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Size-dependent pH effect on calcification in post-larval hard clam *Mercenaria* spp.

George G. Waldbusser[1,*], Heather Bergschneider[1], Mark A. Green[2]

[1]College of Oceanic and Atmospheric Sciences, Oregon State University, COAS Administration Bldg. Corvallis, Oregon 97331-5503, USA

[2]Division of Natural Sciences, St. Joseph's College of Maine, 278 Whites Bridge Road, Standish, Maine 04084, USA

ABSTRACT: Increasing atmospheric carbon dioxide threatens to decrease pH in the world's oceans. Coastal and estuarine calcifying organisms of significant ecological and economical importance are at risk; however, several biogeochemical processes drive pH in these habitats. In particular, coastal and estuarine sediments are frequently undersaturated with respect to calcium carbonate due to high rates of organic matter remineralization, even when overlying waters are saturated. As a result, the post-larval stages of infaunal marine bivalves must be able to deposit new shell material in conditions that are corrosive to shell. We measured calcification rates on the hard clam, *Mercenaria* spp., in 5 post-larval size classes (0.39, 0.56, 0.78, 0.98, and 2.90 mm shell height) using the alkalinity anomaly method. Acidity of experimental water was controlled by bubbling with air–CO_2 blends to obtain pH values of 8.02, 7.64, and 7.41, corresponding to pCO_2 values of 424, 1120, and 1950 µatm. These pH values are typical of those found in many near-shore terrigenous marine sediments. Our results show that calcification rate decreased with lower pH in all 5 size classes measured. We also found a significant effect of size on calcification rate, with the smaller post-larval sizes unable to overcome dissolution pressure. Increased calcification rate with size allowed the larger sizes to overcome dissolution pressure and deposit new shell material under corrosive conditions. Size dependency of pH effects on calcification is likely due to organogenesis and developmental shifts in shell mineralogy occurring through the post-larval stage. Furthermore, we found significantly different calcification rates between the 2 sources of hard clams we used for these experiments, most likely due to genotypic differences. Our findings confirm the susceptibility of the early life stages of this important bivalve to decreasing pH and reveal mechanisms behind the increased mortality in post-larval juvenile hard clams related to dissolution pressure, that has been found in previous studies.

KEY WORDS: Calcification · Acidification · Size-dependent · Hard clam · Post-larval

INTRODUCTION

The potential impacts of ocean acidification on carbonate-depositing organisms due to increasing atmospheric carbon dioxide are receiving considerable attention. Decreased pH may affect calcification by altering carbonate mineral thermodynamics and by effects on other physiological processes of marine calcifiers (Pörtner 2008). Early research on metazoan calcification in benthic habitats focused on coral reefs (Gattuso et al. 1998, Kleypas et al. 1999, Langdon et al. 2000). More recently investigators have recognized that bivalves in temperate coastal waters may also be susceptible to acidification (Green et al. 2004a, 2009, Dove & Sammut 2007, Gazeau et al. 2007, Kurihara 2008, Miller et al. 2009, Talmage & Gobler 2009, Waldbusser et al. 2010). There are many well documented processes that induce pH variability in coastal ecosystems (Soetaert et al. 2007) including daily production–respiration and seasonal bloom cycles (Hinga 2002, Wootton et al. 2008), hydrology and coastal upwelling (Feely et al. 2008, Salisbury et al. 2008, 2009), changes in other atmospheric gases due to human activity such as NO_x and SO_x emissions (Doney et al. 2007), and oxi-

*Email: waldbuss@coas.oregonstate.edu

dation of reduced metabolites in sediments during bioirrigation and particle reworking processes (Green et al. 2004b). As a result, estuarine or coastal pH is far more variable than that of the open ocean and subject to multiple processes making it difficult to parameterize, measure, and predict (Blackford & Gilbert 2007, Soetaert et al. 2007, Borges & Gypens 2010). However, gradual shifting-baseline type decreases in carbonate saturation state of coastal sediment pore waters due to rising atmospheric CO_2 has been noted (Andersson et al. 2006). Determining the potential ecological and economic consequences of acidification on living resources in coastal areas requires additional empirical data of shell production rates, thresholds, and changes to shell growth through various life stages due to variable pH on short and long timescales.

Due to high rates of organic matter remineralization (respiration) in upper sediment layers, coastal marine sediments are often more acidic than overlying waters, and undersaturated with respect to calcite and aragonite (Aller 1982, Green & Aller 1998, 2001)—the calcium carbonate minerals precipitated by bivalves and other calcifiers. The hard clam *Mercenaria mercenaria* has a pelagic larval dispersal stage (and a mostly benthic pediveliger stage) that is utilized until suitable substrate for settlement is found. During the larval and early post-larval stages significant predation risks exist, therefore delayed metamorphosis, settlement, and development to the adult stage increase the risk of predation and represent a population bottleneck (Thorson 1966, Gosselin & Qian 1997). Conversely, burrowing into sediments that are corrosive to shell material has been found to result in significant rates of mortality in post-larval *M. mercenaria* (Green et al. 2009). Carriker (1961) noted possible adaptive strategies in early post-larval *M. mercenaria* that were byssally attached to shell material in the field and transitioned to nearby sediments later in their development. Even with adaptive life history strategies, at some point, infaunal bivalves must burrow into geochemically active sediments that are corrosive to calcium carbonate minerals. During these early life stages, before siphons are fully formed, these organisms must maintain contact between their shell edge and overlying water (Carriker 1961), forcing them to reside in the upper mm of sediment. At this depth in many coastal sediments, these early life stages would be put in direct contact with sediments having the most labile organic matter, thus resulting in exposure to significant amounts of CO_2 due to organic matter remineralization. However, significant microphytobenthic primary production could also help to alleviate some of the corrosion pressure during daylight hours. Infaunal calcifiers, at some point, must be able to precipitate shell under dissolution pressure. Recent works suggest early settlers face significant dissolution pressure and resulting mortality (Green et al. 2004a, 2009); however, the thresholds and sizes at which hard clams may overcome this dissolution pressure are unclear.

