



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

Ryan R. E. Stanley^{1*}, Ian R. Bradbury^{1,2}, Claudio DiBacco³, Paul V. R. Snelgrove¹, Simon R. Thorrold⁴, and Shaun S. Killen⁵

¹Ocean Sciences Center and Biology Department, Memorial University of Newfoundland, PO Box 4200, St John's, NL, Canada A1C 5S7

²Department of Fisheries and Oceans, Northwest Atlantic Fisheries Centre, St John's, NL, Canada A1C 5X1

³Department of Fisheries and Oceans, Bedford Institute of Oceanography, PO Box 1006, Dartmouth, NS, Canada B2Y 4A2

⁴Biology Department, MS #50, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA

⁵Institute of Biodiversity, Animal Health and Comparative Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK

*Corresponding author: tel: +1 709 864 4469; e-mail: rstanley@mun.ca

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We evaluated the influence of environmental exposure of juvenile Atlantic cod (*Gadus morhua*) 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 ($\delta^{13}\text{C}$) and oxygen ($\delta^{18}\text{O}$) isotopes using isotope ratio monitoring mass spectrometry. Temperature and salinity significantly affected all elements and isotopes measured, except 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 $\delta^{13}\text{C}$, 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: elemental fingerprinting, *Gadus morhua*, isotopes, otolith chemistry, salinity, temperature.

Introduction

The application of otolith geochemistry to resolve natal habitats or to reconstruct life histories assumes otolith chemistry corresponds to local environmental conditions and ambient water chemistry, and that these conditions vary to impart a unique geochemical signal (Campana, 1999). Physiological and environmental factors both mediate assimilation of elements and isotopes into otoliths (e.g. Thorrold *et al.*, 1997b; Elsdon and Gillanders, 2002). Prediction of where otolith composition differences might exist, or the reconstruction of fish movements and environmental

histories, requires predictable relationships between environmental variables and otolith chemistry (Elsdon *et al.*, 2008; Reis-Santos *et al.*, 2013). Laboratory studies that have quantified specific relationships between select environmental factors and otolith composition (Hoff and Fuiman, 1995; Bath *et al.*, 2000; Elsdon and Gillanders, 2002, 2003; Martin and Wuenschel, 2006; Miller, 2011; Reis-Santos *et al.*, 2013) highlight a lack of generality across among different species (Gillanders, 2005; Reis-Santos *et al.*, 2008; Barnes and Gillanders, 2013). For instance, Sr:Ca ratios in otoliths vary considerably among species in the absence of differences in

dissolved Sr:Ca in ambient water (Campana, 1999), presumably resulting from interspecific differences in metabolic traits (Kalish, 1991; Campana et al., 2000; Miller, 2011).

Ambient concentrations of elements and isotopes in seawater may also co-vary with temperature and salinity (Epstein and Mayeda, 1953; Elsdon and Gillanders, 2003; Reis-Santos et al., 2013, this study). Retrospective assignment of adults to nursery habitats or environmental conditions during the juvenile period has been accomplished through geochemical analysis of material close to the otolith core, corresponding to the juvenile period (Thorrold et al., 2001; Gillanders, 2005; Farmer et al., 2013). Determining the effects of temperature and salinity on otolith elemental and isotopic incorporation will enhance our ability to predict where significant differences might occur and interpret life history movements (Elsdon and Gillanders, 2002). Although the use of otolith signatures does not require explicit understanding of all details influencing incorporation (Campana et al., 2000), the significant influence of environmental factors adds complexity to the interpretation of geochemical concentrations in isolation of environment (Martin and Wuenschel, 2006).

Atlantic cod (*Gadus morhua*) is a demersal finfish species native to the North Atlantic, spanning from Greenland to Cape Hatteras, NC, USA, to as far east as the Baltic Sea. Spawning occurs in both inshore and offshore environments, between late winter and early spring. Pelagic eggs develop into larvae that later metamorphose into benthic juveniles inhabiting offshore areas as well as shallow inshore regions, often associating with complex habitat (Laurel et al., 2003). The large latitudinal and depth range of Atlantic cod exposes juveniles to a gradient of temperatures and salinities (Dalley and Anderson, 1997), making them ideal candidates for studies on environmental effects on otolith elemental incorporation. Atlantic cod have received considerable research attention, in particular efforts to understand the structure of remaining fragmented populations in the Northwest Atlantic. The pelagic egg and larval phase imparts a large dispersal potential, mediated by spawn timing and ambient conditions (Bradbury et al., 2000; Stanley et al., 2013). Genetic (Ruzzante et al., 2000), tagging (Rose et al., 2011), and otolith chemistry techniques (Campana et al., 1999; Jamieson 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 ($\delta^{13}\text{C}$) and oxygen ($\delta^{18}\text{O}$); 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 ($\sim 11^\circ\text{C}$ and 32 PSU) for up to 137 d 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 d, 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 2 d, 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 l^{-1}) at a pH adjusted to 7.0, for 24 h. 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 pelleted 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-h light cycle.

