Impacts of ocean acidification and mitigative hydrated lime addition on Pacific oyster larvae: implications for shellfish aquaculture

by

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Abstract

Anthropogenic CO_2 emissions are rapidly altering marine chemistry, significantly impacting calcifying organisms' ability to produce and maintain shells. In the Pacific Northwest, shellfish hatcheries have already observed mass die offs of larvae, potentially from ocean acidification. This study aims to elucidate if hydrated lime can be used at the hatchery level to chemically reverse ocean acidification and its negative impacts on larval stages of the Pacific oyster, *Crassostrea gigas*. For 24 days, larvae were exposed to four treatments: ambient - 390 ppm, ocean acidified - 1500 ppm, limed-ambient and limed-ocean acidified treatments to meet pre-industrial levels ~ 280 ppm. Hydrated lime significantly increased larval shell length after two weeks of development, but did not affect survival or percent abnormality between treatments. Investigations into mitigative lime use appear cost effective and feasible for shellfish hatcheries under future CO_2 scenarios, but requires more extensive research at the hatchery level, and for other species.

Keywords: Ocean acidification; Pacific oyster; Larval stages; Hydrated lime; Shellfish aquaculture; Pacific Northwest

Dedication

To my mother, Ann. Through life you have shown me what

real strength is, while giving unconditional love and support.

You are my inspiration. Thank you.

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List of Acronyms

BC	British Columbia
C. gigas	*Crassostrea gigas*, scientific name for Pacific oysters
CO_2	Carbon Dioxide
$CO_2(aq)$	Aqueous Carbon Dioxide
pCO_2	Partial Pressure of Carbon Dioxide
CO_3^{2-}	Carbonate Ion
DIC	Dissolved Inorganic Carbon
EPOCA	European Project on Ocean Acidification
FAO	Food and Agriculture Organization of the United Nations
NIST	National Institute of Standards and Technology
OA	Ocean Acidification
ppm	Parts per million
PSU	Practical Salinity Units
RPD	Relative Percent Difference
SFU	Simon Fraser University
SOP	Standard Operating Procedures
TP	Total Phosphate
TSi	Total Silicate

C NL	Control, No Lime Treatment
C L	Control, L Treatment
OA NL	Ocean Acidified, No Lime Treatment
OA L	Ocean Acidified, Lime Treatment

Glossary

Calcifiers	Organism the produces a calcium carbonate shell
Larvae	Pre-adult form in which many animals hatch from the egg and spend some time during development; capable of independent existence but normally sexually immature. Often markedly different in form from adult, into which it may develop gradually or by a more or less rapid metamorphosis. Often dispersive, especially in aquatic forms. (Thain & Hickman, 2000).
Veliger	Planktonic molluscan larva, developing from trochophore and with larger ciliated bands; adult organs present include foot, mantle and sell (Thain & Hickman, 2000).

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1. Introduction

Ocean acidification has been identified as a significant problem impacting the global ocean by reducing pH as carbon dioxide (CO_2) accumulates in the atmosphere at unprecedented rates (Rost et al., 2008). Ocean acidification (OA) is the process by which CO_2 is absorbed from the atmosphere into the oceans, resulting in an increase of hydrogen ions (H^+) and a decrease in carbonate ions (CO_3^{2-}) in seawater (Figure 1**)** (Feely, 2009). Since the Industrial Revolution, atmospheric CO_2 concentrations have increased from about 280 to just under 400 ppm. One-third of these human-derived CO_2 emissions have been absorbed by the oceans, causing a reduction in the average surface oceanic pH by about 0.1 units from a pH of 8.2 (Feely et al., 2008). Atmospheric CO_2 concentrations are projected to increase to 750 ppm by the end of this century (I.P.C.C., 2007), which could cause oceanic pH to decrease by an additional 0.6 units in response to invasion of anthropogenic CO_2 (Ilyina et al., 2009). A reduction in pH to 7.5 would make the ocean five times more acidic than its pre-industrial value of 8.2.

Elevated CO_2 in the oceans directly impacts the ability of marine organisms to form shell material (integral to their survival) due to lowered saturation indices for both calcite and aragonite ($CaCO_3$) (Feely et al., 2010). Values for aragonite saturation decrease with decreasing ocean pH in response to reduced amounts of free carbonate ions (CO_3^{2-}) available to produce the $CaCO_3$ shell material (Figure 1) (Feely et al., 2008). In theory, shells are more prone to dissolution and organisms will require more energy to maintain shell structure when aragonite becomes undersaturated ($\Omega < 1$; Kurihara, 2008;

Lannig et al., 2010). Indeed, numerous laboratory experiments have demonstrated these effects on a wide range of calcifying marine organisms, including coccolithophores, foraminifera, shellfish, crustose coralline algae, sea urchins, pteropods, and coral reefs (Gazeau et al., 2007, Bijma et al., 1999, Leclercq et al., 2000, Riebesell et al., 2000, and Langdon and Atkinson, 2005). Even tiny changes in the supersaturated levels of aragonite were shown to impact shellfish larvae producing shells, demonstrating how sensitive marine organisms can be to the effects of OA (Salisbury et al., 2008, Talmage et al., 2009, Talmage et al., 2010, Parker et al., 2010, and Gazeau et al., 2011).

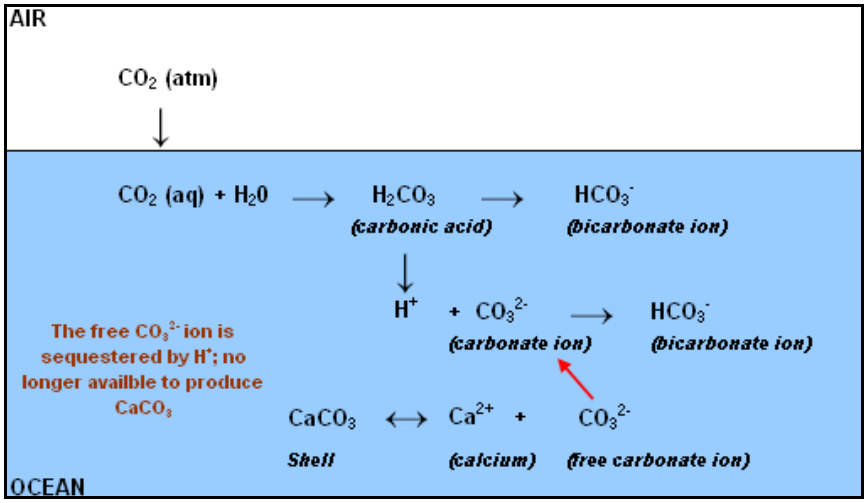

Figure 1: *Chemical Reaction of Ocean Acidification*

More recent studies have focused on the OA influence on early life history stages of marine organisms, such as fertilization and larval development (Parker et al., 2010). For example, larvae of hard clam (*Mercenaria mercenaria*), bay scallops (*Argopecten irradians*), and Eastern oysters (*Crassostrea virginica*), all commercially valuable shellfish on the east coast of North America, were found to have significantly lower survival, reduced growth, and delayed metamorphosis at elevated pCO_2 concentrations

(Talmage & Gobler, 2009). Even current pCO_2 concentrations (390 ppm) show significant impacts on these three shellfish species when compared to those grown at pre-industrial pCO_2 concentrations (250 ppm), demonstrating that OA effects are being observed now (Talmage & Gobler, 2010). Fertilization experiments confirmed negative impacts of OA on the reproduction of shellfish (*Saccostrea glomerata*), copepods (*Acartia steuri*), brittle stars (*Ophiothrix fragilis*) and sea urchins (*Echinometra mathaei* and *Hemicentrotus pulcherrimus*) (Parker et al., 2010, 2012; Kurihara et al., 2004; Dupont et al., 2008; Kurihara and Shirayama 2004; Reuter et al., 2010). For Pacific oyster larvae (*Crassostrea gigas*), laboratory experiments suggest that OA conditions result in increased incidence of abnormality, reduced shell growth, and limited fertilization success, though more research is required to discern OA effects on the transition through larval developmental stages (Kurihara, 2008; Parker et al., 2010; Gazeau et al., 2011).

Understanding and mitigating the effects of OA on shellfish populations in the Pacific Northwest region is particularly important because of rapidly changing oceanic conditions, but also because of the relative importance of the shellfish industry to local economies (Feely et al., 2010). Expansion of the shellfish aquaculture industry has been put forth as an economic strategy to revitalize British Columbia (BC) coastal communities, who have been hit hard by economic declines in other resource sectors (Joyce et al., 2009). The shellfish industry has tripled in BC since 1986 (Joyce et al., 2009). Furthermore, Coastal First Nations are investing considerable time, energy and money to build a shellfish hatchery and assemble a sustainable shellfish industry on the BC coast (Art Sterrit, *Personal communication,* 2011).

In recent years, episodes of mass mortalities of shellfish larvae have occurred in the aquaculture industry in the Pacific Northwest (Welch, 2010). The factors responsible for this decline remain unknown but could include ocean acidification, bacterial infections, unknown environmental conditions, or some combination of these (Amos & Cheney, 2009; The Guardian Express, 2012; BC Shellfish Grower's Association, 2013). The North Pacific Ocean has the most acidic water in the global ocean, and recent modeling work has suggested calcite production will be hardest hit in the mid-to-high latitude regions of the ocean (Fisheries and Oceans, Canada, 2008; Ilyina et al., 2009). For the Pacific region of the US, economic losses to the shellfish industry due to ocean acidification have been estimated at 38 to 599 million US dollars per year by 2060 (depending on discount rates and emission scenarios, (Cooley and Doney, 2009). Therefore finding some means of mitigating the problem of OA in coastal waters could be beneficial to the aquaculture industry.

One strategy proposed to mitigate the effects of increasing atmospheric CO_2 levels and ocean acidification is "liming the ocean" (Society of Chemical Industry, 2008; Lenton & Vaughan, 2009). This strategy involves adding hydrated lime ($Ca(OH)_2$) or quicklime (CaO) to seawater to chemically reverse the effects of OA (Equation 1):

$$CaO + 1.79\,CO_2 + 0.79\,H_2O \longrightarrow Ca^{2+} + 1.62\,HCO_3^- + 0.17\,CO_3^{2-} \qquad (\mathbf{1})$$

Lime addition would increase the buffering capacity of the oceans and enhance absorption of CO_2 from the atmosphere (Society of Chemical Industry, 2008). While modeling experiments suggest that liming the ocean is likely not an effective global-scale means of reducing atmospheric CO_2 levels (Lenton and Vaughan, 2009; Ilyina and Zeebe, 2012; Dr. Ilyina, *personal communication*, 2012), lime addition might be useful to

mitigate the negative effects of OA on local scales in closed basins. Adding lime consumes CO_2 and increases the pH, alkalinity, and free carbonate ions in seawater, therefore increasing the saturation indices of calcite and aragonite (Kheshgi, 1995). Thus, lime addition might be useful at the hatchery level in shellfish aquaculture for mitigating the negative effects of OA on shellfish growth and calcification.

