Environmentally mediated trends in otolith composition of juvenile Atlantic cod (*Gadus morhua*)

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Abstract:

We evaluated the influence of environmental exposure of juvenile Atlantic cod (*Gadus morua*) to inform interpretations of natal origins and movement patterns using otolith geochemistry. Laboratory rearing experiments were conducted with a variety of temperature (~ 5, 8.5 and 12 °C) and salinity (~ 25, 28.5 and 32 PSU) combinations. We measured magnesium (Mg), manganese (Mn), strontium (Sr) and barium (Ba), expressed as a ratio to calcium (Ca), using laser ablation inductively coupled plasma mass spectrometry (ICP-MS), and stable carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) isotopes using isotope ratio monitoring mass spectrometry. Temperature and salinity significantly affected all elements and isotopes measured, with the exception of salinity on Mg:Ca. We detected significant interactions among temperature and salinity for Mn:Ca and Ba:Ca partition coefficients (ratio of otolith chemistry to water chemistry), with significant temperature effects only detected in the 32 and 28.5 PSU salinity treatments. Similarly, we detected a significant interaction between temperature and salinity in incorporation of $\delta^{13}C$, with a significant temperature effect except at intermediate salinity. These results support the contention that environmental mediation of otolith composition varies among species, thus limiting the ability of generalized models to infer life history patterns from chemistry. Our results provide essential baseline information detailing environmental influence on juvenile Atlantic cod otolith composition, punctuating the importance of laboratory validations to translate species-specific otolith composition when inferring *in situ* life histories and movements.

KEYWORDS: Otolith chemistry, temperature, salinity, *Gadus morhua*, elemental fingerprinting, isotopes

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42 **Introduction:**

43 The application of otolith geochemistry to resolve natal habitats or to reconstruct life
44 histories assumes otolith chemistry corresponds to local environmental conditions and
45 ambient water chemistry, and that these conditions vary to impart a unique geochemical signal
46 (Campana, 1999). Physiological and environmental factors both mediate assimilation of
47 elements and isotopes into otoliths (e.g. Thorrold et al., 1997b; Elsdon and Gillanders, 2002).
48 Prediction of where otolith composition differences might exist, or the reconstruction of fish
49 movements and environmental histories, requires predictable relationships between
50 environmental variables and otolith chemistry (Elsdon et al., 2008; Reis-Santos et al., 2013).
51 Laboratory studies that have quantified specific relationships between select environmental
52 factors and otolith composition (Hoff and Fuiman, 1995; Bath et al., 2000; Elsdon and
53 Gillanders, 2002; Elsdon and Gillanders, 2003; Martin and Wuenschel, 2006; Miller, 2011;
54 Reis-Santos et al., 2013) highlight a lack of generality across among different species
55 (Gillanders, 2005; Reis-Santos et al., 2008; Barnes and Gillanders, 2013). For instance, Sr:Ca
56 ratios in otoliths vary considerably among species in the absence of differences in dissolved
57 Sr:Ca in ambient water (Campana, 1999), presumably resulting from inter-specific differences
58 in metabolic traits (Kalish, 1991; Campana et al., 2000; Miller, 2011).

59 Ambient concentrations of elements and isotopes in seawater may also co-vary with
60 temperature and salinity (Epstein and Mayeda, 1953; Elsdon and Gillanders, 2003; Reis-
61 Santos et al., 2013, this study). Retrospective assignment of adults to nursery habitats or
62 environmental conditions during the juvenile period has been accomplished through

63 geochemical analysis of material close to the otolith core, corresponding to the juvenile period
64 (Thorrold et al., 2001; Gillanders, 2005; Farmer et al., 2013). Determining the effects of
65 temperature and salinity on otolith elemental and isotopic incorporation will enhance our
66 ability to predict where significant differences might occur and interpret life history
67 movements (Elsdon and Gillanders, 2002). Although use of otolith signatures does not require
68 explicit understanding of all details influencing incorporation (Campana et al., 2000), the
69 significant influence of environmental factors adds complexity to the interpretation of
70 geochemical concentrations in isolation of environment (Martin and Wuenschel, 2006).

71 Atlantic cod (*Gadus morhua*) is a demersal finfish species native to the North Atlantic,
72 spanning from Greenland to Cape Hatteras, North Carolina to as far east as the Baltic Sea.
73 Spawning occurs in both inshore and offshore environments, between late winter and early
74 spring. Pelagic eggs develop into larvae that later metamorphose into benthic juveniles
75 inhabiting offshore areas as well as shallow inshore regions, often associating with complex
76 habitat (Laurel et al., 2003). The large latitudinal and depth range of Atlantic cod exposes
77 juveniles to a gradient of temperatures and salinities (Dalley and Anderson, 1997), making
78 them ideal candidates for studies on environmental effects on otolith elemental incorporation.
79 Atlantic cod have received considerable research attention, in particular efforts to understand
80 the structure of remaining fragmented populations in the Northwest Atlantic. The pelagic egg
81 and larval phase imparts a large dispersal potential, mediated by spawn timing and ambient
82 conditions (Bradbury et al., 2000; Stanley et al., 2013). Genetic (Ruzzante et al., 2000),
83 tagging (Rose et al., 2011), and otolith chemistry techniques (Campana et al., 1999; Jamieson
84 et al., 2004; D'Avignon and Rose, 2013) have successfully delineated spawning aggregation

spatial structure, but fall short of providing information on critical juvenile habitat and potential links to adult populations.

The use of otolith chemistry for retrospective evaluation of environmental history, or to aid in delineating nursery habitat, requires a baseline understanding of the influence of environment on otolith composition. Controlled laboratory experiments provide a vehicle to evaluate relationships between otolith composition and the environment. Interactive effects of temperature and salinity (e.g. Miller, 2011) necessitate orthogonally designed experiments to fully evaluate environmental drivers on otolith chemistry (Elsdon and Gillanders, 2002). The objective of this study was to evaluate the influence of temperature and salinity on the biogenic incorporation of trace elements in the otoliths of juvenile Atlantic cod, a commercially important species that spans a wide range of these environmental parameters. Specifically, we measured concentrations of magnesium (Mg), manganese (Mn), strontium (Sr) and barium (Ba), expressed as a ratio to calcium (Ca), and stable isotopes of carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$); past studies show some form of environmental mediation in incorporation of all of these elements. Specifically, we utilized controlled laboratory conditions to determine the relative and interactive effects of temperature and salinity on elemental and isotopic concentrations of juvenile cod otoliths.

Methods:

Experimental Design

Juvenile Atlantic cod were obtained from a common broodstock provided by Atlantic Genome Canada and the Dr. Joe Brown Aquatic Research Building (JBARB) at the Ocean Sciences Centre (OSC), Memorial University of Newfoundland (MUN). Newly hatched fish

were reared at ambient conditions (~ 11 °C and 32 PSU) for up to 137 days before they were distributed among the experimental treatments. Forty fish (~ 4 cm standard length) were moved to each experimental aquaria (40 L) and acclimated to desired temperature treatment by adjusting cold room temperatures by 2 °C every 2 days, in order to slowly acclimate fish to the new thermal regime from the common start point of 11 °C. When desired temperature treatments were achieved, salinities were manipulated by 2 PSU every two days, until the new salinity regimes were reached from the common start point of 32 PSU. Once acclimation and treatment levels were achieved, fish were immersed in seawater treated with Alizarin Red-S (ARS) (600 $Mg \cdot l^{-1}$) at a pH adjusted to 7.0, for 24 hours. Staining in Alizarin Red induces a fluorescent tag that clearly indicated the start of the experiment and the relevant otolith material to sample (Figure 1; Beckman and Schulz, 1996). A standardized finfish pelletized diet (EWOS ®) was fed to all fish to minimize any dietary effects of food on otolith chemistry (Walther and Thorrold, 2006; Reis-Santos et al., 2013). Through the experiment fish were fed to saturation *ad-libitum* three times daily and maintained at a 12-hour light cycle.