Three experimental temperature treatments of ~ 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

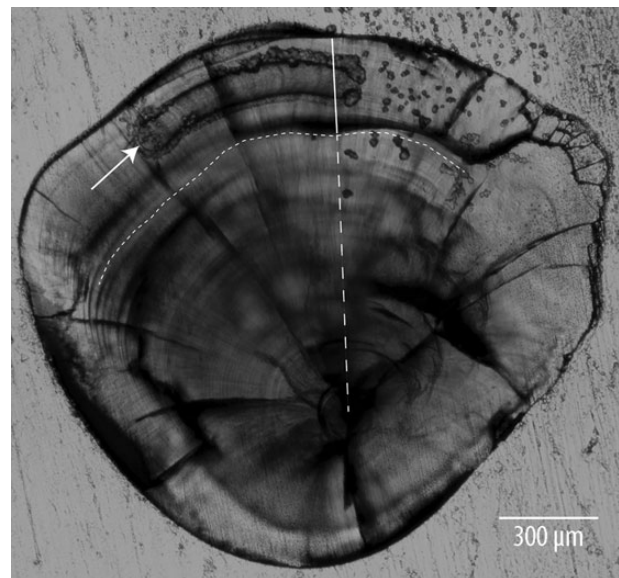


Figure 1. Polished juvenile *G. morhua* sagittal otolith showing laser raster (arrow), otolith growth (solid line), pre-experimental growth (long dashed line), and alizarin tag (short dashed line) denoting beginning of the experiment.

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 h before 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 d (14 July–12 October 2007). Upon completion of the experiment, we removed the fish and recorded standard length. Both sagittal and lapillar 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, before 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 $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ 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 incorporation. We lasered along a line parallel with the otolith edge within a common growth band and outside of the Alizarin mark ($\sim 500 \mu\text{m}$). Laser repetition rate was set at 5 Hz for all analyses, with a scan speed of $5 \mu\text{m s}^{-1}$. We used a certified reference material (CRM) consisting of powdered otoliths (Yoshinaga *et al.*, 2000), dissolved in 2% ultrapure HNO_3 (SeaStar[®]), and diluted to a Ca concentration of $40 \mu\text{g g}^{-1}$, to correct for instrument bias and drift following Thorrold and Swearer (2009). External precision was estimated by analysing a second otolith CRM (Sturgeon *et al.*, 2005), also dissolved in 2% HNO_3 (SeaStar[®]) and diluted to a Ca concentration of $40 \mu\text{g g}^{-1}$, periodically throughout the laser analyses ($n = 55$). Analytical accuracy was determined from the concentrations of Japanese Otolith and NRC standards, averaged across all samples. We found that accuracy was exceeded 98% for all otolith constituents measured, including isotopic ratios described later. We calculated limits of detection (LOD) as five times the mean intensity of blanks (2% HNO_3), run every ten assays for each otolith constituent. Any measurements below the respective LOD were excluded from analysis. We conducted all statistical analyses using otolith elemental compositions converted to molar values and standardized to calcium concentrations ($\text{Me}:\text{Ca}_{\text{otolith}}$). Estimates of external precision based on the relative standard deviation values of the second

CRM were 4.2% for Mg, 17.9% for Mn, 0.40% for Sr, and 1.4% for Ba.

We measured $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values in sagittal otoliths of each individual by milling otoliths with a MicroMill sampler (New Wave Research) or a hand-held dental drill under a stereomicroscope until we obtained 50–200 μg of near-edge material. Stable isotopes were measured using an isotope ratio monitoring mass spectrometer according to methods outlined 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 $\delta^{18}\text{O}$ and ± 0.03 for $\delta^{13}\text{C}$ (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 midpoint of the experiment (day 32). Water intake for the OSC and JBARB comes from ~ 5 m depth with a very stable salinity (± 0.25 PSU, yearly variance) and temperature ($\pm 1^\circ\text{C}$, weekly variance; Danny Boyce, JBARB facilities manager, Memorial University of Newfoundland, St John's, Canada, pers. comm.). 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 analysed for elemental composition. Samples were analysed 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 analysed. Elemental concentrations were standardized to and expressed as element to calcium ratios ($\text{Me}:\text{Ca}_{\text{water}}$), which were used in all subsequent statistical analyses.