The goal of this study is to examine the effects of ocean acidification and mitigative lime addition on the growth, survival and metamorphosis of Pacific oyster (*Crassostrea gigas*) larvae, a commercially valuable shellfish species on the Pacific Coast of North America. Larval stages of this non-native Pacific oyster are particularly important to the shellfish industry because hatcheries are the main supplier to the commercial sector in the Pacific Northwest, where cold temperatures limit natural populations (Barton et al., 2012). The primary objectives for this study are to (a) determine how shellfish larvae will react to seawater enhanced with lime, and therefore an increase in alkalinity and pH; (b) determine whether adding lime provides a temporary solution to combat OA issues in the shellfish industry, until human advances in technology and/or government regulations reduce our dependency on fossil fuels; and (c) assess whether this strategy is cost-effective and efficient to implement at the hatchery level. This study is unique in that it considers experimental impacts of both acidification from elevated CO_2 as well as the possible effects of attempting to neutralize these of effects on the early stages of commercially valuable shellfish larvae of the Pacific Northwest.

2. Methods

Four sets of experiments were run to examine the impacts of lime addition, elevated CO_2, and elevated CO_2 with lime addition on the survival, growth and percent (%) abnormality of the Pacific oyster larvae (*C. gigas*). Experiments were conducted in the COPE Laboratory at Simon Fraser University (SFU) from September 29th to October 23rd, 2012 (duration 24 days). The experimental setup followed EPOCA's *Guide to best practices for ocean acidification research and data reporting* (Riebesell et al., 2010).

2.1. Experimental Setup

Pacific oyster larvae were reared in four carbonate chemistry treatments: Control No Lime (C NL), Ocean Acidified No Lime (OA NL), Control with Lime (C L), and Ocean Acidified with Lime (OA L) (Figure 2). Hydrated lime was used to adjust the carbonate chemistry of present day (C NL) and ocean acidified (OA NL) treatments to values similar to pre-industrial levels (pCO_2 ~ 280 ppm, pH 8.2) for C L and OA L treatments. We used two pCO_2 concentrations: a **control** of 390 ppm (present day levels) and a **treatment** of 1500 ppm (levels predicted for 2250, depending on projection) (Zeebe et al., 2008). CO_2-Air gas mixtures were premixed and certified by Praxair Canada. Four replicate aquaria were randomly assigned to one of four treatments (Figure 2), though one replicate from the OA L treatment was removed early in the experiment due to contamination by cardboard fibers which resulted in large die-offs. All 16 aquaria were

airtight, plexi-glass containers with a total volume of 1.9 L that were housed in an environmental growth chamber and maintained at a temperature of 24.5 °C.

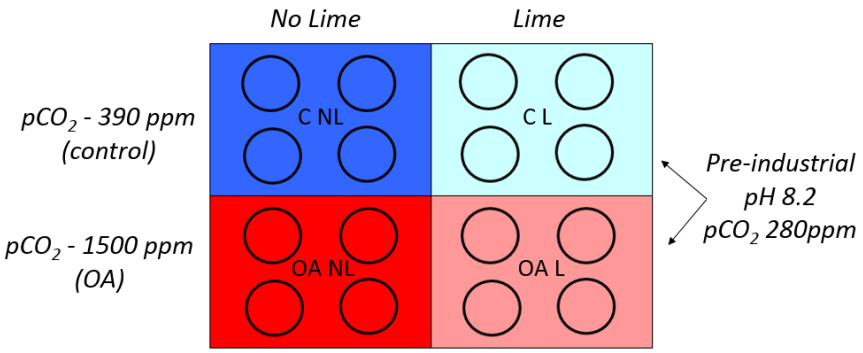

Figure 2: *Using a complete randomized design, 16 aquaria (represented by circles) were randomly assigned to four carbonate chemistry treatments (C NL, OA NL, C L, and OA L) for a total of four replicates per treatment*

Seawater was collected daily from SFU's seawater storage tank, which regularly receives filtered seawater from the Vancouver Aquarium, and throughout the experiment had a constant salinity of 26 Practical Salinity Units (PSU). On collection, 3 gallons of seawater was filtered through a 5 μm water bag and contained in 5 gallon plastic jugs (Strathmann, 1987). Jugs were autoclaved at the start of the experiment to reduce the occurrence of marine pests (zooplankton). Seawater was treated with the assigned pCO_2 concentrations by pre-bubbling the jugs until appropriate pH conditions were met. This typically took 24 hours at a flow rate of 200 ml/min for 3 gallons of seawater. Many related studies bubble culture vessels directly, but air bubbles can cause developmental damage to shellfish larvae, so the decision was made to pre-treat the seawater with the assigned CO_2 levels and liming regimes prior to larval exposure (Strathmann, 1987).

Water was changed daily throughout the experiment. Each container was fitted with a plexiglass tube with 31 μm nitex mesh glued to the base that was used to contain

the larvae and gently decant and transport them to holding containers while aquaria were being cleaned (Strathmann, 1987). Each day, aquaria were completely drained, rinsed aggressively three times with distilled water, and then refilled with seawater to the 1.3 L mark. Therefore, air tight aquaria had a headspace of 0.6 L of treatment gas, and 1.3 L of seawater, comprising its total volume of 1.9 L. In order to prevent the treated seawater from outgassing or sequestering CO_2 during refill, aquaria were first gassed at a high flow rate with the assigned treatment gas for two minutes. A series of tubing connecting the jug to container was used to fill aquaria with seawater, to prevent lab air mixing with the pretreated seawater. Containers assigned to the no-lime treatments were filled first. Then small amounts of hydrated lime were added to the remaining CO_2 treated seawater in the jugs, until a pH of approximately 8.2 was met. Typically, around 15 mg of lime was required to bring the remaining 1.6 gallons of 390 ppm treated seawater to a pH of 8.2; approximately 159 mg of lime was required for the remaining 1500 ppm treated seawater to reach a pH of 8.2. When lime was added to seawater, a small amount of carbonate precipitate formed (Reebs et al., 2011). To prevent this precipitate from interfering with biological measurements, a 31 μm filter was secured to the opening of the jug siphon, to filter large precipitates when refilling aquaria.

Pacific oyster larvae spawned on September 29th, following thermal shocking procedures conducted the day prior (FAO, 2004). Larvae were placed in their appropriate treatments within 24 hours after fertilization (on Day 0), but at the time of inoculation, we noted that some slight shell development had already occurred. Larvae were inoculated at a density of 5 larvae/ml, with the expectation that naturally high mortality would occur (FAO, 2004; Gazeau et al., 2011). *The Hatchery Culture of Bivalve Manual* suggests a density no more than 15 to 20 larvae per ml (FAO, 2004), yet

Strathmann (1987) suggests no more than 2 larvae per ml. Because a high mortality was observed (see results and discussion), higher densities only occurred at the very beginning of the experiment, with much lower densities occurring at the end of the experiment (0.003 larvae/ml, or 1 larvae per 368 mL).

Oyster larvae were fed 5 mL of live *Isochrysis galbana* culture daily, at the start of the experiment. After 2 weeks, the food ration was reduced to 2.5 mL a day, due to lower larval survival. A variety of microscopic marine creatures started to appear in the aquaria after day 7 infecting most of the containers, and may have reduced survivorship or increased larval stress. All aquaria for the OA NL and C L treatment had pests, while 3 out of 4 aquaria for C NL had pests, while no pests were documented in the OA L treatments (*n* =3).

2.2. Chemical and Physical Measurements

Daily measurements of salinity, temperature, and pH were taken from all 16 aquaria before their water change, to summarize the conditions in which the larvae had been reared for the last 24 hours. Daily measurements of salinity, temperature, and pH were also taken from the 2 jugs of pretreated seawater, to summarize the carbonate chemistry system before larval exposure. Measurements of total phosphate (TP), total silicate (TSi) and dissolved inorganic carbon (DIC) were measured on six "chemistry days" (Days 1, 3, 8, 9, 13, and 20) throughout the 24-day experiment (Table 1). These measurements were used to calculate the carbonate chemistry of the aquaria (CO_3^{2+}, HCO_3^-, CO_2) throughout the experiment using the CO2SYS program (Lewis and Wallace, 1998). Because TP, TSi and DIC are time-consuming methods, only two aquaria in each treatment were sampled on each chemistry day, except for October 12th

(Day 13) when all aquaria were sampled. In addition, on each chemistry day, both pretreated jugs of seawater were measured for TP, TSi and DIC before and after lime was added (Table 1).

Table 1: *Calendar of sampling activities throughout experiment (B - biological sampling, C - chemical sampling, X - removed biological data due to equipment failure)*

Treatment	Aquaria #	Day of Experiment																
		0	1	2	3	6	7	8	9	10	11	13	16	17	20	21	22	24
OA L	1	24 hr old larvae inoculated		X	C		X		C		X	C		X			X	X
C L	2			B	C		B		C		B	C		B	C		B	B
OA L	3			B	C		B		C		B	C		B	C		B	B
C L	4			B	C		B		C		B	C		B	C		B	B
OA NL	5			B	C		B		C		B	C		B	C		B	B
OA NL	6			B	C		B		C		B	C		B	C		B	B
C NL	7			B	C		B		C		B	C		B	C		B	B
C L	8		B/C			B		C		B		C	B			B		B
C NL	9		B/C					C		B		C	B			B		B
OA L	10		B/C			B		C		X		C	X			X		X
OA NL	11		B/C			B		C		X		C	X			X		X
C NL	12			B	C		B		C		B	C		B	C		B	B
C NL	13		B/C					C		B		C	B			B		B
OA NL	14		B/C			B		C		B		C	B			B		B
OA L	15		B/C			B		C		B		C	B			B		B
C L	16		B/C			B		C		B		C	B			B		B
Pretreated Jugs																		
Control Before Lime			C		C			C	C			C			C			
Control After Lime			C		C			C	C			C			C			
OA Before Lime			C		C			C	C			C			C			
OA After Lime			C		C			C	C			C			C			

DIC in seawater was measured using a UIC model CM5014 CO_2 Coulometer (version 3) following the Standard Operating Procedure (SOP) 2 from Dickson (2007). The pH and temperature were measured using an OAKTON acorn series 6 pH meter with a pH precision of ± 0.01, and temperature precision of 0.1 degrees Celsius. The pH probe was calibrated using NIST standards (pH 6.86 and 9.18) with a one point calibration on a daily basis, with an occasional two point calibration. Salinity was measured using an ATAGO® Refractometer (GENEQ inc.) with a precision of ± 2‰. TP and TSi were measured using a Hach spectrophotometer and standard colorimetric method (Grasshoff et al., 1999).

Estimates of seawater carbonate chemistry were obtained via the CO2SYS program in Excel 2007 (http://cdiac.ornl.gov/ftp/co2sys/**).** Using this program, carbonate

ion (CO_3^{2-}), bicarbonate ion (HCO_3^-), and CO_2 concentrations could be estimated using the measurements of salinity, temperature, TP, TSi, and two components of the carbonate system (in this case, DIC and pH). Saturation states with respect to calcite and aragonite were calculated using K1 and K2 constants from Dickson and Millero (1987), Dickson's $KHSO_4$ constants, and pH on the NBS scale (Zeebe and Wolf-Gladrow, 2005).

2.2.1. Measurement error and reproducibility

One replicate sample was taken every chemistry day from at least one aquarium for TP, TSi and DIC, following procedural guidelines that for every 12 samples a replicate should be taken (Dickson, 2007). The relative percent difference (RPD) between replicates was calculated for each measured and computed parameter. RPDs were expected to meet a measurement quality objective of less than or equal to 20% (Mitchell, 2006). The RPDs for computed carbonate chemistry parameters ranged from <1% to 14% between replicates, with an average difference of 4%. The RPDs for measured DIC replicates ranged from <1% to 15% between replicates, with an average difference of 5%. In most cases RPDs for measured TSi and TP replicates met with the measurement quality objective of 20% (Mitchell, 2006) with an average difference of 8% and 12%, respectively. In one case a TSi replicate had a difference of 22%, and in two cases TP replicates had differences of 25% and 32%. Because slight differences in TSi and TP concentrations in seawater do not have a strong impact on the relative concentration of carbonate constituents, the exceedance of 20% for TSi and TP were not considered a high concern for the results of these experiments. For example, even if TSi and TP concentrations are doubled (RPD of 67% from initial) the concentration of carbonate constituents do not change.