Three experimental temperature treatments of approximately 5, 8.5 and 12 °C, hereafter referred to as low, medium, and high temperatures, were maintained in three separate cold rooms at the OSC. For each temperature treatment we used three separate salinity treatments of 25, 28.5 and 32 PSU, hereafter referred to as low, medium, and high salinities. Each temperature by salinity treatment was replicated three times for a total of 27 experimental aquaria, and nine unique combinations. Salinities were achieved through dilution of filtered seawater from the JBARB (32 ±0.25 PSU) using non-chlorinated well water from the Marine Institute of Memorial University. Set salinity treatments were pre-mixed and stored in each cold room for 24 hours prior to use in tanks to avoid the need for any

acclimation during daily water exchanges. Partial water exchanges (50%) were performed daily along with siphoning any excess food, waste, or dead fish. Temperature and salinity were checked for consistency daily using a YSI-55 probe. In addition, dissolved oxygen, pH, and ammonia levels were monitored every second day with the YSI probe and ammonia test meters. To minimize ammonia levels, each aquarium was equipped with a Bio-Wheel filter and ammonia filter pads.

Otolith preparation and geochemical analysis

Fish were exposed to experimental treatments for a total of 90 days (14 July – 12 October 2007). Upon completion of the experiment, we removed the fish and recorded standard length. Both sagittal and lapillal otoliths were removed, cleaned with ultrapure water, air dried, and stored in acid-washed glass vials. We later mounted otoliths on glass microscope slides and polished them using 0.3 μm lapping film. Mounted otoliths were cleaned again with a nylon brush, triple rinsed in ultrapure water and sonified for 2 min. We then air dried otoliths in a laminar flow hood, prior to transfer to clean petri dishes, and transport to the Woods Hole Oceanographic Institution Plasma Mass Spectrometry Facility for analysis. One lapillus was randomly selected for laser ablation and one sagitta was randomly selected for $\acute{}^{13}C$ and $\acute{}^{18}O$ analysis.

We measured otolith elemental composition using a Thermo Finnigan *Element2* inductively coupled plasma mass spectrometer (ICP-MS) coupled with an ArF excimer 193-nm laser ablation system. We chose to quantify five elements (^{25}Mg, ^{43}Ca, ^{55}Mn, ^{88}Sr, and ^{138}Ba,) that were consistently higher in the otolith samples than in HNO_3 blanks during preliminary analyses and have shown potential for environmentally mediated otolith

153 incorporation. We lasered along a line parallel to the otolith edge within a common growth
154 band and outside of the Alizarin mark (~ 500 μm). Laser repetition rate was set at 5 Hz for all
155 analyses, with a scan speed of 5 μm s^{-1}. We used a certified reference material (CRM)
156 consisting of powdered otoliths (Yoshinaga et al., 2000), dissolved in 2% ultrapure HNO_3
157 (SeaStar®) and diluted to a Ca concentration of 40 $\mu g \cdot g^{-1}$, to correct for instrument bias and
158 drift following Thorrold and Swearer (2009). External precision was estimated by analyzing a
159 second otolith CRM (Sturgeon et al., 2005), also dissolved in 2% HNO_3 (SeaStar®) and
160 diluted to a Ca concentration of 40 $\mu g \cdot g^{-1}$, periodically throughout the laser analyses (n = 55).
161 Analytical accuracy was determined from the concentrations of Japanese Otolith and NRC
162 standards, averaged across all samples. We found that accuracy was exceeded 98% for all
163 otolith constituents measured, including isotopic ratios described later. We calculated limits of
164 detection (LOD) as 5 times the mean intensity of blanks (2% HNO_3), run every 10 assays for
165 each otolith constituent. Any measurements below the respective LOD were excluded from
166 analysis. We conducted all statistical analyses using otolith elemental compositions converted
167 to molar values and standardized to calcium concentrations ($Me:Ca_{otolith}$). Estimates of
168 external precision based on the relative standard deviation values of the second CRM were
169 4.2% for Mg, 17.9% for Mn, 0.40% for Sr, and 1.4% for Ba.

170 We measured $\delta^{13}C$ and $\delta^{18}O$ values in sagittal otoliths of each individual by milling
171 otoliths with a MicroMill sampler (New Wave Research) or a handheld dental drill under a
172 stereomicroscope until we obtained 50 to 200 μg of near edge material. Stable isotopes were
173 measured using an isotope ratio monitoring mass spectrometer according to methods outlined
174 by Ostermann and Curry (2000). We report values of both isotopes relative to Vienna Pee Dee

Belemnite (VPDB ‰). Long-term precision estimates based on the use of NBS-19 were ±0.07 for $\delta^{18}O$ and ±0.03 for $\delta^{13}C$ (Ostermann and Curry, 2000).

Water analyses

We used ICP-MS to measure the chemical composition of water samples taken from each experimental treatment (n=27) at the mid-point of the experiment (day 32). Water intake for the OSC and JBARB comes from approximately 5 m depth with a very stable salinity (±0.25 PSU, yearly variance) and temperature (± 1 ^{o}C, weekly variance) (Danny Boyce, JBARB facilities manager, Memorial University of Newfoundland, St. John's Canada, A1C 6N3, personal communication). Low variance in water condition indicates a relatively stable intake water mass, with no expected major changes in ambient water chemistry during the course of the experiment. Because water for all treatments was obtained from a common filtered and stable source, we assume samples collected represent the average conditions experienced over the course of the experiment aside from any temperature or salinity effects. For each treatment, a 50-ml aliquot of water was sampled, acidified with ultrapure nitric acid, and subsequently analyzed for elemental composition. Samples were analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) (Varian Vista Pro). Methods for analysis were adapted from EPA Method 200.7 (ICP-OES). Elemental calibration standards were prepared from 1000 mg l^{-1} reference solutions and original stocks are NIST-Traceable. Precision estimates are not provided because only one water sample from each treatment was analyzed. Elemental concentrations were standardized to and expressed as element to calcium ratios ($Me:Ca_{water}$), which were used in all subsequent statistical analyses.

196 *Growth*

197 Upon experiment completion, we recorded standard length for each fish. Because all
198 fish came from the fertilization event, and were harvested at the same time, we used standard
199 length of the fish as measure of somatic growth. We also measured otolith deposition using
200 the Alizarin stain as a marker for the beginning of the experiment. Otolith length was defined
201 as the length of a transect radiating out from the beginning of the alizarin stain to the outer
202 edge of the otolith. In addition, we measured pre-experimental growth from the otolith core to
203 the beginning of the alizarin stain (Figure 1). Pre-experimental otolith length provides an
204 index scoring the relative size of individuals placed into experimental treatments.

205 *Statistical Analysis*

206 Elemental ($Me:Ca_{otolith}$ & $Me:Ca_{water}$) and isotope data were inspected for normality
207 using Q-Q plots and clear outliers (i.e., more than two times the standard deviation from the
208 mean of a given metric) were removed. In total, less than 3% of observations met these
209 exclusion criteria. Partition coefficients provide a complementary metric to compare otolith
210 incorporation rates across experimental treatments accounting for both $Me:Ca_{otolith}$ and
211 $Me:Ca_{water}$ (Reis-Santos et al., 2013), and are especially useful for comparisons among species
212 and studies (Martin and Wuenschel, 2006). We calculated partition coefficients (D_{me}) for each
213 element by dividing the measured otolith calcium ratio ($Me:Ca_{otolith}$) by the observed water
214 ratio ($Me:Ca_{water}$) of the treatment (Morse and Bender, 1990). The majority of field studies
215 only provide baseline information based on observed otolith composition because the ambient
216 water chemistry is often missing and therefore partition coefficients cannot be calculated (but
217 see, Thorrold et al., 1998). For this reason, we present analyses of otolith composition
218 ($Me:Ca_{otolith}$) and partition coefficients (D_{me}) in tandem.