Differences exist in the mineral form of calcium carbonate precipitated by larval and post-larval bivalves of some species, such as the eastern oyster *Crassostrea virginica* with aragonite (more soluble) in the larval stage and calcite (less soluble) in the adult stage (Stenzel 1963, 1964). *Mercenaria mercenaria* is primarily aragonite throughout its entire life history (Fritz 2001, and references therein). More recently, significant amounts of amorphous calcium carbonate (ACC) were documented in larval bivalve shells (Weiss et al. 2002). Given that ACC is more soluble than aragonite, it may be surmised that the early life stages of bivalves, and the retained prodissoconch portions of their shells, are most susceptible to moderate decreases in pH. The shells of bivalves and other calcifying species are not entirely composed of mineral calcium carbonate. Shells have a protective outer cuticle type layer, the periostracum, and an organic matrix into which the mineral phase is deposited (Levi-Kalisman et al. 2001). These organic components likely provide the shell some protection from dissolution, relative to pure mineral. Ground biogenic calcium carbonate minerals have been found to dissolve faster than pure mineral phases due to differences in surface area for dissolution and the bacterial degradation of the organic matrix into which carbonate minerals are precipitated (Glover & Kidwell 1993, Cubillas et al. 2005). As a result, the saturation indices used to determine solubility of pure calcium carbonate minerals only approximate the response of shell material in live bivalves and other metazoan calcifiers.

Calcification, and by extension shell growth, is an integral component of organism growth in mollusks (Wilbur & Saleuddin 1983 and references therein); however, it should be noted that calcification (mineral deposition) and shell growth (mineral plus organic deposition) are not identical processes. The formation of the organic proteinaceous material of the shell occurs at a significant metabolic cost (Palmer 1992), and Hautmann (2006) surmised that in thick shelled species, such as hard clams, mineral deposition also required a significant metabolic expense. Considerable attention has been given to growth of adult hard clams (Arnold et al. 1991, Carmichael et al. 2004, Henry & Nixon 2008) as well as recent work on larval growth of *Mercenaria mercenaria* and other larval bivalves in relation to pH (Miller et al. 2009, Talmage & Gobler 2009). Although some classic works have documented metamorphosis and general growth patterns in the early life stages of *M. mercenaria* (Carriker 1961, 2001 and references therein), little is known about

abiotic controls on growth and development in the early post-larval stages. Furthermore, differences in shell growth follow complex responses to environmental factors when genotype and hybridization of *Mercenaria* spp. are taken into account (Dillon & Manzi 1989, Arnold et al. 1996, 1998). Understanding pH-shell growth dynamics at this early post-larval stage is vital, as the post-larval settlers will be exposed to relatively lower pH within sediments than at the pelagic larval stage, and growth through this post-larval stage is crucial for recruitment to adult populations.

We developed an experimental system to measure differences in calcification of the hard clam *Mercenaria mercenaria* in response to pH (ranging from ~7.40 to 8.00). These measurements were made on size classes from 0.39 to 2.90 mm shell height. Our objectives were to (1) determine if relatively small changes in pH affect calcification of *M. mercenaria*, (2) measure the response of calcification through the early post-larval stages, (3) identify possible mechanisms responsible for changes in calcification with size and pH, and (4) highlight the potential impacts of acidification on this infaunal dwelling bivalve.

MATERIALS AND METHODS

Experimental organisms. Juvenile hard clams of *Mercenaria* spp. were obtained from Cherrystone Aqua Farms (Cheriton, Virginia) and Southern Cross Sea Farms (Cedar Key, Florida). Although we intended on using *M. mercenaria* in our experiments, Arnold et al. (2009) documented that *M. mercenaria* hybridizes with *M. campechiensis* in the Southern Cross Sea Farms hatchery, with roughly 25% of the hard clams having the hybrid genotype and the remainder being pure *M. mercenaria*. Differences exist in growth rates among genotypes and vary with environmental factors (Dillon & Manzi 1989, Arnold et al. 1996, 1998), with hybrids typically growing faster. The comparison of hatchery source of hard clams was not originally intended to be part of the experimental design, but limitations on organism availability required that we utilize 2 sources for experimental organisms. The size classes from the 2 different hatcheries were as follows: 0.39, 0.78, and 2.90 mm clams were from the Virginia hatchery (VA), while the 0.56 and 0.98 mm clams from the Florida hatchery (FL). Juveniles were kept in separate holding tanks based on size and origin, with daily water changes. A diet of cultured *Isochrysis* spp. (Tiso) strain CCMP1324 supplemented with Shellfish Diet 1800 (Reed Mariculture) was fed in batches for up to 4–5 h, d^{-1}. At the termination of feeding, organisms were removed from the feeding media and returned to their holding tanks. Clams were rinsed daily with deionized water for roughly a minute to help control bacterial growth and disease. The seawater used in the experiments was collected from the University of Delaware, Lewes Seawater Facility. Plastic drums of water were transported back to Chesapeake Biological Laboratory in Solomons, Maryland where it was treated with 0.5 ml of bleach per l of seawater following procedures in Palin (1983) and sealed until ready for use. Approximately 1 wk prior to use, the drum was heavily aerated with a large air stone and dechlorinated with 0.167 g sodium thiosulfate per ml of bleach added. Several days after dechlorination, we tested pH and alkalinity and amended the water with sodium bicarbonate and sodium carbonate as needed to return alkalinity and pH to roughly 2.225 mmol l^{-1} and 8.00, respectively.

