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Growth rate and age effects on *Mya arenaria* shell chemistry: Implications for biogeochemical studies

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Abstract

The chemical composition of bivalve shells can reflect that of their environment, making them useful indicators of climate, pollution, and ecosystem changes. However, biological factors can also influence chemical properties of biogenic carbonate. Understanding how these factors affect chemical incorporation is essential for studies that use elemental chemistry of carbonates as indicators of environmental parameters. This study examined the effects of bivalve shell growth rate and age on the incorporation of elements into juvenile softshell clams, *Mya arenaria*. Although previous studies have explored the effects of these two biological factors, reports have differed depending on species and environmental conditions. In addition, none of the previous studies have examined growth rate and age in the same species and within the same study. We reared clams in controlled laboratory conditions and used solution-based inductively coupled plasma mass spectrometry (ICP-MS) analysis to explore whether growth rate affects elemental incorporation into shell. Growth rate was negatively correlated with Mg, Mn, and Ba shell concentration, possibly due to increased discrimination ability with size. The relationship between growth rate and Pb and Sr was unresolved. To determine age effects on incorporation, we used laser ablation ICP-MS to measure changes in chemical composition across shells of individual clams. Age affected incorporation of Mn, Sr, and Ba within the juvenile shell, primarily due to significantly different elemental composition of early shell material compared to shell accreted later in life. Variability in shell composition increased closer to the umbo (hinge), which may be the result of methodology or may indicate an increased ability with age to discriminate against ions that are not calcium or carbonate. The effects of age and growth rate on elemental incorporation have the potential to bias data interpretation and should be considered in any biogeochemical study that uses bivalves as environmental indicators.
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Keywords: Bivalve shell; Carbonate chemistry; Element incorporation; Growth rate; *Mya arenaria*

1. Introduction

Marine bivalves have been used successfully as indicators of environmental properties for several decades (e.g. Dodd, 1965; Klein et al., 1996; Boisson et al., 1998). Bivalves are ideally suited for this purpose given their sedentary nature after recruiting to the benthos, high abundances, relatively large sizes, longevity, and hardiness (Phillips, 1977). In particular, bivalve shells have proven useful for environmental reconstructions since shell and ambient water elemental concentrations exhibit a monotonically increasing relationship (Wilbur, 1972). This relationship is sometimes affected by environmental properties such as temperature and salinity; researchers exploit this characteristic to explore environmental conditions that occurred during shell development (e.g. Rucker and Valentine, 1961; Dodd, 1965; Lerman, 1965; Dodd and Crisp, 1982; Pitts and Wallace, 1994; Lazareth et al., 2003). In studies where bivalves are used as indicators of environment, whole shells or portions of individual shells are analyzed for their elemental composition, and then related to spatial or temporal variation in elemental concentrations, temperature, or salinity of the seawater of formation.

Although the relationship between shell composition and water chemistry has been studied in the past, there are no consistent results across different species. For instance, Sr:Ca in molluscan shell has been reported as correlating both positively

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(Dodd, 1965; Smith et al., 1979; Stecher et al., 1996) and negatively (Zacherl et al., 2003) to temperature, depending on the species examined. These differences likely originate in the physiological underpinnings of shell formation and deposition, such as regulation of extrapallial fluid composition by the mantle, which differs depending on species (Wilbur, 1972). Such contradictory results highlight the need for understanding biological factors that potentially influence elemental composition of shell.

Despite some uncertainty about the physiological processes that drive the monotonic relationship between shell chemistry and water composition, many fields of study take advantage of the relationship to answer questions about the environment and the ecosystem as a whole. For instance, environmental managers use bivalves as bioindicators of metal contamination in coastal habitats by measuring the trace metal content of shells (Lindh et al., 1988; Bourgoin, 1990; Fuge et al., 1993; Pearce and Mann, 2006). Another use of bivalve shell composition is as a recorder of habitat use. For instance, population biologists are exploring the use of geographically variable chemical signatures recorded in larval shell as natural tags to track dispersal (Zacherl, 2005; Becker et al., 2007). Shell chemistry also can be used as a proxy for changes in water properties. Biological monitors of water chemistry, such as bivalves, are especially important in habitats that are difficult to extensively sample on relevant time scales, such as deep-sea hydrothermal vents (Hart and Blusztajn, 1998), or in paleoclimatic studies of salinity or temperature change on millennial time scales (e.g. Bourgoin and Risk, 1987).

In this study, we were most concerned with biological factors that might affect elemental incorporation into shell, specifically the effect of growth rate and age. Declines in elemental incorporation into the shell's crystal latice have been reported to occur with age for Pb in the abalone *Haliotis* spp. (Hirao et al., 1994; Arai et al., 2003) and for Sr and Mg in the bivalve *Mytilus* spp. (Dodd, 1965). In addition to age, growth rate may impact elemental incorporation into shell. Individuals with rapid growth rates have been shown to incorporate higher amounts of elements into carbonate compared to slower-growing individuals of the same species (Wilbur, 1972). This effect has been observed in coral skeletons (Marshall and McCulloch, 2002; Mitsuguchi et al., 2003), fish otoliths (Hamer and Jenkins, 2007), and adult bivalve shells (Stecher et al., 1996; Gillikin et al., 2005; Carre et al., 2006). These two biological factors have the potential to decouple relationships between ambient water properties and biogenic carbonate composition. As such, they must be understood and taken into account during any attempts to reconstruct environmental conditions or habitat use based on shell chemistry.

Although the studies above have explored the effects of growth rate and age on elemental incorporation into carbonate, none have explored the effects of these two biological factors in the same species and within the same study. The goal of this study was to understand the relationships between growth rate and age on shell chemistry in the commercially important softshell clam, *Mya arenaria*. First, we examined the effects of growth rate on shell elemental composition by comparing shells of clams from the same cohort, reared in the same conditions, but with different final sizes and therefore different growth rates. Second, we compared different regions of shell within the juvenile stage to explore the effects of age on incorporation. Previous studies have focused on one or two elements for exploring the relationship between biological factors and elemental incorporation into shell (e.g. Hirao et al., 1994; Gillikin et al., 2005; Carre et al., 2006). Here we look at five elements found to be useful indicators of environmental properties in previous studies. In addition, we used controlled laboratory conditions to distinguish between biological factors and environmental factors that vary in natural settings, such as temperature and salinity. Our results have implications for biogeochemical studies that use bivalve shell elemental composition as an indicator of environmental parameters. If age or growth rate affects incorporation of elements into carbonate, investigators are obliged to take these factors into consideration when interpreting environmental variables based on shell chemistry.