Statistical analyses were conducted in R-stats version 3.1.0 (R Development Core Team, 2014). Differences in growth, water chemistry, otolith chemistry, and partition coefficients, as a function of experimental treatment, were analysed as general mixed-effects models (LME) using the "*lme4*" package in R. Response variables (i.e. D_{me}) were $\log_{10}$ transformed and treated as continuous variables in response to our categorical fixed treatments, temperature and salinity. To account for growth effects, we constructed a temperature by salinity growth model, using the same mixed-effects model outlined previously, and employed the residuals as an index of growth variability which accounts for correlations with environment. Growth residuals were incorporated into LME models as continuous covariates. Replicate tanks, within each treatment, were treated as a nested random categorical factor for all models. We did not report the non-significant random tank effect because it was unimportant to the statistical question posed in the analysis (Bolker et al., 2009). Each element and isotope was analyzed individually, thus creating 6 separate statistical tests. To control for family-wise error rates and multiple comparisons, the significance of each test was adjusted using a *Benjamini-Hochberg* (Benjamini and Hochberg, 1995) correction and compared to a significance level set at ± = 0.05. In instances where we detected significant differences, post-hoc pairwise tests using the '*multcomp*' package in R determined which treatments differed.

Random effects

For all mixed-effects models we found no significant replicate tank effect. All p-values exceeded 1.2 except otolith ´ ^{18}C which approximated 0.08.

241 *Ethics*

242 All experimental procedures and fish handling were approved and conducted in
243 accordance to the Canadian Council of Animal Care Guidelines and Memorial University of
244 Newfoundland animal utilization protocols.

245 **Results:**

246 *Rearing conditions*

247 Experimental conditions were consistent throughout the trials for low (5.0 ± 0.2 °C and
248 25.1 ± 0.1 PSU; Mean ± SD), intermediate (8.6 ± 0.3 °C and 28.5 ± 0.2 PSU; Mean ± SD),
249 and high (12.2 ± 0.3 °C and 31.8 ± 0.2 PSU; Mean ± SD) treatment levels (Figure 2). All
250 water was sourced from a common intake and filtration system, but variation in ambient water
251 chemistry occurred as a function of experimental conditions (Table 1). Salinity significantly
252 affected Me:Ca_{water} ratios (Table 2), however, we detected no temperature, temperature-
253 salinity interactions, or tank replicate effects in Me:Ca_{water}. Mg:Ca and Sr:Ca_{water} were
254 positively correlated and Mn:Ca and Ba:Ca_{water} were negatively correlated with treatment
255 salinity (Figure 3).

256 *Otolith Chemistry*

257 Temperature significantly influenced elemental-calcium (Me:$Ca_{Otolith}$) and isotope
258 ratios (Tables 2 & 3 respectively; Figure 4). Salinity significant influenced on all elements and
259 isotopes tested, with the exception of magnesium (Mg:$Ca_{Otolith}$). We detected no interactive
260 effects for any elemental ratio or for either stable isotope variables. Both Mg:Ca and Mn:Ca
261 were significantly higher at the warmest temperatures. Mg:Ca and Sr:Ca ratios increased with
262 salinity, whereas Mn:Ca and Ba:Ca ratios decreased (Figure 4), corresponding to trends
263 observed in Me:Ca_{Water} (Figure 3). Similarly, temperature and salinity significantly affected

isotope ratios. We did not find significant interactive effects of temperature and salinity on $\acute{}^{18}O$ ratios, but did find a significant interaction on ratios of $\acute{}^{13}C$ (Table 3). Both $\acute{}^{13}C$ and $\acute{}^{18}O$ associated positively with salinity across all temperature treatments. $\acute{}^{13}C$ values were significantly lower at the low temperatures compared to intermediate and high temperature treatments, except at the intermediate salinity treatment (thus the significant interaction between temperature and salinity). Conversely, we observed significantly higher $\acute{}^{18}O$ in the low temperature treatment compared to intermediate and high temperatures (Figure 4).

Partition Coefficients

Ranges of elemental partition coefficients, D_{Me} (Table 1), were similar to those reported in previous studies (Martin et al., 2004; Martin and Thorrold, 2005; Martin and Wuenschel, 2006; Miller, 2011; Reis-Santos et al., 2013). Estimates of D_{Me} for all elements showed a significant influence of temperature and a similar effect for salinity for all elements except D_{Mg}. We found significant interactive effects between temperature and salinity for D_{Mn} and D_{Ba} (Table 2, Figure 5). D_{Mg} showed a weakly significant positive association with temperature only. Temperature significantly influenced D_{Mn} only at the highest salinity treatment. D_{Mn} associated positively with salinity for all temperature treatments, especially at the highest temperature. Salinity influenced D_{Sr} coefficients, particularly at low temperatures, where the intermediate salinity treatment had a significantly lower mean D_{sr} value than low or high salinity treatments. D_{sr} associated negatively with temperature, with the largest mean D_{Sr} in the lowest temperature treatments (Figure 5). For D_{Ba}, we observed a temperature effect only in the high salinity treatment where coefficients were significantly greater than the low temperature treatment. Barium partition coefficients tracked positively with salinity in all

temperature treatments with highest coefficients associated with the highest salinity. We observed temperature effects in D_{Ba} only in the high salinity treatment.

Growth

We found no significant difference among experimental treatments in pre-experimental otolith length, confirming that all individuals were similar in size before experimental rearing (ANOVA: F=0.99 p=0.38, F=0.44 p=0.64, for temperature and salinity respectively). Somatic and otolith growth were highly correlated (Pearson r =0.58, p<0.0001) and temperature affected both variables significantly, with lowest growth in the coldest temperature treatment (Figure 6). Collectively, otoliths grew proportionately faster than the fish themselves, resulting in proportionally smaller otoliths in faster growing fish (log linear model: slope = 1.4 t=12.7, p=<0.00001). Salinity did not significantly affect growth rate singularly, but we did observe a weakly significant interaction between temperature and salinity for somatic growth (Table 4). In low temperature treatments, growth was significantly higher at intermediate salinities.

Mixed-effects models revealed significant relationships for Me:Ca and D_{Me} with Mg, Mn, and Sr, as well as ratios of $\acute{}^{13}C$, with treatment growth residuals (Tables 2 and 3). All estimated slopes were positive except for D_{Sr} and Sr:Ca, and we observed no significant relationships between growth residuals and Ba:Ca, D_{Ba} and $\acute{}^{18}O$. These trends mirror directionality and significance of correlative relationships among otolith constituents and growth across treatments (Supplementary Table 1).

Discussion:

Otolith geochemistry provides potentially powerful methodology for identifying natal habitats and inferring environmental histories of juvenile Atlantic cod, potentially allowing individuals in adult populations to be linked to specific juvenile or natal nursery habitat. Reliable prediction and evaluation of fish environmental history requires, however, an understanding of how the environment mediates incorporation of ambient elements and isotopes into fish otoliths. Broadly speaking, the influence of temperature and salinity on otolith elemental composition remains ambiguous, with laboratory studies (e.g. Bath et al., 2000; Elsdon and Gillanders, 2002; Elsdon and Gillanders, 2003; Miller, 2011; Reis-Santos et al., 2013) highlighting strong species-specific relationships (Reis-Santos et al., 2008; Melancon et al., 2009), likely attributable to variable physiology (Miller, 2011). Indeed, there are few examples of consistent relationships between otolith chemistry and environment among fish species (Campana, 1999). Laboratory validations thus provides an important and necessary step in evaluating biotic and abiotic influences on otolith incorporation rates (Kalish, 1991; Miller, 2011) for specific elements and isotopes prior to field application (Reis-Santos et al., 2013). Our experiment represents an important contribution towards understanding the application of otolith microchemistry to environmental reconstructions of juvenile Atlantic cod (*Gadus morhua*) and similar coastal marine fishes.

Our experiments demonstrate significant influences of both temperature and salinity on otolith composition of juvenile Atlantic cod (*Gadus morhua*). Gradients of temperature, salinity, and ambient chemical conditions (Me:Ca_{water}), driven by variation in upwelling and mixing of marine and freshwater inputs (Reis-Santos et al., 2013) often characterize coastal nursery habitats in which fish reside or move. Our experiment included a range of

temperatures experienced by juvenile Atlantic cod in the Newfoundland inshore environment (Dalley and Anderson, 1997; Craig and Colbourne, 2004), therefore providing a realistic template on which to test the relationship between environment and otolith composition.