Experimental system. The experimental system consisted of a CO_2 tank plumbed through a flask containing deionized water to hydrate the dry gas (Fig. 1). The gas was then sent to a header where it was split into 2 lines, one each for the medium and low pH treatments. The high pH treatment was bubbled with ambient air only. All tubing used was low gas permeability tygon tubing. Each CO_2 line ran through a gas flow control valve (Gilmont Microflow Meter) and then was spliced into air lines with small-gauge hypodermic needles. The pH in the medium- and low-pH treatment groups was controlled by adjusting the CO_2 flow at the flow control valves. The tubing carrying air–CO_2 blends were then run to gang valves that split the flow into replicate experimental flasks. Each pH treatment group had one control flask with no organisms to monitor pH over the

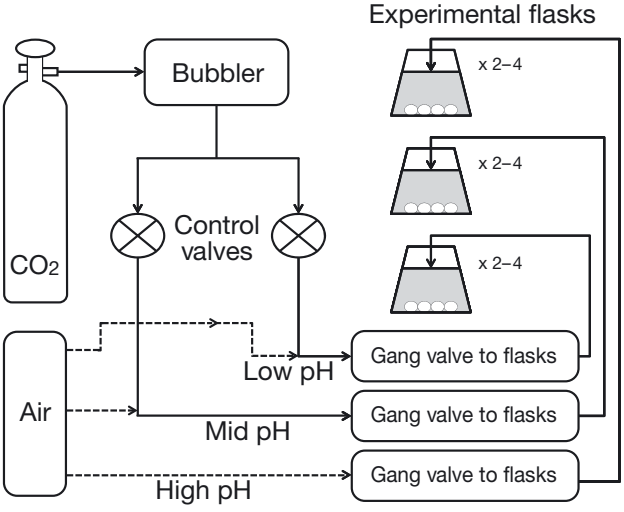

Fig. 1. Experimental system. Number of flasks varied with each run of different sized organisms, but all experiments had at least 2 flasks, 1 with clams and 1 control flask. Only 1 flask depicted here per pH treatment, with clams as circles in bottom of flasks. Flasks were kept in a constant temperature water bath

course of the experiment. A controlled temperature water bath was used to maintain a temperature of 20°C in flasks. The flasks used for experiments were 55 ml Erlenmeyer Pyrex that were acid washed, triple rinsed with deionized water, and dried prior to use. Before each experimental run, the system was set up and gas flows adjusted until stable pH readings (see 'Experimental design') on seawater within flasks were obtained for a several hour period. Once the system had stabilized, ~50 ml of new seawater was added to new pre-weighed flasks, flasks were weighed to obtain water weight, clams were added to flasks, flasks were placed in the water bath, and bubbling with gas blends was initiated. The pH in the new flasks typically stabilized within 30 min, values were verified and spot checked on the control flask for each treatment throughout the experiments.

Experimental design. Separate experimental runs were conducted for each size class of hard clams. In a series of pilot studies, we determined between 1 to 2 g live weight of clams in 50 ml of seawater typically gave a response of alkalinity change of ~200 to 400 µmol within 8 h in the high pH treatment. An 8 h timeframe was long enough to allow us to complete all alkalinity titrations between sampling periods, but short enough to minimize container effects, as longer runs or larger changes in alkalinity typically resulted in asymptotic relationship between alkalinity and time. The time for each experimental run was constrained by the total change of alkalinity within treatments. We limited total alkalinity change to 0.5 mmol l^{-1}; however, in all but one experimental run (2.90 mm clams), we terminated the experiments after a change of 0.2 to 0.3 mmol l^{-1}. The number of replicate flasks in each experimental run (or each size class) was constrained by the amount of experimental organisms available and the biomass needed to elicit a significant alkalinity change in high pH flasks over several hours. We used triplicates for the clams with a shell height of 2.90 and 0.98 mm, 4 replicates for the 0.78 mm clams, and only 1 replicate for the 0.39 and 0.56 mm sizes because roughly 50 000 to 70 000 organisms were required at this size to attain a live weight of 1 to 2 g per flask. We simply could not attain the number of organisms needed for replicates at this size from one hatchery source at one time. When replicate flasks were used, flasks within a treatment group were calibrated to have pH within 0.05 units of the target pH by adjusting gang valves, measuring pH during set up, and comparing bubble rates in flasks.

Clam sizing and live weights. We received organisms from the hatcheries based on a nominal sieve size of which clams would pass through. Clams were therefore sized using a digital camera mounted on an Olympus dissecting microscope and image analysis. Between 50 and 100 individuals were randomly measured for each size class within 1 to 5 d of each experimental run. Photographs were taken with a scale bar placed in the field of view to calibrate each image using ImageJ software v1.42q. Shell height (SH) was measured as the distance from umbo to the leading posterior edge of the shell. Unless otherwise noted, all sizes reported herein are shell height. The live weight (LW) of clams within each experimental flask was determined at the end of the experimental run by carefully pouring clams onto a weigh boat, decanting water, and blotting the remaining water with a kimwipe. Blotting was done by tipping the weigh boat and blotting water that ran out from the bunched clams to prevent adhesion of clams to the kimwipe. Extreme care was used to remove all possible water without desiccating organisms.

Calcification rates and analytical methods. Calcification was measured using the alkalinity anomaly method (Smith & Key 1975) as in Waldbusser et al. (2010) where a 2 eq decrease in water alkalinity is equal to a 1 mol increase in shell $CaCO_3$ (Smith & Key 1975). Thus, the rate of new shell production is inversely related to the decrease in alkalinity within experimental flasks. Conversely, increases in alkalinity are due to dissolution of shell in the same proportion. Although we assumed that other biogeochemical processes contributing to a change in alkalinity are negligible relative to precipitation/dissolution of CaCO3 (Gazeau et al. 2007), alkalinity change due to other processes is a concern of this method. Primarily, the production of ammonium through respiration could have potentially contributed to the change in total alkalinity. However, based on previous rate measurements of ammonium production measured on hard clams (Srna & Baggaley 1976), the total estimated contribution of ammonium to alkalinity change in our experiments was <1% and can be considered negligible.

Experiments were run for ≤8 h (see above) with at least 3 alkalinity samples taken over that time period. Sampling for alkalinity was conducted without replacing water and 3 to 4 ml of water were drawn into syringes and remained sealed until titration, usually within 20 min. Due to the lack of replacement of water samples, we corrected the total alkalinity in flasks over time to what the total alkalinity would have been had a sample not been removed. The pH in control flasks was measured over the course of the experiment to verify that there were no changes in gas or air flow resulting in pH change. Flow meters on CO_2 flow control valves were visually checked several times over the course of the experiment. Additionally, pH was measured on each sample prior to the alkalinity titration to ensure consistent pH values over the course of the experiment.

