcock et al. 2012) and in *A. japonicus* this fractionation is possibly negatively affected by temperature. Therefore, it may be that Mg is under a temperature dependent discrimination in *A. japonicus* that is not the case in other species of teleost, highlighting the species-specific response on some elements.#

Sr and Ba in fish otoliths are commonly used markers of salinity but our findings suggest salinity is also modified by temperature. Significant interactions between the extrinsic or environmental factors of salinity and temperature suggest that the otolith element and salinity relationship is being modified by temperatures that occur within the normal range of the study species. An interaction between salinity and temperature for Sr:Ca in otoliths of *A. japonicus* occurred in both stocks and for Ba:Ca in the NSW stock. A similar interaction has been described for another estuary associated species, *Acanthopagrus butcheri* (Elsdon and Gillanders 2002). Most studies testing salinity and temperature differences have found no significant interaction between these variables (e.g. Martin and Wuenschel 2006). Our findings are similar to Elsdon and Gillanders (2002) where *A. butcheri* also showed an increasing concentration in Sr:Ca and Ba:Ca with increasing temperature at low salinities. Unfortunately Elsdon and Gillanders (2002) did not test salinities higher than 30 ‰ limiting a comparison of higher salinities. Although we did not detect an interaction between temperature and salinity for Ba:Ca in WA fish otoliths, there was a significant effect of salinity. This result was also found in *Lutjanus griseus* when tested for a salinity and temperature interaction (Martin and

370 Wuenschel 2006). Our results show that hypersalinity caused an increase in Ba:Ca reflecting the
371 water chemistry and as such suggest that Ba incorporation is not just a function of bioavailability at
372 low salinities. Even within stocks it appears that the full range of environmental conditions that fish
373 may be exposed to should be considered when reconstructing environmental histories.
374
375 For *A. japonicus* Sr is a useful marker of salinity changes when other variables (extrinsic and intrinsic)
376 are taken into account. When salinity was the only factor tested, Sr:Ca increased with increasing
377 salinity for *A. japonicus*. A positive increase in otolith Sr:Ca with increasing salinity has been reported
378 in some studies (Radtke et al. 1988; Kalish 1990; Limburg 1995) but not others (Hoff and Fuiman
379 1995). In the present study, differences in Sr:Ca concentrations were driven by the most saline
380 treatment (50 ‰) which is at the upper limit of the species natural occurrence. The few studies that
381 have examined the affect of hypersalinity (> 40 ‰) on Sr:Ca otolith chemistry have generally shown
382 a trend that is consistent with these findings (but see Gillanders and Munro 2012). Martin and
383 Wuenschel (2006) reared fish in the laboratory and included a salinity treatment level of 45 ‰; they
384 found that salinity significantly affected otolith Sr:Ca but only at relatively high temperature
385 treatments (28 and 33 °C) and they did not specify which salinity treatment levels drove the
386 differences (their graphs suggest it could have been 45 ‰). In the wild, hypersalinity (> 40 ‰)
387 similarly increased Sr incorporation into the otolith (Diouf et al. 2006). Fish in hypersaline
388 environments may experience physiological stress which inhibits their ability to osmoregulate. Thus,
389 there may be increased elemental uptake into the endolymph and otolith (Secor et al. 1995; Diouf et
390 al. 2006) with increasing salinity. Similarly, while not statistically significant, our results suggest that
391 Ba:Ca and Mg:Ca otolith concentrations are elevated in hypersaline environments, reflecting the
392 surrounding water chemistry, which is consistent with Gillanders and Munro (2012).
393
394 We have demonstrated that element incorporation is modified by intraspecifc genetic differences
395 where the uptake of some elements into the otoliths of fish is the result of complex interactions