Otolith chemistry

Both temperature and salinity significantly affected almost all of the $Me:Ca_{Otolith}$ and D_{Me} variables. Only $Mg:Ca_{otolith}$ and D_{Mg} were influenced by temperature alone. Previous laboratory experiments evaluating the effects of environment on elemental incorporation found significant effects of both temperature and salinity both with (Secor et al., 1995; Elsdon and Gillanders, 2002; Barnes and Gillanders, 2013) and without (e.g. Martin and Thorrold, 2005; Martin and Wuenschel, 2006) an interaction. Significant interactions between temperature and salinity brings into question generalities drawn from assessments where uncontrolled factors such as temperature or salinity in coastal environments may add environmental heterogeneity and bias otolith signals (Elsdon and Gillanders, 2002).

Temperature and salinity both significantly influenced Sr:Ca ratios and partition coefficients. We observed highest $Sr:Ca_{otolith}$ values in the highest salinity treatments (~32 PSU). The result was expected as $Sr:Ca_{Otolith}$ commonly occurs in proportion to ambient availability (Farrell and Campana, 1996; Campana et al., 1999) and measurements of water chemistry showed the highest $Sr:Ca_{Water}$ ratios at the highest salinity across all temperature treatments. By calculating partition coefficients, we were able to examine the effect of temperature and salinity on $Sr:Ca_{Otolith}$ after accounting for differences in $Sr:Ca_{Water}$. While several studies found a positive effect of temperature on D_{Sr} (e.g. Bath et al., 2000; Martin et al., 2004), we found the opposite, with highest D_{Sr} values in the low temperature treatment,

similar to trends reported for larval Pacific, *Gadus marcrocephalus* (DiMaria et al., 2010), and Atlantic cod (Townsend et al., 1995). At low temperatures fish apparently have a reduced ability to discriminate Sr incorporation into the otolith (Townsend et al., 1995), which mirrors our finding of higher Sr:Ca and D_{Sr} at the lowest temperature treatment. We found a dome-shaped effect of salinity on Sr partition coefficients, with lowest D_{Sr} values at intermediate salinities. Martin et al. (2004) also noted a significant effect of salinity (and hence dissolved Sr concentrations in the ambient water) on D_{Sr} and suggested that mutual inhibition of Ca and Sr ions across intestinal membranes may have generated this result. Our results are more complicated, as this inhibition would need to be complex and non-linear to generate the relation between salinity and D_{Sr} that we observed. Correspondingly Brown and Severin (2009) reviewed published otolith work and noted that marine species in general often exhibit equivalent or greater variation in $Sr:Ca_{Otolith}$ ratios relative to diadromous and freshwater species, despite less variable ambient concentrations; these results suggest an equivocal contribution of $Sr:Ca_{Water}$ levels to $Sr:Ca_{Otolith}$. Strontium is typically used as a chemical tracer of salinity, often defining transitions between freshwater and marine habitats (e.g. Bradbury et al., 2008). Studies that successfully developed otolith strontium and salinity relationships often utilized a larger gradient than used in our study (e.g. Martin and Wuenschel, 2006), and potentially spanned a greater range than that used as coastal juvenile cod habitat. The use of otolith strontium as an environmental tracer in Atlantic cod appears tenuous in isolation, though in combination with other elements might provide a contextual relationship to environment and a tracer for ambient Sr:Ca chemistry.

Barium incorporation correlated positively with temperature and negatively with salinity. $Ba:Ca_{Otolith}$ ratios were significantly lower at the lowest temperature treatment with

374 decreasing levels at increasing salinity, as seen in previous studies with black bream (Webb et
375 al., 2012). As expected, $Ba:Ca_{Water}$ did not differ significantly across temperature treatments,
376 but did decrease significantly with salinity. Controlling for ambient water chemistry a
377 different pattern emerges, with a significant temperature by salinity interaction influence on
378 D_{Ba}. We observed higher D_{Ba} coefficients and detected temperature effects only at the highest
379 salinity. Previous studies reported interactive influences of temperature and salinity on otolith
380 Ba:Ca ratios in Chinook salmon (Miller, 2011) and European seabass (Reis-Santos et al.,
381 2013). Changes in $Ba:Ca_{otolith}$ and D_{Ba} patterns may be a product of water chemistry. Indeed,
382 manipulations of ambient barium uniformly showed a clear influence on $Ba:Ca_{otolith}$ ratios
383 (Miller, 2009; Collingsworth et al., 2010). Facilitation between Ba and Sr ions may also
384 explain our observations. Previous studies highlighted elevated incorporation of Ba with
385 increased Sr ratios (de Vries et al., 2005). Though our study did not manipulate Sr
386 concentrations directly, we did observe the higher dissolved Sr concentrations, and lowest
387 dissolved Ba concentrations, in the highest salinity treatment. A complementary hypothesis to
388 Sr facilitation proposes that increased ambient water concentrations of Ba may inhibit
389 incorporation of Ba into otoliths, based on the fact that Ba approaches proportional
390 incorporation with the ambient water at low concentrations (Bath et al., 2000; de Vries et al.,
391 2005).

392 Magnesium may offer promise as temperature proxy for juvenile Atlantic cod because
393 only temperature significantly affected incorporation, measured by both Mg:Ca and D_{Mg}.
394 Increasing temperature led to increasing Mg:Ca ratios in otoliths, both pooled across and
395 within salinity treatments. This result confirms trends reported by Barnes and Gillanders
396 (2013) and Elsdon and Gillanders (2002), who also found a significant positive relationship

between Mg:Ca and temperature but no effect of salinity. Mg:Ca is believed to be primarily under biological control (Martin and Thorrold, 2005), with otolith Mg concentration regulated by physiological fractionation between blood and endolymphatic fluids surrounding fish otoliths (Melancon et al., 2009). Temperature-mediated control of $Mg:Ca_{otolith}$ ratios observed in our study and others may represent a product of thermal influence on the fractionation of Mg from blood to endolymphatic fluids (Barnes and Gillanders, 2013). Responses of otolith Mg:Ca ratios to temperature vary across taxa, with reports of non-significant (Martin and Thorrold, 2005; Martin and Wuenschel, 2006) and negative effects of temperature (Fowler et al., 1995). Variation in physiological response to temperature and its impact on fractionation likely contributes to among-species differences (Barnes and Gillanders, 2013).

Temperature and salinity significantly increased otolith manganese incorporation. Both $Mn:Ca_{Otolith}$ and D_{Mn} were positively associated with temperature, with highest values in the warmest treatment pooled across salinity treatments. The influence of salinity was more varied with significantly lower $Mn:Ca_{Otolith}$ and higher D_{Mn} ratios at higher salinities only when the temperature exceeded 5 ^{o}C . Considering partition coefficients, we found a significant temperature and salinity interaction on D_{Mn}, similar to previous work with juvenile spot, *Leiostomus xanthurus* (Martin and Thorrold, 2005). Temperature had a significant positive effect on D_{Mn} in high salinity treatments. As in Martin and Thorrold (2005), we found greater differentiation in D_{Mn} across salinity treatments at the warmest temperatures. Differences between Me:Ca and D_{Me} are often attributed to water chemistry variation across treatments (Martin and Wuenschel, 2006), and indeed we observed a significant negative association between $Mn:Ca_{Water}$ and salinity as previously documented (Martin and Thorrold, 2005). However, past studies have not reliably linked ambient ratios of Mn ($Mn:Ca_{Water}$) to otolith

chemistry (Elsdon and Gillanders, 2003; Collingsworth et al., 2010). The signal-to-noise ratio of Mn is high relative to other elements, given sensitivity to changes in water chemistry and low otolith concentrations. Nonetheless, our observations of inverted trends with $Mn:Ca_{Otolith}$ and D_{Mn} potentially indicate limitation, however, without mediation of ambient conditions we cannot fully partition the influence of variable physiology and ambient availability on Mn incorporation. Despite a lack of consensus on the mechanisms underlying concentrations in fish otoliths, Mn has proven useful in discrimination analyses (Thorrold et al., 1998; Reis-Santos et al., 2008; D'Avignon and Rose, 2013). For Atlantic cod, Mn could potentially be used as a useful tracer for temperature; however, reliable reconstructions would contingent upon some prior knowledge of salinity based on our results.