Growth

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

Statistical analysis

Elemental ($\text{Me}:\text{Ca}_{\text{otolith}}$ and $\text{Me}:\text{Ca}_{\text{water}}$) and isotope data were inspected for normality using Q–Q plots and clear outliers (i.e. more than two times the standard deviation from the mean of a given metric) were removed. In total, $<3\%$ of observations met these exclusion criteria. Partition coefficients provide a complementary metric to compare otolith incorporation rates across experimental treatments accounting for both $\text{Me}:\text{Ca}_{\text{otolith}}$ and

Me:Ca_{water} (Reis-Santos *et al.*, 2013), and are especially useful for comparisons among species and studies (Martin and Wuenschel, 2006). We calculated partition coefficients (D_{me}) for each element by dividing the measured otolith calcium ratio (Me:Ca_{otolith}) by the observed water ratio (Me:Ca_{water}) of the treatment (Morse and Bender, 1990). The majority of field studies only provide baseline information based on observed otolith composition because the ambient water chemistry is often missing and therefore partition coefficients cannot be calculated (but see, Thorrold *et al.*, 1998). For this reason, we present analyses of otolith composition (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 log₁₀ 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 analysed individually, thus creating six 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 with a significance level set at $\alpha = 0.05$. 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 $\delta^{18}\text{C}$ which approximated 0.08.

Ethics

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

Results

Rearing conditions

Experimental conditions were consistent throughout the trials for low ($5.0 \pm 0.2^\circ\text{C}$ and 25.1 ± 0.1 PSU; mean \pm s.d.), intermediate ($8.6 \pm 0.3^\circ\text{C}$ and 28.5 ± 0.2 PSU; mean \pm s.d.), and high ($12.2 \pm 0.3^\circ\text{C}$ and 31.8 ± 0.2 PSU; mean \pm s.d.) treatment levels (Figure 2). All water was sourced from a common intake and filtration system, but variation in ambient water chemistry occurred as a function of experimental conditions (Table 1). Salinity significantly affected Me:Ca_{water} ratios (Table 2); however, we detected no temperature, temperature–salinity interactions, or tank replicate

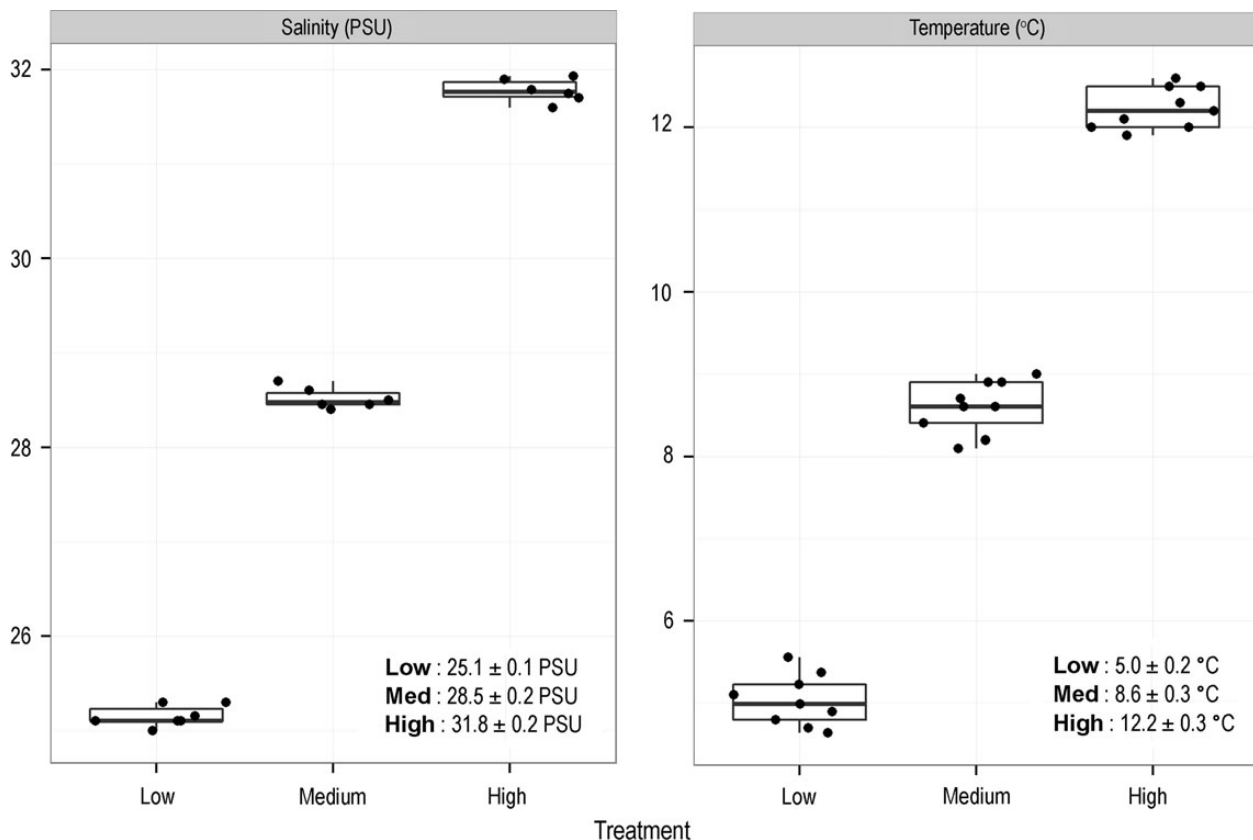


Figure 2. Summary of experimental treatments conditions. Means values are reported ± 1 standard deviation.

effects in Me:Ca_{water}. Mg:Ca and Sr:Ca_{water} were positively correlated and Mn:Ca and Ba:Ca_{water} were negatively correlated with treatment salinity (Figure 3).