2. Methods

2.1. Clam rearing

Adult *M. arenaria* with ripe gonads were obtained from Cotuit, Massachusetts in April 2006 and transported to the Environmental Systems Laboratory (ESL) at Woods Hole Oceanographic Institution, where they were placed in mesh bags and suspended in a 750 L tank with filtered seawater. Spawning activity commenced approximately 1 h later, and the tank was left undisturbed to allow spawning to complete and for fertilization to take place. After 5 h the adult clams were removed, and the tank contents were filtered through a 35 μm synthetic nylon mesh sieve to concentrate the larvae into a small volume (~20 L). We counted a subsample of larvae and obtained a total estimate of 16 million trochophore larvae.

Trochophore larvae were placed into 12 L high-density polyethylene tanks (three tanks per experiment) at a density of approximately 45 larvae mL^{-1}. Clams whose shells were intended for the growth rate experiment (see explanation of experiments below) were reared in water with salinity ~22.5‰ at 20 °C. Clams whose shells were intended for the age experiment were reared in undiluted seawater (~30‰) at 24 °C. Temperatures and salinities were within the range experienced by this species in temperate tidal estuaries and were chosen because the tanks were in use as part of a larger set of experiments; no comparisons were made between shells of clams reared in different salinities. Tanks were placed into large water baths to maintain experimental temperatures. The 20 °C water bath received a continuous supply of 20 °C water, which was regulated for the facility's seawater supply line by large-volume chillers and heaters. The water bath for 24 °C tanks was maintained using a 120-volt tank heater regulated by a thermostat (Process Technology EasyPlug™ Heater with Digital Controller, 1800 W). Seawater for tanks was obtained from the in-house supply line, which pumps water from Vineyard Sound 100 m offshore at a water depth of 4 m. All

seawater was filtered to remove particles >1 μm before use. Salinity was reduced to 22.5‰ by adding ultrapure H_2O to Vineyard Sound seawater. Tank temperatures and salinities were measured every other day, and water samples were taken weekly to determine ambient elemental ratios over the course of the experiment (see Section 2.2).

Clams were raised for 60 days under treatment conditions, with complete water changes every 2 days. New tank water was adjusted to the appropriate temperature and salinity before clams were added. Larvae metamorphosed into juveniles within 2 weeks of spawning. Larvae and juveniles were fed a mix of live *Isochrysis* sp. and concentrated algae (Instant Algae© Shellfish Diet 1800) one to two times daily based on clearance rates. All tanks received the same food mixture over the course of the experiment. After 60 days, clams ranged in size from 0.8 mm to 7.1 mm; they were removed from their tanks and frozen until cleaned and prepared for analysis. There were >200 surviving clams in each of the six tanks, and we analyzed 25, 27, and 25 individuals from the three growth rate experiment tanks and 7, 5, and 5 individuals from the three age experiment tanks. These sample sizes were chosen based on statistical tests that determined ideal sizes of 25 and 5 for solution and laser ablation ICP-MS, respectively. We prepared two additional samples from each tank when possible to account for losses during the shell cleaning process. If samples remained intact after cleaning, we kept the resulting larger sample size to improve statistical power.

2.2. Seawater analysis

We quantified variability in seawater composition by measuring elemental ratios in each tank for each week of the experiment ($n=9$ per tank). Thus it was possible to distinguish differences in elemental incorporation due to variable seawater chemistry versus ontogeny or growth rate. Samples were vacuum filtered using acid-washed plastic funnels with 0.2 μm cellulose nitrate membrane filters. Samples were then transferred to acid-washed HDPE bottles, acidified to pH ~2 using ultrapure HNO_3, and refrigerated for up to two months before analysis. Seawater samples were prepared for analysis by diluting 50-fold with ultrapure 2% HNO_3.

Water samples were analyzed using a Thermo-Finnigan MAT Element2 magnetic sector field inductively coupled plasma mass spectrometer (ICP-MS). To correct for mass bias and instrument drift, a 2% HNO_3 blank solution and CASS-4 Nearshore Seawater Reference Material (National Research Council Canada Certified Reference Material) were run periodically. During analyses we monitored ^{25}Mg, ^{48}Ca, ^{55}Mn, ^{88}Sr, ^{138}Ba, and ^{208}Pb in low resolution mode. These elements were chosen because of their previous use as environmental tags (Kalish, 1989; Campana, 1999; Elsdon and Gillanders, 2003; Zacherl et al., 2003). Molar ratios of each element to Ca were calculated using mass bias corrections calculated from calibration standards. Limits of detection (LOD, 3σ of blank) were as follows: 27 μg g^{-1} for Mg; 7.7 μg g^{-1} for Ca; 5.6 μg g^{-1} for Mn; 5.9 μg g^{-1} for Sr; 0.42 μg g^{-1} for Ba; and 0.50 μg g^{-1} for Pb. We tested for differences in seawater chemistry among tanks and over time within a tank using a two-way analysis of variance (ANOVA). Significant differences in seawater composition were accounted for by using partition coefficients to report shell elemental incorporation. Partition coefficients, or the ratio between Element:Ca values in carbonate and ambient water, are used in biogeochemistry literature to relate seawater elemental ratios to those of carbonate (Lea and Spero, 1992). We conducted all analyses for growth rate and age effects using both elemental ratios and partition coefficients. Both methods yielded the same results, and we report only elemental ratios hereafter.