Stable C and O isotope chemistry offers a useful tool for reconstructing environmental histories of aquatic organisms because both isotope systems vary geographically in coastal and ocean waters (Thorrold et al., 1997a). Carbon isotopes ($´^{13}C$) in otoliths reflect a mixture of ambient dissolved inorganic carbon (DIC) and dietary carbon (Kalish, 1991; Schwarcz et al., 1998) sources in a ratio that is likely mediated by physiology (Thorrold et al., 1997a). Reported otolith ratios between ambient DIC and metabolic carbon vary from approximately 20% (Weidman and Millner, 2000) to 30% (Hoie et al., 2004) for Atlantic cod. We found a non-linear effect of temperature on $´^{13}C$ values, with decreases between intermediate and high temperature treatments as expected (Weidman and Millner, 2000) but with the lowest $´^{13}C$ values found at the coolest temperatures (~ 5 °C). We held juvenile cod in the low temperature treatment at the bottom of their expected thermal range (Brander, 1995). Correspondingly, somatic and otolith growth were significantly reduced in the low temperature treatments. The result is, nonetheless, similar to that of Hoie et al. (2003) who observed higher $´^{13}C$ values

than low growth rate fish in larval and early juvenile Atlantic cod (<21 mm) with higher growth rates and presumably higher metabolism. Like Hoie et al. (2003), our observations cannot be attributed to diet, as we did not vary feeding among treatments, and all cultures were fed to saturation. Previous studies reported behavioural differences both in respiration rate (McConnaughey et al., 1997) and swimming alterations of metabolic activity (Bjornsson, 1993; Hoie et al., 2003) that correlated negatively with $\acute{}^{13}C$. We lacked a direct measure of either index but would not expect either behaviour to increase at lower temperatures. Considering only the intermediate and high temperature treatments, where we observed no significant difference in size, $\acute{}^{13}C$ declined on average -0.14 ‰/°C ($p=0.001$, $r^2=0.37$), similar to rates derived from seasonal otolith records of wild caught Atlantic cod ~-0.16 ‰/°C (Weidman and Millner, 2000). This rate is similar to that previously reported for bearded rock cod (Kalish, 1991), Atlantic croaker (Thorrold et al., 1997a), as well as various species of foraminifera and mollusks (Grossman and Ku, 1986) (-0.18,-0.22, -0.11, and -0.13 ‰/°C, respectively).

Otolith $\acute{}^{18}O$ decreased with temperature, confirming patterns previously reported for Atlantic cod (Hoie et al., 2004) and other biogenic aragonites (Grossman and Ku, 1986). Past studies documented negative associations of otolith $\acute{}^{18}O$ and temperature for a variety of species, and demonstrated the reliability of Atlantic cod as an indicator of temperature to within 1 °C (Weidman and Millner, 2000) when adequately constraining the $\acute{}^{18}O$ of the ambient water.

Despite evidence linking salinity to variable isotope incorporation in fish otoliths (e.g. Schwarcz et al., 1998; Elsdon and Gillanders, 2002), no previous studies tested salinity effects

on otolith isotope concentrations in cod. We found a significant positive association between otolith isotopes and salinity. The positive association between salinity and $\delta^{18}O$ confirms previous studies documenting proportional otolith incorporation with ambient conditions (Thorrold et al., 1997a) and a positive relationship between water $\delta^{18}O$ and salinity (Gao, 2002).

Relationships with growth

Somatic and otolith growth rate can influence incorporation of elements and isotopes in fish otoliths (Hoie et al., 2003; Martin and Thorrold, 2005). Fish with faster growth rates generally have proportionally smaller otoliths (Worthington et al., 1995). In particular, past work found a negative correlation between otolith size and growth rate in juvenile Atlantic cod (Otterlei et al., 2002); our study shows that otoliths grow proportionally faster (~1.4) than fish length, confirming this trend. Diet and changes in physiology often associated with growth rate and size, influence carbon in particular (Hoie et al., 2003) as our data also showed. Somatic growth rate might be more important for larval and juvenile fishes where more variable growth is expected (Martin and Wuenschel, 2006). Our experiment revealed strong correlations between somatic growth and temperature (Bolland, 2008), which precluded a full evaluation of the combined effects of somatic growth with environmental condition resulting from limited variation individual growth within a treatment. A three-way temperature, salinity and feeding (growth) experiment similar to the nested design employed by Hoie et al. (2003) would most clearly elucidate the effect of variable growth rate on otolith incorporation (Martin and Wuenschel, 2006).

Though growth was not controlled orthogonally to environment in our experiment, we nonetheless observed variation in growth within treatments. Residuals from a temperature by

488 salinity growth model offer a tool to evaluate the effect of growth while accounting for
489 environmental correlates. Our study found significant positive relationships between growth
490 and otolith magnesium and manganese (Me:Ca and D_{Me}), similar to observations of D_{Mn} in
491 Atlantic cod (Limburg et al., 2011) and other groundfish species (Limburg et al., 2014).
492 Strontium (Sr:Ca and D_{Sr}) and growth were significantly and negatively related, as reported in
493 juvenile striped bass (*Morone saxatilis*) (Secor et al., 1995). Incorporation of barium (Ba:Ca
494 and D_{Ba}) was growth independent, as reported in adult Atlantic cod (Thorisson et al. (2011)
495 and juvenile spot croaker (*Leiostomus xanthurus*) (Bath et al., 2000). Similarly, we observed
496 an overall negative but non-significant relationship between oxygen ($\acute{}^{18}O$) incorporation and
497 growth, suggesting growth independent incorporation, mirroring patterns previously reported
498 for juvenile Atlantic cod (Hoie et al. (2003). Carbon ($\acute{}^{13}C$) ratios and growth were
499 significantly and positively related, echoing similar trends in previous studies (Thorrold et al.,
500 1997a). At low temperatures, growth was highest at intermediate salinities, where we also
501 observed the highest $\acute{}^{13}C$ values. This relationship with growth likely drove the significant
502 interaction between temperature and salinity on $\acute{}^{13}C$ incorporation. As with $\acute{}^{18}O$, the positive
503 association between $\acute{}^{13}C$ and growth mirrors patterns observed for temperature and juvenile
504 Atlantic cod (Hoie and Folkvord, 2006). Indeed all observed relationships between growth
505 and otolith chemistry mirror temperature patterns. Collectively our results support the
506 assertion that variation in physiology and otolith deposition associated with growth influence
507 trace element incorporation (Secor et al., 1995), further highlighting the need for experimental
508 studies that nest growth within temperature.

Conclusion

Ultimately, otolith geochemical composition provides a template to discern environmental histories of juvenile Atlantic cod, potentially providing novel information about this critical period. Analyses of otolith composition provides one method to assign juvenile residency in Atlantic cod, offering a feasible alternative to tagging studies that are logistically difficult or impossible in larval and juvenile fishes given their small size and relatively high mortalities (Thorisson et al., 2011). Spatial and temporal differences in otolith chemistry among geographic regions may reflect ambient water chemistry and environmental condition, noting the modest effect that diet alone has on otolith incorporation for most fish (Hoff and Fuiman, 1995; Milton and Chenery, 2001; Walther and Thorrold, 2006; Webb et al., 2012; Woodcock et al., 2012), with some notable acceptations (Buckel et al., 2004). The wide range of environmental conditions experienced by Atlantic cod in nearshore nursery habitat necessitates a comprehensive understanding of temperature and salinity effects on otolith chemistry. Moreover, these complex environments demand a more diverse suite of otolith trace elements and isotopes to ensure successful reconstruction. Results from our study provide a comprehensive assessment of temperature and salinity effects on a substantial suite of both elemental and isotopic constituents in juvenile Atlantic cod otoliths. Temperature and salinity both significantly affected all elements and isotopes we measured except Mg, which was apparently incorporated into otoliths independent of salinity. Collectively our results highlight the potential utility of otolith geochemistry to identify change and reconstruct environmental life histories of Atlantic cod, but they also highlight key considerations and limitations of the application when considering environmental variables in isolation.