Two DIC standards of 500 μmol/L and a 2500 μmol/L DIC concentrations were prepared in the lab, following standard operating procedures (SOP14, Dickson, 2007). These standards were prepared the day prior to or the day of chemistry analysis and used for a maximum of four days after preparation. The measured 500 μmol/L DIC standard had an average percent error of 21% compared to the expected calculated DIC concentration. The measured 2500 μmol/L DIC standard had an average percent error of 8% compared to the expected calculated DIC concentration.

2.3. Biological Measurements

Survival rate, development stage, number of deformities, and shell growth were measured from each container over a two-day period six times throughout the experiment (Days 1/2, 6/7, 10/11, 16/17, 21/22 and 24, see Table 1). Two containers from each treatment were measured on one day, with the remaining containers measured the next day (Table 1). To concentrate larvae in a small volume of seawater for microscopic viewing, the inner tubes housing larvae (within their aquaria) were gently decanted and poured into a 150 ml beaker containing a small amount of seawater to prevent damaging shells during the pouring process. The procedure concentrated larvae into a smaller volume of seawater which was again filtered through a small 31 μm sieve submerged in a seawater filled beaker. Lifting the sieve from the beaker prevented damage that would otherwise occur if larvae were poured and filtered onto a sieve. Larvae were then concentrated within a small area within the sieve, allowing for small aliquots of seawater (with concentrated larvae) to be pipetted out and placed on a Petri dish for microscopic viewing.

Over a thirty minute period, each container was measured for larval survival (dead or alive), developmental stage (D-Stage, early veliger, early umbonate, and late umbonate), deformities (deformed or normal) and shell length under 100x magnification using a compound microscope. Photographs were taken with a digital camera of every larvae observed, through the microscope eyepiece. Shell lengths of live individuals were measured from these photos using Image J software at a later date. A 50 division scale was placed in the eyepiece of the microscope, and calibrated with a 1000 μm stage micrometer. Therefore, each photograph had a scale, and was scaled individually using Image J software prior to taking the shell length measurement.

Under ideal situations, shell length was measured parallel to the hinge at the widest point of the shell (Figure 3a). However, because larvae were free-swimming during biological measurements, this movement introduced some additional uncertainty into the shell length measurements. Photographs would often capture images of larvae swimming upright or in various other positions (Figure 3b, c). In cases where larvae were swimming upright in the photographs, measurements were taken across the shell (see Figure 3b). Additional measurement uncertainty was introduced when larvae would occasionally come to rest at the edge of a water droplet, or when images were blurry and shell edges hard to define. In order to determine the level of error of imperfect larval images, detailed information was collected from each photograph on whether larvae were in a **diagonal** position (disallowing proper 2D measure for the 3D reality), were located on a **water edge**, or if image was **blurry**. Diagonal, water edge, and blurry images had a percent error of 1 to 19% compared to photographs that had no observable error for shell length measurement. As well, no significant difference was

observed between images with underlying imperfections versus ideal photographs (ANOVA), therefore imperfect images were included in all shell length analysis.

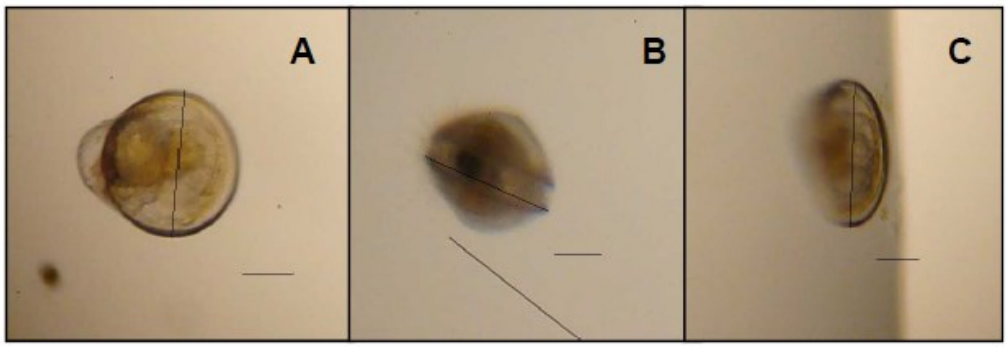

Figure 3: *Shell length measurements of 3 oyster larvae in various positions: A - Normal, B - Upright, C - Water Edge. Bottom right corner of each image contains a 50 μm scale bar.*

Detailed methodology for measuring larval abnormality is described in His et al. (1997), but requires larvae to be preserved in formalin before recording data. We were unable to completely follow this protocol, as it was necessary to keep the culture alive. Therefore, abnormality was measured subjectively by observing obvious shell irregularities of live individuals following deformities presented in His et al., 1997 (Figure 4).

The experiment continued until Day 24 (October 23rd) when survival reached 0 to 7 individuals per container (average 3.5 individuals), and the remaining larvae appeared to have stopped growing. On the final day of the experiment (October 23rd), the remaining larvae were filtered from each container and microscopically viewed and measured on a 31 μm mesh.

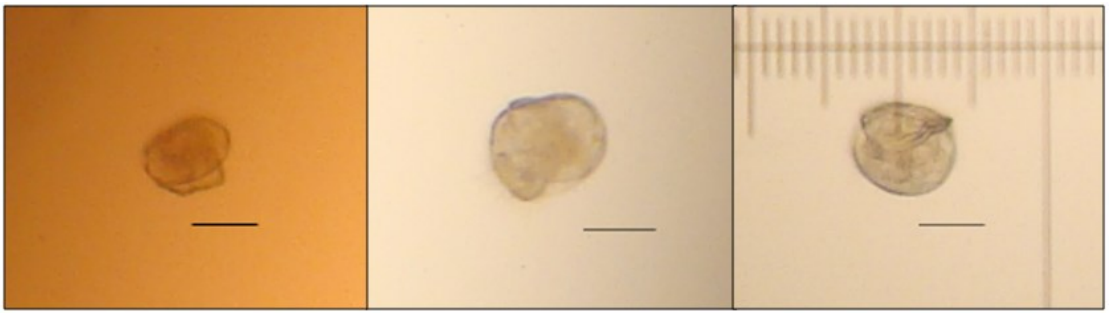

Figure 4: *Examples of obvious shell abnormalities. Bottom right corner of each image contains a 50 μm scale bar.*

2.4. Statistical Analysis

A Kruskal-Wallis one way ANOVA was used to compare differences in survival, developmental stage, and deformities between the four treatments. As shell length data were normally distributed and homoscedastic, an ANOVA was used to compare differences between treatments. Regression analysis was used to statistically compare biological measurements (e.g., shell length, survival, and deformities) with carbonate chemistry parameters (e.g., pH, alkalinity, Ω aragonite, CO_3^{2-}) for Days 1/2, 6/7, 10/11, 16/17, 21/22, and 24 when biological measurements were conducted. For the regression analysis, the chemistry results collected over the entire experiment for each container were averaged and used as the independent variable. Examination of carbonate chemistry over the six measurement days shows that the chemical parameters did not change substantially over time during the experiment within each container (Appendix).

3. Results

3.1. Chemistry

In both sets of lime addition treatments, adding hydrated lime to ocean acidified and present day seawater resulted in lower pCO_2 concentrations and increased values of pH, calcite and aragonite saturation, carbonate ion concentrations (CO_3^{2-}) and total alkalinity (Table 2; Figures 5-6). Specifically, lime addition reduced pCO_2 to pre-industrial levels, from 1229 ppm (OA NL) to 270 ppm (OA L) in the OA treatments and from 385 ppm (C NL) to 288 ppm (C L) in the control treatments (Figure 5a). The pH of ocean acidified seawater increased from 7.67 (OA NL) to 8.32 (OA L), and the control pH of 8.14 (C NL) increased to 8.25 (C L) due to the addition of lime (Figure 5b). Aragonite was undersaturated in the ocean acidification (OA NL) treatment (Ω 0.72) but supersaturated in all other treatments (C NL Ω 1.99, C L Ω 2.53, OA L Ω 3.29) (Figure 5f). Calcite was near saturation in the ocean acidified (OA NL) treatment (Ω 1.12) and reached highest values in the OA L treatment (Ω 5.14; Figure 5e). The concentration of free carbonate ions followed the same trend as aragonite and calcite saturation, ranging from 44 μmol/kg in the OA NL treatment to 200 μmol/kg in the limed ocean acidified (OA L) treatment (Figure 6a). Due to the small volumes of lime needed to increase the pH of present day seawater to pre-industrial conditions, total alkalinity only slightly increased from 1755 to 1795 μmol/kg due to the addition of Ca^{2+}. Alkalinity was lowest in the ocean acidification treatment (1666 μmol/kg) and highest in the limed ocean acidified (OA L) seawater (1987 μmol/kg) due to the larger amounts of lime needed to increase

the pH to pre-industrial levels (Figure 5c). The pCO_2 values for ocean acidification treatment only reached values 1229 ppm, despite bubbling with a 1500 ppm gas mixture (Figure 5a). Minor outgassing or lack of full equilibration between the seawater and carbon dioxide may be reasons for the lower than expected pCO_2.

Dissolved inorganic carbon (DIC) was relatively unchanged between C NL and C L treatments (1584 and 1573 μmol/kg, respectively; Figure 5d). Ocean acidified seawater had higher DIC concentrations due the increase of CO_2 in the system (1634 μmol/kg). DIC was substantially higher in the limed ocean acidified seawater (1707 μmol/kg), and it was later determined that the $Ca(OH)_2$ particles had absorbed CO_2 from the air, creating small amounts of $CaCO_3$ in the powder (Dr. Andrew Dickson, *personal communication* 2012). Through DIC analysis of the hydrated lime, we found between 3 to 7% of $Ca(OH)_2$ had converted to $CaCO_3$ depending on air exposure in the sealed container, which explains the increase of DIC for the OA L treatment.

In general, the measured and calculated chemical parameters from the pretreated jugs of seawater (before larval exposure) were in the same range as the chemistry of seawater sampled after larval exposure (for 24 hours). The OA L treatment was an exception, with the pretreated jugs of seawater having higher pCO_2 and lower carbonate ion concentration, pH, calcite and aragonite saturations than the OA L seawater the next day (after 24 hrs) (Figure 5-6). The difference in chemistry is due to the chemical conversion of $Ca(OH)_2$ into $CaCO_3$ over time, as it is a time limiting step that depends on temperature, availability of carbon dioxide, and the particle size of lime (Locke et al., 2009). Although the chemistry variables were sampled thirty minutes after the lime had been added to the seawater, these results indicate that more time was needed for the carbonate system to reach equilibrium with the lime.