2.3. Growth rate experiment

Since all clams were from the same spawning event and were allowed to grow for 60 days after fertilization, differences in final shell size were attributed to different growth rates. We recorded the final length along the longest axis of the shell to the nearest 0.01 mm of each individual. This length was converted to growth rate (μm day^{-1}) by assuming a linear relationship between size and age. Typically, the nonlinear Von Bertalanffy equation is used to describe bivalve growth rate (Brousseau, 1979; Appeldoorn, 1983). This equation, however, is most applicable to clams much older than 60 days. The portion of the Von Bertalanffy curve which accounts for clam growth during the first 60 days is approximately linear (Von Bertalanffy, 1938), therefore the assumption of linear growth was appropriate for our purposes.

Shells were cleaned thoroughly using techniques developed for foraminiferan tests (Boyle, 1981) with modifications specifically for *M. arenaria*. The most notable modification was removal of the reductive cleaning step because it dissolved the proteinaceous structure of the shell so that it could not be prepared for laser ablation. Clam shells were placed in individual acid-washed vials using acid-washed plastic forceps, and sonicated briefly to remove organic matter. Individuals were rinsed three times with ultrapure water, and then soaked for 10 min at 80 °C in 1% H_2O_2 solution buffered in 1 N ultrapure NaOH to remove organic material. Afterward, shells were rinsed three times with ultrapure water, transferred to clean, acid-washed vials, rinsed four times with ultrapure water, then left to dry overnight in a laminar flow hood. After shells were dry, they were weighed to the nearest 0.1 mg and dissolved in 2% ultrapure HNO_3 to achieve a 20,000-fold dilution of calcium carbonate for each clam based on weight. All shell cleaning and preparation was done in a Class 100 clean room.

Shell material was analyzed using ICP-MS with corrections for mass bias and instrument drift as for seawater analyses using two solution-based standards, an aragonitic otolith reference material (Yoshinaga et al., 2000) and the certified reference material FEBS-1 (Sturgeon et al., 2005). The same elements noted above were analyzed in low resolution mode and converted into molar ratios relative to Ca using mass bias corrections calculated from calibration standards. Limits of detection were as follows: 0.218 μg g^{-1} for Mg; 0.819 μg g^{-1} for Ca; 1.36 μg g^{-1} for Mn; 0.632 μg g^{-1} for Sr; 0.150 μg g^{-1} for Ba; and 0.122 μg g^{-1} for Pb. Elemental ratios that were more than two standard deviations from the mean of all sample

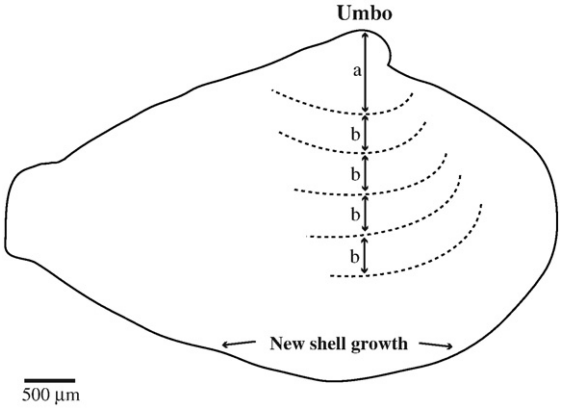

Fig. 1. Diagram of juvenile shell showing positions of the laser tracks for the age experiment. The distances a = 700 μm and b = 360 μm. Lines ablated along growth rings (dashed) are 1400 μm each.

intensities for a particular element (i.e. the global mean) were eliminated from further analysis, and the entire shell was eliminated if three or more elemental ratios met the exclusion criteria. We removed 17/380 (4.5%) of the elemental ratios for the age experiment, plus all data from one entire clam shell, before further analyses. There was no evidence of matrix effects for any element based on plots of E:Ca versus ^{48}Ca intensity.

To determine whether growth rate affected elemental incorporation into shell, ideally we would pool data from each replicate tank of the growth rate experiment to increase statistical power. However because clam sizes differed among tanks, we first performed regression analysis on growth rate versus elemental ratios for each tank separately (analysis of covariance). We then compared slopes among tanks to determine the validity of pooling using equations from Zar (1999). In the case where slopes were not significantly different (i.e. $F<F_{crit}$), we calculated the regression slope for all three tanks combined.

Our method assumes a linear relationship between growth rate and elemental incorporation into shell. If the relationship is instead nonlinear, then slope differences among tanks may result from the different ranges in growth rates represented in each tank. To test for this possibility, we repeated the regression analysis described above with the data selected to include only the range in growth rates observed in all three tanks (0.040–0.064 mm day^{-1}). If a nonlinear relationship was responsible for slope differences among tanks, we would expect those differences to disappear when only selected data are used.

2.4. Age experiment

All clams were of the same age (60 days) when the experiment was terminated. To determine variability in elemental incorporation with age, we measured and compared elemental composition at different growth rings along the shell of an individual clam, which correspond to different clam ages. Growth rings located closer to the umbo were laid earlier in the clam's life, and rings further from the umbo were laid more recently. We chose individuals that were of similar size to minimize the effects of differences due to growth rate. Exploratory statistics showed that there were no significant correlations between elemental composition and growth rate for any of the individuals analyzed for the age experiment, allowing us to interpret the effects of age without the confounding effects of growth rate.

Shells were cleaned as in the growth rate experiment, and their lengths were recorded to the nearest 0.01 mm. Individual shell valves were then mounted on glass slides using Devcon© Super Glue. We were not able to polish shells to an even plane for analysis as this would have resulted in excessive sample loss due to the combined effects of the shells' curvature and thinness (<500 μm). Shell material was analyzed using ICP-MS coupled to a New Wave Research UP213 laser. We ablated material by tracking the laser along five concentric growth increments of juvenile clam shells radiating out from the umbo. Each ring ablated was 1400 μm in length and traced one of the growth increments of the shell. Measurements were taken at the same distances from the umbo for all shells, with the assumption that the resulting measurements represented similar ages among individuals. This assumption was valid since growth rates did not differ significantly. The first ring measured was 700 μm away from the umbo as measured along the axis of growth, and the remaining four rings measured were spaced 360 μm from one another (Fig. 1). The laser was set to 80% output (0.12 mJ per pulse), with a 10 Hz repetition rate, 30 μm spot size, and a scan speed of 10 μm s^{-1}.