Otolith chemistry

Temperature significantly influenced elemental-calcium (Me:Ca_{Otolith}) and isotope ratios (Tables 2 and 3, respectively; Figure 4). Salinity significantly influenced on all elements and isotopes tested, except magnesium (Mg:Ca_{Otolith}). We detected no interactive effects for any elemental ratio or for either stable isotope variables. Both Mg:Ca and Mn:Ca were significantly higher at the warmest temperatures. Mg:Ca and Sr:Ca ratios increased with salinity, whereas Mn:Ca and Ba:Ca ratios decreased (Figure 4), corresponding to trends 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 $\delta^{18}\text{O}$ ratios, but did find a significant interaction on ratios of $\delta^{13}\text{C}$ (Table 3). Both $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ associated positively with salinity across all temperature treatments. $\delta^{13}\text{C}$ values were significantly lower at the low temperatures compared with intermediate and high temperature treatments, except at the intermediate salinity treatment (thus the significant interaction between temperature and salinity). Conversely, we observed significantly higher $\delta^{18}\text{O}$ in the low-temperature treatment compared with 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

Table 1. Means, standard deviations (s.d.), and ranges for each element and isotope *G. morhua* otolith constituent measured across all treatments.

Parameter	Mean	s.d.	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
$\delta^{18}\text{O}$	−3.68	0.79	−5.3 to −1.87	‰	116
$\delta^{13}\text{C}$	−0.57	0.98	−2.57 to 1.46	‰	116
D_{Me}					
Mg	3.81	2.1	1.43–11.3	$\times 10^{-6}$	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

Trace elemental data are standardized as a ratio of calcium; units for elements are per mol Ca, isotopic ratios of $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ are expressed in ‰ relative to VPDB, and partition coefficients are ratios of otolith chemistry to ambient water chemistry.

Table 2. Results of linear mixed-effects models comparing water chemistry (Md:Ca_{water}), otolith chemistry (Me:Ca_{Otolith}), and elemental partition coefficients (D_{Me}) as a function of temperature (T) and salinity (S) with replicate tank controlled as a random variable.

	Source of variation	d.f.	Mg		Mn		Sr		Ba	
			F	p-value	F	p-value	F	p-value	F	p-value
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

Residuals from the temperature \times salinity growth relationship were evoked as a continuous covariate. To control for multiple comparisons, p -values are adjusted according to the *Benjamini–Hochberg* transformation.