Table 2: *Physical and chemical variables within each treatment (mean ± SE), measured from aquaria and pretreated jugs.*

Measured Variables	Treatment			
	Control, No Lime C-NL (*n = 20*)	Control, Lime C-L (*n = 23*)	Ocean Acidified, No Lime OA-NL (*n = 21*)	Ocean Acidified, Lime OA-L (*n = 21*)
Temperature (°C)	24.5 ± 0.2	24.6 ± 0.1	24.5 ± 0.2	24.5 ± 0.1
Salinity (o/oo)	26.1 ± 0.1	26.1 ± 0.1	25.9 ± 0.1	26.0 ± 0.2
pH	8.13 ± 0.01	8.25 ± 0.01	7.67 ± 0.01	8.32 ± 0.03
DIC (µmol kg^{-1})	1583 ± 17	1573 ± 14	1634 ± 28	1707 ± 26
Total Phosphate (µmol kg^{-1})	1.89 ± 0.10	1.80 ± 0.04	1.83 ± 0.11	1.70 ± 0.10
Total Silicate (µmol kg^{-1})	14.46 ± 0.27	13.84 ± 0.39	14.25 ± 0.21	13.40 ± 0.41
Computed Variables				
pCO_2 (ppm)	386 ± 8	288 ± 5	1229 ± 44	270 ± 18
Total Alkalinity (µmol kg^{-1})	1754 ± 19	1795 ± 16	1666 ± 26	1987 ± 37
Ω Aragonite	1.99 ± 0.05	2.53 ± 0.04	0.72 ± 0.02	3.29 ± 0.22
Ω Calcite	3.11 ± 0.07	3.96 ± 0.07	1.13 ± 0.03	5.14 ± 0.34
$[CO_3^{2-}]$ (µmol kg^{-1})	121 ± 3	154 ± 3	44 ± 1	200 ± 13
$[HCO_3^-]$ (µmol kg^{-1})	67 ± 4	58 ± 5	121 ± 5	104 ± 5
$[CO_2]$ (µmol kg^{-1})	11.60 ± 0.25	8.62 ± 0.16	37.05 ± 1.38	8.11 ± 0.56

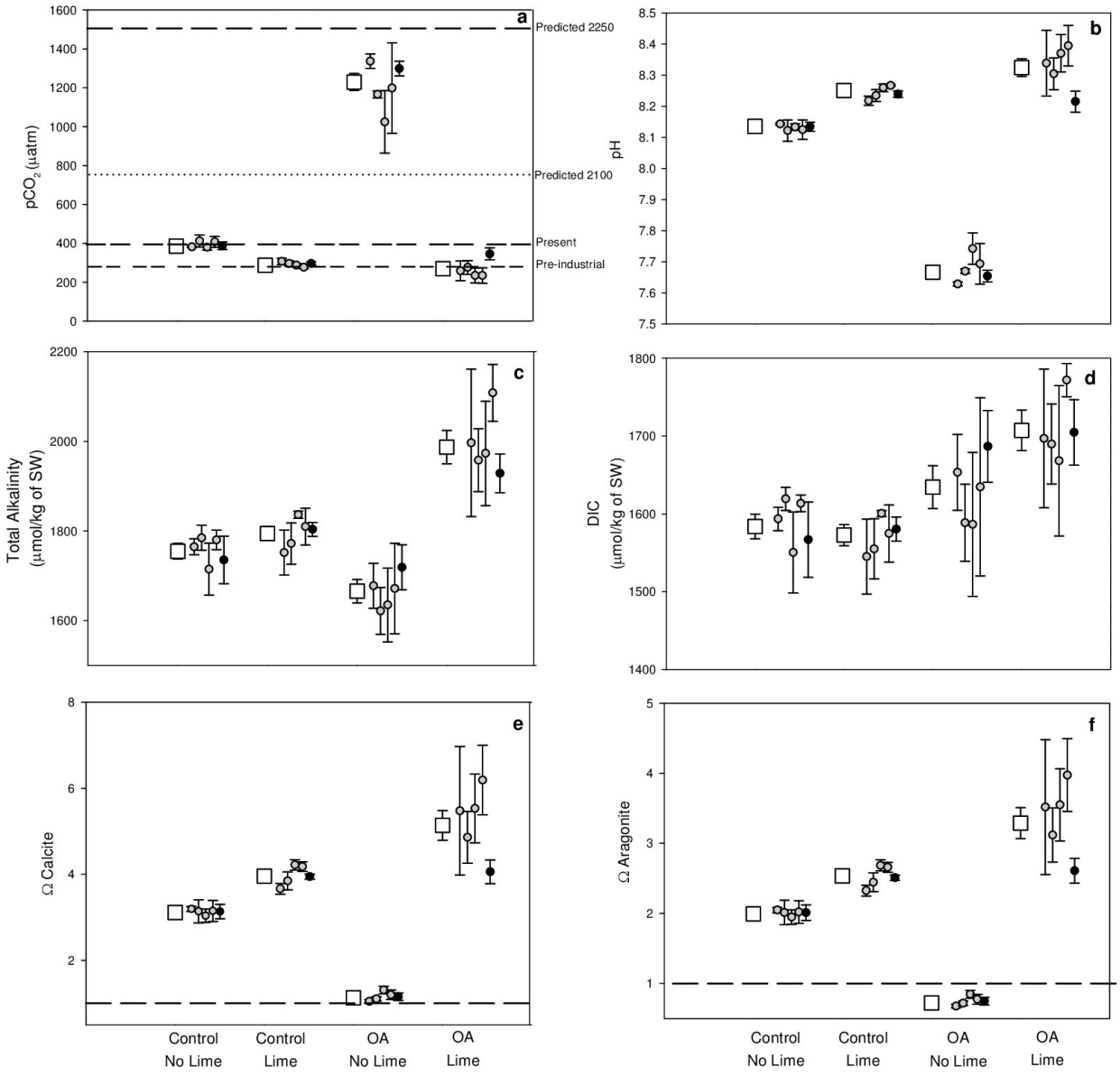

Figure 5: Chemistry variables per treatment (□ symbol, ± SE), and per container (○ symbol, mean ± SE). Black circles are concentrations of pre-treated jugs before larvae exposure. Grey circles are concentrations in replicated containers (n = 4) after larvae exposure for one day.

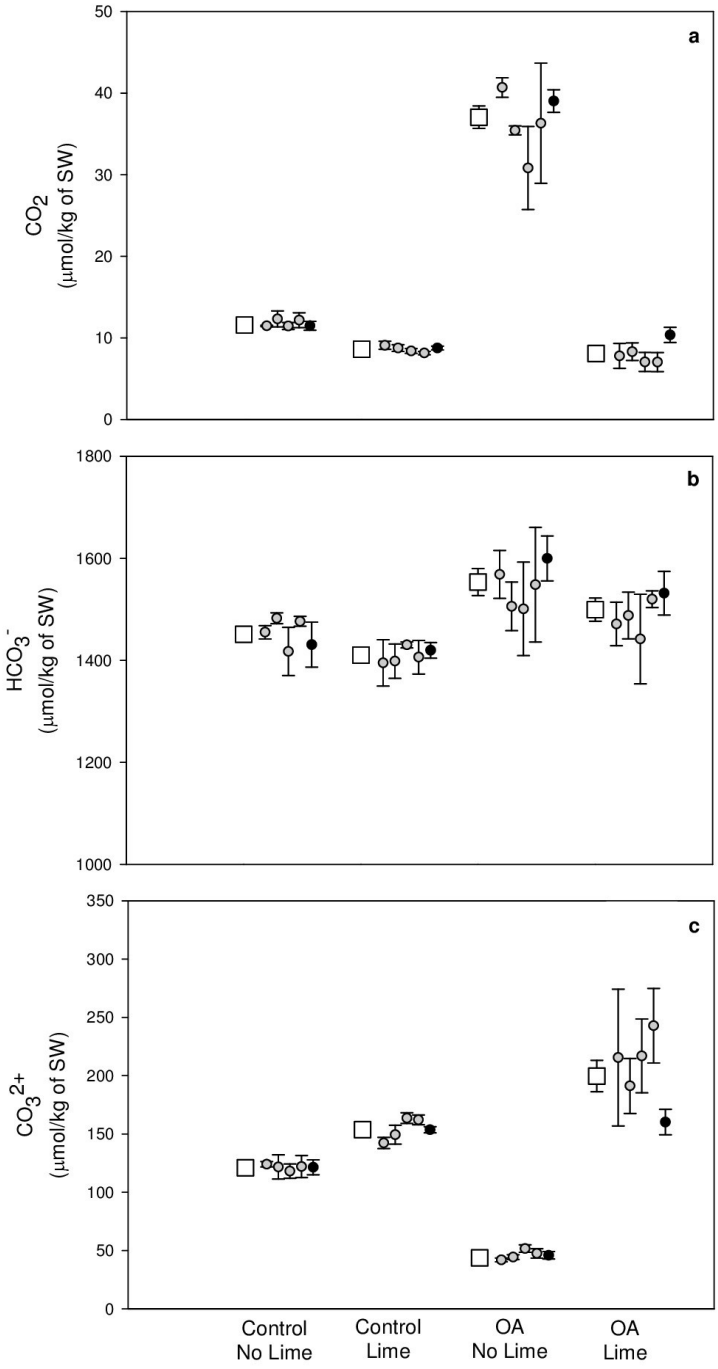

Figure 6: *Carbonate parameters per treatment (□ symbol, ± SE), and per container (○ symbol, mean ± SE). Black circles are concentrations of pre-treated jugs before larvae exposure. Grey circles are concentrations in replicated containers (n = 4) after larvae exposure for one day.*

3.2. Biological Response

Larval shell lengths were increased significantly after two weeks of growth in the ocean acidified seawater treatment enhanced with lime (OA L). When fitted with a Michaelis-Menton function, shell length versus CO_3^{2-} concentration had a significant correlation on Day 17 ($r^2 = 0.49$, $p < 0.01$), Day 22 ($r^2 = 0.53$, $p<0.05$), and on the final day of the experiment Day 24 ($r^2 = 0.57$, $p < 0.01$) (Figure 7). Very similar results were observed between shell length and aragonite and calcite saturation, as both are strongly correlated with CO_3^{2-} concentration ($r^2 = 0.99$, p value < 0.0001). No significant relationships were observed between shell length versus DIC, HCO_3^-, or total alkalinity.

Scatterplots of shell length versus pH, pCO_2 and CO_2 (aq) showed two distinct groups (group 1: OA L, C L, and C NL, and group 2: OA NL) though no statistically significant relationships were observed between shell length and these chemical parameters (Figure 8, as an example). When ANOVAs are used to compare shell lengths between the four treatments as categorical variables, the only significant result is observed for the last day of the experiment, with oyster larvae from the control group (C NL) having significantly longer shells than oyster larvae from the ocean acidified group (OA NL) ($p < 0.05$).

No significant differences in oyster larval survival, development stage, and abnormality were found between any of the four treatments throughout the experiment. Average survival rate throughout the experiment was low, with approximately 50% of the larvae surviving within each container from the previous day of measurement (Figure 9). An estimated 7000 larvae were inoculated in each container at the beginning of the

experiment, and only approximately 1% survived over the first 48 hours. Therefore, the instantaneous mortality rate ($M = \ln(N_o/N_t)/-t$) was extremely high over this period (inoculation to D-stage veligers) ranging from -1.85 $days^{-1}$ (C NL) to -2.04 $days^{-1}$ (OA L). Yet, the instantaneous mortality rates calculated for the duration of the experiment (Days 1 to 24, veliger to late umbonate) had similar values to those determined by Quayle (1964) for *C. gigas* (veliger to spat, 18 days) of -0.26 $days^{-1}$, with -0.11 $days^{-1}$ (OA NL) to 0.21 $days^{-1}$ (C L) (Rumrill, 1990). Furthermore, the mortality rates between treatments were similar, implying that lime addition was not the cause for the high mortality, but was due to some other factor felt across treatments. Nonetheless, due to the high mortality rates throughout the experiment, interpretation of the results must be done with caution.