Vaporized material from the ablation was transported via a helium gas stream to the dual-inlet quartz spray chamber where it was mixed with 1% HNO_3 aerosol from a self-aspirating PFA 20 μL min^{-1} nebulizer. The analyte was then transported to the

Table 1
Mean temperature, salinity, and dissolved ambient seawater elemental ratios to calcium (±SE) for tanks from both experiments

	Temp	Salinity	Mg:Ca	Mn:Ca	Sr:Ca	Ba:Ca	Pb:Ca
	°C	‰	(mol mol^{-1})	(μmol mol^{-1})	(mmol mol^{-1})	(μmol mol^{-1})	(nmol mol^{-1})
Growth rate experiment							
Tank 1	19.4±0.04	22.4±0.07	5.09±0.04	18.6±4.94	8.56±0.03	14.9±1.38	15.3±4.12
Tank 2	19.4±0.04	22.4±0.05	5.09±0.03	13.6±3.55	8.64±0.03	15.1±1.41	9.37±2.27
Tank 3	19.5±0.04	22.3±0.05	5.05±0.02	14.7±3.98	8.57±0.03	15.1±1.19	9.32±1.53
Age experiment							
Tank 1	23.9±0.05	29.5±0.02	5.03±0.01	12.8±3.26	8.58±0.03	14.9±0.68	11.0±2.04
Tank 2	23.8±0.05	29.7±0.06	5.03±0.02	11.6±3.66	8.61±0.03	14.5±0.17	8.31±1.91
Tank 3	23.9±0.05	29.5±0.25	5.01±0.03	12.6±2.65	8.61±0.04	15.6±1.03	9.45±2.26

Table 2
Results of ANOVA testing for differences of seawater elemental ratios due to week of experiment or tank

Source	DF	Mg:Ca			Mn:Ca			Sr:Ca			Ba:Ca			Pb:Ca		
		MS	*F*	*p*	MS	*F*	*p*	MS	*F*	*p*	MS	*F*	*p*	MS	*F*	*p*
Growth rate experiment																
Week	8	0.017	**9.441**	**0.000**	479.173	**18.409**	**0.000**	0.012	**3.303**	**0.020**	17.085	1.405	0.267	186.687	**6.899**	**0.001**
Tank	2	0.003	1.769	0.202	2.699	0.104	0.902	0.011	3.101	0.073	0.211	0.017	0.983	61.525	2.286	0.134
Error	16	0.002			26.029			0.004			12.164			26.917		
Age experiment																
Week	8	0.006	1.884	0.134	183.184	**5.282**	**0.002**	0.011	1.625	0.194	4.193	0.878	0.555	82.594	**4.936**	**0.003**
Tank	2	0.001	0.485	0.624	3.680	0.106	0.900	0.001	0.174	0.842	2.713	0.568	0.578	16.065	0.960	0.404
Error	16	0.003			34.681			0.007			4.778			16.732		

Bold *F* statistics and *p* values are significant.

ICP-MS via an argon carrier gas. We corrected for mass bias and instrument drift using standards as in the growth rate experiment. Elements were measured in medium resolution mode, and molar ratios of each element to ^{48}Ca were calculated using mass bias corrections as above. LOD were as follows: 0.153 μg g^{-1} for Mg; 0.581 μg g^{-1} for Ca; 0.836 μg g^{-1} for Mn; 1.04 μg g^{-1} for Sr; 0.095 μg g^{-1} for Ba; and 0.169 μg g^{-1} for Pb. Elemental ratios that were more than two standard deviations from the global mean were eliminated from further analysis, and results for an entire growth ring were removed if three or more of the elemental ratios met the exclusion criteria. We removed 14/450 (3.1%) of the elemental ratios for the age experiment, plus all data from two entire clam shells, before analyses. There was no evidence of matrix effects for any element based on plots of E/Ca versus ^{48}Ca intensity. To test for differences in elemental incorporation due to age, we compared shell composition between growth rings within a clam using multivariate repeated measures ANOVA (Winer, 1971; Barcikowski and Robey, 1984). Data were log-transformed to achieve a normal distribution; log-transformed data were used for the plots and for all regression calculations.

3. Results

3.1. Seawater analyses

Although seawater for all tanks originated from the same source, ambient elemental composition varied over time for tanks in both experiments (Tables 1 and 2). Temporal variability in seawater chemistry for the growth rate experiment was not expected to affect our analyses since we compared only the bulk shell composition with the time-averaged water composition. For the age experiment, however, temporal variability might affect our analyses since we measured areas of shell that correspond to specific time periods. Results from ANOVA and plots of tank elemental ratios over the course of the age experiment showed that tanks differed over time for Mn:Ca and Pb:Ca (Table 2, Fig. 2). Elemental ratios for weeks 1–3 were