Alkalinity was determined with a 2-point pH titration (Edmond 1970), with diluted HCl. The concentration of HCl used in the titrations was calibrated against

a known alkalinity standard. An Orion 938007MD micro temperature probe, Thermo-scientific 8103BN combination semi-micro pH electrode, and Thermo-scientific 5 star pH meter (Thermo Fisher Scientific) were used to measure pH and temperature of samples. A 3 point calibration curve was used to calibrate the pH electrode using National Bureau of standards (NBS) buffers. The pH 7.0 buffer was measured at the beginning and end of each set of alkalinity determinations to ensure the electrode did not drift during measurements. Over a set of alkalinity samples, pH remained within 0.02 pH units of the buffer value, and the electrode was recalibrated if pH drifted by more than 0.05 units. Additionally, at each sample time, an alkalinity standard was measured to ensure analytical consistency. Reported values of pH are on the seawater scale, converted from NBS scale using CO2SYS in Matlab. Partial pressure of CO_2 and saturation state of aragonite were calculated using CO2SYS in Matlab (van Heuven et al. 2009) using the Merbach (refit by Dickson & Millero 1987) carbonic acid dissociation constants and Dickson's sulfate dissociation constants.

Representative pH profiles. Representative pH profiles were obtained from mudflats within 3 coastal embayments in the northeast USA: Long Island Sound (LIS) (New York), South Portland and Falmouth (Maine). The 3 sites had sediment properties characteristic of hard clam habitat, with porosity of 0.9 for LIS and Falmouth and 0.8 for South Portland. The organic content, by loss on ignition, was determined to be 3.0% (wt/wt) for LIS and Falmouth, and 2.0% for South Portland. A large subcore (14.5 cm inner diameter; Cellulose acetate butyrate [CAB] tube) was removed from sediment at each location and stored at *in situ* temperature for transport back to the laboratory. Once in the laboratory (within 6 h of core retrieval), cores were sectioned under nitrogen in a glove bag, and porewater separated at in situ temperature without air contact using centrifugation (5 min, 5000 rpm, 20 to 40 ml pore water). pH was immediately measured in pore water using a Corning combination electrode standardized between each measurement using pH 4 and 7 NBS-traceable buffers.

Data analysis. Calcification rates were analyzed by fitting a general linear model (GLM) to the calcification data. Due to the unequal replication among size classes and to avoid artificially inflating significance by fitting a model with multiple observations at each dependent variable level, we took the mean values at each pH and size for both sources and fit the GLM to the mean values. We have included error bars on graphs in order to visualize the variability within pH-size treatments. Additionally, running the model on the means versus replicated data did not change the inferences from the model; the only differences were *F*-values and decreased p-values when replicate values were included. A stepwise model selection method was used to identify the best fitting GLM (variables of Source, Size, pH and Interactions) by minimizing fit statistics such mean square error, Bayesian Information Criteria (BIC), and Cp Mallow's index. As noted above, the variable Source was not an *a priori* factor in the experimental design. Additionally, plots of residuals were used to assess model fits. The final model that was determined by best fit statistics, meeting assumptions, and preventing over-parameterization was:

$$\text{Calcification rate} = \text{Source} + \beta_1(\text{Source} \times \text{Size}) + \beta_2(\text{pH}) \quad (1)$$

where Source is the hatchery from which the clams were obtained, β_i is the parameter estimate (slope) for variable *i*, Size is mean SH for the cohort, and pH is on the seawater scale. Source was a categorical variable, and Source × Size is a hybrid interaction term, resulting in separate slopes for the size effect based on source. The model was fit without an implicit intercept to prevent over-parameterization using the factor variable of Source to fit 2 separate intercepts, one for each source of organisms. These 2 intercepts are offset from one another by the overall effect of the 2 different sources. Statistical difference between the FL and VA clams was determined by a *t*-test ($\alpha = 0.05$) of the least squares means of calcification rates by Source. The Source × Size effect was also included, as separate intercepts failed to generate a model that adequately predicted differences in shell growth based on hatchery source. Significant differences between Source × Size values was determined from the 95% confidence interval around each parameter estimate. Assumptions of linearity, independence, and homoscedascity were verified by visual inspection of residuals plotted against predicted values and independent variables. The assumption of normality was confirmed with Shapiro–Wilk's statistic. We confirmed there were no overly influential data points by examining the studentized residuals. Statistical analyses were conducted in SAS v.9.2 using PROC GLM for final model fitting and PROC GLMSELECT for stepwise model selection and evaluation.

RESULTS

Experimental and analytical consistency

Using simple equipment and manually controlled flow valves, we were able to maintain replicate pH treatments consistently among experimental runs (Table 1). The variability in pH within treatments, across experimental runs (SD of 0.02 to 0.05) was not

Table 1. *Mercenaria mercenaria.* Experimental conditions for calcification experiments. Mean ± SD for pH and alkalinity across experimental trials. All trials were conducted at salinity of 30 and 20°C. pH was converted to seawater scale using CO2SYS (van Heuven et al. 2009). Mean ± SD of pCO_2 and saturation state of aragonite were calculated using CO2SYS program for each treatment across experimental runs

pH treatment	pH	Alkalinity (mmol l^{-1})	pCO_2 (µatm)	ΩAragonite
High	8.02 ± 0.02	2.236 ± 0.050	424 ± 26	2.52 ± 0.14
Mid	7.64 ± 0.05	2.226 ± 0.030	1120 ± 146	1.18 ± 0.14
Low	7.41 ± 0.05	2.226 ± 0.033	1950 ± 240	0.72 ± 0.09

much larger than the precision of the electrode (0.01). However, it should be noted that even small variability in pH propagates large variance in the calculations of pCO_2 and saturation state due to the sensitivity of these values (particularly carbonate ion concentration) to pH. Therefore, the uncertainty around the mean values should be considered when comparing across studies. Our analytical precision for the alkalinity titrations within experimental runs, as measured by the SD of alkalinity standards, averaged 0.015 mmol l^{-1}, ranging from 0.007 to 0.034 mmol l^{-1}. The average alkalinity variability in the control (no clam) flasks for all treatments within experimental runs was 0.03 mmol l^{-1}. Additionally, pH variability (SD) in control flasks within a treatment and experimental run averaged 0.026 pH units across all treatment/runs with a range of 0.005 to 0.083 within experimental runs. Therefore, pH was relatively consistent across and within experimental runs.