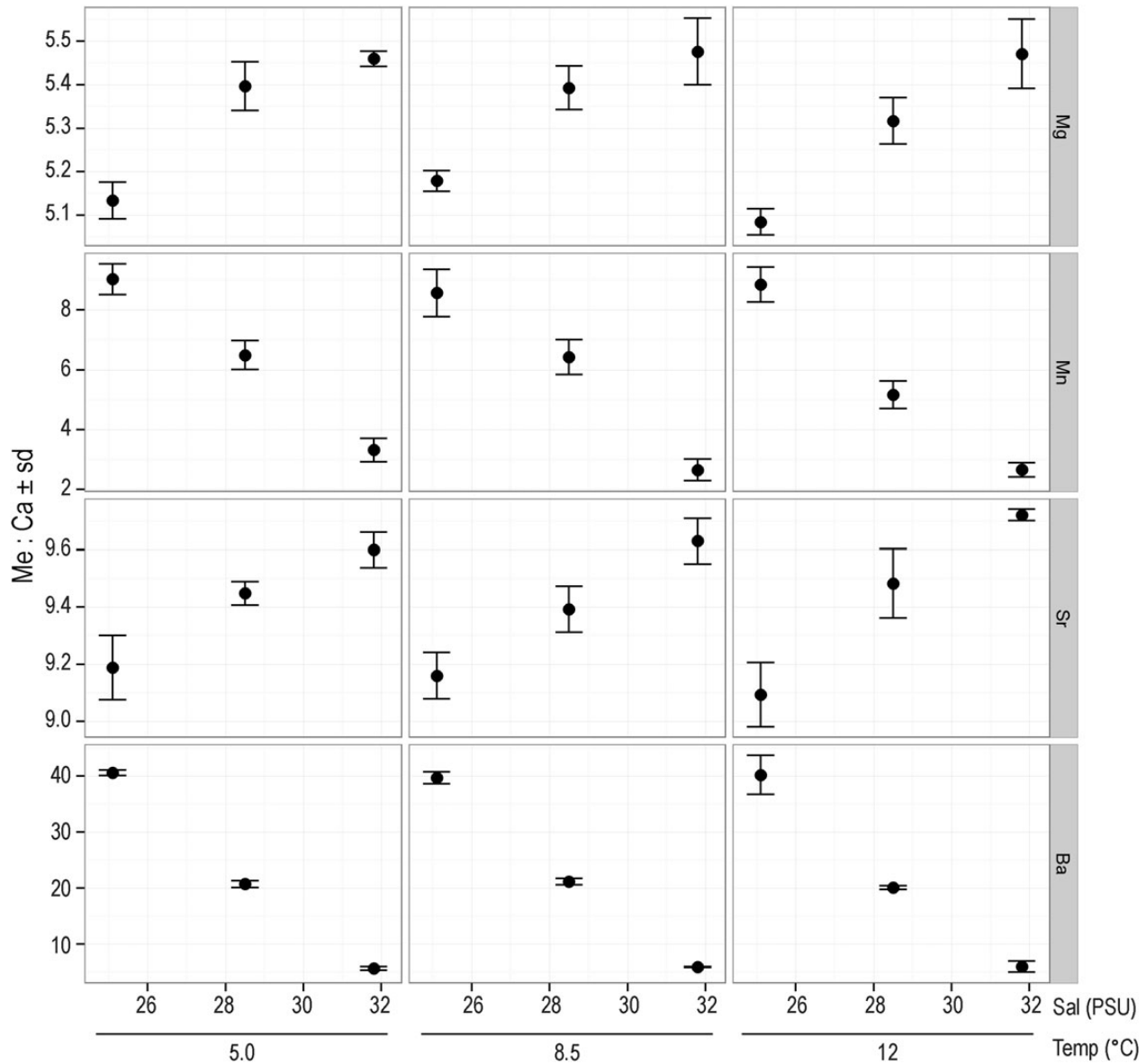


Figure 3. Mean ratios of Me:Ca_{water} among temperature and salinity experimental treatments. Mg:Ca values are in mol·mol⁻¹, Mn:Ca and Ba:Ca are μmol·mol⁻¹, and Sr:Ca is mmol·mol⁻¹.

(Pearson's $r = 0.58$, $p < 0.0001$) 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, $t = 12.7$, $p \leq 0.00001$). 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 $\delta^{13}C$, 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 $\delta^{18}O$. These trends mirror directionality and significance of

correlative relationships among otolith constituents and growth across treatments (Supplementary Table S1).

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, 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

Table 3. Results of linear mixed-effects models comparing otolith isotopes carbon and oxygen ratios among temperature (T) and salinity (S) treatments with replicate tank controlled as a random variable.

Source of variation	d.f.	$\delta^{18}\text{O}$		$\delta^{13}\text{C}$		
		F	p-value	F	p-value	
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

Residuals from the temperature \times salinity growth relationship were evoked as a continuous covariate. To control for multiple comparisons, p-values are adjusted according to the Benjamini–Hochberg transformation.

species (Campana, 1999). Laboratory validations thus provide an important and necessary step in evaluating biotic and abiotic influences on otolith incorporation rates (Kalish, 1991; Miller, 2011) for specific elements and isotopes before 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 (*G. morhua*) and similar coastal marine fish.

Our experiments demonstrate significant influences of both temperature and salinity on otolith composition of juvenile Atlantic cod (*G. 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