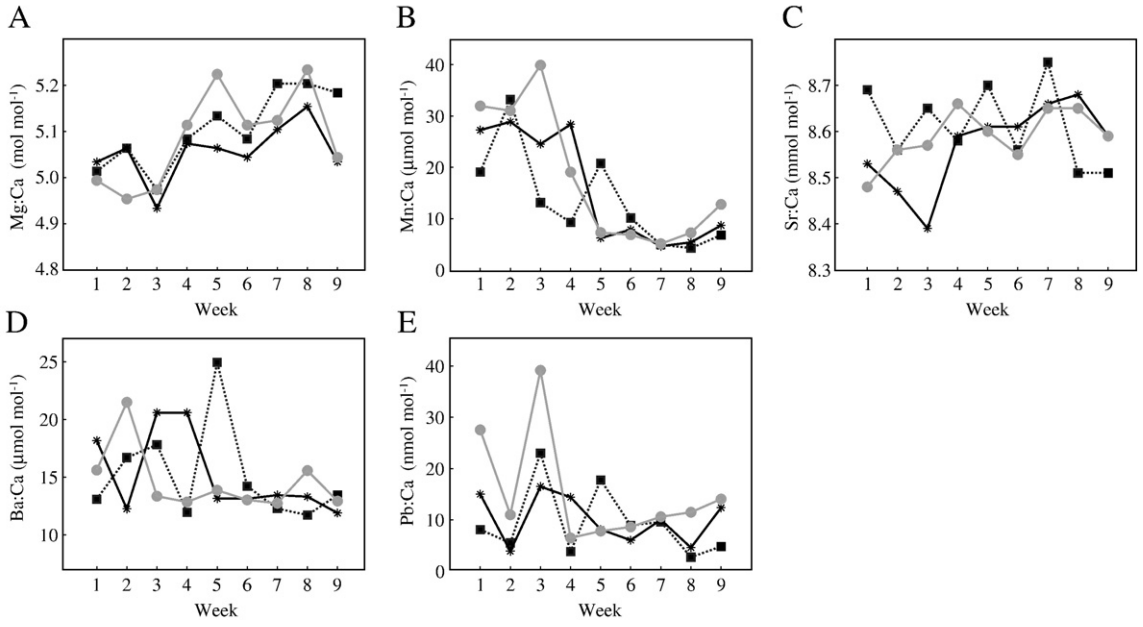

Fig. 2. Seawater elemental ratios over 9 weeks for each tank in the age experiment for (A) Mg:Ca, (B) Mn:Ca, (C) Sr:Ca, (D) Ba:Ca, and (E) Pb:Ca (tank 1 = circles; tank 2 = squares; tank 3 = crosses).

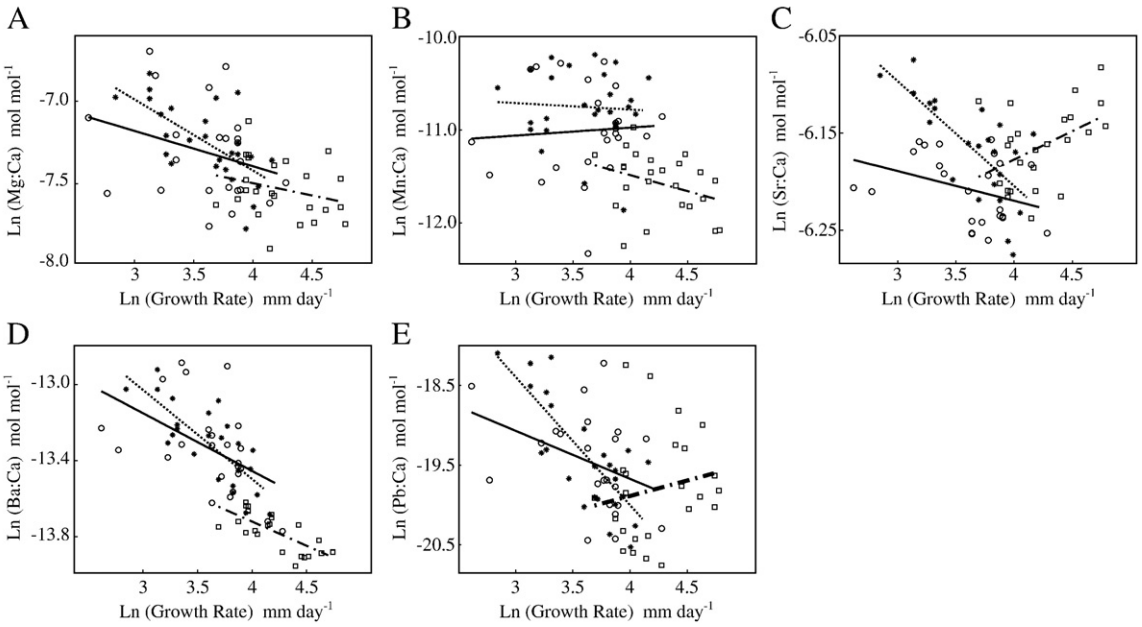

Fig. 3. Elemental ratios of individual clam shells for *Mya arenaria* growing at different rates for (A) Mg:Ca, (B) Mn:Ca, (C) Sr:Ca, (D) Ba:Ca, and (E) Pb:Ca. Data are from three replicate tanks (tank 1 = circles; tank 2 = squares; tank 3 = stars). Regression lines are for each tank separately: tank 1 = solid; tank 2 = dotted; tank 3 = dashed and dotted.

higher than for weeks 6–9 for Mn, and week 3 had higher Pb:Ca than other weeks ($p<0.01$, Tukey–Kramer pairwise tests).

3.2. Growth rate effects

We used solution-based ICP-MS analysis of juvenile clam shells from three replicate tanks ($n=25$, 27, 25) to test the effects of growth rate on elemental incorporation into shell (Fig. 3). Growth rates ranged from 0.014 to 0.119 mm day^{-1} (0.050±0.023 mm day^{-1}, mean±SD). To determine the relationship between shell elemental incorporation and growth rate, we plotted elemental ratios versus growth rates for each tank separately (Fig. 3). Values of R^2 ranged from 0.005 to 0.634, with 7 of the 15 regression slopes significantly differing from zero ($p<0.05$, Table 3). There was a general trend of decreasing elemental ratios with increasing growth rate, with the exception of Sr:Ca and Pb:Ca from tank 3 (Fig. 3).

Slopes significantly differed among replicate tanks for two of the five elemental ratios ($p<0.001$, Table 4). For Sr:Ca, the slope of tank 3 differed from the other two, and for Pb:Ca the slopes of tanks 2 and 3 differed. As a result of the differences among tanks for these two elements, we were not able to draw conclusions from the regression statistics for combined tanks. For the remaining three elemental ratios (Mg:Ca, Mn:Ca, Ba:Ca), slopes did not differ significantly among tanks, so we calculated the common slope for each element with equation 18.30 in Zar (1999) using data from all three tanks (Table 4).

We performed the same regression analyses on selected data to test whether significant differences in slopes were due to a nonlinear relationship between growth rate and elemental incorporation into shell. However data truncation resulted in the elimination of ~50% of data points ($n=10$, 9, 9), and as a consequence we lost statistical power and were not able to detect significant relationships for any tanks or elements. We could not, therefore, use the selected data to pool values from replicate tanks, and the issue of nonlinearity remains unresolved.