396 between extrinsic and intrinsic factors. This study has shown that environmental variables (i.e.
397 salinity and temperature) significantly influence otolith chemistry, but these are not the only
398 influencing factors. While the influence of genetic differences on otolith chemistry may strengthen
399 the application of otolith chemistry for delineating patterns of stock structure of wild fish,
400 differences in otolith element incorporation among stocks and variable patterns of interaction with
401 environmental factors will render the use of otolith chemistry for the reconstruction of
402 environmental histories challenging (at least for the study species). However, if genetic stocks are
403 considered and a multifactorial approach used reconstructions may be possible (Perrier et al. 2011).
404 We caution environmental reconstructions without validating basic assumptions about the relative
405 contributions of environmental and genetic factors of otolith element incorporation. For example
406 the study species is an estuary associated marine fish that potentially encounters different levels of
407 salinity (fresh to hypersaline) at different stages of its life history. Ideally we could assess the
408 animal's use of different habitats based on levels of Sr and Ba. However, inferences of movement or
409 environmental histories based on fine scale salinity gradients (e.g. brackish to marine) could be
410 erroneous due to the possible influence of factors other than salinity (e.g. temperature and
411 genetics). This may not be an issue when studying fish which experience broad changes in salinity
412 such as those experienced by anadromous fish due to salinity being the main influence and other
413 factors slightly modifying otolith chemistry. Otolith chemistry may still be useful for aiding spatial
414 identification of management units, but for environmental reconstructions elemental responses to
415 extrinsic and intrinsic influences should be validated for study species. It may also be pertinent to
416 use otolith chemistry in tandem with other techniques (e.g. genetics, morphometrics and dart
417 tagging).
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Table 1. Results of permutational ANOVA comparing the Sr:Ca, Ba:Ca and Mg:Ca elemental ratio in otoliths of NSW and WA *A. japonicus* among salinity (S), temperature (T) and stock (St) treatments.

		Sr:Ca			Ba:Ca			Mg:Ca		
Source	df	MS	F	p	MS	F	p	MS	F	p
Stock	1	1.5159	38.0930	0.001	3.7161E-7	0.70158	0.404	9.6017E-2	3.3236	0.095
Salinity	3	2.9024	72.9260	0.001	1.3404E-5	25.2310	0.001	3.0005E-2	1.0350	0.423
Temp.	1	0.6183	15.5380	0.002	3.9604E-6	7.4770	0.014	0.6049	20.9400	0.003
St x S	3	7.663E-2	1.9254	0.177	6.8038E-6	12.8070	0.001	4.6681E-2	1.6101	0.225
St xT	1	0.1288	3.2357	0.084	1.0053E-6	1.8979	0.187	2.1790E-4	7.5427E-3	0.929
S x T	3	8.6913E-2	2.1838	0.138	1.8942E-6	3.5655	0.042	3.1047E-2	1.0709	0.389
St x S x T	3	0.2798	7.0301	0.004	6.0891E-7	1.1462	0.360	1.0641E-2	0.3670	0.772
Residual	212	3.9097E-2			2.266E-7			8.9821E-3		

Note: df, degrees of freedom; MS, mean squares; F, F ratio; p significance ($p < 0.05$).

Table 2. Results of permutational ANOVA comparing the effects of treatment salinities (S) on otolith Sr:Ca, Ba:Ca and Mg:Ca for NSW *A. japonicus* reared at 20 °C.

		Sr:Ca			Ba:Ca			Mg:Ca		
Source	df	MS	F	p	MS	F	p	MS	F	p
Salinity	6	0.4820	5.8848	0.007	1.8454E-6	3.6077	0.072	1.9351E-2	1.2351	0.400
Tank (S)	7	8.1926E-2	2.7069	0.014	5.1169E-7	3.7067	0.001	1.5672E-2	2.4222	0.027
Residual	116	3.0266E-2			1.3805E-7			6.4703E-3		

Note: df, degrees of freedom; MS, mean squares; F, F ratio; p significance ($p < 0.05$).

564 **Table 3.** Results of permutational ANOVA comparing the Sr:Ca, Ba:Ca and Mg:Ca ratio in otoliths of NSW *A.*

565 *japonicus* among salinity (S) and temperature (T) treatments.