Supplementary material

Additional supplementary material is available at ICES Journal of Marine Science online and the online version of this manuscript. Supplementary data is comprised of a table with the full temperature by salinity partitioned correlations between otolith constituents and fish growth, both otolith and somatic.

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552 **Tables:**

553 Table 1. Means, standard deviations (sd) and ranges for each element and isotope *Gadus*
554 *morhua* otolith constituent measured across all treatments. Trace elemental data is
555 standardized as a ratio of calcium; units for elements are per mol Ca, Isotopic ratios of $\delta^{18}O$
556 and $\delta^{13}C$ are expressed in ‰ relative to VPDB, and partition coefficients are ratios of otolith
557 chemistry to ambient water chemistry.

558

Parameter	Mean	Sd	Range	Units	n
$Me:Ca_{Water}$					
Mg	5.31	0.57	(5.06-5.53)	Mol	24
Mn	5.99	2.60	(2.26-9.50)	µmol	24
Sr	9.40	0.23	(8.87-9.74)	mmol	24
Ba	22.98	14.8	(5.23-44.25)	µmol	24
$Me:Ca_{Otolith}$					
Mg	20.19	11.15	(7.27-61.7)	µmol	278
Mn	2.62	1.05	(1.02-6.07)	µmol	273
Sr	3.08	0.76	(1.68-5.05)	mmol	273
Ba	2.49	0.79	(1.32-4.54)	µmol	273
$\delta^{18}O$	-3.68	0.79	(-5.3--1.87)	‰	116
$\delta^{13}C$	-0.57	0.98	(-2.57-1.46)	‰	116
D_{Me}					
Mg	3.81	2.1	(1.43-11.3)	$*10^{-6}$	269
Mn	0.50	0.27	(0.129-1.72)	-	267
Sr	0.28	0.08	(0.18-0.53)	-	265
Ba	0.18	0.14	(0.033-0.73)	-	269

559

560 Table 2: Results of linear mixed effects models comparing water chemistry (Md:Ca_{Water}), otolith chemistry (Me:$Ca_{Otolith}$) and
561 elemental partition coefficients (D_{Me}) as a function of temperature (T) and salinity (S) with replicate tank controlled as a random
562 variable. Residuals from the temperature x salinity growth relationship were evoked as a continuous covariate. To control for multiple
563 comparisons p-values are adjusted according to the *Benjamini-Hochberg* transformation.
564

	Source of Variation	df	Mg F	Mg p	Mn F	Mn p	Sr F	Sr p	Ba F	Ba p
Me:Ca_{Water}	T	2	1.8	0.354	5.4	0.042	0.5	0.879	0.3	0.904
	S	2	238.0	**<0.0001**	194.5	**<0.0001**	324.4	**<0.0001**	1233.0	**<0.0001**
	T:S	4	0.2	1.000	1.9	0.337	0.6	0.879	0.2	0.924
Me:$Ca_{Otolith}$	T	2	6.8	**0.003**	14.2	**<0.0001**	27.7	**<0.0001**	27.5	**<0.0001**
	S	2	1.3	0.343	14.4	**<0.0001**	6.2	**0.004**	17.2	**<0.0001**
	G	4	4.7	**0.046**	15.0	**<0.0001**	31.6	**<0.0001**	0.3	0.728
	T:S	9	0.1	0.984	0.6	0.799	0.1	0.984	1.8	0.192
D_{Me}	T	2	7.2	**0.002**	34.8	**<0.0001**	28.0	**<0.0001**	25.9	**<0.0001**
	S	2	0.3	0.888	117.6	**<0.0001**	4.2	**0.029**	742.4	**<0.0001**
	G	4	4.7	**0.049**	15.0	**<0.0001**	31.6	**<0.0001**	0.3	0.606
	T:S	9	0.1	0.974	2.7	**0.041**	0.1	0.974	2.6	**0.046**

565 Table 3: Results of linear mixed effects models comparing otolith isotopes Carbon and
566 Oxygen ratios among temperature (T) and salinity (S) treatments with replicate tank
567 controlled as a random variable. Residuals from the temperature x salinity growth relationship
568 were evoked as a continuous covariate. To control for multiple comparisons p-values are
569 adjusted according to the *Benjamini-Hochberg* transformation.

	Source of Variation	df	$\delta^{18}O$ F	$\delta^{18}O$ p	$\delta^{13}C$ F	$\delta^{13}C$ p
$Me:Ca_{Otolith}$	T	2	61.39	**<0.0001**	43.72	**<0.0001**
	S	2	23.23	**<0.0001**	21.70	**<0.0001**
	G	4	0.02	0.914	0.95	**0.030**
	T:S	9	1.72	0.208	4.67	**<0.0001**

570

571 Table 4: Results of linear mixed effects models comparing somatic and otolith growth as a
572 function of temperature (T) and salinity (T). Tank replicate treated as a random factor.

Source of variation	df	Somatic F	Somatic p	Otolith F	Otolith p
T	2	102.4	**<0.0001**	34.0	**<0.0001**
S	2	1.7	0.23	2.9	0.06
S x T	4	5.3	**0.001**	1.7	0.16