3.3. Age effects

We analyzed shell along five growth rings of juvenile clams from three replicate tanks ($n=7$, 5, 5) to determine whether elemental incorporation varied as the individual aged (Fig. 4).

Table 3
Results of regression analysis for growth rate experiment for individual tanks

Source	β_0	β_1	R^2	F	p
Mg:Ca					
Tank 1	−6.537	−0.214	0.089	2.057	0.166
Tank 2	−5.696	−0.431	0.434	**18.374**	**0.0003**
Tank 3	−6.896	−0.150	0.070	1.662	0.211
Mn:Ca					
Tank 1	−11.311	0.084	0.005	0.103	0.751
Tank 2	−10.510	−0.079	0.004	0.106	0.766
Tank 3	−10.124	−0.338	0.109	2.682	0.116
Sr:Ca					
Tank 1	−6.098	−0.031	0.120	3.010	0.097
Tank 2	−5.775	−0.108	0.518	**22.558**	**0.0001**
Tank 3	−6.410	0.0583	0.199	**5.483**	**0.029**
Ba:Ca					
Tank 1	−12.245	−0.303	0.243	**7.051**	**0.014**
Tank 2	−11.629	−0.469	0.606	**33.861**	**0.000**
Tank 3	−12.710	−0.255	0.566	**27.619**	**0.000**
Pb:Ca					
Tank 1	−17.262	−0.602	0.144	3.363	0.082
Tank 2	−13.570	−1.608	0.634	**41.490**	**0.000**
Tank 3	−21.435	0.387	0.032	0.737	0.400

The coefficients β_0 and β_1 correspond to the regression equation, where $y=\beta_0+\beta_1 x$. Bold F statistics and p values are significant.

Table 4
Results of statistic testing for differences among regression functions of replicate tanks

Elemental ratio	F	p	β_1
Mg:Ca	1.320	0.273	−0.271
Mn:Ca	0.745	0.465	0.078
Sr:Ca	**12.936**	<0.0005	−0.032
Ba:Ca	1.439	0.244	−0.347
Pb:Ca	**8.010**	<0.001	−0.692

Bold F statistics are significant and indicate that slopes are different among tanks, making it invalid to pool data.

Repeated measures multivariate ANOVA indicated that there were significant differences in elemental ratios among growth rings for Mn:Ca, Sr:Ca and Ba:Ca (Table 5). In all three cases, the first growth ring (0.70 mm away from the umbo) differed from the remaining four growth rings for individuals in one or more replicate tanks (Tukey–Kramer pairwise comparisons). There were also significant differences among measurements of clam shells from the same tank for Mn:Ca, Ba:Ca, and Pb:Ca. Finally, we detected a tank effect for one elemental ratio; mean Ba:Ca of shells in tank 1 was higher than in tanks 2 or 3.

Although clam shells of similar size were chosen for the age experiment, total lengths ranged from 3.36 to 6.45 mm (5.32±0.77 mm, mean±SD), as measured along the shell approximately perpendicular to the axis of growth. The difference in size after 60 days resulted in a range of growth rates from 0.056 to 0.107 mm day^{-1} (0.089±0.013 mm day^{-1}, mean±SD). We tested for a correlation between growth rate and elemental incorporation for each clam (averaged over growth rings) analyzed in the age experiment and found none (R^2 ranged from 0.0007 to 0.124; p values ranged from 0.15 to 0.91). This lack of correlation suggests that the range of growth rates for clam shells was sufficiently small so as not to impact elemental incorporation.

4. Discussion

4.1. Growth rate effects

Previous studies have suggested that the rate of calcium carbonate crystal formation, which is closely tied to growth rate, influences elemental incorporation in bivalves (Stecher et al., 1996; Gillikin et al., 2005; Carre et al., 2006). Intuitively, a higher growth rate might be expected to result in more crystal defects during shell formation: increased active transport of Ca^{2+} molecules into the extrapallial fluid would lead to higher rates of inclusion of non-Ca^{2+} ions that are of similar size and charge (Wilbur and Saleuddin, 1983). Indeed, higher growth rates have been reported as corresponding to increased inclusion of Mg, Mn, and Ba in fish otoliths (Bath Martin and Thorrold, 2005; Hamer and Jenkins, 2007), and bivalve shells (Stecher et al., 1996; Carre et al., 2006). However in our study, we found that elemental ratios of Mg:Ca, Mn:Ca, and Ba:Ca were all negatively correlated to growth rate in *M. arenaria*. One explanation for our disparate results may be that the organism's physiological ability to discriminate between Ca^{2+} and other ions improves with size. Clams with higher growth rates would have reached the threshold size for improved discrimination for Ca^{2+} sooner than those with slower growth rates. As a consequence, proportionally more shell would have been laid with lower elemental ratios to calcium over our two-month study, resulting in the negative correlation that we observed. Although such an age effect has not been observed in *M. arenaria*, previous studies of molluscs have reported decreased elemental incorporation with size (Dodd, 1965; Hirao et al., 1994; Arai et al., 2003).