		Sr:Ca			Ba:Ca			Mg:Ca		
Source	df	MS	F	p	MS	F	p	MS	F	p
Salinity	3	2.8093	56.5300	0.001	7.0346E-6	11.3960	0.001	6.9062E-3	0.3006	0.820
Temp.	2	0.5140	10.3440	0.003	1.1563E-5	18.7410	0.002	1.1280	49.1160	0.001
S x T	6	0.2987	6.0092	0.005	2.5557E-6	4.1360	0.022	1.6494E-2	0.7170	0.634
Tank (S x T)	12	4.9736E-2	1.2044	0.287	6.1913E-7	2.6553	0.002	2.3057E-2	3.5532	0.001
Residual	202	4.1295E-2			2.3316E-7			6.4890E-3		

566 **Note:** df, degrees of freedom; MS, mean squares; F, F ratio; p significance ($p < 0.05$).

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569 **Table 4.** Results of permutational ANOVA comparing the Sr:Ca, Ba:Ca and Mg:Ca elemental ratio in otoliths of

570 WA *A. japonicus* among salinity (S) and temperature (T) treatments.

		Sr:Ca			Ba:Ca			Mg:Ca		
Source	df	MS	F	p	MS	F	p	MS	F	p
Salinity	3	1.1025	41.9470	0.001	1.0634E-5	25.1700	0.003	5.5951E-2	1.8162	0.220
Temp.	1	7.3170E-2	2.7681	0.109	3.9038E-7	0.9319	0.338	0.25148	8.2457	0.024
S x T	3	0.1548	5.8909	0.032	5.4928E-7	1.3002	0.358	1.622E-2	0.5265	0.676
Tank (S x T)	8	2.5984E-2	0.70774	0.701	4.2951E-7	2.4308	0.022	3.1422E-2	3.3590	0.004
Residual	78	3.6714E-2			1.7670E-7			9.3548E-3		

571 **Note:** df, degrees of freedom; MS, mean squares; F, F ratio; p significance ($p < 0.05$).

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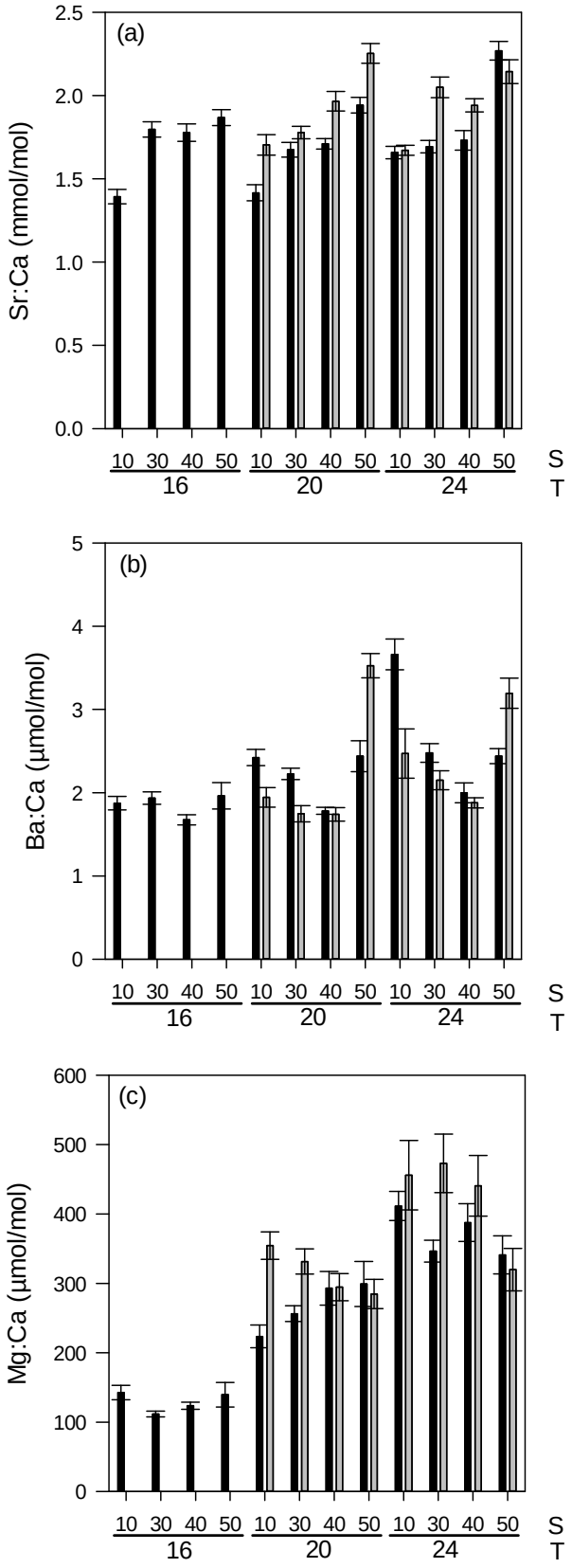
Figure legends