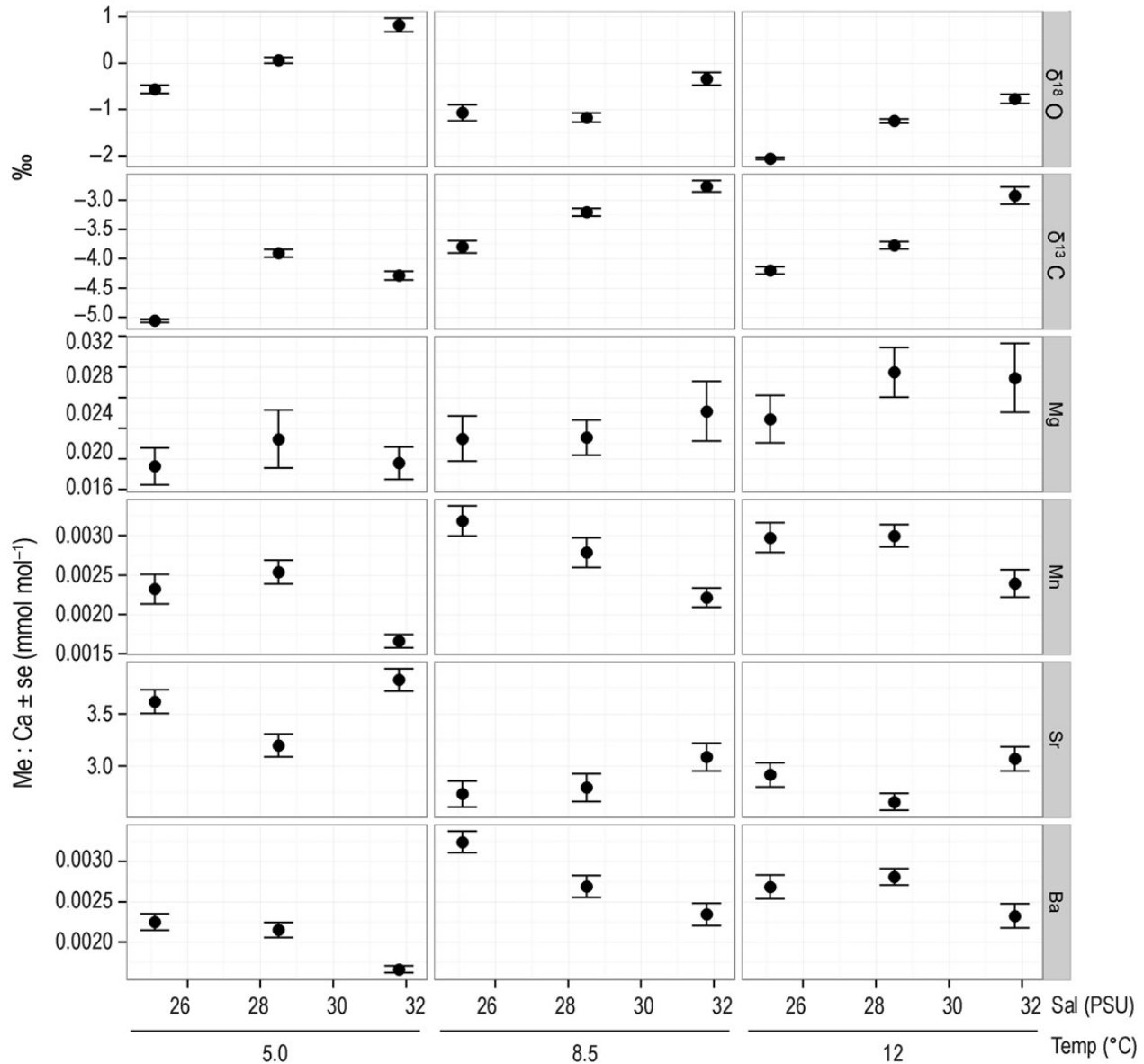


Figure 4. Mean Me:Ca_{Otolith} and isotopic ratios for temperature and salinity treatments ($n = 3$, respectively) of juvenile *G. morhua* otoliths. Isotopes in ‰ relative to VPDB. Error bars represent ± 1 standard error.