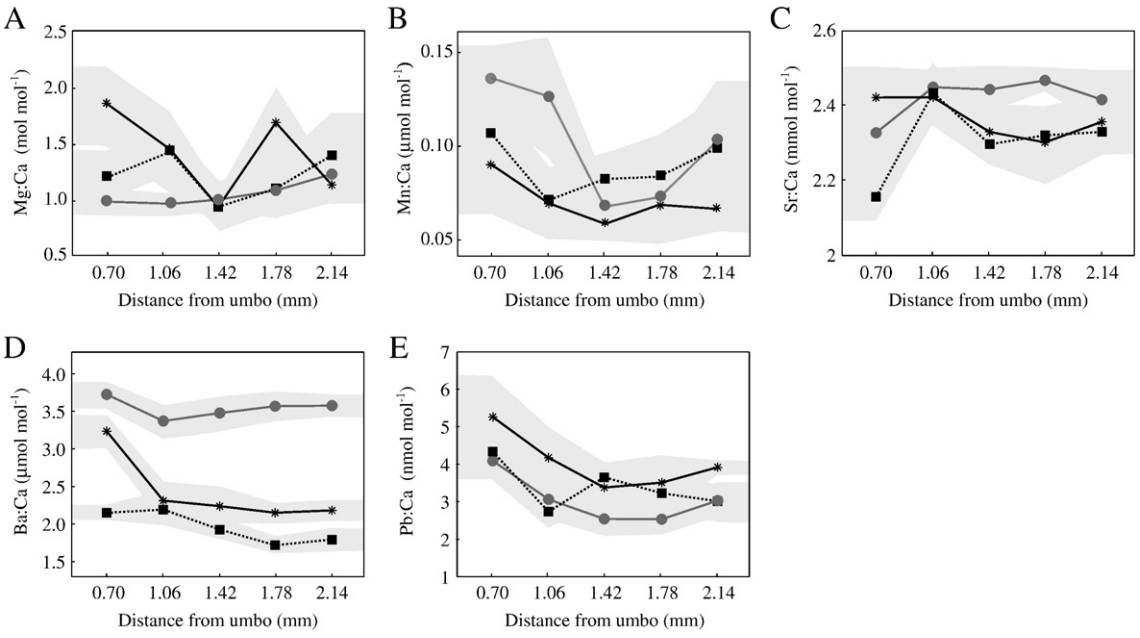

Fig. 4. Mean elemental ratios for *Mya arenaria* from three replicate tanks for shell measurements taken at different distances from the umbo for (A) Mg:Ca, (B) Mn:Ca, (C) Sr:Ca, (D) Ba:Ca, and (E) Pb:Ca (tank 1 = circles; tank 2 = squares; tank 3 = stars). Shaded regions are ±1 SD of mean.

Table 5
Results of multivariate repeated measures ANOVA testing for differences between growth rings in three replicate tanks

		Mg:Ca			Mn:Ca			Sr:Ca			Ba:Ca			Pb:Ca		
Univariate	DF	MS	*F*	*p*	MS	*F*	*p*	MS	*F*	*p*	MS	*F*	*p*	MS	*F*	*p*
Among tanks	2	0.33	0.93	0.42	0.01	0.68	0.53	0.07	1.92	0.19	9.11	**25.29**	**0.0001**	10.1	2.36	0.13
Within tanks																
Among rings	2	0.22	0.86	0.49	0.01	**3.25**	**0.02**	0.05	2.65	0.09	0.72	**4.44**	**0.01**	5.02	**3.48**	**0.03**
Ring × Tank	8	0.14	0.53	0.79	0.01	1.33	0.25	0.03	1.80	0.16	0.25	0.53	0.21	0.83	0.80	0.74
Multivariate	DF	λ	*F*	*p*	λ	*F*	*p*	λ	*F*	*p*	λ	*F*	*p*	λ	*F*	*p*
Among rings	4	0.70	1.06	0.42	0.38	**4.05**	**0.03**	0.27	**5.41**	**0.02**	0.31	**4.44**	**0.04**	0.52	2.56	0.10
Ring × Tank	8	0.74	0.40	0.91	0.49	1.07	0.42	0.24	2.06	0.10	0.47	0.92	0.52	0.49	1.18	0.35

Bold *F* statistics and *p* values are significant.

Although every attempt was made to assure environmental conditions were consistent across tanks, we found a wide range of growth rates for clams among the three replicate tanks. Our analyses indicated no significant differences in water chemistry, temperature, or salinity (Tables 1 and 2), however there may be additional factors we did not account for that are the source of the variability in growth rates. For instance, different biological conditions were present in each of the tanks owing to a variety of processes potentially occurring, such as algal growth and microbial activity. Although we sampled the seawater weekly to quantify the changes in its chemistry over time, we did not attempt to identify or quantify biological activity. This biological activity might in turn affect clam growth and elemental incorporation into shell. For example, increases in food supply due to algal growth would result in faster growth rates, or the presence of additional oxygen-consuming organisms might result in decreased oxygen supply to the clams and therefore reduced metabolic and growth rates.

The most notable tank effect was seen in the relationships between growth rate and Sr:Ca and Pb:Ca; we found positive or negative correlations depending on tank. Our mixed results are particularly interesting for Sr:Ca since previously reported relationships indicated positive correlations in bivalves (Stecher et al., 1996; Gillikin et al., 2005) and corals (Weber, 1973). Sr:Ca correlations to growth rate from otoliths are mixed (Kalish, 1989; Arai et al., 1996; Bath et al., 2000; Martin et al., 2004) but studies generally report negative correlations between Sr:Ca and growth rate (Sadovy and Severin, 1992, 1994; Hamer and Jenkins, 2007; Lin et al., 2007). Since Sr ions are of the same charge as Ca ions and only slightly larger, they are substituted directly into the aragonite crystal lattice (Speer, 1983). As a result, Sr:Ca ratios should decrease with calcification rate (i.e. growth rate) since higher Ca concentrations in the extrapallial fluid would dilute Sr ions (Sinclair, 2005). Consequently we expected to find negative correlations for Sr:Ca and growth rate in all of the replicate tanks, and the mixed results suggest that other factors are influencing Sr incorporation into bivalve shell.

We cannot attribute our results to variable Sr:Ca available in seawater since we found no significant differences in Sr:Ca among tanks (Table 2). The more likely cause is the different ranges in growth rate depending on tank. Tank 3 growth rates were higher on average than those of tanks 1 and 2, and tank 3 is the only replicate tank with clams having growth rates higher than 75 μm day^{-1}. Indeed, shells from tank 3 had Sr:Ca and Pb:Ca that negatively correlated with growth rate, while shells from the other two tanks positively correlated. There may be different physiological processes operating as the clam grows larger, causing a shift in the correlation between growth rate and elemental ratios with size. For instance, Carre et al. (2006) found that curved shell sections in bivalves had higher Sr:Ca than flat sections. This occurs because there is more organic matrix in the curved sections of shell than in flat sections, and therefore also more binding sites for Ca^{2+} and its competing ions (Rosenberg and Hughes, 1991). As the clam grows, proportionally more shell is composed of flat sections, and whole-shell analysis of a larger individual would have lower Sr:Ca and Pb:Ca than a smaller individual.