Figure 1. Mean concentrations (± standard error) of trace elements in otoliths of NSW (black) and WA (grey) *A. japonicus* reared under experimental treatments of salinity (S) and temperature (T).

Figure 2. Mean concentrations (± standard error) of trace elements in otoliths of NSW *A. japonicus* reared under experimental treatments of salinity at a fixed temperature (20 °C); * denotes significant tank effect; different colour bars distinguish the two tanks.

Figure 3. a) Mean concentrations (± standard error) of Ba:Ca in otoliths of NSW (black) and WA (grey) *A. japonicus* reared under the experimental treatment of salinity and b) mean concentrations (± standard error) of Mg:Ca in otoliths of *A. japonicus* reared under the experimental treatment of temperature.


(a)
Sr:Ca (mmol/mol)
2.5
2.0
1.5
1.0
0.5
0.0
10 30 40 50 10 30 40 50 10 30 40 50
16 20 24
S
T
(b)
Ba:Ca (µmol/mol)
5
4
3
2
1
0
10 30 40 50 10 30 40 50 10 30 40 50
16 20 24
S
T
(c)
Mg:Ca (µmol/mol)
600
500
400
300
200
100
0
10 30 40 50 10 30 40 50 10 30 40 50
16 20 24
S
T

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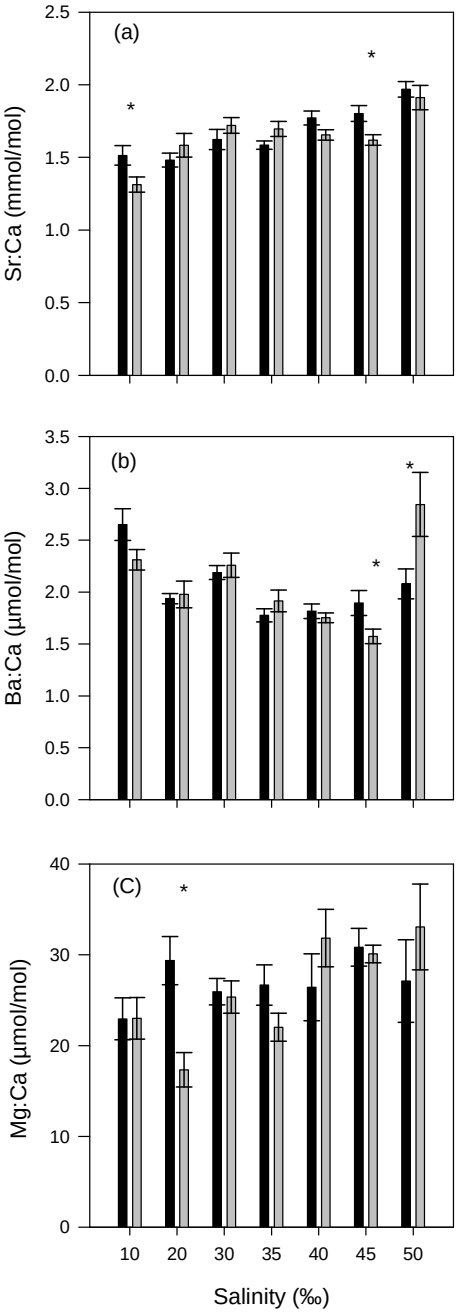
(a)
Sr:Ca (mmol/mol)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
(b)
Ba:Ca (µmol/mol)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
(C)
Mg:Ca (µmol/mol)
40
30
20
10
0
*
10
20
30
35
40
45
50
Salinity (‰)