In all treatments, larvae developed into early veliger larvae within the first week, and developed into early and late umbonate stages over the course of the final two weeks. Larvae in the ocean acidification treatment appeared to have trouble growing after day 17 with most larvae staying in the early umbonate stage, though no significant difference was observed. Abnormalities were more apparent in the very beginning of the experiment for all treatments, with approximately a quarter of the surviving population becoming deformed (Figure 10). Overtime, deformed larvae died, with only 3% of the abnormal larvae surviving to day 17, and no deformed larvae observed at the end of the experiment.

Detection of dead larvae during biological measurements was significantly lower in the OA NL treatment compared to the C L treatment on day 7 ($p < 0.05$), and the C NL treatment on day 11 ($p < 0.05$). No other significant relationships were observed between treatments for the count of dead larval shell.

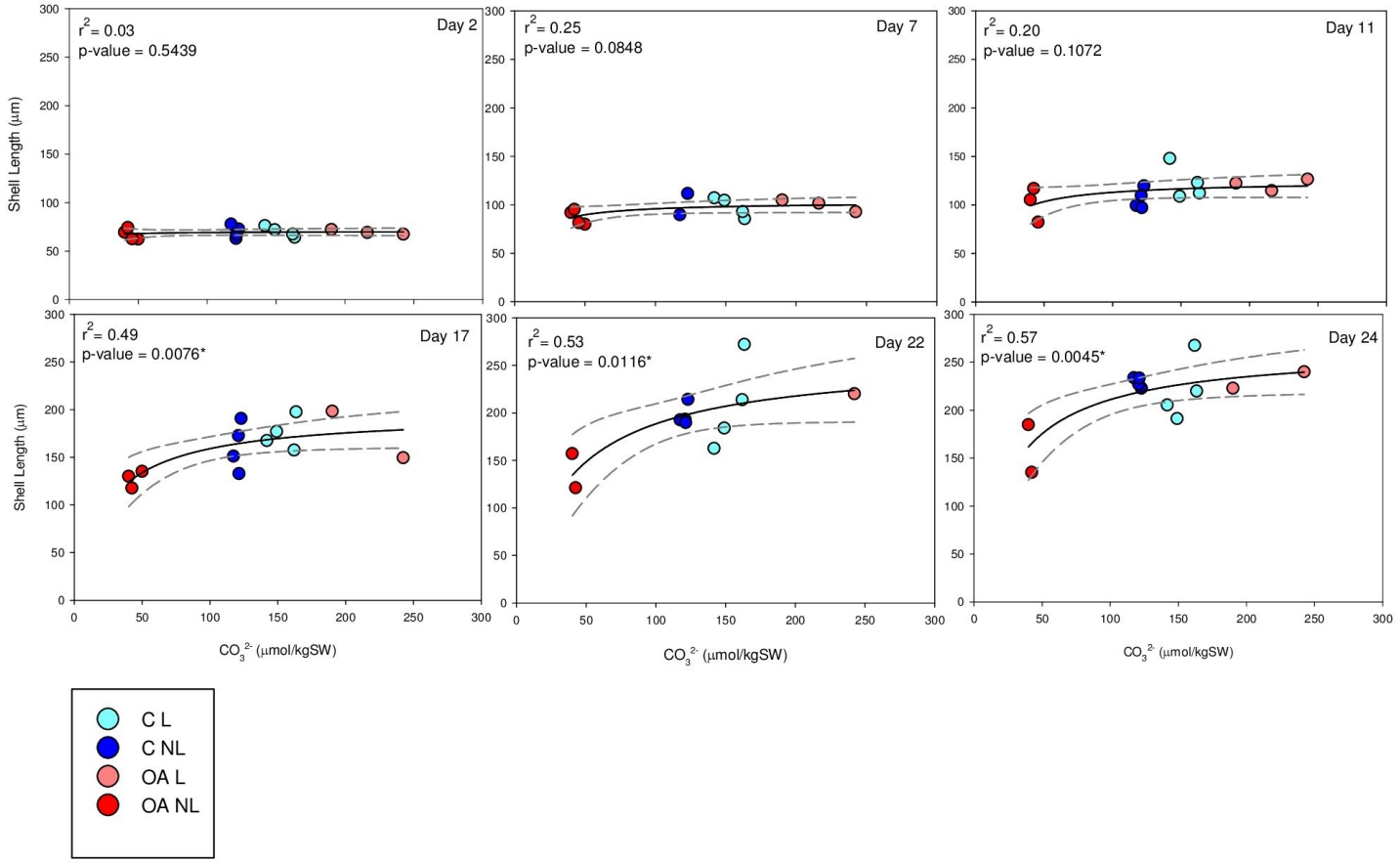

***Figure 7**: Shell length versus carbonate ion concentration, fitted with Michaelis-Menton equation and 95% confidence interval (grey dashed lines). Each circle represents the average shell length and carbonate ion concentration per container.*

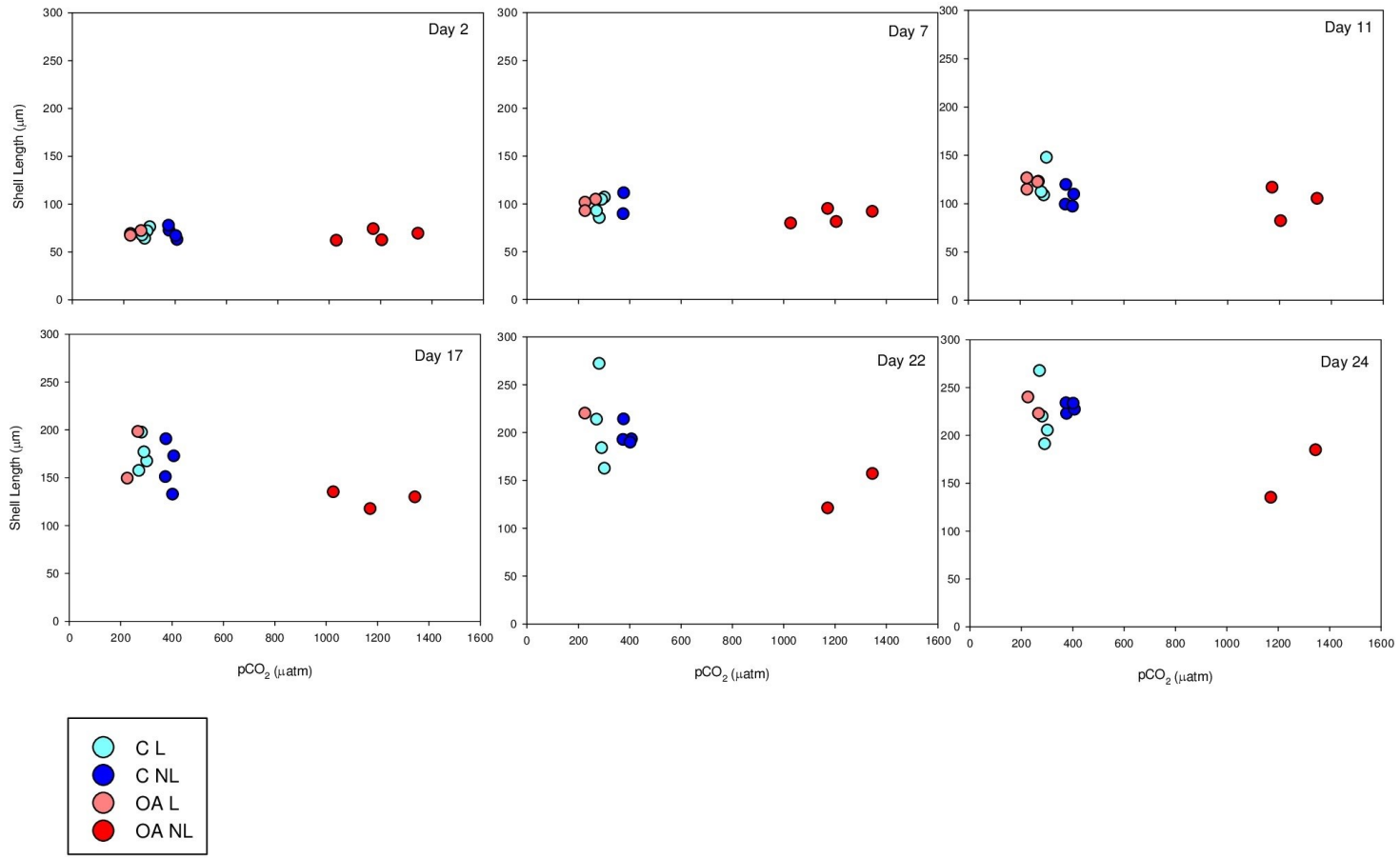

***Figure 8**: Shell length versus pCO_2 (ppm). No observable fit, but two distinct groups noticeable. Each circle represents the average shell length and pCO_2 (ppm).*