In addition to a wide range of growth rates for clams among tanks, we found a wide range within tanks as well. Explanations for the observed range within tanks cannot be attributed to biological activity or chemical differences since these factors would affect all of the individuals of a particular tank. The source of variability within tanks is therefore likely to be due to physiology at the individual clam level. Metabolic rate and growth rate are closely linked, and differences in metabolic rate among clams might result in different growth rates (Bayne and Newell, 1983; Rosenberg and Hughes, 1991). Variable metabolic rates, and therefore growth rates, may originate from genetic variability. If the genetic types of larvae produced in our laboratory spawning varied widely in their metabolic rates, then we might expect to see differences among individuals within a given tank.

4.2. Age effects

Based on significant differences among growth rings within individual clams, age significantly influenced the incorporation of Mn, Sr and Ba into *M. arenaria* juvenile shell. This result is most likely attributable to increased variability in elemental ratios with decreasing distance from the umbo. Visual inspection of data plots indicates that variability in elemental ratios is highest near the umbo (Fig. 4). Indeed, statistical analyses confirm that all significant differences among growth rings disappear when the first growth ring measured is removed from analyses. This result may be an artifact of the laser ablation method used. Although laser parameters are consistent over all

measurements, the time period that ablated shell material represents is not known. For instance, if a clam produces more material per day at week 4 compared to previous weeks, then measurements taken before week 4 will represent a larger time period sampled, and therefore may result in larger variability in the elemental ratios (see Elsdon and Gillanders, 2003 for a complete discussion of problems associated with the analysis of elements in calcified structures). This hypothesis contradicts our assumption of linear growth over the course of our experiment, however the data are not sufficiently conclusive to render our assumption false. Another explanation for increased variability in the first growth ring measured might be that seawater varied more at the beginning of the experiment (Fig. 2). However this hypothesis is not testable since we were not able to match shell growth rings to particular experimental weeks, and therefore to specific water chemistry. This is because growth patterns in molluscan shell are a result of complex interactions between physiology and environment, especially during early shell formation, preventing accurate estimates of the shell formation timeline in daily or weekly increments (see Lutz and Rhodes, 1980 for a complete discussion). Furthermore, based on observations over the course of the experiment, the first growth ring measured was likely accreted around week four, at which point seawater chemistry variability was in decline.

Our results for Mn:Ca are at least in part attributable to changes in seawater Mn:Ca over time. Mn:Ca significantly differed in tank seawater over the course of the experiment (Table 2, Fig. 2). There are two possible explanations for this pattern. First, organic ligands may be in higher quantities later in the experiment due to excretion by clams or biological activity relating to food input; these ligands may bond to Mn and remove it from solution (Libes, 1992). We would detect a drop in the Mn:Ca ratios in seawater over time as a result, even though the input water Mn:Ca is unchanged. Another explanation is that some event occurred between seawater samples taken at weeks 4 and 5 that caused a decrease in Mn:Ca in the seawater. The unknown event might have been natural, affecting the source of seawater to the supply line, or it may have occurred within the supply infrastructure (e.g. the pipes were flushed to remove a blockage, causing seawater chemistry to change). Clams grew to ~1 mm in length (as measured perpendicular to the axis of growth) until around week 4, which corresponds to approximately 0.8 mm in width along the axis of growth. It is therefore likely that only the first growth ring measured, located 0.70 mm from the umbo, would have been affected by the elevated Mn:Ca ratios seen in the seawater. Indeed, the highest Mn:Ca level for seawater was during week 3 in tank 1, where clam shells also had the highest two Mn:Ca ratios measured, for growth rings located 0.70 and 1.06 mm away from the umbo.

4.3. Conclusions and future directions

In this study, we found that both growth rate and age significantly affect elemental incorporation into the shell of *M. arenaria*. Growth rate significantly correlated negatively with elemental ratios for 7 of the 15 analyses, which was surprising since previously published studies tended to report positive correlations. There were significant tank effects in Sr:Ca and Pb:Ca correlations to growth rate, potentially due to disparate biological activity occurring in the three replicate tanks. We found that growth rates varied widely within tanks, indicating that there is a physiological factor that we did not control for in our experiment, potentially originating at the genetic level. Our analysis of the effects of age on elemental incorporation into *M. arenaria* shell revealed higher variability closer to the umbo than further away, which caused significant differences with age for clams. When the first growth ring measured was removed from analyses, these significant differences disappeared. We hypothesize that the age effect is primarily a result of the shell's more pronounced curvature near the umbo, which affects physiological processes such as uptake of Ca^{2+} relative to other ions of similar size and charge.

The effects of size on elemental incorporation have the potential to bias data interpretation in any biogeochemical study that uses bivalves as indicators of environment. Juvenile bivalves are ideal candidates for environmental indicators: predictable spawning behavior allows one to be sure what season their shell is laid, and they are more susceptible to some environmental contaminants, making them an early indicator of problems that might not yet affect adults. However studies that use juveniles in this capacity must be careful to take into account the variability in shell composition with age and growth rate. Variability in shell chemistry could be interpreted as indicating shifting environmental conditions when in fact composition may reflect individual or ontogenetic variability in physiological processes. As our data show, clams reared in identical environments may incorporate different elemental signatures in their shells depending on their age or growth rate. Care must be taken to understand the potentially confounding effects of physiology for this and other bivalve species before making inferences about the environment based on shell chemistry.

This study was the first to explicitly examine the effects of variable growth rates and age in bivalves on incorporation of a suite of elements into shell, and in the same species during the same study. Although the variability we found is not cause for dismissing bivalves as useful environmental indicators, further studies should focus on the cause of variable incorporation within a cohort, especially with relation to the variables we were not able to control for in this set of experiments. The bioavailable levels of elements in the seawater should be measured to ascertain whether the source of variability in ratios is due to water chemistry or physiology. The sizes of clams should be tracked and recorded over the entire experiment to assess individual growth rates rather than averages, and to identify exactly when individual growth rings are accreted so they can be matched to the correct water sample taken. Parental analysis would be useful to establish genetic contributions of individuals and to determine if certain genetic combinations lead to higher or lower growth rates, explaining the variability that we saw within tanks.

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