Hard clam calcification

Variability in calcification rates of the hard clam *Mercenaria* spp. was relatively small among replicates in a given size and pH treatment (Fig. 2); however, we were unable to replicate the 2 smallest size classes due to the number of organisms required (see Materials and Methods). The GLM, as described above, was significant ($F_{5, 10}$ = 44.01, p < 0.0001, R^2 = 0.93), with Source, Source × Size, and pH as significant sources of variance in the model (Table 2). Unfortunately, only 2 size classes were measured from the FL hatchery; therefore, the parameter estimate for Source × Size for this hatchery should be viewed cautiously (given this is a slope estimate based on 2 points). However, if a common size effect parameter was fit to the model, calcification rates were under and over predicted for the larger and smaller clams, respectively, from the FL hatchery. Both the Source × Size and Source values between the 2 hatcheries were significantly different from one another (Fig. 3). The hybridized *Mercenaria*

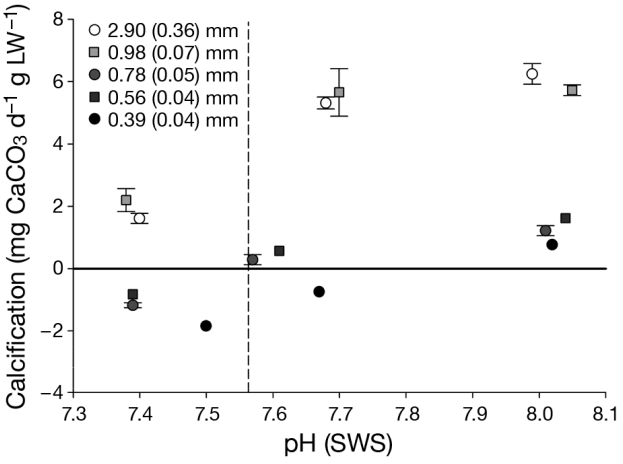

Fig. 2. *Mercenaria mercenaria.* Calcification rates vs. pH (SWS: seawater scale) for 5 size classes. Grey scale from black to white = increasing size. Error bars: SD of replicate flasks for a given size and pH treatment. Shell heights in key: mean size (SD). Clams from Virginia (circles) or Florida (squares) hatcheries. Dashed vertical line: approximate aragonite saturation threshold based on average alkalinity conditions across all data (lower pH waters are undersaturated, higher pH waters are supersaturated). Solid horizontal line: transition between net calcification and net dissolution. LW: live weight

calcified faster with increasing size at 9.34 versus 2.02 mg d^{-1} g^{-1} per mm in SH for the pure *M. mercenaria*. The 95% confidence limit around the Source × Size parameter estimate for the hybridized (FL) and non-hybridized (VA) *Mercenaria* was 5.83 to 12.84 and 1.48 to 2.57, respectively. Since each Source × Size parameter estimate falls outside of the confidence interval of the other, these are significantly different at α = 0.05. Additionally, the least squares means of calcification for the FL and VA clams were significantly different from one another (t_{13} = 7.05, p ≤ 0.0001). The difference between the intercepts or offset was 4.94.

Table 2. *Mercenaria mercenaria.* Parameter estimates and significance of variables in the general linear model of Source, Source × Size, and pH effects on calcification rate in hard clams. Ndf, ddf: numerator and denominator df for test of significance. FL: Florida, VA: Virginia. SE values in parentheses

Source of variance	Parameter estimate	ndf, ddf	*F*	p
Source		2, 10	22.79	0.0002
FL	−42.13 (6.40)			
VA	−38.86 (6.34)			
Source × Size		2, 10	51.97	<0.0001
FL	9.34 (1.57)			
VA	2.02 (0.24)			
pH	4.86 (0.82)	1, 10	35.07	0.0001

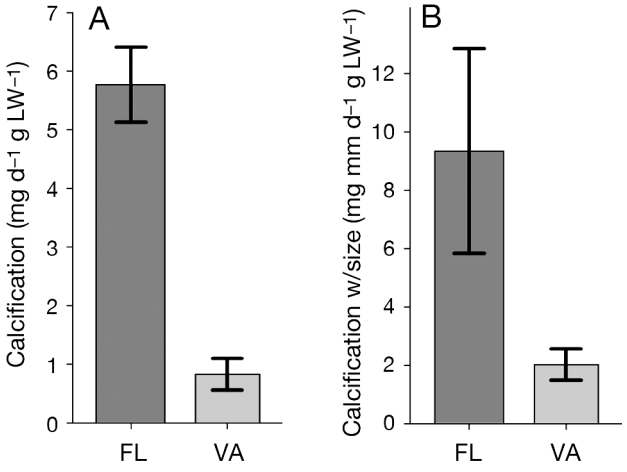

Fig. 3. *Mercenaria mercenaria.* Source effects on calcification rate. (A) Least square means of overall calcification rate by hatchery source (error bars = SE); units: calcification rate. (B) Rate of calcification increase with size by hatchery source with 95% confidence limit as error bars; units: calcification rate per shell height. FL: Florida, VA: Virginia, LW: live weight

Given the unequal sample sizes between the 2 sources, lack of replication at the 2 smallest sizes, and different distribution of measurements across pH and size, it is best to view the statistical differences between the hatchery sources of clams cautiously.