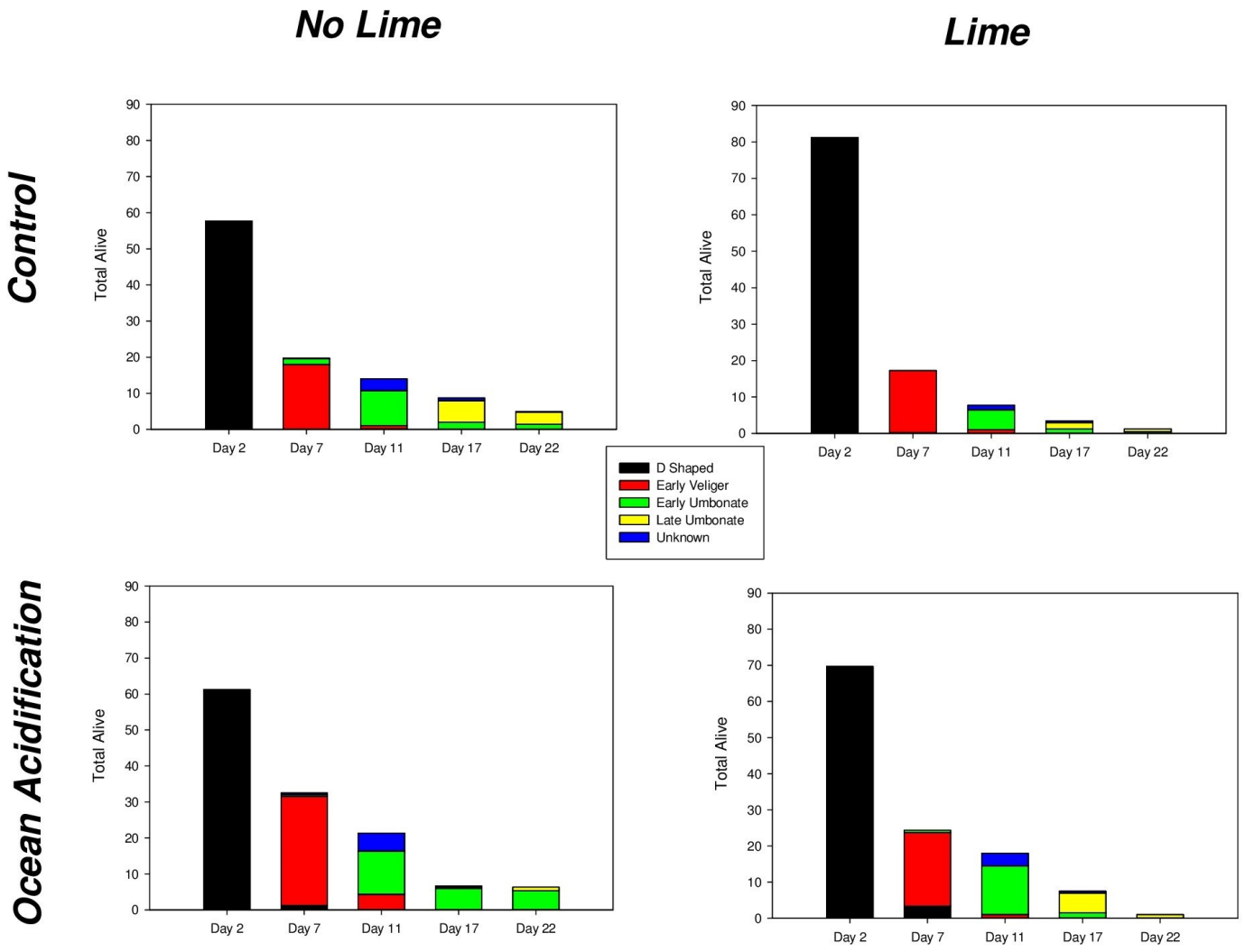

***Figure 9**: Total alive larvae (including normal and deformed), values averaged over all replicates per treatment.*

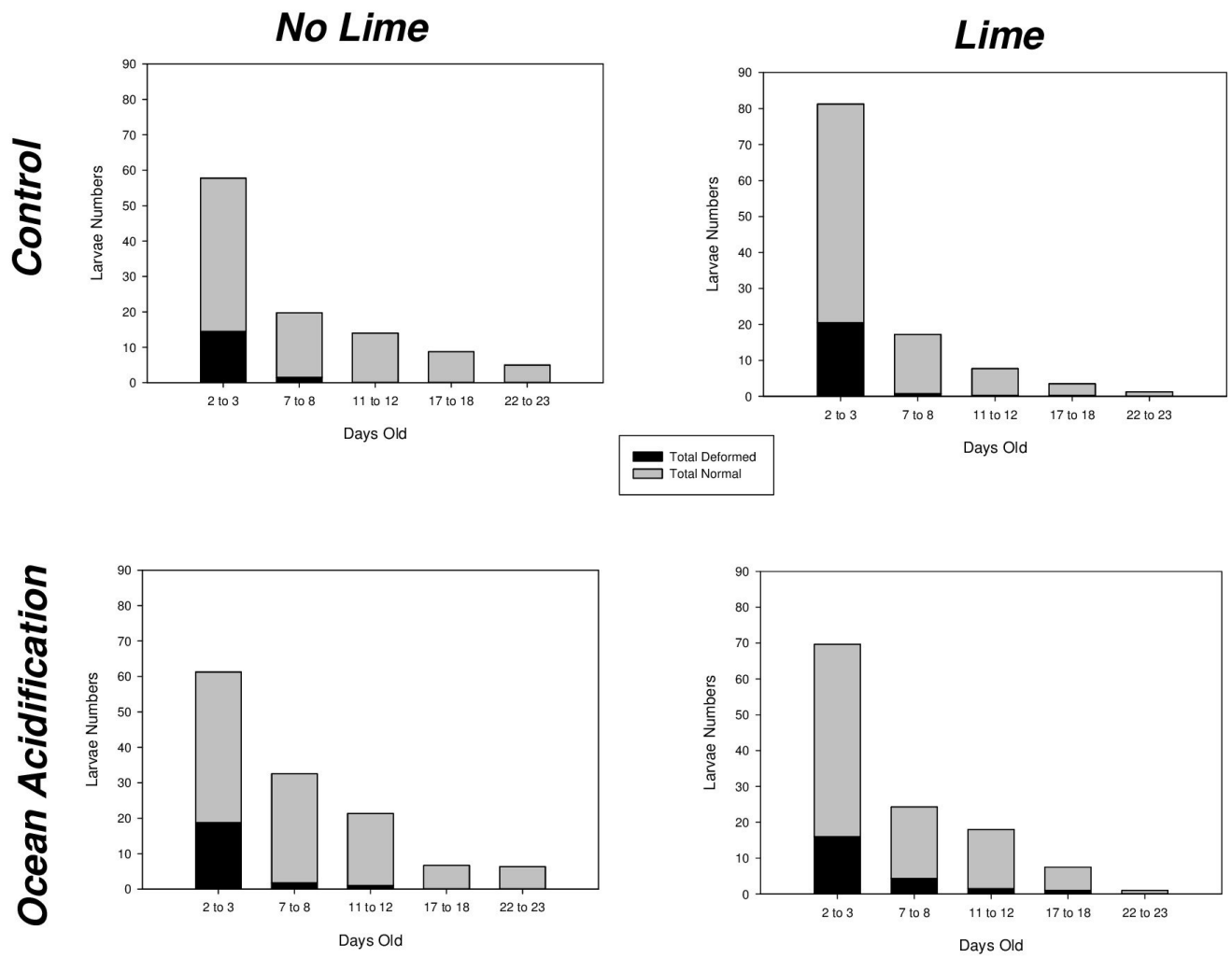

Figure 10: *Total normal and deformed larvae, values averaged over all replicates per treatment.*

4. Discussion

4.1. Explanation of Results

As expected, the results from our chemical analysis of samples suggest that hydrated lime can be used to chemically reverse ocean acidification to pre-industrial conditions in laboratory cultures. Overall, lime addition lowered pCO_2, and increased pH, concentration of carbonate ions, and the saturation of aragonite and calcite, while increasing the total alkalinity of the system. In response, *C. gigas* larvae grew significantly longer (anterior-posterior) in the limed and control treatments versus the ocean acidification treatment, after two weeks of development.

Our results seem to support previous research with *C. gigas* larvae suggesting that increases in carbonate ion concentrations drive enhanced growth (Gazeau et al., 2011). Gazeau et al. (2011) demonstrated that shell lengths of *C. gigas* larvae were highly correlated with carbonate ion concentrations, but not pH or aragonite saturation. Our results are similar to those of Gazeau et al. (2011), but also suggest that shell length is correlated to aragonite and calcite saturations. However, these correlations may be an artifact of the high correlation between carbonate ion concentration and aragonite and calcite saturation states of seawater, whereas Gazeau et al. (2011) avoided carbonate to aragonite/calcite correlations by adjusting alkalinity with a strong acid (HCl) and salts ($CaCl_2$ and $NaHCO_3$) in various treatments.

Compared to other laboratory studies, our results showed a delayed response of *C. gigas* shell length to ocean acidification, as no statistically significant impact of ocean

acidification occurred until after two weeks of development (into the umbonate stage). Many small scale laboratory experiments on *C. gigas* larvae (tanks < 5 L) show ocean acidification significantly impacting shell length within the first couple days of development, at the D-Stage (Kurihara et al., 2007, Kurihara et al., 2008, Gazeau et al., 2011, and Parker et al., 2010). In contrast, our results are more in line with those of large scale studies (tanks > 450 L), where *C. gigas* were reared at the hatchery level (e,g, Barton et al., 2012; Dineshram et al., 2012). Barton et al., (2012) monitored seawater carbonate chemistry and *C.gigas* biological responses at an Oregon-based shellfish hatchery in Summer, 2009. They found that larval shell length only showed signs of decreased growth, due to aragonite undersaturation, upon entry into their midstage growth (~120 to 150 μm), or the umbonate stage (Barton et al., 2012). The authors concluded that the larvae may have been surviving on their egg yolk reserves, which can provide nutrition up to the midgrowth stage and thereby provide the energy to grow shell even in suboptimal conditions (Barton et al., 2012, Khedar et al., 2010a, and Rico-Villa et al., 2009). Similarly, Dineshram et al. (2012) conducted a large scale experiment and found that shell length of *C. gigas* larvae was not hindered by lower pH levels until after day four of the experiment (Dineshram et al., 2012). The authors propose that the oyster larvae may enter into a preparatory phase for further calcification growth after four days, at which point shell growth is affected by surrounding conditions (Dineshram et al., 2012).

In the present study, the delayed response of shell length to the OA treatment may be due to larvae surviving on their yolk sac. However, another explanation could be that larvae were not inoculated into their treatments until after some shell development occurred, due to the unexpected spawning event that was capitalized on at the time.

Other recent work has suggested that the timing of initiation of the experiment had a significant influence on *C. gigas* shell length (Parker et al., 2010). In this study, gametes fertilized in ambient conditions and later inoculated into ocean acidification conditions were larger than those fertilized in ocean acidified conditions (Parker et al., 2010).

The later inoculation of larvae may also explain why no differences in the proportion of abnormalities were observed between treatments. At the beginning of the experiment, approximately 25% of the larvae were deformed in each treatment, and this 25% subsequently died off completely over the duration of the experiment. In contrast, two small-scale studies showed increased % abnormalities of *C. gigas* larvae under ocean acidified conditions within the first three days of development (Kurihara 2007, Parker et al., 2010). Kurihara et al. (2007) reported that only 5% of D-Stage larvae were normal in ocean acidified conditions, compared to 68% in control conditions. Parker et al. (2010) found significant increases in % abnormality with increasing levels of pCO_2. The timing of inoculation into treatments has also been shown to affect the percentage of abnormalities that occur throughout the ocean acidification experiments (Parker et al., 2010). Gametes that were directly fertilized under ocean acidified conditions were on average 13% more abnormal than gametes that were inoculated into the ocean acidification treatment thirty minutes after fertilization in ambient conditions (Parker et al., 2010). In our experiment, larvae spent at least 24 hours in ambient conditions before inoculation into treatments. Given that a mere thirty-minute difference in treatment exposure can influence the rate of abnormality formation, it is quite possible that rearing larvae for at least 24 hrs in ambient conditions prior to ocean acidification exposure would result in similar levels of deformities across all treatments. We note, however, that a 24-hour holding period in other experiments with shellfish larvae (*Mercenaria*

mercenaria, Argopecten irradians, and *Crassostrea virginica*) did not result in shell development before inoculation into treatments (Talmage et al., 2009). However, % abnormality was not measured to compare with our results (Talmage et al., 2009). Thus, it is recommended that future OA research on shellfish larvae should start at the fertilization stage to avoid confounding effects of untreated seawater on larvae development throughout the experiment.

Overall, survival rate in the present study was extremely low at 1% of initial density, a survival rate that is similar to expectations for wild populations (Gazeau et al., 2011). The mortality rate was extremely high over the first 48 hrs, and may be due to the inoculation process, as gametes were pipetted through a tube into the aquaria in order to not disturb the carbonate chemistry, and this could have damaged embryos. Those surviving the first 48 hour had similar instantaneous mortality rates as measured for *C. gigas* by Quayle (1964). Yet, in general, the level of survivorship was much lower than what was presented in Talmage et al. (2009), who followed stricter water filtration guidelines and used antibiotics.