573

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Figures

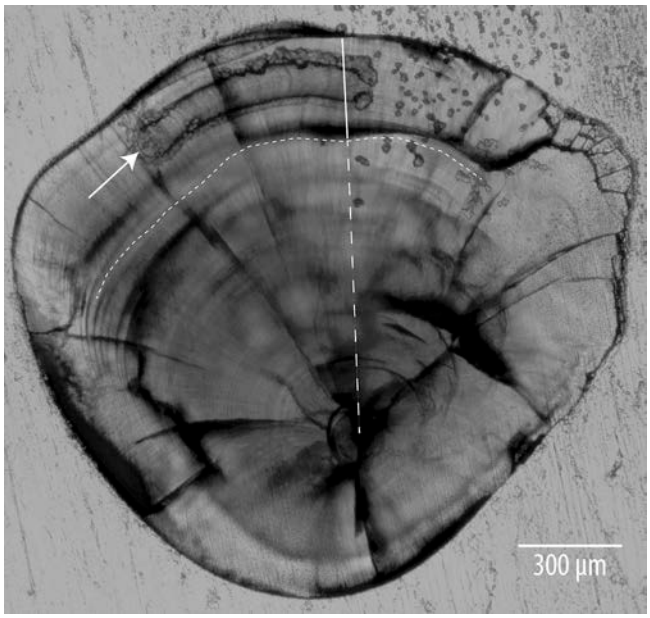
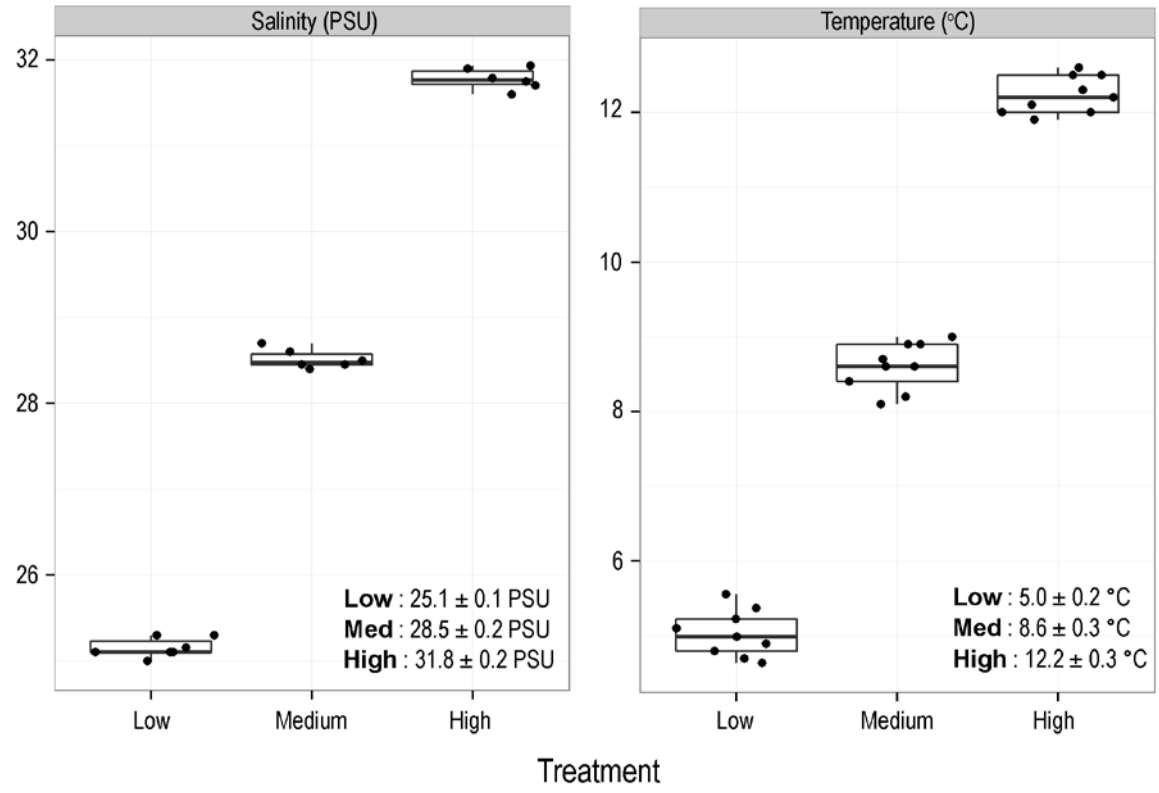
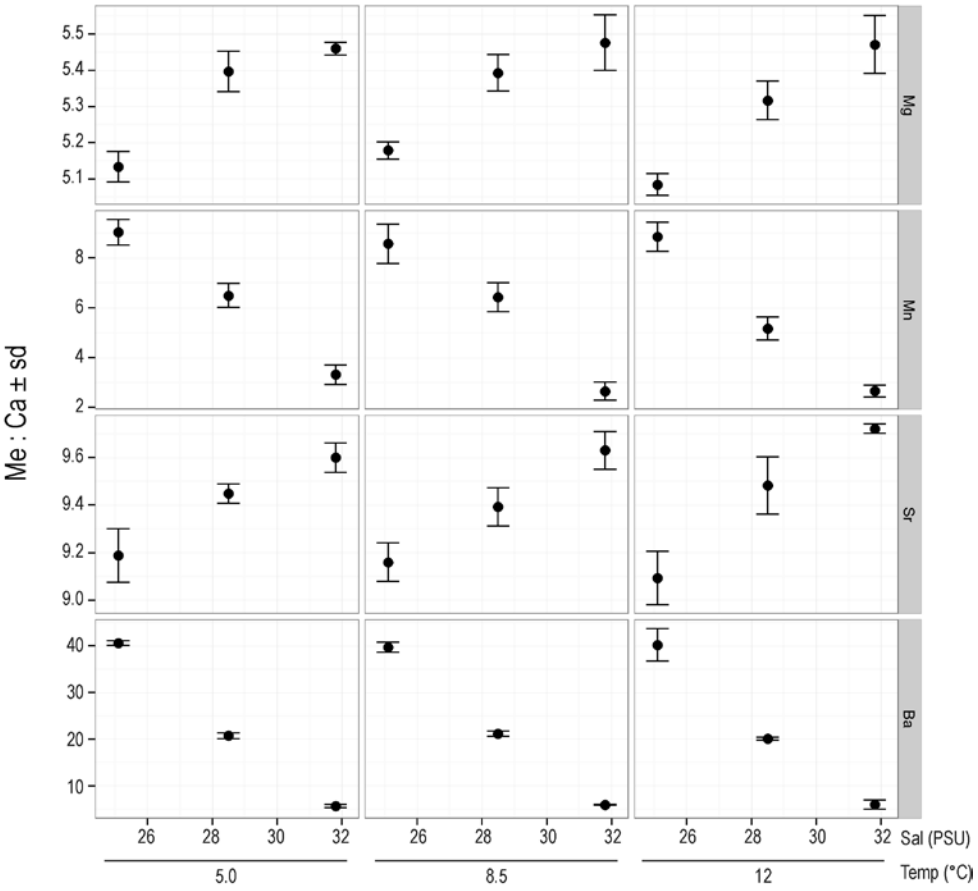


Figure 1: Polished juvenile *Gadus morhua* sagittal otolith showing laser raster (arrow), oltolith growth (solid line), pre-experimental growth (long dashed line) and alizarin tag (short dashed line) denoting beginning of the experiment.