A common pH effect parameter was determined through the model fitting (Table 2) indicating that although differences in overall growth with size varied, the pH effect was consistent across Source and Size. It should be noted that this conclusion is only applicable across the size and pH range we measured, and it is likely that above these sizes, this linear relationship may not follow. However, based on our data and model, the size dependent effect appears to be related more to the increase in overall growth rate with size, rather than a decreased sensitivity to pH. In other words, it does not appear that dissolution is increased at smaller sizes; rather, that calcification is increased at larger sizes. Since the pH effect in the model was consistent, and calcification rate increased with size, the pH effect becomes more important to the smaller sizes as a decrease in calcification is more likely to result in net dissolution. A decrease in pH does result in slower net calcification rates in the larger sizes; however, baseline rates of calcification are high enough to overcome dissolution pressure. The estimate of the pH effect on these post-larval hard clams indicates that a drop in pH of 1 unit would result in a drop of almost 5 mg $CaCO_3$ d^{-1} g LW^{-1}, which is close to the maximum calcification rates measured here, suggesting that even the largest sizes would at some point be unable to net calcify with decreasing pH. The 2 largest size classes (0.98, 2.90 mm) were also able to net calcify in conditions of aragonite undersaturation, and the next largest size class (0.78 mm) appeared to very slightly net calcify just below the saturation threshold for aragonite (Fig. 2).

DISCUSSION

Calcification in post-larval hard clams of the species *Mercenaria mercenaria* decreased with relatively small declines in pH, and larger post-larval juveniles were less sensitive to relative moderate pH decline. It is important to note that the lack of replication at the 2 smallest sizes, due to experimental limitations, is somewhat problematic. The small variability within size and pH treatments, however, provides us with confidence that the general trends are valid, but further measurements are needed to verify the current findings. At the lowest pH treatments, the smaller clams experienced net shell dissolution, whereas the larger clams generally were still able to net calcify, albeit at a diminished rate (Fig. 2).

The significant Source and Source × Size terms in the GLM (Table 2) highlights the potential role of genotype in regulating calcification rates, and possibly overcoming acidification impacts on shell growth. Although the general calcification response to pH was determined to be the same from the 2 hatcheries (Table 2), the significantly higher calcification rate of the FL clams suggests that they would reach a size at which they could overcome dissolution pressure sooner. It is clear, however, that a pH drop of 0.5 units results in decreased calcification in all sizes of hard clams, regardless of source. Importantly, pH may affect physiological processes other than shell production (Pörtner 2008, Pörtner et al. 2004); therefore, the effects of pH on calcification may be directly related to the mineralization of shell or indirectly to overall physiology. Our data suggest that the dissolution pressure is similar across size, but the increased rate of calcification with size allows the larger post-larval clams to overcome this dissolution pressure. This would support a hypothesis that within the pH range of our experiments, there was little direct physiological impact on hard clam; rather, the effect was related to a balance between shell growth and dissolution. Additional work is much needed in this general area to elucidate these types of questions.

One concern of short-term exposure experiments, such as these, is the 'shock effect' resulting in calcification values that are inconsistent with values obtained in experiments where organisms are given time to acclimate. However, holding organisms that are typically found in variable conditions under stable conditions may also provide an incomplete picture of

response. Other researchers have shown significant changes in sediment porewater redox conditions over hours (Wenzhofer & Glud 2004, Stahl et al. 2006) driven by diurnal production–respiration cycles at the sediment–water interface. The low pH values we used in these experiments are not abnormally low for porewater pH and well within values often found in coastal sediments (Fig. 4; Stahl et al. 2006). We therefore suggest that both short-term experiments that capture responses and longer-term experiments that measure baselines are needed to quantify response of shell-forming species to changes in pH.

Coastal and estuarine sediments are habitats of significant remineralization of organic matter, and thus generate CO_2 (e.g. Green & Aller 1998, 2001, Andersson et al. 2006). The rapid equilibrium thermodynamics of the carbonate system and diffusion-dominated transport of solutes in organically rich sediments result in higher concentrations of CO_2 and lower pH of sediment porewater versus overlying water, especially in shallow, well-mixed water bodies (Fig. 4). Furthermore, porewater chemistry may vary over small spatial and temporal scales, as illustrated by rapid hourly changes in both oxygen (Wenzhofer & Glud 2004) and pH (Stahl et al. 2006) with diurnal cycles. Early post-larval bivalves settle to these types of sediments that are often undersaturated with respect to aragonite (Green et al. 2009; Fig. 4), and must grow shell under thermodynamically unfavorable conditions. Most recent efforts to study acidification effects on near-shore temperate shell bearing benthic fauna have examined the larval stages (Clark et al. 2009, Miller et al. 2009, Todgham & Hofmann 2009), which are believed to be the most susceptible stage to decreases in pH, primarily due to the solubility of larval shell (Weiss et al. 2002). The early post-larval stage is the link between larval and adult stages, retains the larval shell section, and must use significant energy reserves to successfully complete metamorphosis and organogenesis (Rodriguez et al. 1990, Videla et al. 1998); its growth and survival plays a significant role in population success (Gosselin & Qian 1997). Despite the thermodynamically unfavorable conditions for $CaCO_3$ preservation *Mercenaria* spp. and many other aragonitic bivalves are found in, they persist in these environments. It is clear that infaunal bivalves, and other shell bearing infauna, must have evolved to cope with the naturally variable and lower pH in marine sediments (relative to overlying waters). However, our results corroborate previous findings that dissolution pressure may play a significant role in infaunal bivalve growth (Ringwood & Keppler 2002) and survival (Green et al. 2004a, 2009), and this effect is mitigated with size through the early post-larval stages. What is unclear is the extent of pH change in marine sediments due to anthropogenic impacts on biogeochemical cycles over the course of weeks during bivalve early life history and corresponding chronic and acute thresholds under non-laboratory conditions.