Unfortunately, it is not possible to compare our mortality rates with many studies, as most were interested in growth and % abnormality, but not survivorship, and therefore killed and preserved larvae prior to taking measurements (Gazeau et al., 2011, Kurihara et al., 2007, Parker et al., 2012). While it is possible that an unknown water quality issue or pests may have affected this experiment, we note that the sample specimens from all treatments moved through development stages in a manner consistent with ranges presented in the FAO's manual on *Hatchery Culture of Bivalves* (FAO, 2004). The one exception is that larvae appeared to stop growing after three weeks, never reaching the pediveliger stage. To our knowledge, no study has looked at whether *C. gigas* larval

development is delayed at elevated pCO_2. However, this type of delayed development would be consistent with what has been observed for larval stages of the eastern oyster (*Crassostrea virginica*), hard clam (*Mercenaria mercenaria*) and bay scallop (*Argopecten irradians*) and sydney rock oyster (*Saccostrea glomerata*) (Talmage et al., 2009; Parker et al., 2010).

Timing of inoculation may also explain why differences were not observed between treatments for survival for *C. gigas* larvae. However, relating our results to other studies is complicated because the few studies that have looked specifically at *C. gigas* survival in ocean acidified conditions draw somewhat contradictory conclusions. Contrary to our results, Parker et al. (2010) observed a reduction in the number of viable D-Stage *C. gigas* larvae with increasing pCO_2 conditions (Parker et al., 2010). Similar to our results, Gazeau et al. (2011) found that the numbers of viable D-Stage *C. gigas* larvae were not significantly different between the control and ocean acidified conditions with chemical parameters similar to those in our experiments. The one exception occurred when alkalinity was drastically reduced from approximately 2440 μmol/kg to 1094 μmol/kg to draw down carbonate ion concentrations, in which only 19% of D-Stage larvae were viable, significantly lower than the 90% found in all other treatments (Gazeau et al., 2011).

4.2. Implications for Pacific Northwest Shellfish Aquaculture

Our results are basically consistent with Gazeau et al. (2011), who conclude that negative impacts to *C. gigas* larvae may only occur when carbonate ion concentrations are reduced below the aragonite saturation level. This finding could be cause for

concern for the shellfish aquaculture industry using non-native Pacific Oysters for commercial distribution in the Pacific Northwest (Barton et al., 2012). Given that the North Pacific Ocean already holds some of the most acidic waters in the global ocean, future increases in atmospheric CO_2 concentrations could reduce aragonite saturation to critical levels for these commercial populations (Fisheries and Oceans Sciences Canada, 2008). At present, the global surface ocean has already lost approximately 16% of carbonate ions due to human activities over the last 200 years (Barton et al., 2012, Doney et al., 2009), and time-series data show that the North Pacific Ocean surface waters are already losing 0.34% aragonite saturation per year on average. Furthermore, an upward migration of the saturation horizon depth of one to two metres a year is being observed in the North Pacific Ocean, primarily due to CO_2 uptake from anthropogenic activities (Feely et al., 2012). If CO_2 emissions continue as projected over the next century, model simulations predict an additional 50% drop in carbonate ion concentrations in the global surface ocean, which would likely amplify the current problems in the North Pacific Ocean (Feely et al., 2012, Kleypas et al., 2006).

One caveat of these predicted changes to ocean chemistry is that they are derived from open ocean observations, and extrapolating these changes to coastal oceans where oyster populations actually live is somewhat difficult. Predicting climate change impacts in coastal environments is difficult because spatial and temporal variability is high due to the complexity of physical and biogeochemical processes (Wootton et al., 2008; Feely et al., 2012). Yet, some studies have demonstrated that the highly dynamic coastal ecosystems in the Pacific Northwest may enhance the effects of ocean acidification such as upwelling of deep CO_2-rich waters, acidic freshwater

discharge from rivers, and proximity to human activities that results in nutrient and strong acid/base pollution (Doney et al., 2007; Feely et al., 2010; Salisbury et al., 2008).

In relation to our study, carbonate chemistry measurements collected from the surface waters of the Puget Sound, Washington in August 2008 had similar values to our experimental results, with pH ranging from 7.77 to 8.25 and aragonite saturation from 1.01 to 2.79 (Feely et al., 2010). Furthermore, recent modeling and field work in the Strait of Georgia have already measured intermittent events with pH values below 8 and associated aragonite undersaturation in summer months since 2007 when data collection began (Moore-Maley et al., personal communication, 2013). These changes are likely the result of low-alkalinity inputs associated with the Fraser River freshet. The fact that aragonite undersaturation events are already occurring here suggest that carbonate ion concentrations will continue to dip below aragonite saturation levels in the Pacific Northwest in the future. Given that the summer months are when Pacific Oysters and many other shellfish species spawn (FAO, 2004), these future low-saturation events could have serious consequences for larval development associated with both the natural shellfish populations and the local shellfish aquaculture industry.

4.3. Usefulness of Lime Addition to Shellfish Hatcheries

Though further research is required, this study suggests that lime addition may prove beneficial to shellfish hatcheries under future, high-CO_2 emissions scenarios because it will help to enhance the size, survivability and energy efficiency of the growing stock. First, this study demonstrated that lime addition enhanced the growth of *C. gigas* larvae, and the size of larvae influences their survivability and the time required to reach market size (Parker et al., 2010). Smaller larvae are more prone to starvation

because they encounter and clear less food from the water column than larger larvae (Parker et al., 2010, Kurihara et al., 2007 Hart et al., 1995). Second, the shell protects larvae from predation, provides buoyancy control, and helps regulate internal pH, which can all be impacted from weaker and smaller shell sizes due to ocean acidification and therefore potentially counteracted via lime (Kurihara, 2008). Third, the physiological constraints on building shell material is energetically costly, possibly consuming three quarters of the total energy needed for somatic growth (Lannig et al., 2010). Thus, conditions with higher aragonite saturations are more energetically favorable because biomineralization involves active intracellular regulation of inorganic carbon, Ca^{2+} and H^{+} (Williamson and Turley, 2012). Furthermore, early oyster larval stages are known to use amorphous calcium carbonate (ACC) at the beginning stages of shell development, which is thirty times more soluble than the aragonite or calcite that becomes more prevalent in the shell composition at later stages (Ross et al., 2011). Maintaining the deposited shell from dissolution and erosion also consumes energy (Lannig et al., 2010). Kurihara et al. (2007) argue that dissolution may play as much as a role as synthesis for the limited growth observed in early larval stages under ocean acidified conditions. As observed in our study, the reduced count of dead larval shell in the ocean acidification treatment may have been due to shell dissolution in the undersaturated conditions. Researchers have yet to observe a compensatory mechanism to counteract ocean acidification in oyster larvae or other early life stages of (Dineshram et al., 2012), but the addition of lime in this study suggests a means by which the negative effects of OA can be counteracted. Thus, addressing the effects of ocean acidification on early larval stages is of crucial importance to the shellfish industry.

Using lime to maintain the shell length of early larval stages may have positive impacts on later post settlement stages of bivalve cultures. For example, when juvenile bivalve *M. mercenaria* were reared in sediments undersaturated with aragonite, those with reduced size had significantly higher mortality rates than larger individuals (Green et al., 2009). Given that this study suggests that lime addition produces larger C. gigas larvae, juvenile C. gigas may experience similar benefits during out-planting if the larval stages are raised in hatcheries that buffered with lime addition. However, this idea remains to be tested with future experiments.

Hatcheries may also consider using lime to enhance growth of later stages of shellfish, as elevated pCO_2 concentrations have been observed to reduce the size of *C. gigas* pediveliger larvae, spat (post settlement) and adult oysters (Parker et al., 2010; Kurihara 2008; Gazeau et al., 2007). In another example, mudflats buffered with crushed *Mya arenia* shells ($CaCO_3$) had three times the recruitment of juvenile bivalves than unbuffered plots, even though sediment aragonite saturation only increased from 0.25 to 0.53 due to the addition of crushed shell material (Green et al., 2009). Though the use hydrated lime ($Ca(OH)_2$) outside of hatchery operations requires further study to investigate the potential toxicological impacts on phytoplankton and benthic organisms, sectors of the aquaculture industry that rear spat to adult stages on the foreshore may consider adding crushed shells to the sediment to enhance the survival of their stock under future ocean acidified conditions. Often, whole or chipped oyster shells are already used as settlement substrates for oyster larvae, and generally aquaculture sites have an abundant supply of left-over shell material from harvested stock which could be used to buffer foreshore sediments with $CaCO_3$, and at a very low cost as only machinery to grind the shells into powder would be required (FAO, 2004, and Green et

al., 2009). As there has been only one study proving the positive use of powdered $CaCO_3$ on developing juvenile shellfish growing in the foreshore, it is recommended that shellfish aquaculturists interested in this technique conduct further studies to determine its usefulness.

A new contribution of this study is its analysis of any detrimental impacts on larvae in response to mitigative lime addition. These results suggest that adding hydrated lime did not have any detrimental impacts on larval survival or level of deformities. In fact, the addition of hydrated lime resulted in an overall enhanced growth of larvae, most likely due to the effect lime addition has on elevating the carbonate ion concentrations of the treatments (Gazeau et al., 2011). Although unlikely, there is a chance that late inoculation of *C. gigas* to their treatments may have reduced any observable toxicological response from the lime addition, as it may have done for ocean acidification.

4.4. Feasibility of lime use for hatcheries

Hydrated lime is already used in the shellfish aquaculture industry on the east coast of Canada, as a relatively environmentally benign means to control fouling of equipment by invasive tunicates (Locke et al., 2009, and Reebs et al., 2011). Hydrated lime is not a registered pesticide in Canada, and the practice of using it for tunicate control in the vicinity of cultured species has been deemed safe for human consumption (Locke et al., 2009). Furthermore, a recent west coast study showed no toxic effects for adult pacific oysters when exposed to very high levels (4%) of hydrated lime for short periods of time (5 mins) for the removal of invasive tunicates (Rolheiser et al., 2012) However, further investigations would be needed to assess its safety at the hatchery

level. Because hydrated lime is already used in the aquaculture industry and has been assessed as relatively safe, the practice of using it at the hatchery level to enhance larval stock under future ocean acidified conditions makes the proposition of using lime seem very feasible. In fact, Taylor Shellfish Farms is already using sodium carbonate to treat acidic seawater with the addition of carbonate ions with demonstrated success, though sodium carbonate is less abundant and more expensive than hydrated lime (Eudeline and Sehaffnit, 2012; Rau, 2009; Kheshgi, 1995).

Economically, the use of hydrated lime should be cost effective for hatcheries, as very small amounts of lime are needed to chemically reverse ocean acidification. For example, in the present study, approximately 94 mg of lime was needed to increase the pH from 7.67 (OA NL) to 8.32 (OA L), in one gallon of seawater. Thus, treating 100,000 gallons of seawater a day would require approximately 9.4 kg of lime a day, at a cost of approximately five dollars or $2000 a year (given the current price of a 20 kg bag of hydrated lime at ten dollars). These costs are extremely low in comparison to the predicted annual losses to the aquaculture industry by 2060 of upwards of $599 million (Cooley and Doney, 2009). Our financial estimate is a very simplified calculation of the expected costs, as the amount of lime used depends largely on the seawater temperature, carbon dioxide concentration, particle size of the lime, and the amount of water that is treated (Locke et al., 2009). Yet, for our experiments, we reared oyster larvae at temperatures typically used in hatcheries (20 to 25 ^{o}C) and dissolved CO_2 levels expected from future scenarios, in order to test the feasibility of using fine powdered lime for legitimate hatchery conditions.