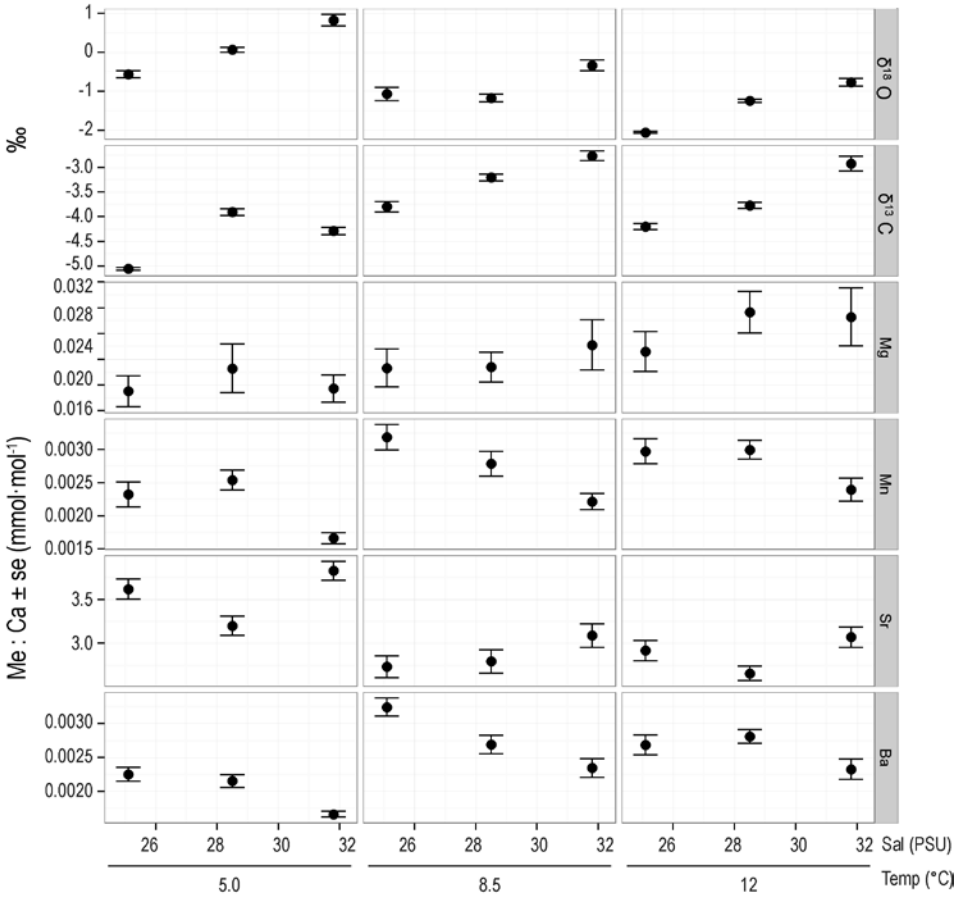


583 Figure 2: Summary of experimental treatments conditions. Means values are reported ± 1
584 standard deviation.



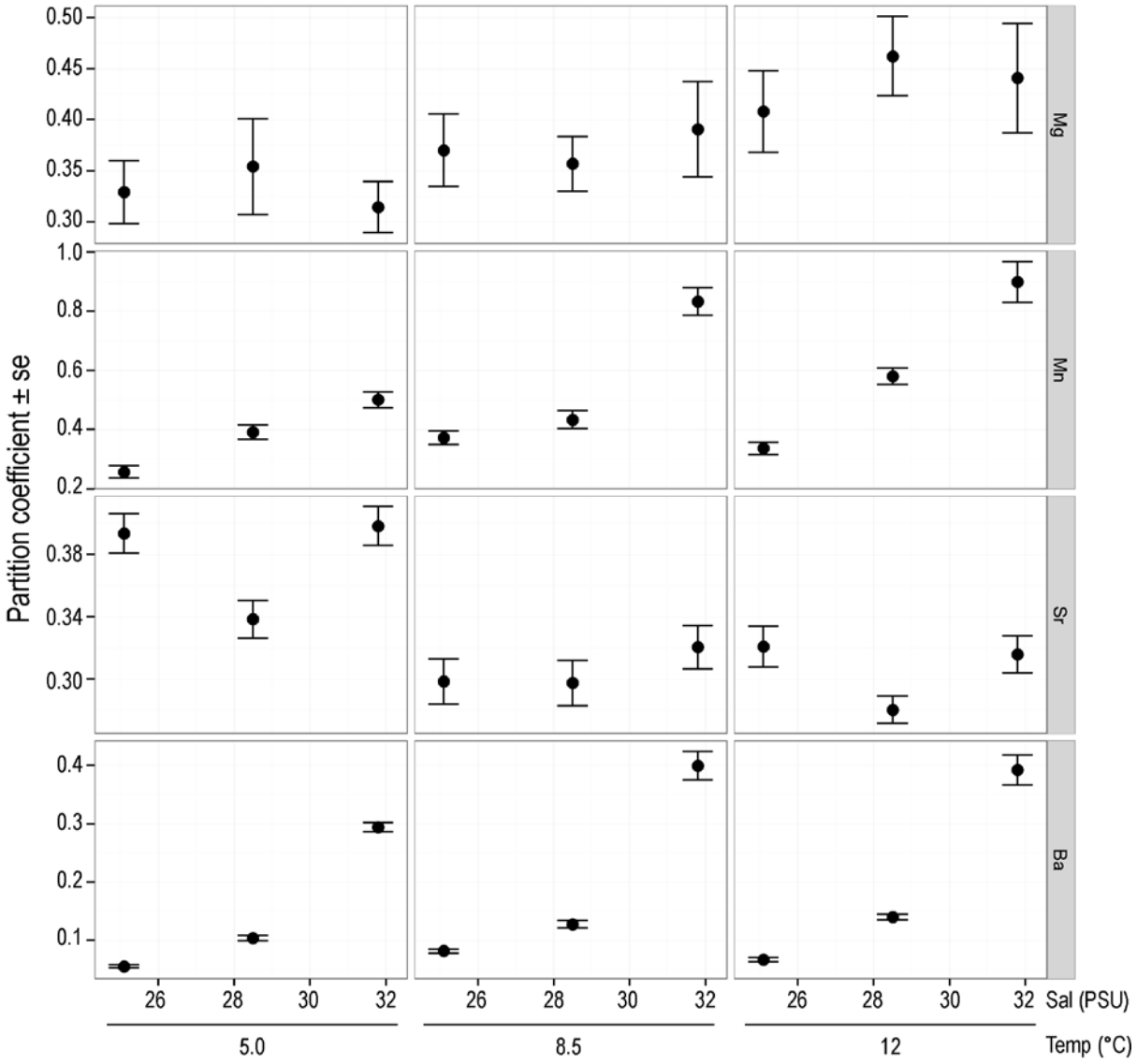
585

586 Figure 3: Mean ratios of Me:Ca_{water} among temperature and salinity experimental treatments.
587 Mg:Ca values are in mol·mol^{-1}, Mn:Ca and Ba:Ca are µmol·mol^{-1}, and Sr:Ca is mmol·mol^{-1}.



588

589 Figure 4: Mean Me:$Ca_{Otolith}$ and isotopic ratios for temperature and salinity treatments (n=3
590 respectively) of juvenile *Gadus morhua* otoliths. Isotopes in ‰ relative to VPDB. Error bars
591 represent ± 1 standard error.Figure 5: Mean partition coefficients (D_{Me}) for juvenile *Gadus*
592 *morhua* otoliths reared under temperature and salinity experimental treatments). Mean D_{Mg}
593 values are multiplied by 10^5.



594

595 Figure 5. Mean partition coefficients (D_{me}) for juvenile *G. morhua* otoliths reared under
596 temperature and salinity experimental treatments. Mean D_{mg} values are multiplied by 10^5.

597

598 Figure 6. Summary of somatic and otolith growth throughout the experiment. Boxplot fill
599 colours denote results of within treatment Tukey's post-hoc tests ($\pm < 0.05$).

Supplementary material:

Table S1. Correlations between growth and elemental partition coefficients and isotope values in juvenile *Gadus morhua* grown under experimental conditions. Pearson correlation coefficients presented with *Benjamini-Hochberg* adjusted ± controlling for multiple comparisons * ± <0.05, ** ± <0.01. Isotopes in ‰ relative to VPDB. Sample size of each treatment and element is denoted by n.

	Temp	Low			Med			High			Pooled
	Sal	Low	Med	High	Low	Med	High	Low	Med	High	
D_{Mg}	Somatic	0.15	0.03	-0.01	0.29	0.36	-0.27	0.1	0.05	-0.17	**0.21***
	Otolith	0.17	-0.2	0.6	0.36	0.27	0.26	0.22	-0.17	-0.22	**0.19***
	n	*37*	*21*	*28*	*31*	*33*	*27*	*29*	*38*	*25*	*269*
D_{Mn}	Somatic	0.26	-0.52	-0.19	0.41	0.42	0.12	0.06	0.14	0.02	**0.33***
	Otolith	0.41	-0.17	0.28	0.45	**0.66***	**0.55***	0.36	0.16	0.05	**0.34***
	n	*39*	*21*	*25*	*28*	*31*	*29*	*31*	*39*	*24*	*267*
D_{Sr}	Somatic	-0.32	0.87	-0.39	-0.3	-0.4	-0.51	-0.14	-0.01	-0.47	**-0.56***
	Otolith	-0.42	-0.25	0.01	-0.48	**-0.74***	**-0.42**	**-0.56***	-0.28	-0.21	**-0.57***
	n	*37*	*22*	*24*	*27*	*32*	*29*	*29*	*38*	*27*	*265*
D_{Ba}	Somatic	-0.05	-0.63	0.21	0.17	0.05	0.33	0.03	-0.04	-0.1	0.03
	Otolith	0.08	0.15	0.17	0.22	0.2	0.33	0.36	0.32	0.39	0.13
	n	*33*	*23*	*28*	*27*	*32*	*29*	*31*	*39*	*27*	*269*
$\delta^{13}C$	Somatic	0.19	-0.03	-0.42	0.08	-0.04	0.01	0.26	0.7	0.22	**0.45***
	Otolith	0.31	0.07	-0.6	-0.07	-0.35	0.22	0.26	-0.5	0.01	**0.37***
	n	8	15	16	13	15	15	11	10	14	116
$\delta^{18}O$	Somatic	-0.27	0.31	-0.24	0.38	-0.33	-0.06	-0.69	0.35	0.27	**-0.41***
	Otolith	0.11	0.11	0	0.06	0.1	0.29	**-0.86***	0.11	0.08	**-0.34***
	n	*14*	*14*	*16*	*11*	*13*	*15*	*9*	*10*	*14*	*116*

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