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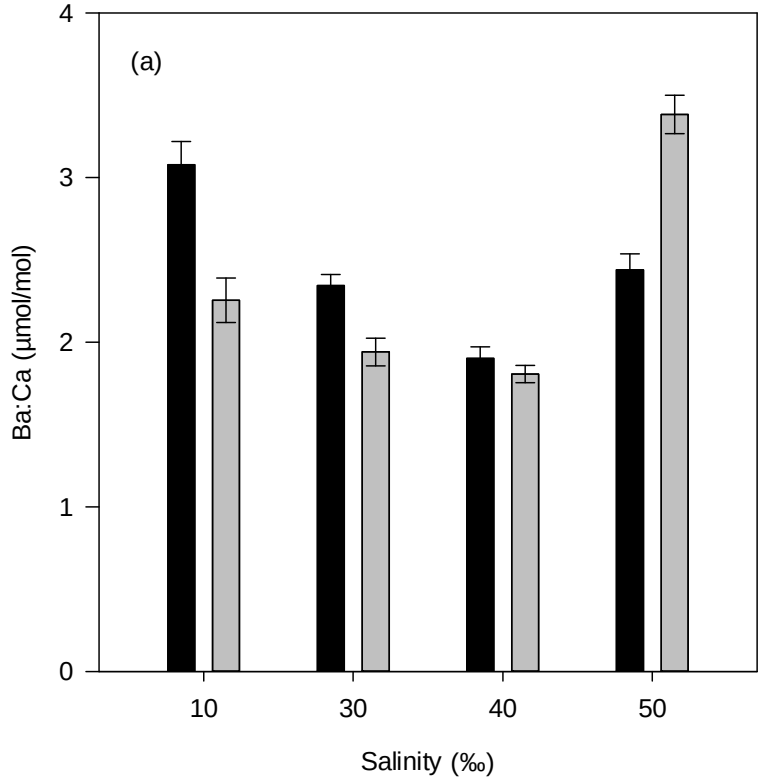
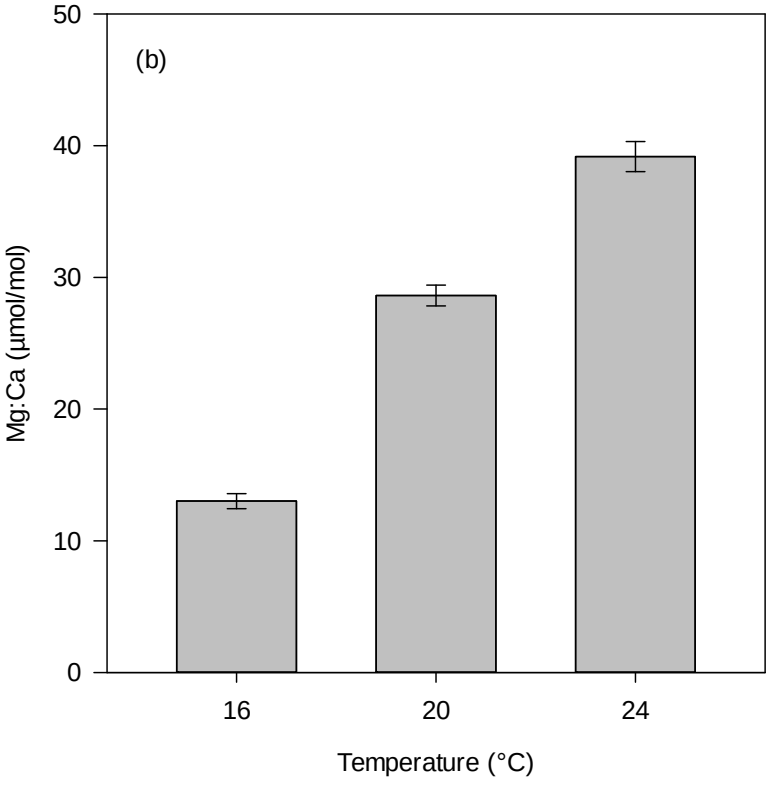

(a)
Ba:Ca (µmol/mol)
0
1
2
3
4
10
30
40
50
Salinity (‰)


(b)
Mg:Ca (µmol/mol)
0
10
20
30
40
50
16
20
24
Temperature (°C)

600

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