Some additional practical concerns may still need to be addressed to facilitate use of hydrated lime in aquaculture. Hatcheries may have to consider allocating funds

for increased filtration of lime treated seawater, as precipitates of carbonates are produced during the chemical reaction, and could build up like a plaque in a hatchery's flow-through systems. Another potential cost is the impact lime may have on phytoplankton used to feed shellfish larvae, as it could pose a toxicological impact to the food source at the time of feeding. Both of these concerns would have to be addressed through exploratory research at the hatchery, before the use of lime is implemented.

Government regulations may require hatcheries to conduct an environmental impact assessment before allowing the use of lime at the hatchery level, as wastewater pumped out of the hatchery will have increased alkalinity and could have negative impacts to the local marine environment. Little research has been conducted on the environmental impacts of using lime (Locke et al., 2009). However, when lime addition has been used for tunicate control on the east coast, field observations suggest that the addition of seawater with pH values of 12 have not produced negative responses in marine organisms, even when they were engulfed by the cloud of carbonate precipitates (Locke et al., 2009). The dumping of this high-pH water produced a limited pH footprint of approximately 1m around the dumping site, and the pH quickly reduced to a pH of 8.3 to 9.0 (Locke et al., 2009).

The pH values of lime-filled waste water that is already permitted for dumping in east coast estuaries far exceed the pH values anticipated from mitigative lime addition required to assist with hatchery growth. This implies that hatcheries may not need to treat lime-treated hatchery waste water. Due to the large quantities that may be emitted into the marine environment, the aquaculture industry would have to at least ensure that the waste water pH does not exceed the government guidelines (for example, pH cannot be ≥ 8.7 in marine environment under CCME guidelines, 1999). However, in our

experiments, ocean acidified seawater enhanced with lime fell within natural oceanic conditions and did not exceed the CCME pH guideline, and therefore may not be a huge impact. Furthermore, in well protected harbours where water movement is stagnant and sediments accumulate, precipitated carbonates that are not previously filtered from the waste water may build up on the sediment floor and could produce behavioral responses from benthic organisms. One such response has been observed in sand shrimp which avoid lime covered sediments in laboratory studies (Reebs et al., 2011). These precipitates could also have unanticipated toxic effects. For example, quicklime (CaO) has been observed to create lesions on the surface of seastars (Locke et al., 2009, Loosanoff et al., 1942). While quicklime is known to have more toxic properties than hydrated lime (Locke et al., 2009), these types of adverse effects from precipitates require further investigation prior to implementation of hydrated lime addition.

4.5. Other strategies to investigate

Strategies that could assist hatcheries in their adaptation to future issues with ocean acidification fall into two categories: (a) investing in technologies that adjust the carbonate chemistry of seawater to maintain growth of existing shellfish breeds and species, or (b) investing in research on shellfish adaptability to facilitate selection of shellfish populations that are genetically better able to cope with the combined stresses associated with ocean acidification and climate change. The present study has focused on one method of altering the chemical environment of aquaculture systems, but others may exist. Other approaches may involve reducing the negative impacts of CO_2 on broodstock by employing degassing technologies that strip CO_2 from seawater. This technology has been implemented successfully at Island Scallops shellfish hatchery (Mr.

Robert Saunders, *personal communication,* 2011). While potentially requiring more capital costs than hydrated lime addition, this system would have the advantage of avoiding issues associated with the formation and environmental regulation of lime precipitates, but it could also remove dissolved oxygen from the system (Dr. Christopher Harley, *personal communication*, 2011).

The latter category of strategies involves exploring the adaptability of shellfish species employed by the shellfish industry. Recent work suggests that *C. gigas* larvae may survive future climate change conditions better than other shellfish species such as the sydney rock oyster larvae *S. glomerata*, which experience significantly more lethal effects under increasing pCO_2 and temperatures conditions (Parker et al., 2012). The eastern oyster larvae (*Crassotrea virginica*), which are closely related to *C. gigas,* appear less sensitive to CO_2 changes than other shellfish larvae, and their survival was only impacted at pCO_2 concentrations of 1500 ppm. In contrast, hard clam (*M. mercenaria*) and bay scallop (*A. irradians*) larval survival was drastically reduced by pCO_2 of ~650 ppm (Talmage et al., 2009).

Given these variable reactions to elevated concentrations of CO_2, hatcheries may want to put efforts in selective breeding of broodstock that are more adaptable to high-CO_2 stress. For example, recent work has shown that wild *S. glomerata* larvae are less resilient to elevated CO_2 concentrations than selectively bred *S. glomerata* (Parker et al., 2012). Although both populations are affected, the selectively bred *S. glomerata* population experiences a stronger increase in standard metabolic rate in response to higher CO_2 concentrations when compared with wild populations, thus demonstrating a potential adaptation mechanism (Parker et al., 2012). Adult *C. gigas* have also been observed to increase metabolic activity at elevated pCO_2 conditions with acute warming.

However, this reallocation of energy sources could have negative consequences, such as the inability to meet the new energy demand (Lannig et al., 2010). Hatcheries may even want to employ simple techniques such as reproductively conditioning oysters at elevated pCO_2, which was shown to produce larger and faster developing *S. glomerata* larvae compared to larvae from than parents conditioned in ambient conditions. This was due to maternal carry-over effects, such as increasing energy per offspring, though no difference was observed for survival (Parker et al., 2012). The Pacific Oyster may be a model organism for research on adaptation to ocean acidification because the genome has recently been sequenced making it easier to isolate and study the genes that respond to elevated CO_2 stress, potentially allowing for genetic improvement (Zhang et al., 2012).

5. Conclusion

The current research has confirmed previous studies that demonstrate a link between carbonate ion concentrations, calcite and aragonite saturation, and shell growth in a Pacific oyster that is used extensively in aquaculture. After 24 days in laboratory culture, larvae from the species *C. gigas* demonstrated a significant reduction in shell length in treatments with lowered pH and carbonate ion concentrations, relative to ambient and limed conditions. This research has also demonstrated the potential for using hydrated lime to chemically reverse the effects of ocean acidification, and the positive influence it has on cultured shellfish within the shellfish aquaculture industry. In the experiments presented here, the addition of hydrated lime changed carbonate chemistry to those in line with pre-industrial conditions. It resulted in longer shell lengths, and produced no significant differences in rates of mortality or deformity.

Our results suggest that lime addition may provide a cost-effective means combating increased acidification in coastal waters. However, more research is needed to determine if other shellfish species larvae grow in seawater enhanced with lime, and whether it impacts their survival and level of deformities. It is highly recommended that a similar study be conducted with gametes fertilized in treatment conditions, to remove errors related to the time-lag of inoculating into treatments. While this small-scale study has demonstrated the potential, large-scale studies (on a hatchery scale) which incorporate lime-addition will help to address more of the questions involving its practical implementation as a mitigative option. In addition, this research should extend into

outplanting studies to determine if use of hydrated lime in early larval stages results in enhanced survival and growth for adults reaching market size (3 to 5+ years). This could be done in conjunction with treatments that buffer the foreshore with powdered $CaCO_3$ versus unbuffered plots.

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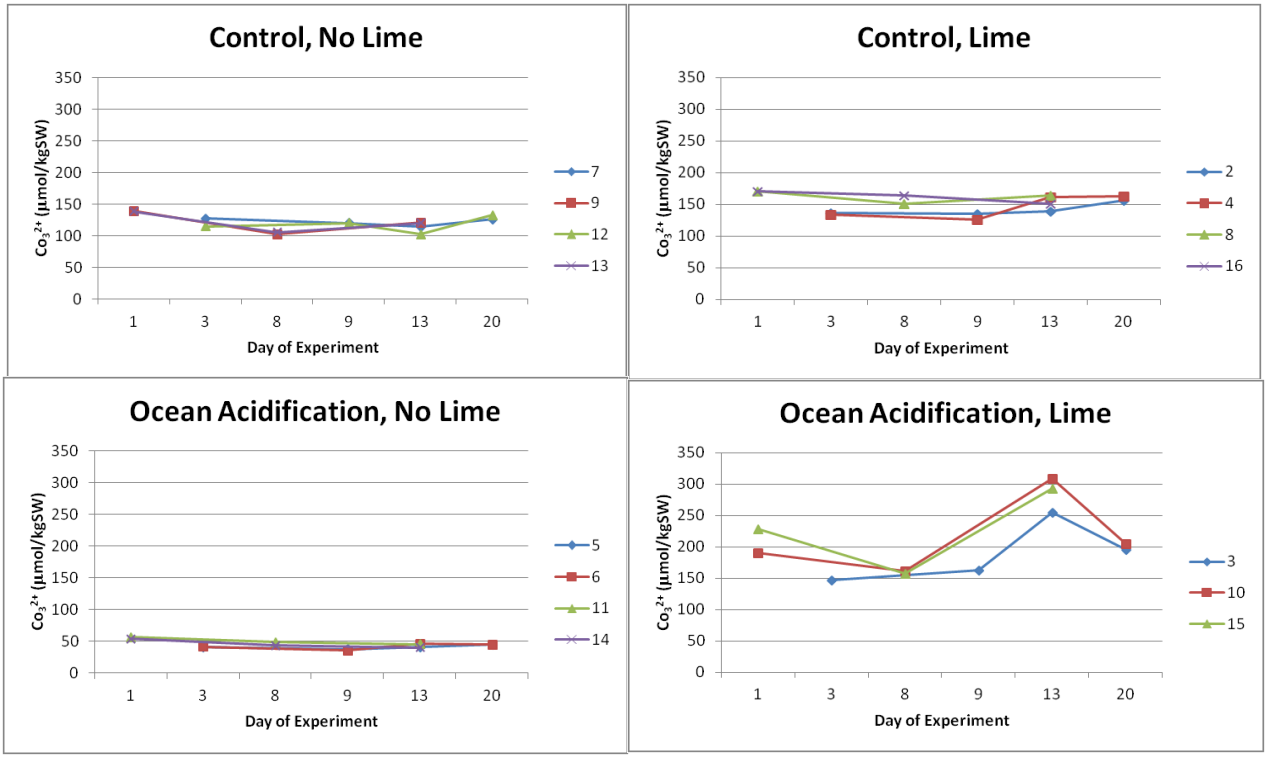
Appendices

Appendix A

Supplemental Table: Carbonate ion concentrations measured overtime for each container (Day 1 to 20). Last two columns summarize the average and SE

Treatment	Container	Day of Experiment						Avg.	SE
		1	3	8	9	13	20		
Control, No Lime	7		127.46		120.09	113.69	126.09	122.96	2.74
	9	139.18		102.67		121.08		120.98	10.54
	12		114.70		120.07	102.19	132.59	117.39	6.30
	13	138.66		105.46		119.98		121.37	9.61
Control, Lime	2		136.91		134.83	139.65	156.17	141.89	4.86
	4		134.35		125.57	161.20	162.24	149.12	8.26
	8	170.06		150.21		163.77		163.52	4.68
	16	170.36		163.82		150.21		162.05	4.24
Ocean Acidification, No Lime	5		40.69		37.08	40.48	44.70	40.00	1.65
	6		41.35		34.97	45.79	44.33	42.45	2.04
	11	56.12		48.48		45.20		49.93	3.24
	14	53.52		43.47		39.88		45.62	4.08
Ocean Acidification, Lime	3		147.24		162.66	254.96	195.84	190.17	23.86
	10	190.83		161.44		308.27	204.34	216.22	31.96
	15	228.01		156.99		292.39		242.45	32.33



Supplemental Figure: Graphical representation of values in table above.
Carbonate ion concentration measured overtime for each container (Day 1 to 20)