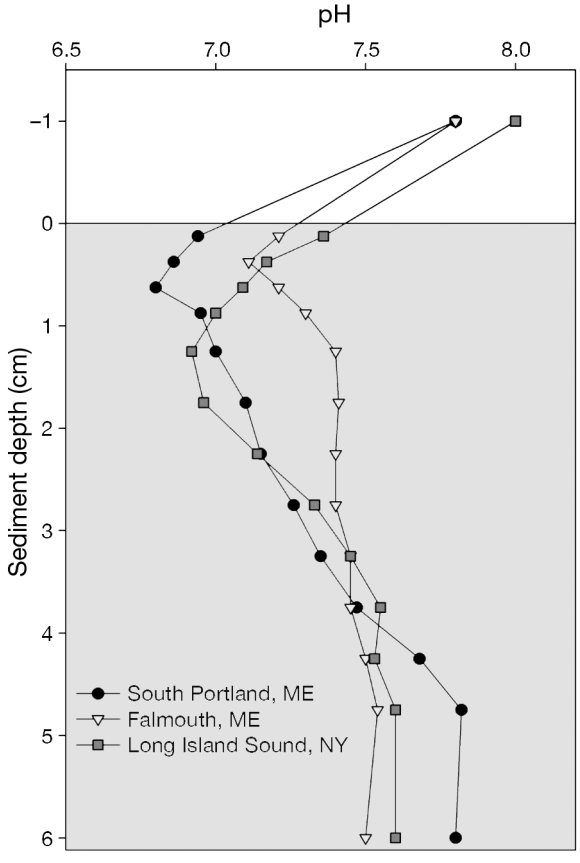

Fig. 4. *Mercenaria mercenaria.* Porewater pH profiles from 3 geographic locations often inhabited by hard clams and other infaunal bivalves. Profiles were measured in early summer from South Portland and Falmouth, Maine, and Long Island Sound, New York

The size range of hard clams in our experiments (0.39 to 2.90 mm) corresponds to the post-larval stages in which significant morphological development of siphons, mantle, and shell occurs (Carriker 1961, 2001) and mortality is high (Thorson 1966). We have compiled information from Carriker (2001) into a table highlighting significant morphological changes to hard clams with approximate size from larval through early post-larval stages (Table 3). At the smallest sizes we studied, 0.39 mm SH, the hard clam has not yet formed a definitive inhalant siphon and pumps water through the pedal opening, which must remain in contact with overlying water, limiting the depth to which these sizes can burrow (Carriker 1961, Zwarts & Wanink 1989). The pedal opening will eventually fuse to form the mantle folds, which is the location of the periostracal groove, which produces the outer protective shell layer called the periostracum (Saleuddin & Petit 1983,

Table 3. *Mercenaria mercenaria.* Early development of hard clams, adapted from Carriker (2001; and references therein). Shell height is approximate, as variability exists in timing and size of these changes in shell stage and development. Prodissoconch stages are larval; Dissoconch stages are post-larval

Shell stage	Shell height (mm)	Comments
Prodissoconch I	0.078	First shell material, 24 h post-fertilization
Prodissoconch II	0.098	Formation of mantle cavity begins
Dissoconch I	0.210	Typical settlement, early metamorphosis
	0.275	Fusion of mantle lobes begins
	0.300	Metamorphosis
Dissoconch II	1.000	Early development inhalant siphon Inner fold of mantle formation
	4.000	Definitive inhalant siphon

Checa 2000). Additionally, the mantle allows the bivalve to seal off the calcifying fluid from the surrounding seawater. At the Dissoconch II stage, between SH of ~1 to 4 mm, the inhalant siphon forms, allowing the hard clam to more effectively pump water into and out of the mantle cavity (for feeding and respiration).

The early development of the siphon and mantle folds may then represent a developmental bottleneck of recruitment to adult populations in regards to lower pH. During the post-larval stage significant reorganization of the body plan is underway (including changes from larval to adult feeding organs), and the metabolic costs of metamorphosis coupled with energetic demands of shell formation (Palmer 1992, Hautmann 2006) could likely be partially mitigated by quickly developing food capturing organs. If requisite feeding organs are not yet developed during the early life stages (Table 3), and exposure to lower pH environments requires more energy to pump protons out of the calcifying fluid to counter dissolution pressure, then it seems that a clam's post-larval development stage in relation to sediment geochemistry is a crucial point for success or failure. It appears that successful and rapid metamorphosis (also affected in the larval stage by acidity, sensu Talmage & Gobler 2009) and growth should allow hard clams to overcome some level of dissolution pressure by developing key organ systems associated with feeding and shell growth, thereby limiting the reliance on larval lipid reserves.

Solving the GLM from the results above for pH when calcification is zero, over the size range used in our experiments allows us to estimate a size threshold at which these clams may overcome dissolution pressure. The solution estimates at ~1 mm in SH (Fig. 5) or the Dissoconch II stage, these organisms can overcome dissolution pressure. Although, this estimate is only for the clams from the VA hatchery (due to limited sizes from the FL hatchery), it provides a testable bench mark for other studies. A horizontal reference line on Fig. 5 notes the approximate saturation state threshold with respect to aragonite at the alkalinity in our experiments (2.2 mmol l^{-1}). The intersection of the net-zero calcification line with the saturation state threshold indicates size at which these clams are predicted to be able to overcome dissolution pressure. However, some important caveats are required with this prediction. (1) It is unclear from current experimental data, and other published studies, if this relationship would hold up over varying salinity and temperature, as these appear to be important covariates on calcification rate in other juvenile bivalves (Waldbusser et al. 2010). (2) The differences in calcification response between the 2 hatcheries suggest that this predictive approach may only be reliable within species. (3) This threshold is estimated for groups of clams, so individual responses are likely to vary within a cohort; therefore, it should be seen as a probable number rather than a strict delineation. With these caveats, the predicted threshold size of ~1 mm corresponds closely to the beginning of Dissoconch II, at which siphons and mantle folds become more fully developed, assisting in food capture and assimilation. Interestingly, changes in carbon isotopic fractionation across the axis of shell growth