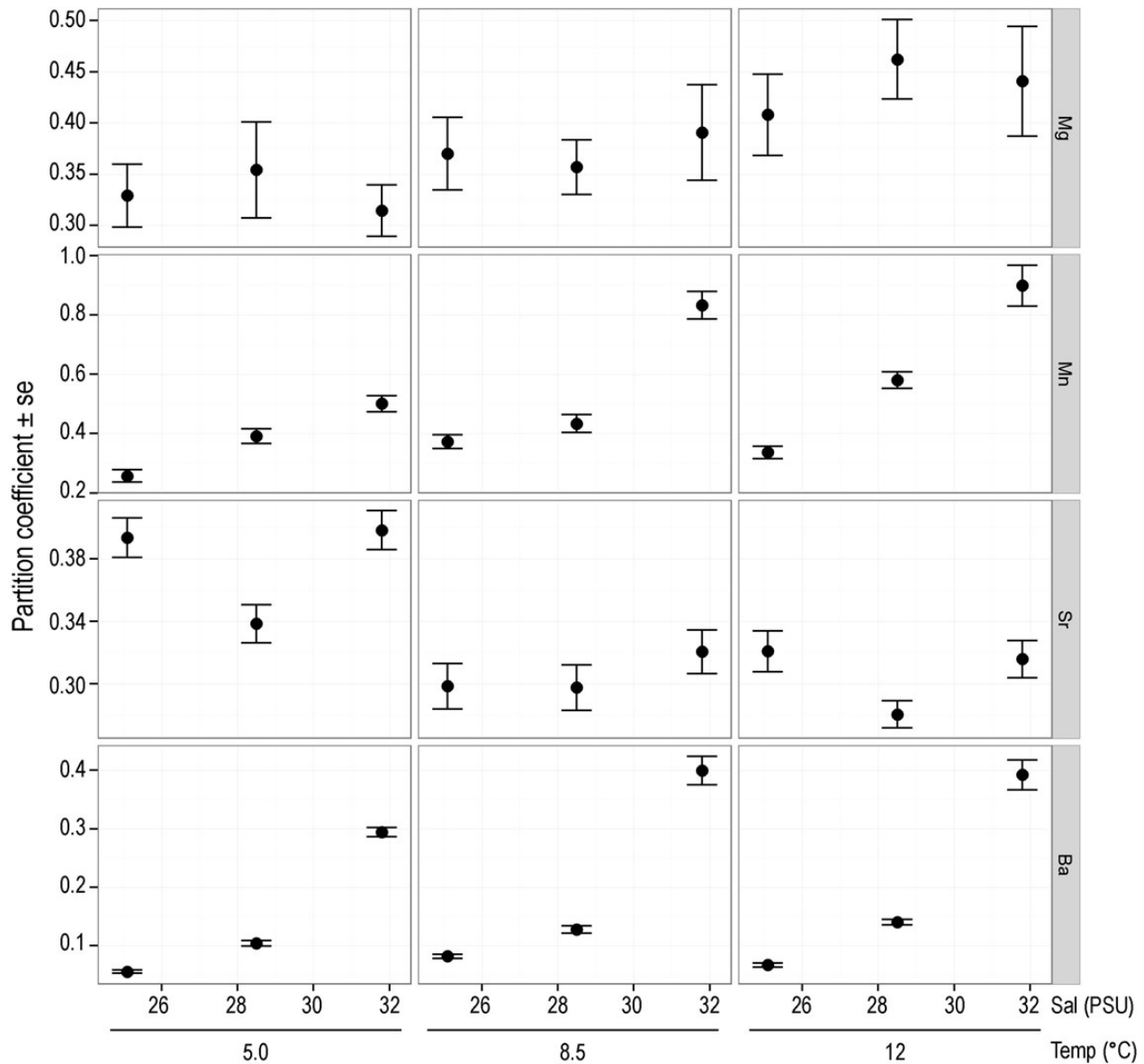


Figure 5. Mean partition coefficients (D_{Me}) for juvenile *G. morhua* otoliths reared under temperature and salinity experimental treatments. Mean D_{Mg} values are multiplied by 10^5 .

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 bring 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 macrocephalus* (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