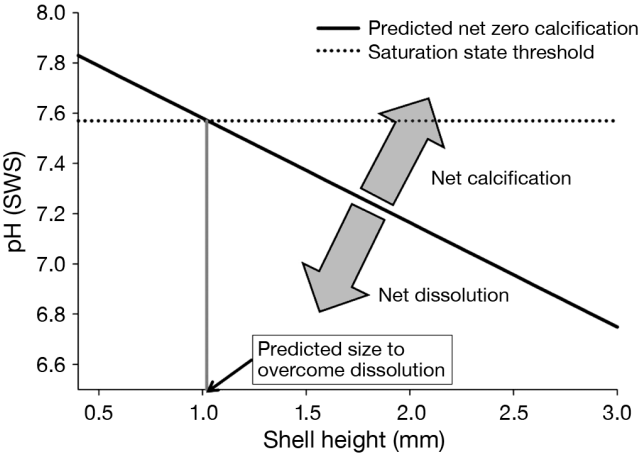

Fig. 5. *Mercenaria mercenaria.* Predicted pH (SWS: seawater scale) thresholds for size of *Mercenaria* from the general linear model fit to the Virginia hatchery data only. Dotted line: pH value at which waters become undersaturated with respect to aragonite at the average experimental alkalinity, salinity, and temperature (just <7.6). Net zero calcification line: pH at size below which dissolution is predicted to occur. It is important to note that these predictions are only applicable to range of sizes and at the salinity/alkalinity of the current experiments. Additionally, had this relationship been determined for the Florida hatchery *Mercenaria* hybrids (based on the limited data we have), a smaller size at which dissolution pressure could be overcome would have been found

in hard clams indicate that larger/older clams utilize more respired CO_2 for calcification versus smaller/younger clams (Elliot et al. 2003, Lorrain et al. 2004, Gillikin et al. 2007). Although to date little is known about the isotope ratios in early juvenile shells, the increase in respired C in shell material deposited with age suggests the potential importance of feeding physiology on calcification.

The current findings strongly support the 'death by dissolution' hypothesis of Green et al. (2009), suggesting that sediment pore-water carbonate thermodynamics is a structuring factor for infaunal bivalve populations. Green et al. (2009) found significant mortality, attributed to dissolution, over several days at a pH 7.0 for hard clams of nominal sieve sizes from 0.2 to 0.6 mm. These sieve sizes correspond to ~0.4 to 0.9 mm shell length in the current experiments, based on empirical relationships determined with the current study organisms. When pH was slightly higher at 7.3, the 0.2 mm nominal sieve sized (or ~0.4 mm SH) clams experienced significant mortality over days, while the 0.6 mm sieve sized (~0.9 mm SH) clams did not (Green et al. 2009).

An alternative or complementary mechanism driving the size effects is the ontological change in mineral composition from ACC to aragonite (Weiss et al. 2002). ACC is more soluble than aragonite; therefore, this may also be a mechanism for the size dependent effect of pH on calcification in this species. Significant changes occur in shell composition over the course of the larval stage; as the larvae grow, they deposit more aragonite on the ventral margin relative to ACC. Weiss et al. (2002) also found that ACC may be the precursor to aragonite in the shells of *Mercenaria mercenaria*. In scanning electron microscopy images of post-larval *M. mercenaria* subject to low pH, Green et al. (2009) noted the extensive deterioration of the umbo section of the shell. The area noted in Fig. 7 of Green et al. (2009) also closely corresponds to the prodissoconch sections (larval) of the shell that would have proportionally more ACC than aragonite. The SH of the area illustrated in Green et al. (2009) is ~0.1 mm, corresponding to the approximate transition between Prodissoconch I and II (Goodsell & Eversole 1992). The findings of Weiss et al. (2002) support the speculation that a shift in calcification occurs between the Prodissoconch I and II stages, with considerably more aragonite in the Prodissoconch II stage (thus affording the hard clam better ability to withstand lower pH). Our findings show that at the smaller post-larval size, these infaunal bivalves cannot net calcify under conditions typical for terrigenous coastal sediments. Whether these organisms are still calcifying at the same rate and cannot build shell faster than it is dissolving, or if physiological processes are slowed and the rate of new shell growth slows is unknown.

The differences in calcification between clams from the hatchery sources used in our experiments indicate that genotypic differences among similar species may play a significant role in determining susceptibility to acidification effects. Although this was not an *a priori* factor in our experimental design, the Source differences provide important insight for future studies. Genetic analyses of northern hard clams *Mercenaria mercenaria* from the Florida hatchery have shown that nearly 25% of the hard clams in the hatchery are hybrids with the southern hard clam *M. campenchiensis* (Arnold et al. 2009). The southern hard clam is generally believed to have a higher growth rate, and this would support our findings of higher calcification in the clams from the FL hatchery; however, the differences in growth among northern, southern, and hybrid clams are complex and vary with habitat (Arnold et al. 1998). Conversely, Goodsell & Eversole (1992) found that hard clam hybrids from *M. mercenaria* lines had the highest rates of shell growth during the Prodissoconch II stage (just prior to metamorphosis). Relatively little is known about possible differences in organogenesis in the early post-larval stages between the 2 species. We cannot definitively state whether the genotypic differences are responsible for the differences in calcification rates; however, we suggest that determining genotypic effects on calcification rates is an important research avenue that requires further investigation. Extensive work has shown differences in growth rates of natural populations of *M. mercenaria* with geographic location and other environmental variables (Arnold et al. 1991, Carmichael et al. 2004, Henry & Nixon 2008, Kraeuter et al. 2009). It would therefore be naive to suggest that pH is the only factor that would have significant effects on shell growth in this species and other bivalves, as salinity and temperature also alter calcification (Waldbusser et al. 2010). However, the differences in hatchery calcification rates suggest that selective breeding programs may be one possible mitigation strategy for commercial bivalve stocks.

Predicting the change in pH in coastal and estuarine waters due to atmospheric CO_2 and other anthropogenic impacts on biogeochemical cycles is a daunting task (Andersson et al. 2006, Blackford & Gilbert 2007, Wootton et al. 2008). Hard clams and other benthic infaunal calcifiers are ecologically and commercially important to many coastal ecosystems, their sediment habitats are typically more corrosive than overlying waters, and these ecosystems are likely to be altered by increased CO_2 and acidification in complex ways (Andersson et al. 2006, Borges & Gypens 2010, Waldbusser et al. 2010). Therefore, it is important to quantify the responses of sediment dwelling calcifying organisms to changes in pH, while designing experi-

ments that more closely mimic the highly dynamic nature of these habitats. Our experiments continue to build upon mounting evidence that altering pH in estuarine and coastal waters could have significant impacts on populations and communities of calcifying organisms in temperate coastal waters.

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