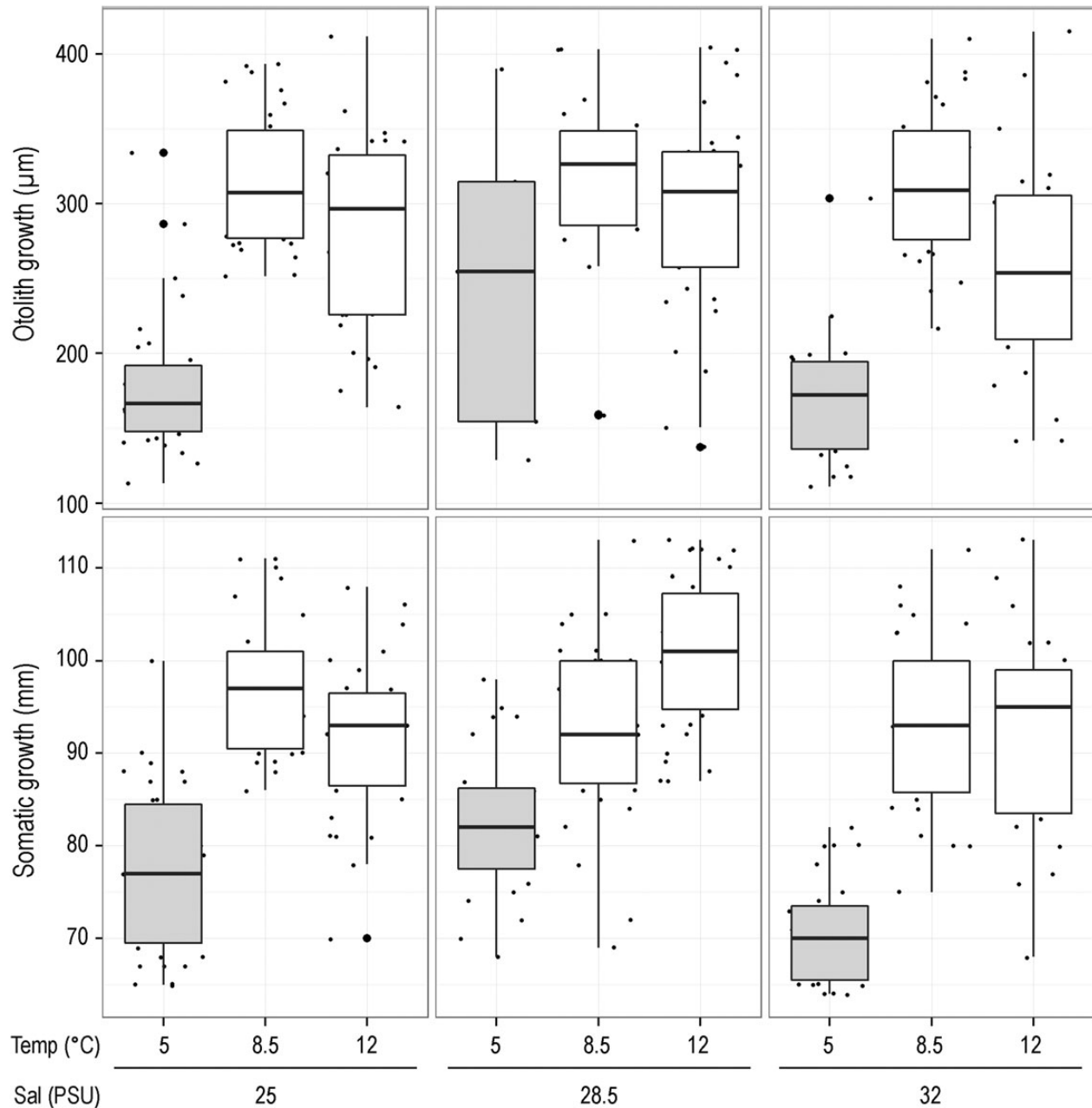


Figure 6. Summary of somatic and otolith growth throughout the experiment. Boxplot fill colours denote results of within treatment Tukey's *post hoc* tests ($\alpha < 0.05$).

Table 4. Results of linear mixed-effects models comparing somatic and otolith growth as a function of temperature (T) and salinity (S).

Source of variation	d.f.	Somatic		Otolith	
		F	p -value	F	p -value
T	2	102.4	<0.0001	34.0	<0.0001
S	2	1.7	0.23	2.9	0.06
$S \times T$	4	5.3	0.001	1.7	0.16

Tank replicate treated as a random factor.

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

Magnesium may offer promise as temperature proxy for juvenile Atlantic cod because only temperature significantly affected incorporation, measured by both Mg:Ca and D_{Mg} . Increasing temperature led to increasing Mg:Ca ratios in otoliths, both pooled across and within salinity treatments. This result confirms trends reported by Barnes and Gillanders (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°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 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 ($\delta^{13}C$) 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 ~20% (Weidman and Millner, 2000) to 30% (Hoie et al., 2004) for Atlantic cod. We found a non-linear effect of temperature on $\delta^{13}C$ values, with decreases between intermediate and high temperature treatments as expected (Weidman and Millner, 2000) but with the lowest $\delta^{13}C$ 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 $\delta^{13}C$ 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 $\delta^{13}C$. 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, $\delta^{13}C$ declined on average $-0.14 \text{ ‰ } ^\circ\text{C}^{-1}$ ($p = 0.001$, $r^2 = 0.37$), similar to rates derived from seasonal otolith records of wild-caught Atlantic cod approximately $-0.16 \text{ ‰ } ^\circ\text{C}^{-1}$ (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 molluscs (Grossman and Ku, 1986; -0.18 , -0.22 , -0.11 , and -0.13 ‰ °C⁻¹, respectively).

Otolith $\delta^{18}\text{O}$ 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 $\delta^{18}\text{O}$ 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 $\delta^{18}\text{O}$ 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 $\delta^{18}\text{O}$ confirms previous studies documenting proportional otolith incorporation with ambient conditions (Thorrold *et al.*, 1997a) and a positive relationship between water $\delta^{18}\text{O}$ 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 fish 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 salinity growth model offer a tool to evaluate the effect of growth while accounting for environmental correlates. Our study found significant positive relationships between growth and otolith magnesium and manganese (Me:Ca and D_{Me}), similar to observations of D_{Mn} in Atlantic cod (Limburg *et al.*, 2011) and other groundfish species (Limburg *et al.*, 2014). Strontium (Sr:Ca and D_{Sr}) and growth were significantly and negatively related, as reported in juvenile striped bass (*Morone saxatilis*; Secor *et al.*, 1995). Incorporation of barium (Ba:Ca and D_{Ba}) was growth independent, as reported in adult Atlantic cod (Thorisson *et al.*, 2011) and juvenile spot croaker (*L. xanthurus*; Bath *et al.*, 2000). Similarly, we observed an overall negative but non-significant relationship between oxygen ($\delta^{18}\text{O}$) incorporation and growth, suggesting growth independent incorporation, mirroring patterns previously reported for juvenile Atlantic cod (Hoie *et al.*, 2003). Carbon ($\delta^{13}\text{C}$) ratios and growth were significantly and positively related, echoing similar trends in previous studies (Thorrold *et al.*, 1997a). At low temperatures,

growth was highest at intermediate salinities, where we also observed the highest $\delta^{13}\text{C}$ values. This relationship with growth likely drove the significant interaction between temperature and salinity on $\delta^{13}\text{C}$ incorporation. As with $\delta^{18}\text{O}$, the positive association between $\delta^{13}\text{C}$ and growth mirrors patterns observed for temperature and juvenile Atlantic cod (Hoie and Folkvord, 2006). Indeed, all observed relationships between growth and otolith chemistry mirror temperature patterns. Collectively, our results support the assertion that variation in physiology and otolith deposition associated with growth influence trace element incorporation (Secor *et al.*, 1995), further highlighting the need for experimental 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 provide 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 fish, 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 exceptions (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 data

Supplementary material is available at the ICESJMS online version of this manuscript.

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